Geographic Variation in Trace Element Profiles of Shrimp and Viability of Elemental Profiling as a Traceability Tool

by

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Abstract

The increased concern about the environmental consequences of shrimp culture in importing markets has led to a greater emphasis on sustainability of the products. Traceability is an important component of making claims about product sustainability, and therefore traceability tools are being explored in seafood supply chains. One tool that has been identified is elemental profiling, which is the process of identifying pre-determined groups with classification models based on element concentrations in tissues. Here, we explored the potential for elemental profiling in shrimp from aquaculture ponds. Shrimp from five production countries were collected from shrimp farms and successfully classified based on element profiles with an overall accuracy of 91%. This provides evidence that unique within country profiles exist for shrimp from aquaculture ponds. Shrimp from retail stores were collected in the USA and Europe with the purpose of identifying country of origin identified on the labels. Shrimp labeled as originating in Ecuador, India, Indonesia, Thailand, and Vietnam were collected and classified to labeled country of origin with moderate success (71% overall accuracy). A second classification procedure with the samples from farms within the same countries was attempted where the samples from retail stores were used as a testing dataset, but this was much less accurate (40%). This suggests that the samples from retail stores are incompatible with samples from farms, and a possible confounding factor is the exposure to salts and other chemicals at the processing plants. To investigate this possible phenomenon, samples were obtained from farms in Thailand and Ecuador that were paired with samples from farms pre and post processing. The element profiles of samples in Ecuador showed no practical differences, while the samples in Thailand were markedly different because of the salt bath during processing. Finally, sources of variation that may affect elemental profiles were investigated, namely laboratory variation and shrimp tissue.

In a ring-test of three laboratories, 2 of 5 elements were significantly different, and a homogenized sample replicated 20 times in one laboratory had high coefficients of variance for elements that were above detection limits. Two farms in Alabama were used to analyze variance in tissue samples from shrimp. A multivariate comparison showed an interaction between tissue type and location, suggesting that mineral compartmentalization is geographically dependent. Altogether these results show 1): elemental profiling is a viable tool in shrimp, 2) retail products likely need to be profiled with other retail products or post-processed shrimp, 3) processing interferes with the elemental profile of shrimp products, and 4) shrimp tissues are variable across geographies and laboratory analysis can play a role in increasing variation in element profiles. This work will improve future efforts in elemental profiling and shows that it is a viable tool for traceability in seafood products, specifically shrimp.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Fraudulent labeling in seafood has been identified as a recurring problem, particularly in importing markets like the United States and the European Union. Jacquet and Pauly (2008) identified a number of fisheries products that have been mislabeled for profit gains, and to lose less desirable names for more familiar friendly sounding names (e.g., the Patagonian Toothfish being sold as Chilean Sea Bass). Several studies have identified that around 30% of seafood products are mislabeled in some way, with either false species names, misleading names, or incorrect product origin labeling (Jacquet and Pauly 2008, Christiansen et al. 2018, Pardo et al. 2016). The mislabeling of seafood is to the detriment producers who cannot be adequately compensated for their product and consumers who are being misinformed about their food purchasing choices.

The shrimp industry in Southeast Asia has encountered criticisms in recent years for alleged human rights abuses within the supply chain and has historically been scrutinized for the impacts of industry practices on local environment and communities (Boyd and McNevin 2015). The abuses, in part, were enabled by poor supply chain management. Specifically, many exporters are not aware of where their products are produced, and instances of the practice of transhipping have been documented. In this context, transhipping can be defined as labeling a product as being produced in a certain country when it was produced in another, and then subsequently being exported to a market often in a third country. These practices led to lawsuits in the US for importing companies in which slave labor issues have been documented (Hodal et al. 2014), and caused the European Union to issue a yellow card to Thailand in 2015 (Kelly 2018).

Fundamentally, the lack of traceability in shrimp supply chains not only enables these practices, but also enables the use of chemical therapeutants and poor production practices because of a lack of accountability.

Trace element profiling has been put forth as a potential tool for improving traceability in seafood supply chains (Leal et al. 2015). The process involves collecting samples that belong to different, predetermined groups (e.g., county of origin, organic vs conventional production, wild vs cultured), analyzing tissues for elemental concentrations, and using statistical procedures to delineate the groupings. Exposure of trace elements in a culture system could come through two primary sources: feed and the environment (water or soil in the culture unit). The method has been used repeatedly in the literature on a conceptual basis (see Li et al. 2016) however, it remains relatively underutilized in practical applications. One of the first attempts in the literature with the use of trace elements to discern geographic origin in shrimp was Smith and Watts (2009). The procedure described therein was being used to discern the origins of retail products being shipped to the US (as will be discussed below).

Limited applications of trace element profiling have been made in a practical sense, with the only well documented application with seafood products outside of an academic setting occurring in 2008-2009 when the United States Customs and Border Patrol attempted to use elemental profiling to determine the origins of a shipment of shrimp by CP Prima from Indonesia (Seafood Source Staff 2009, US Customs and Border Patrol 2010). US CBP later published their method (Smith and Watts 2009), but at the time the underlying analysis was unknown (Seafood Source Staff 2009). While several other studies with aquaculture products have been produced since, (Li et al. 2016, Gopi et al. 2019a), the method remains relatively unverified and there is some uncertainty as to the validity of the procedure in a practical application. The goal of the

proposed work herein is to understand factors that may affect the viability of elemental profiling as a traceability tool and pilot an application with retail samples.

1.2 Overview of Shrimp Aquaculture

Shrimp farming as an industrial practice is a relatively new activity. In some Asian countries, there was a history of capturing small wild shrimp in manmade mangrove ponds in the tidal zones during attempts to capture milkfish *Chanos chanos,* however these were not widespread and did not result in much production. The earliest attempts to culture shrimp in an aquaculture sense began in Japan with Kuruma Prawns *Marsupenaeus japonicus* in the mid-20th century (Chamberlain 2010). Japan is too cold for shrimp production most of the year, but the developments in culturing Kuruma prawn were adopted in Taiwan in the 1970's and 1980's where commercial production began in earnest with black tiger shrimp *Penaeus monodon*. The farms in Taiwan were quickly intensified and subsequently the farms collapsed due to disease, but the technologies began to spread to other Asian countries. At roughly the same time, the production of whiteleg shrimp began in the tidal areas in Ecuador, which would become the major producer of shrimp in the Americas. This consolidated the production of shrimp to two major areas in the world, Southern and Southeast Asia and Latin America, and ultimately would consolidate the production of shrimp into two species, whiteleg shrimp and black tiger shrimp, which account for about 95% of the worlds Penaeid shrimp production. The shrimp industry has undergone several rises and crashes related to the rises of viral diseases like Taura Virus, Whitespot Syndrome, and Early Mortality Syndrome (Walker and Winton 2010), however, the production shrimp began to explode with the introduction of specific pathogen free (SPF) larvae around 2003 (see Figure 1). As it stands, the countries that produce the most shrimp on an annual basis are Ecuador, India, Thailand, Indonesia, Vietnam, and China (Table 1). The majority of this

production is for export markets in developed countries, with the exception of China where a majority of the production is consumed domestically (Zhang et al. 2017). The markets that dominate international imports for shrimp products are the European Union, United Kingdom, United States, Japan, and Korea (Figure 1). In the US, the Department of Commerce reported that over 698 million kg of shrimp were imported in 2019, with India being the highest importer at 287 million kg (NOAA 2020).

1.3 Environmental Issues in Shrimp Aquaculture

The rise of aquaculture was initially heralded as the "blue revolution", much like the green revolution of the early-mid 20th century that was associated with agriculture. However, as aquaculture came to prominence, so too did the criticisms of the practice. The devastation that aquaculture caused in the mangrove tidal areas is well documented. Indeed, some estimate that as much as 50% of global mangrove coverage was lost in the 20th century, and while aquaculture was a major contributor (Hamilton 2013), it was not entirely responsible for the loss of mangroves. The rise of shrimp aquaculture did correspond and in many cases cause mangrove loss, however modern farms have begun to move out of the mangrove areas (Boyd et al. 2017, Boyd et al. 2018) because of the lack of suitability of the habitat for intensified shrimp production (Tho et al. 2011). It is often overlooked that many governments encouraged the spread of shrimp aquaculture as economic development, and some still do. In Vietnam, a practice known as integrated mangrove aquaculture is utilized in the Mekong delta by small stakeholders that is ostensibly extensive production, but involves maintaining a certain percentage of mangrove cover on the property leased from the government (Joffre et al. 2015). These systems are often used to grow black tiger shrimp and are held up as environmentally sustainable even though they fragment mangroves.

Another source of criticism has been the resources spent on intensified shrimp production in aquaculture (Naylor et al. 1998, Naylor et al. 2000). Aquaculture feeds traditionally had a high fishmeal and fish oil inclusion rate, however as fishery stocks have struggled and fishmeal increased in cost, plant ingredients (namely soy derived meals) have replaced a portion of fishmeal in aquaculture feeds. The percentage of fishmeal in shrimp feeds has steadily declined as nutritional needs of shrimp become better defined (Boyd and McNevin 2015), and diets without any fishmeal have been derived in laboratory settings (e.g., Amaya et al. 2007).

The localized environmental impact of shrimp production has also been bemoaned. Poor production practices and disease outbreaks led to prophylactic use of antibiotics in shrimp, which in turn led to the presence of antibiotic residues in the surrounding environment (Binh et al. 2018, Di Cesare et al. 2013, Holmstrom et al. 2003, Swapna et al. 2012). Shrimp farmers have indeed grown to understand the divisiveness of antibiotics. Despite the ongoing detection of antibiotics in local environs, recent surveys of shrimp farms revealed not one farmer who was willing to admit using antibiotics (Boyd et al. 2017, Boyd et al. 2018). Effluent discharge is another issue that is well documented. Intensive shrimp production often produces nutrient loaded effluent waters, which when released back to the water body from which the water was obtained, can decrease overall availability of dissolved oxygen in the water and causes eutrophication (Jones et al. 2001, Paez-Osuna 2001, Paul and Vogl 2011, Ramesh 2001). In previous decades, shrimp farms were also blamed for the excessive use of freshwater from local aquifers, which caused the drawdown of the aquifer, and sometimes the intrusion of saltwater or brackishwater into the water table (Kongkeo 1997).

These issues highlight the history of criticism of shrimp farming from environmental groups. These issues are indirectly responsible for the rise of certification schemes, which

attempt to separate best actors in the industry from worst performing actors and reward them with competitive market advantages in exporting markets. Of the many standards for shrimp , the two most popular are the Aquaculture Stewardship Council (ASC) and the Global Aquaculture Alliance's Best Aquaculture Practice (BAP) standards. In the United States, the BAP holds the vast majority of the certified shrimp product with little no presence by the ASC, while in Europe both the ASC and BAP are prevalent in grocery stores. Sustainability standards like the ASC and BAP were developed to address many of the criticisms of shrimp aquaculture including resource use, local environmental impacts, and social impacts to the local community and workers at the farm (Aquaculture Stewardship Council 2014). Standards like the ASC have been criticized as "green washing" (that is to have the environmentalist community endorse practices that are deemed by other environmentalists as destructive) the aquaculture industry (Belton et al. 2010). Altogether, the foundation of certification standards and of retail products in general is the claims making of the products; the claims of environmental responsibility, social responsibility, etc. These claims are dependent on the traceability and validity of the product.

1.4 Traceability and Shrimp Supply Chains

Olsen and Borit (2013) provide a review of definitions of traceability and the practical application of these definitions. They define traceable food commodities as having four key parts, summarized as follows: 1. traceable resource units – items that can be grouped with similar properties; 2. identifiers – unique keys that can be assigned to the traceable units never to be reused; 3. metrics - recorded properties linked to the identifiers; 4. access – the information must have accessibility. In this context, they derive the definition of traceability in food commodity context as follows: "The ability to access any or all information relating to that which is under

consideration throughout its entire life cycle, by means of recorded identifications". The shrimp supply chain will be explored through this definition.

Shrimp supply chains in Latin America tend to be different than shrimp supply chains in Southeast and South Asia. The shrimp industry in Asia consists of mostly disaggregated small farmers, with centralized hatchery reared larvae, feed manufacturing, and processing of harvested shrimp. To this point, it is believed there is between 10-20 thousand shrimp farms in Thailand, but less than 200 processing plants (Seafood Tip 2021, https://seafood-tip.com/sourcing-intelligence/countries/thailand/shrimp/). There are also so-called "Agents", middlemen that act as brokers and suppliers for various stages in the supply chain (e.g., farms and processors on behalf of the processor) who aggregate products for upstream supply chain actors (Dietsche 2009), which in turn makes the acquisition of raw materials easier. In the context of traceability, brokers present a challenge for aquaculture suppliers in that products from multiple farms, for example, may be aggregated and mixed, which increases granularity of the product, which is an important determination in the overall traceability (Karlsen et al. 2011). Instead of being able to identify a product to the farm level, now only one of several farms can be identified. Agents are common at other steps in the supply chain, including hatcheries to farms, fisherman to fishmeal plants, and processors and exporters (Dietsche 2009). The aggregation of products from several farms, boats, processors at different steps in the supply chain impairs traceability in shrimp aquaculture in the context of Olsen and Borit's (2013) definition.

Supply chains in Latin America can be less complicated, but have similar issues. Latin American farms tend to be much larger than Asian farms. In recent field surveys conducted by the World Wildlife Fund, the average farm size in farms in India, Thailand, and Vietnam was

7.65 ha while the average farm in Ecuador was 149 ha (Boyd et al. 2017, Boyd et al. 2018, Boyd 2021). The larger farms tend to have their own processing plants and will sometimes process of the shrimp of smaller neighboring farms. Because the size of the farms is much greater, the processing can be done more easily in identifiable batches, where farms or even ponds at some larger farms do not need be aggregated to keep the processing plant operating. Altogether, the challenges of traceability are less in Latin America due to the corporatized nature and size of the farms.

1.5 Seafood Fraud in Importing Markets

The issue of mislabeled seafood not only is derived because of issues with production practices at the farms and processors but also because of issues in the foreign markets where the products are sold. Jacquet and Pauly (2008) summarized several relevant research items on seafood fraud. From their findings several conclusions can be reached. Seafood products are often mislabeled to improve marketability (i.e., change a less desirable sounding name to a more desirable sounding name), substitute farmed fish for wild fish, and substitute low value fish for higher value fish. Other examples of fraud have been tied to improving the perceived environmental responsibility of a product (Miller et al. 2012). In the US, shrimp have been mislabeled, especially in southern states to substitute farmed products for wild gulf shrimp (Korzik et al. 2019), which is a higher value product. Several attempts have been made to widely classify the occurrence of seafood, and generally they reach the conclusion that 20-30% of seafood products are mislabeled (Christiansen et al. 2018, Naaum et al. 2016, Pardo et al. 2016, Luque and Donlan 2019).

Several reviews have been conducted to identify tools that can improve the traceability of food products (Leal et al. 2015, Gopi et al. 2019a, Hassoun et al. 2020). One possible solution

that has been identified is elemental profiling. More broadly applied to agricultural products because of the direct uptake of minerals by plants from the soil, elemental profiling is a traceability tool that has been applied in a wide variety of products and contexts. Elemental profiling is generally described as the process of using elemental concentrations in selected tissues to discriminate samples into pre-determined groupings. The underlying concept of elemental profiling is that environmental exposure leaves patterns or "fingerprints" in animal tissues as a result of locale specific variations in exposure. In aquatic organisms this is primarily by two means, exposure and uptake through water and uptake of minerals through the digestion of food.

1.6 Elemental Profiling in Seafood Products

Elemental profiling has been applied to a wide variety of species and settings in seafood. Anderson et al. (2010) found that salmon flesh from wild caught salmon and farm raised salmon could be discriminated with >92% accuracy. Rearing locations of several species have been successfully delineated (Albuquerque et al. 2016, Ricardo et al. 2017, Bennion et al. 2019). Swearer et al. (2003) found that elemental concentrations in otoliths could be used to separate five different fish species from each other in localized estuarine environments. It has been used on a wide variety of species including salmonids (Loewen et al. 2015, Watson et al. 2018), marine fishes (Arechavala-Lopez et al. 2013), cephalopods (Arbuckle and Wormuth 2014), crustaceans (Ortea and Gallardo 2015, Luo et al. 2019), molluscs (Becker et al. 2005) and seaweeds (Hattori et al. 2009).

The attempts to identify predefined groupings in shrimp are summarized in Table 2. The first attempt to separate shrimp populations was in wild caught Northern King Prawns *Penaeus plebejus* in the Australia (Courtney et al. 1994). Watts and Smith collected shrimp from 10

different countries, with an overall accuracy of >90%. The methods and samples collected in Watts and Smith were the basis of a validation process that was used as evidence against CP Prima in a US Customs and Border Patrol cases. CP Prima shipping containers filled with frozen shrimp were seized on the grounds that they were suspected to be transshipped, which in this context means that the shrimp were grown in one country and processed and labeled in a different country before export to the end port destination. Ultimately, the CP shrimp were released and allowed to be shipped (Seafood Source Staff 2009). Li et al. (2014) found that shrimp from three farms in the USA were distinguishable from each other and that different tissues in the shrimp were also distinguishable. More recently, projects by Li et al. (2017) and Gopi et al. (2019b) have distinguished shrimp reared in different locations in Southeast Asia, and Li et al. (2019) distinguished shrimp products in China raised in low salinity and high salinity ponds.

1.7 Objectives

There are several knowledge gaps in elemental profiling with shrimp that remain unresolved. There has been little detail on the elemental profiles of shrimp from Latin America, even though Ecuador produces at least 10% of the worlds whiteleg shrimp production. In a practical sense, the application of elemental profiling has remained limited to the attempt by US Customs and Border Patrol. To this end, preliminary data from the Word Wildlife Fund found that it was difficult to discriminate retail samples to geographic origin with samples from farms as the data used to train the discriminant model (Author's unpublished data). There is a possibility that there are incongruences between the elemental profiles of shrimp harvested directly from shrimp farms and shrimp from retailers, although this remains unknown. Shrimp from farms get exposed to chemicals during harvest and at the processing plant, namely sodium

metabisulfite and sodium chloride (Boyd and McNevin 2015). It is possible that the salt baths at the processing plants are changing the profiles of the shrimp.

More broadly, several practical matters remain uninvestigated with regards to elemental profiling. In shrimp specifically, Li et al. (2014) was able to discern differences in different tissue types, however, did not attempt to understand the interaction between tissue type and geographic variation, therefore it is unknown if tissues of different types can be mixed into the same classification without impacting the results. Several studies have used different tissue combinations (e.g., Bennion et al. 2019 used seven different tissue combinations) to analyze the same data, but they did not draw any conclusions about the importance of element compartmentalization into different tissues. In the analytical process it is generally accepted that the results of a particular laboratory are not applicable to other laboratories and that laboratory variation is prominent enough to cause issues with elemental profiling. In a practical application, this needs to be considered when establishing an elemental database that would be used to analyze unknown samples.

Therefore, the overall purpose of this study is to assess the validity of using elemental profiling to identify the origin of retail products. The chapters listed below are designed to inform this overall objective in some way. Summarized, in brief, the objectives of this dissertation are to:

1. *Assess the strength of discriminant analysis in seafood items with trace elements through meta-analysis*

2. *Create a database of elemental profiles for farms Ecuador from the primary shrimp producing regions*

3. *Assess the viability of utilizing element profiling for identifying retail samples independent of product labeling, with samples from the EU and USA*

4. *Assess the effect of industrial scale processing on element profiles in shrimp*

5. *Determine sources of possible variation in shrimp element profiles by analyzing the following:*
    1. *A laboratory ring test*
    2. *Analyze the effect of digestion matrix*
    3. *Variation in tissue element concentrations*

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Table 1. The average annual production of Penaeid shrimp between 2014-2018, according to FAO's Fishstat data. All members of the family Penaeidae in the Fishstat database are included in this summary.

Country	Annual Production (tons)	Percent (%)	Cumulative Percent (%)
China	1879037	35.83	35.83
Indonesia	741885	14.15	49.98
Viet Nam	665561	12.69	62.67
India	544909	10.39	73.06
Ecuador	423000	8.07	81.12
Thailand	324963	6.20	87.32
Mexico	130622	2.49	89.81
Bangladesh	79386	1.51	91.32
Brazil	63280	1.21	92.53
Philippines	60602	1.16	93.69
Other	331178	6.31	

Table 2. A summary elemental studies in penaeid shrimp aiming to discern geography.

Author	Year	Species	Region	Classifier Level	Classification Accuracy
Courtney et al.	1994	Northern King Prawns *Melicertus Plebejus*	Australia	Region	100%
Smith and Watts	2009	Whiteleg Shrimp *Litopenaeus vannamei* and Black Tiger Shrimp *Penaeus Monodon*	Asia and Latin America	Country	> 90 %
Li et al.	2014	Whiteleg Shrimp *Litopenaeus vannamei*	Southeast United States	Farm	100%
Ortea and Gallardo	2015	Multiple Penaeid Species	Global	Country	51-100 %
Li et al.	2017	Whiteleg Shrimp *Litopenaeus vannamei*	Southeast Asia	Country/ Region	97%
Gopi et al.	2019	Black Tiger Shrimp *Penaeus Monodon*	Southeast Asia and Polynesia	Country	82-100%
Li et al.	2019	Whiteleg Shrimp *Litopenaeus vannamei*	China	Region	100%

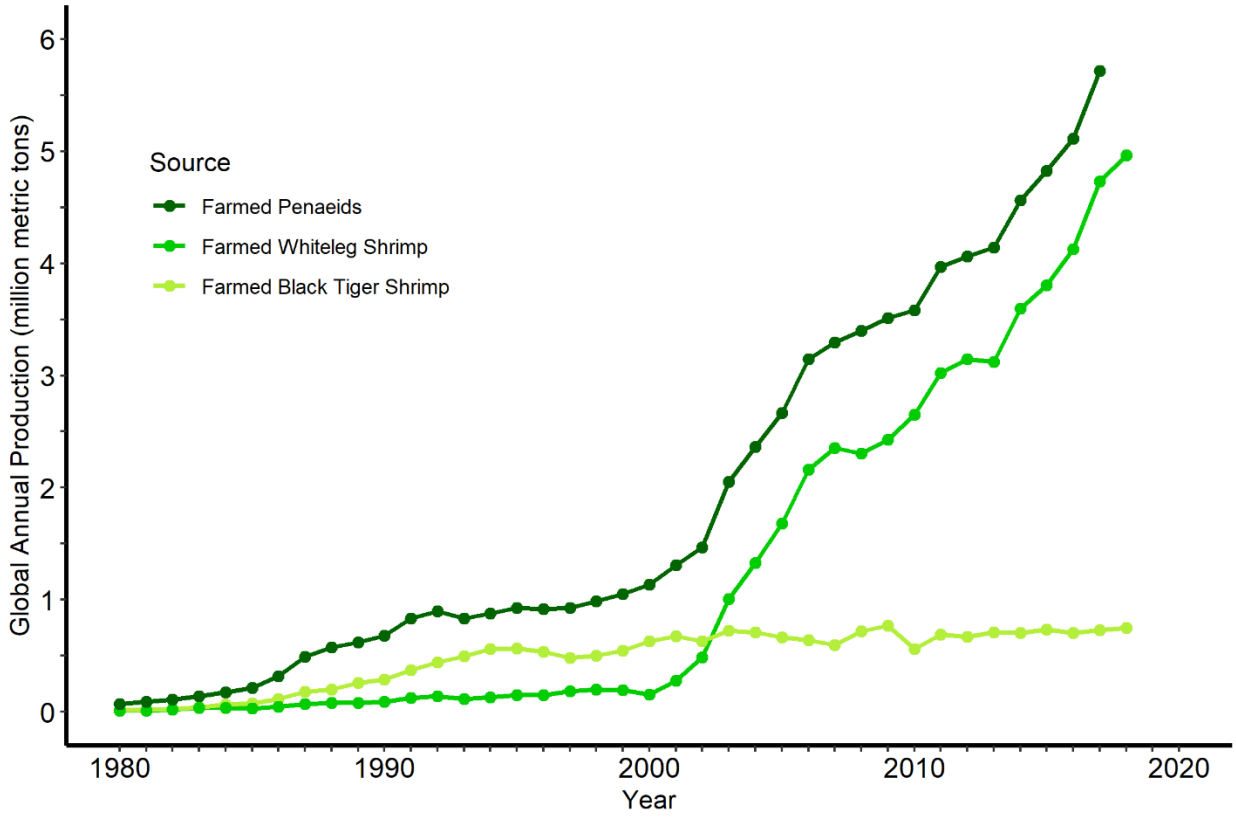

Figure 1. Global farmed shrimp production between 1980 and 2018, according to FAO data.

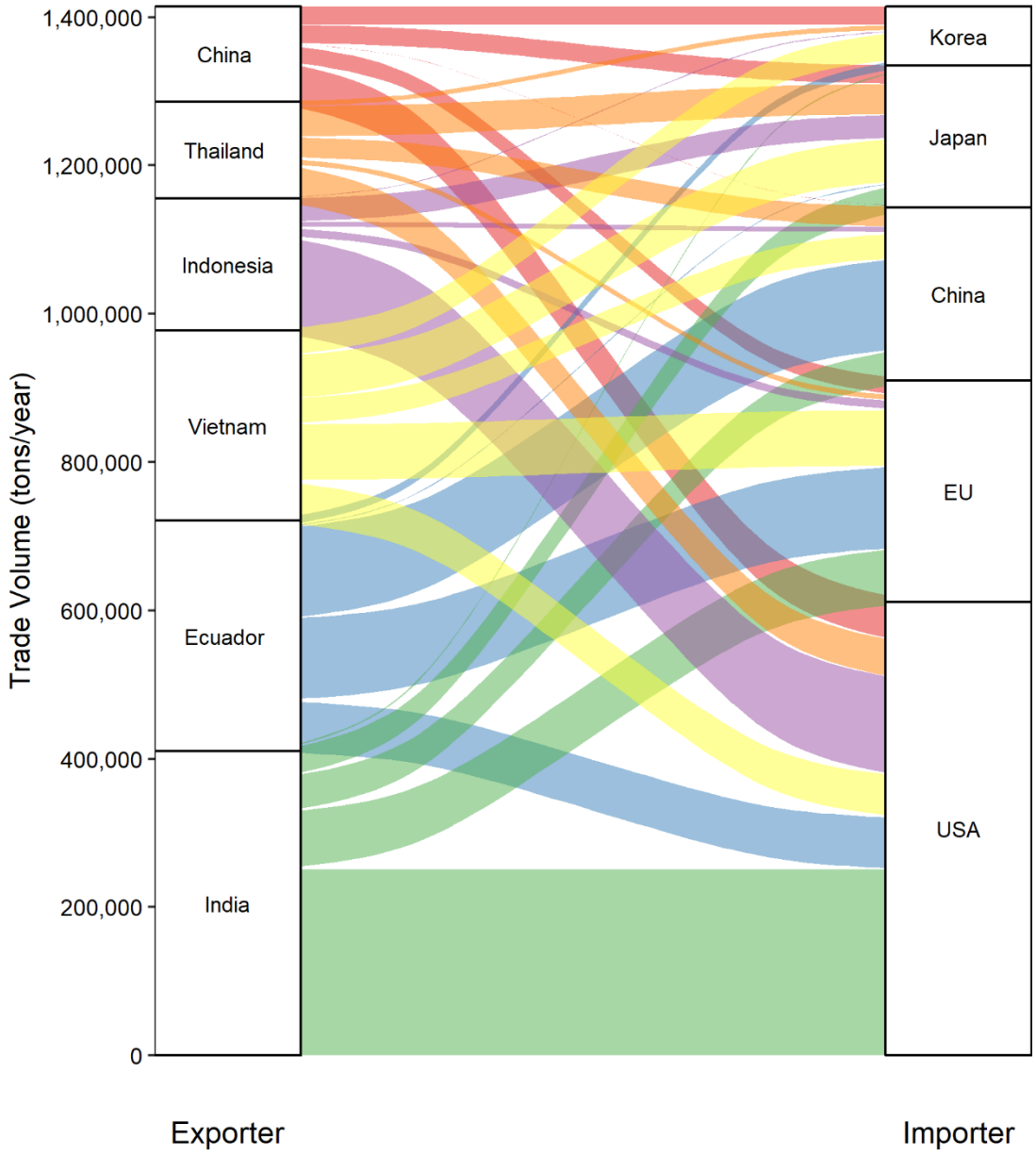

Figure 2. The trade flows of major shrimp importers and exporters for the year 2019.

CHAPTER 2

THE UTILITY OF DISCRIMINANT ANALYSIS TO DETMERINE THE GEOGRAPHIC ORIGIN OF COMMERICIALLY IMPORTANT SEAFOOD AND AQUACULTURE SPECIES: A META-ANALYSIS

2.1 Abstract

Elemental profiling is a process where element concentrations in tissues are used to discern group membership between *a priori* groups, such as geographic location. The goal of this meta-analysis was to determine the effectiveness of elemental profiling to discern the geographic origin of seafood products when coupled with discriminant analyses. Cohen's Kappa of discriminant analyses were calculated based on reported accuracies and calculated expected accuracies. A systemic literature review was conducted which generated 86 effect sizes from 43 studies. A random effects model was used to estimate Cohen's Kappa, and the average effect observed was 0.83 (± 0.036 95% CI) with a p value of <0.001. Moderators were investigated as part of this analysis. There were no significant differences based on production method or geographic range, but differences were observed based on the type of tissue analyzed and taxa groups. Meta-regression was used to analyze the relationship between the effect size and the number of elements included in each analysis or sample size. There was a weak observed relationship between Cohen's Kappa and the number of elements analyzed and no relationship with sample size. Based on these results, recommendations for future studies that utilize elemental profiling are included.

2.2 Introduction

The world's population is projected to grow to 9 to 10 billion people by 2050 and the need for edible proteins is estimated to increase at an even higher rate (FAO 2009). Seafood is an important source of protein and healthy fats (e.g., Omega 3's) for many people and constitutes roughly 23% of current global meat production (Edwards et al. 2019). Seafood products were traditionally derived mostly from capture fisheries, however, capture fisheries have plateaued (FAO 2018) whereas aquaculture continues to expand and recently surpassed fisheries production in terms of volume for human consumption.

Despite the importance of seafood to the human food supply chain, seafood products are plagued by fraudulent labeling which has been well documented in the United States and the European Union. Mislabeling occurs to manipulate prices (Jacquet and Pauly 2008), improve marketing (i.e., "sustainability") (Miller et al. 2012), and substitute farmed species for wild species (Korzik et al. 2019), among other causes. In aquaculture shrimp products, transshipping has been an issue in the past, resulting in incorrectly labeled country of origin among retail products. Beyond mislabeling in seafood, the identification of discrete wild fish stocks is important for proper management (Begg et al. 1999). Discrete stocks can require different courses of action to be appropriately managed even within the same population (Stephenson 1999).

Elemental profiling has been identified as a tool to increase traceability in seafood products (Li et al. 2016, Leal et al. 2015, Gopi et al. 2019a), and to identify fisheries stocks (e.g., Loewen et al. 2015, Avigliano et al. 2019, Chouvelon et al. 2017). Elemental profiling (or elemental fingerprinting, trace element analysis, etc.) involves analyzing samples from *a priori* groupings for element contents to determine if differences in the element concentrations can be

used in conjunction with discriminant analyses to discern group membership. Elemental profiling has been used to determine geographic origins (e.g., Gopi et al. 2019b, Li et al. 2017, Albuquerque et al. 2016), production origin (Anderson et al. 2010, Chaguri et al. 2017), and salinity of shrimp ponds (Li et al. 2019) with a high degree of success. While there are numerous examples in the literature of successful elemental profiling, it is often said to be a novel approach or "exploratory" (e.g, Ortea and Gallardo 2015). To this point, very few practical examples exist, where elemental profiling was applied as a tool for traceability in a regulatory capacity. Studies in the literature serve as a proof of concept- predetermined groups are discernable through their mineral contents- but they have not gone as far as to validate unknown samples, except in a few cases. One such example that does exist occurred when the United States Customs and Border Control seized shrimp from CP Prima in Indonesia on the basis that their elemental profiles suggested the shrimps were transshipped (US Customs and Border Patrol 2010), which was based on the data and analytical procedure in Smith and Watts (2009).

Elemental profiling is not a new technique, and therefore a body of literature exist on the topic, especially for aquatic species. The goal of this study is to characterize the effectiveness of elemental profiling in determining the geographic origin of commercially important fisheries and aquaculture species in the scientific literature through a meta-analysis. Based on the results herein, a secondary objective was to identify factors that are related to the success of elemental profiling studies and make recommendations for future studies that plan to utilize the technique in the future.

2.3 Methods

2.3.1 Literature Search

To obtain studies related to elemental profiling in commercially important aquatic species, a structured literature search was conducted in August of 2019 based on the PRISMA guidelines developed by Moher et al. (2009). An information scientist was consulted as part of developing the search. The search was conducted in Web of Science, Agricola, Medline, and American Fisheries Science Abstracts with the proper combinations of the following search terms for each data base; trace element profiling, elemental fingerprinting, elemental distribution, chemical profile, trace element composition, element microchemistry, isotope, geography, origin, location, country, area, fish, crustacean, shrimp, mollusk, seafood, clam, catfish, and sand eel. An example of the search in a database such as Web of Science would be as follows, (trace element profil* OR trace mineral profil* OR elemental profil* OR elemental fingerprint* OR trace metal profil* OR element distribution OR chemical profil* OR trace elemental OR trace element composition OR (element* AND isotop*) OR microchemistry) AND (geograph* OR origin* OR location* OR countr* OR area*) AND (fish OR fishes OR crustacea* OR shrimp OR mollus* OR seafood OR clam* OR catfish OR sandeel). All years and languages were considered. Studies were included based on the following criteria: i. the study was on an aquatic species that is cultured or harvested as a food product, either locally or on a commercial scale, ii. the study was not meant to discern the natal origins of the species of interest, iii. the study used elemental or isotopic concentrations from a tissue as the means for discriminant analysis, iv. the data analysis utilized some discriminant procedure (e.g., a linear discriminant analysis, random forest, etc.) that reported an overall accuracy or a confusion matrix style table, v. the study attempts to discern the geographic origin of the organisms in the study. Studies that were not

complete in their reporting were not considered for this study. In addition to the structured literature search in the online databases, the first 100 results of Google Scholar were scanned as it is standard practice to limit the number of results scanned in Google Scholar (Haddaway *et al.* 2015), and references in the studies included deemed appropriate for inclusion were cross-referenced to find additional studies not found in the literature search.

2.3.2 Data Collection and Analysis

After studies were collected and screened for inclusion, the authors obtained the following information for each study: i. species included, ii. taxonomic membership (e.g., fish, crustaceans, mollusks, etc.), iii. production type (fisheries vs. aquaculture), iv. the purpose of the study (traceability/ identifying fraud or mislabeling vs. stock identification), v. analytical method (e.g., ICPMS, ICPAES), vi. the number of elements used in the discriminant analysis, vii. the geographical range of the analysis (coded by the authors), viii. The type of tissue used in the analysis (coded by the authors), ix. the overall sample size, x. the number of groups in each discriminant analysis conducted, xi. the accuracy of the test. The geographic range of the study was coded as follows; 'local' – within the same watershed (e.g., Ricardo et al. 2015), 'regional' – within the same country (e.g., Ortea and Gallardo 2015), and 'country' - groupings were at the country level (e.g., Li et al. 2017). For capture fisheries studies, stocks that were off the coast of the same country were considered regional and stocks that were from locations that were located off the coasts of different countries were considered to be on the "country" level (e.g., Farabegoli et al. 2018). Tissue types were grouped as the following: 'hard' - calcified tissues (e.g., bone, shells, scales, fin rays, otoliths), 'soft' - muscle or other tissues that are not calcified, 'mixed' - an analysis that included both hard and soft tissues, and 'algae' - tissues from aquatic macro-algae. Several studies reported multiple discriminant analyses. A given study could have multiple

results included in this meta-analysis if the results were based on different element, tissue, and group membership combinations (e.g., Tournois et al. 2013 had 7 effect sizes included in this study). Finally, if a study reported an accuracy from the statistical procedure as well as a cross-validation (e.g., Adey et al. 2009), the accuracy from the cross validation is reported here for the overall accuracy of the analysis.

The effect size used in this meta-analysis was Cohen's Kappa (Kappa). Originally intended to be used as a measure of interrater agreement between two observers (Cohen 1960), it has been used to access the accuracy of discriminant procedures as well (Titus et al, 1984). Cohen's Kappa corrects for incidences of agreement based on chance, and is calculated with the following formula :

$$Cohen's\ Kappa = \frac{\mathrm{Pr}_a - Pr_e}{1 - Pr_e}$$

Where Pr_a is the percentage of accurate classifications, and Pr_e is percent of correct classifications by chance as decimals. A generalized formula for calculating Pr_e is found in McHugh (2012). The standard error of Kappa was calculated with a formula in McHugh (2012):

$$SE_{Kappa} = \sqrt{\frac{Pr_a * (1 - Pr_a)}{n * (1 - Pr_e)^2}}$$

Where n is the total number of samples in the discriminant procedure. The variance of classifications where 100% accuracy were estimated by solving the formula for SE_{kappa} with 99% accuracy.

After Kappa and variances of the analyses were calculated, a random effects model was used to estimate the overall efficacy of discriminant functions. A random effects model accounts for variability in between study error as well as within study variation. Heterogeneity in the meta-analysis was assessed based on the Q and I^2 statistic. A Q test assesses between study heterogeneity, while I^2 is the percentage of variation that is due to heterogeneity across studies

and not random chance. A rank correlation test and Eggers test were used to assess publication bias (Begg and Mazumdar 1994, Egger et al. 1997). Both tests are meant to assess the significance of any relationship between effect size and the variance of the corresponding effect size. Moderator analysis (i.e., a multiple regression in a meta-analytical context) was conducted to determine the effect of taxonomic group, tissue type analyzed, scale (local vs. regional vs. country), and production type (aquaculture vs. fisheries). The variation explained among moderators was assessed based on the τ^2 (a measure of dispersion) computed r^2 (Borenstein et al. 2011). *Post hoc* linear contrasts were used to compare all means in each subgroup analysis in the r package "glht" (Hothorn et al. 2008). The procedure in this package is robust to small sample or uneven sample sizes (Herberich et al. 2010). Meta regression (i.e., linear regression in a meta-analytic context) was used to determine the relationship between Kappa and the number of elements used in the discriminant analysis and Kappa and the overall sample size. All analyses were conducted in R version 3.6.2 (R Core Team 2019) using the metafor package (Viechtbauer 2010).

2.4 Results

2.4.1 Literature search

A total of 4839 records were screened after duplicates were removed (Figure 1). Of the 104 records accessed for full text inclusion, 43 were deemed appropriate for this meta-analysis. The most prevalent reason for exclusion was studies that were designed to discern natal origins in wild fish populations, which excluded 29 studies. Other reasons for exclusion were a lack of information to calculate the effect size (n=13), studies that did not separate geographies (n = 9), insufficient data (n = 2), confounding study design (n = 2), species was not a food item or of commercial significance (n= 3), and the study did not use a discriminant analyses (n =3). A total

of 86 effect sizes representing 34 biological families were included in the meta-analysis. The median accuracy of the discriminant analyses reported herein was 90% with 83% and 97% representing the 25^{th} and 75^{th} percentile, respectively. The average sample size here was 119 and the average group size was 31. Of the 86 effect sizes reported here, 15 utilized a nonparametric test (e.g., Random forest or KNN), and 71 used a parametric discriminant analysis (e.g., LDA, QDA).

2.4.2 Meta Analysis

The result of the random effect model to determine the average efficacy of discriminant functions can be seen in Figure 2. The overall effect was 0.83 with a standard error of 0.0185. Publication bias was assessed in this dataset through visual means and statistical means. The funnel plot can be seen in Figure 3. While visual inspection of the plot shows a fairly symmetrical distribution about the mean, the Eggar's test ($z = -7.952$, $p < 0.0001$) and rank correlation test both (Kendall's $\tau = -0.405$, $p = <0.0001$) were significant. There are a large number of points that fall outside the 95% confidence region when visually inspecting the plot.

Subgroup analysis was next conducted to determine significant differences between factors that could affect the outcomes of a discriminant analysis (Table 1). Crustaceans had a significantly higher kappa than fish (estimate = 0.149, $z = 2.864$, $p = 0.0376$). With regards to tissues, discriminant analyses with mixed tissues had a significantly higher kappa than hard tissues (estimate = 0.144, $z = 2.971$, $p = 0.014$), and soft tissue also had significantly higher kappa scores (estimate = 0.165, $z= 3.67$, $p = 0.001$). In production, analysis involving samples that were from aquaculture were statistically better than fisheries, but both were similar to analysis with mixed sources. No significant differences were found based on geographical range.

The subgroups analyzed here had a combined r^2 of 0.152, or 15.2% percent of the heterogeneity in the study.

Meta regression was used to analyze the relationship between kappa and sample size, and kappa and the number of elements analyzed (Figures 4 and 5, respectively). A significant relationship was detected between the effect size and the number of elements included in the analysis (Kappa = 0.749 + 0.0079*number of elements, $\beta_0 = p < 0.0001$, $\beta_1 = p = 0.0014$). No relationship was also detected between the effect size and sample size (model = kappa= 0.826 + 0.00004 (n), $\beta_0 = p < 0.0001$, $\beta_1 = p = 0.743$).

2.5 Discussion

Seafood is an important part of the protein landscape in food production. Nearly 160 million metric tons of seafood were produced in 2018, with roughly half coming from fisheries and half coming from aquaculture (Edwards et al. 2019). Due to monetary gains, marketing, regulation, or convenience, seafood is routinely fraudulently labeled in supermarkets and restaurants (Lagasse et al. 2014, Christiansen et al. 2018, Pardo et al. 2016). The first step to accountability is traceability, and therefore discerning the origin of seafood is important for consumers, retailers, producers, and fringe industries that support the production of wild fisheries and aquaculture products. This meta-analysis aims to understand the efficacy of elemental profiling to determine the origins of seafood products, and species that are important as food species.

The notion that elemental profiling is unexplored, or novel put forth in some studies should be reconsidered based on the results of this study. Over 40 studies were included here and another 58, which were technically about elemental profiling but not within the scope of this study, were also found during this literature search. Additionally, numerous examples exist in

agriculture beyond fisheries and aquaculture species that show that elemental profiling can be an effective technique at discerning the geographic origin of biological organisms (e.g., Krivachy et al. 2015). Therefore, describing this technique as novel or unresolved is somewhat misleading, as this is a widely used practice which has been used by customs agencies to discern the geographic provenance of products in the past (US Customs and Border Patrol 2010). Additionally, this study showed that elemental profiling has been conducted on a wide variety of aquatic organisms as there are 34 families included here, providing a wide base of information is available on the technique.

Many meta-analyses rely on Cohen's definitions of 'small', 'medium', and 'large' with regards to the measured effect size to determine the magnitude of the impact, however there are flaws with these categories which were discussed by Correll et al. (2020). Similarly, Landis and Koch (1977) provide categories for measures of agreement based on Kappa, where 1-0.81 are considered "almost perfect", 0.61-0.80 as "substantial", on down to < 0, which would imply that a classification was less accurate than random chance. By the definition of Landis and Koch (1977), 60% of the studies in this meta-analysis are "almost perfect" and another 28% are "substantial". Given that 88% of the data here fall within the two best categorizations, an alternate approach will be used to discuss the importance of individual analyses. The recommendations of Correll et al. (2020) will be followed here, and the 75^{th} percentile will be considered a large effect, the median a medium effect, and the 25^{th} percentile a small effect. The median effect across all groups was a kappa of 0.867. A small effect was 0.730 and a large effect was 0.951. However, nearly all these studies report accuracies that utilize the same data to create and test the model, which likely means that the accuracies are overly optimistic. Still, the values

above can be used as guidelines when deciding how effective a model is when assessing the validity of the results in aquatic species.

The results herein suggest some underlying methodological issues with the reported data on elemental profiling in aquatic organisms. While the funnel plot in Figure 3 shows a fairly symmetrical distribution when visually checked, both statistical tests used to evaluate publication bias were significant. Even though the funnel plot is somewhat symmetrical about the mean value, there are several studies that fall outside the funnel shape that represents the 95% confidence limits. Another issue present in the data is studies with small sample sizes reporting high accuracies (e.g., Bennion et al. 2019, Zhao and Zhang 2016, Guo et al. 2013). This may be a form of publication bias, but not in the traditional sense. The dataset is likely made of overly positive results, not dissimilar to p-hacking (Head et al. 2015). This conclusion is based on several observations about the data. There are no effect sizes in this study that are negative (this would be the result of a classification performing as well as random chance), and only one study contains confidence intervals that cross zero. While there is a relationship between effect size and sample size, the assessment of fit (r^2) is less than 0.01, and an examination of the scatter plot of average group size and the effect size finds no obvious relationship either (Figure 6). This is even more so true for studies with small samples. Many studies with small sample sizes are likely overly optimistic in the reported discriminations.

Moderator analyses showed that some taxa groups had a significantly higher score than others. This result suggests the ease of which some organisms are discriminated is different compared to others. The geographic scale of the studies involved was shown to be insignificant, and authors across the literature are reporting values at finer, local scales that are similar at wider geographic ranges. Intuitively, this is perplexing, as one could assume that the finer geographic

scale would make it more difficult to accurately discriminate samples from one another. This is another piece of evidence that suggests researchers who work in this area are reporting overly positive results, and therefore it is possible that the "file drawer problem" is evident here (Rosenthal 1979). Beyond this, very little of the methodological assumptions of the technique have been explored, and with a relatively low r^2 from the moderator analyses, other factors besides the one analyzed here could be at play in determining the overall effectiveness of elemental profiling.

One of the goals of meta-analysis is to provide recommendations to future studies (Moher et al. 2009). The decision on how many elements to analyze is one of the first key questions a researcher must ask when conducting elemental profiling. The studies contained herein can serve as guideposts based on the species and tissue the researcher chooses to analyze. Due to the abundant nature of the number of studies, and the variety of species and tissues covered, this study can serve as a first glance as to which elements are generally important in deciding which elements to analyze. For example, 3 studies in this report attempted to classify Penaeid shrimps, and one could use these as the basis for a future analysis. Based on this study, there is likely not a great deal gained by analyzing a large quantity of elements (e.g., 25) instead it is better to identify important elements that will have an impact on the analysis, especially if the analysis is costly and limits sample size.

The type of tissue to use in a discriminant analysis is one of the key questions to assess before conducting sampling. Here, analyses with mixed tissues (contain both hard and soft tissues), tended to have higher kappa than hard tissues alone. This is possibly due to the compartmentalizing of elements into different tissues in organisms (Li et al. 2014), and mixed tissues capturing a greater amount of variability among geographic locations. Many of the

studies involving fish used otoliths and laser ablation to discern geographic origin. This is a lethal method, however is proven to work (e.g., Campana et al. 1994, Tournois et al. 2013) and has robust guidelines for methodologies (Campana 1999, Campana et al. 2000, Elsdon et al. 2008, Tanner et al. 2016). Researchers seeking non-lethal methods should be encouraged by the results in this study, as scales and fin clips may be adequate tissues for elemental profiling, and several studies have shown effective results while using these tissues (e.g., Ramsay et al. 2011, Clarke et al. 2007, Flem et al. 2018).

One of the issues encountered in doing this meta-analysis was inconsistent reporting across studies. The methods section of study conducting elemental profiling should report the number of elements used in the discriminant procedure, as these are sometimes different from the number analyzed and can be in other locations in the manuscript (e.g., Li et al. 2017 reports the number of elements analyzed in the methods but the number used in the analysis in the results as part of the analysis was selecting important elements). A graphic depiction of the dispersion of the data in multi-dimensional space (e.g., a PCA) aids in the understanding of the underlying data structure (see Li et al. 2017 for an example). Additionally, the results should report a confusion matrix for the analyses conducted, for completeness and for comparison purposes, and the sample size of each group included in each discriminant analysis.

2.6 Conclusions

This study analyzed the effectiveness of elemental profiling as a means to classify samples to their geographic origin. Overall, this study demonstrates that within the literature there is an abundance of studies of elemental profiling being a successful tool for traceability in the literature. Based on the results of this meta-analysis however, caution should be taken in interpreting this finding because of complications with the data. The literature on this topic may

paint a flawed picture as no studies were included in this analysis where the discriminant analysis was less accurate than random chance. In the context of frequentist hypothesis testing, this is equivalent to finding no studies with non-significant results. Moving forward, researchers undertaking this type of research should be aware that the potential for inconclusive results is likely much higher than published research suggests.

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Table 1. Results of the moderator analysis. The letter superscripts represents significant differences within each subgroup as a result of post hoc contrasts. Significance level was $\alpha = 0.05$

Moderator	n	Kappa	SE	Significance
Tissue Type				
Hard	58	0.78^{a}	0.021	<0.0001
Soft	14	0.94^{b}	0.039	<0.0001
Mixed	12	0.92^{b}	0.044	<0.0001
Plant	2	1.00^{ab}	0.103	<0.0001
Taxa				
Cephalopod	1	0.81^{ab}	0.178	<0.0001
Crustacean	12	0.94^{a}	0.046	<0.0001
Echinoderm	1	1.00^{ab}	0.156	<0.0001
Fish	45	0.79^{b}	0.025	<0.0001
Mollusk	25	0.83^{ab}	0.034	<0.0001
Plant	2	1.00^{ab}	0.110	<0.0001
Geographic Range				
Local	10	0.82^{a}	0.047	<0.001
Regional	62	0.82^{a}	0.054	<0.001
Country	14	0.84^{a}	0.022	<0.001
Production Method				
Aquaculture	22	0.91^{a}	0.035	<0.001
Fisheries	62	0.80^{b}	0.021	<0.001
Mixed	2	0.85^{ab}	0.120	<0.001

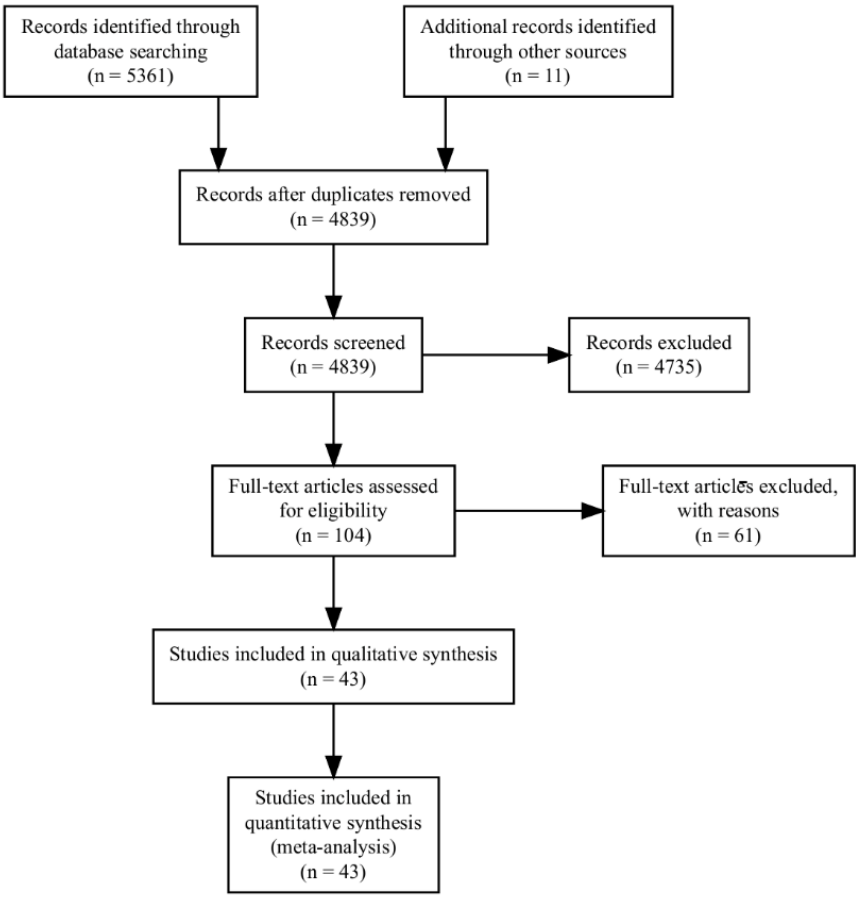

Figure 1. A PRISMA flow chart depicting the results of the literature search for this study.

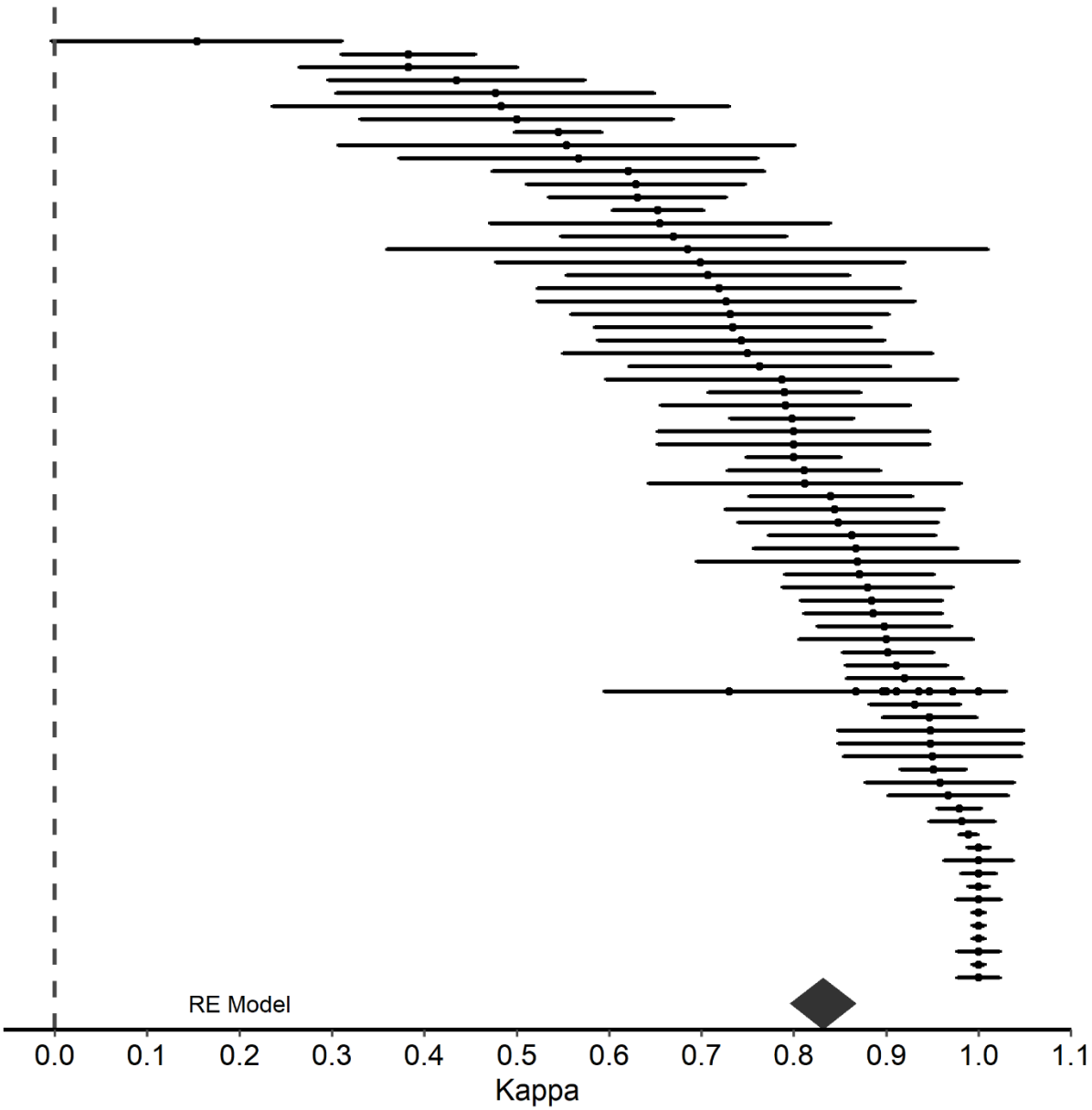

Figure 2. A caterpillar plot displaying all effect sizes in this study. The dashed line at X = 0 represents no effect. The horizontal error bars are the 95% Confidence interval. The diamond at the bottom of the figure represents the overall effect (peak) and it's 95% confidence interval (end points).

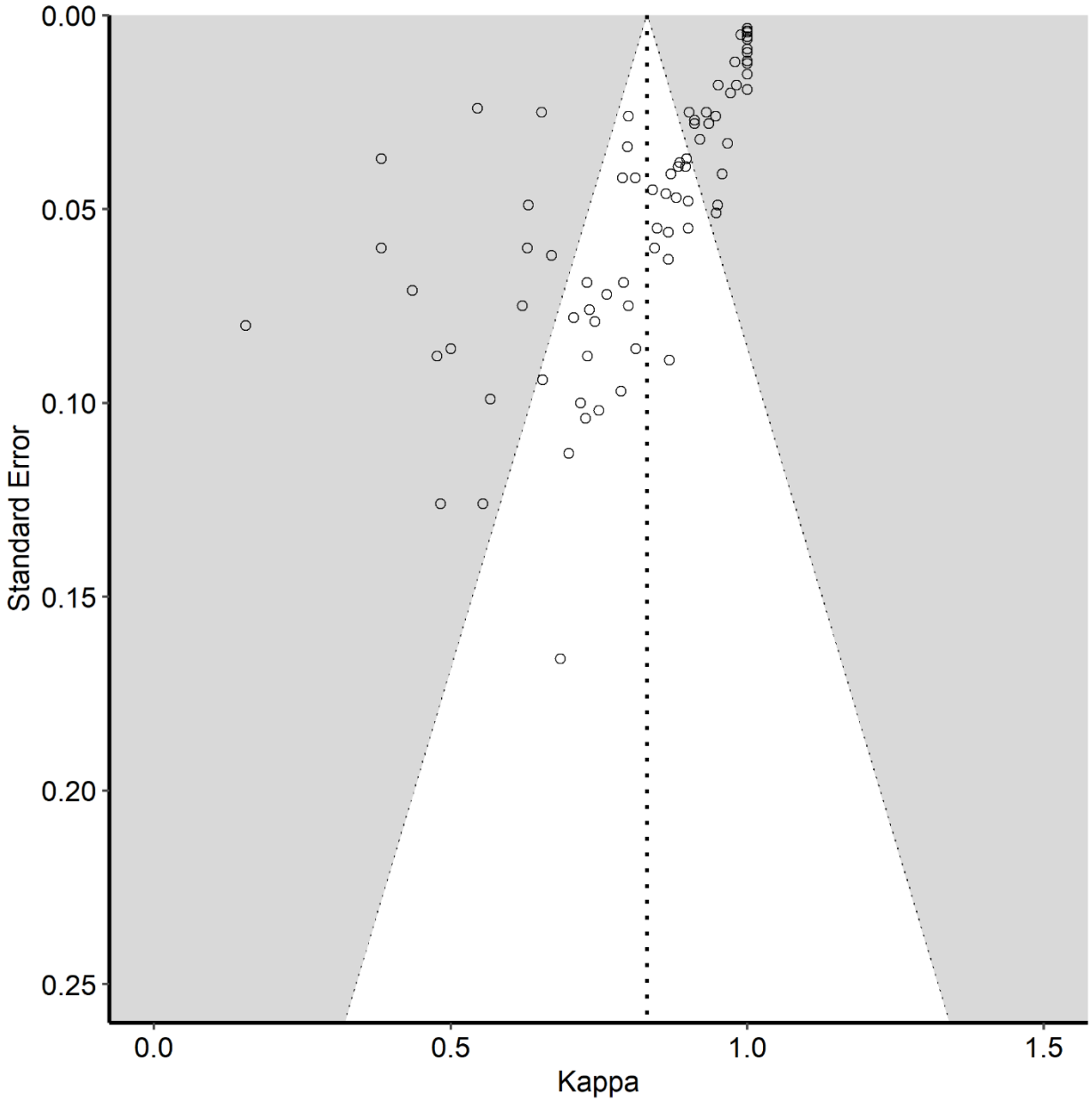

Figure 3. A funnel plot of the effect sizes in this study. The white area represents the 95% confidence region about the mean.

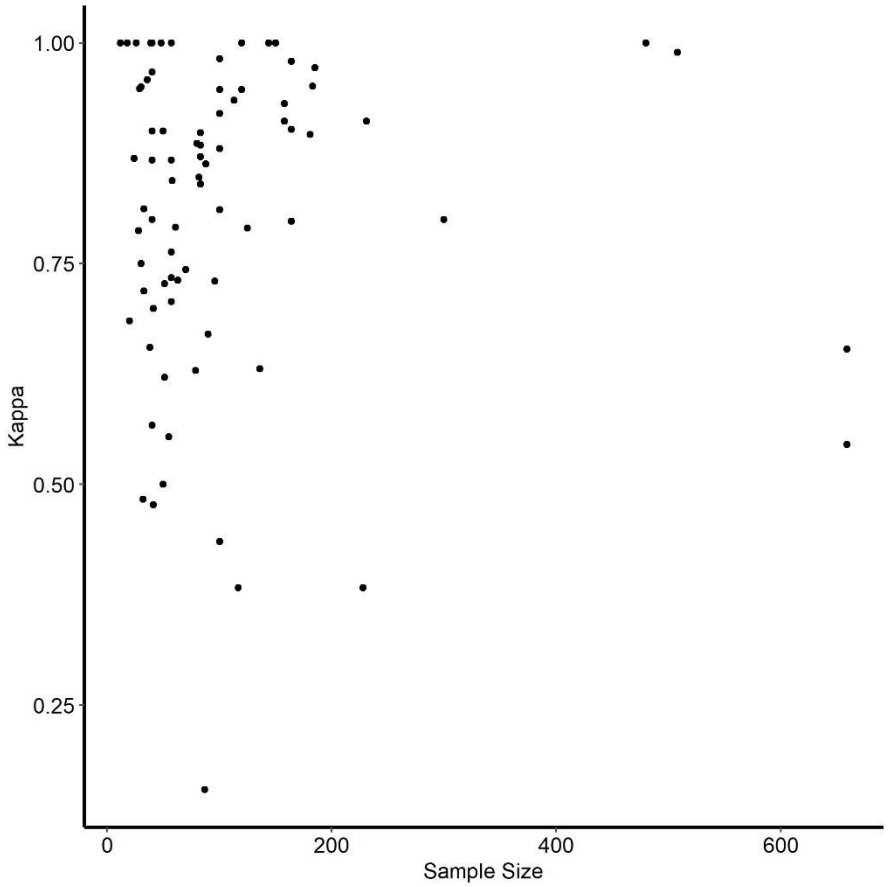

Figure 4. The relationship between overall sample size and effect size (Kappa) in the data included in this study.

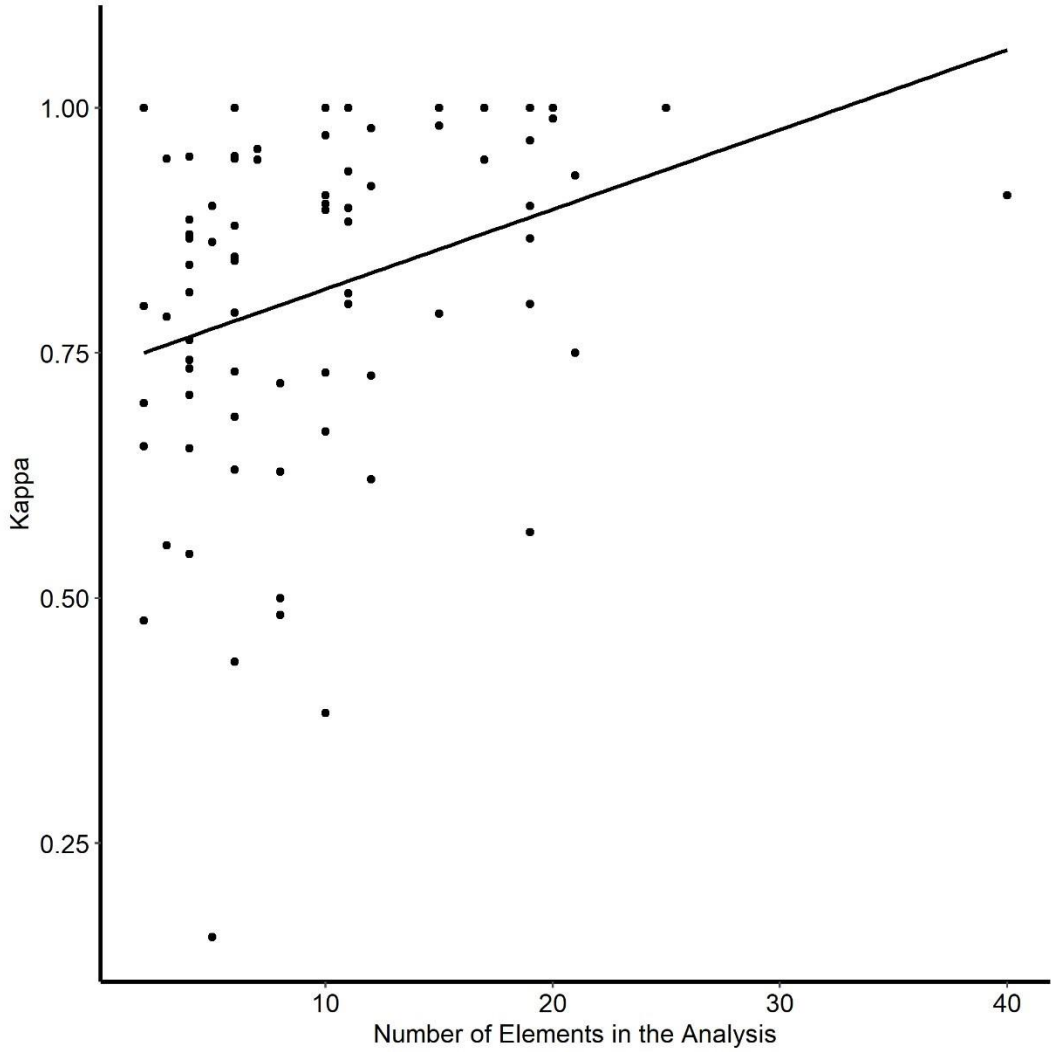

Figure 5. The relationship between the number of elements included in the discriminant analysis and the effect size (Kappa) in this study.

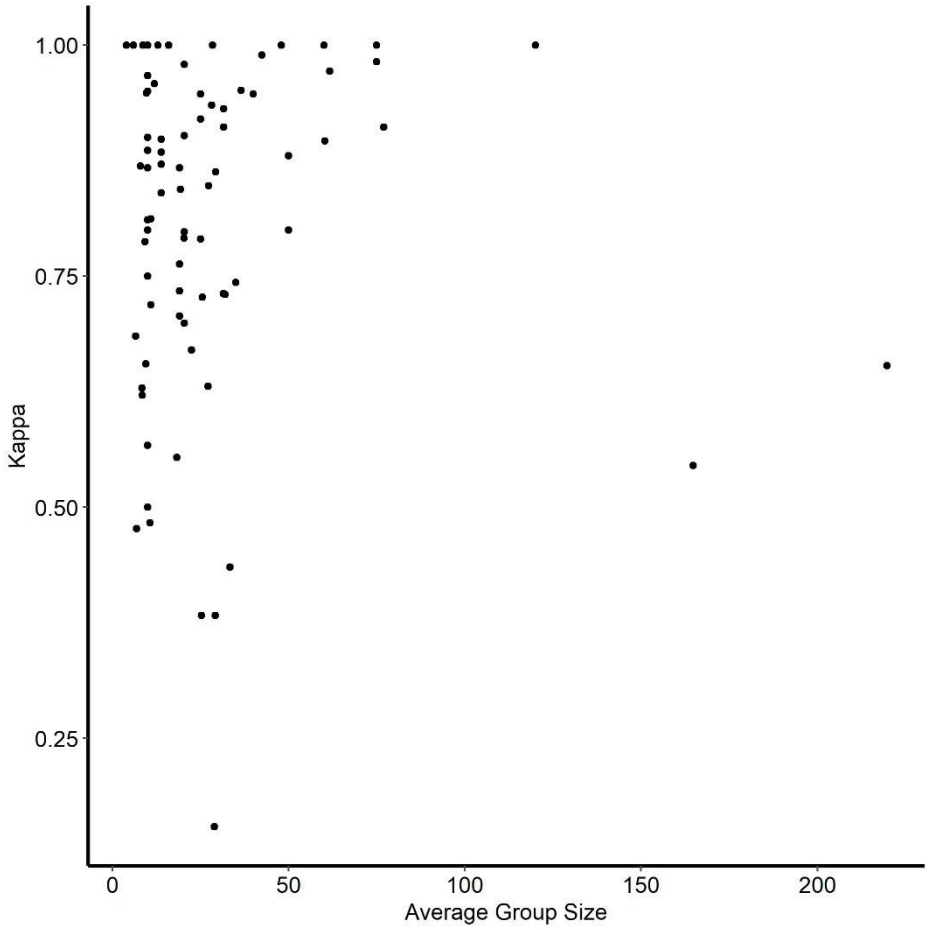

Figure 6. The relationship between average group size and the effect size (Kappa) of the data in this study.

CHAPTER 3

ASSESSING THE VARIABILITY AND DISCIRIMINATORY POWER OF ELEMENT FINGERPRINTS IN WHITELEG SHRIMP *Litopenaeus vannamei* FROM MAJOR SHRIMP PRODUCTION COUNTRIES

3.1 Abstract

Whiteleg shrimp *Litopenaeus vannamei* collected from farms in Ecuador, India, Indonesia, Vietnam, and Thailand were classified to country of origin based on element concentrations in muscle tissue. Shrimp were dried, digested, and analyzed via ICP-MS. Out of the 42 elements investigated, 33 are reported. Twenty-eight elements were statistically different across countries, and of these 28, Ecuador had unique group membership in 17. A random forest classification model utilizing 16 elements had an overall accuracy of 91% of correctly classified samples to country of origin. A canonical discriminant analysis was conducted to understand the variation in the data and identify elements that were important to differentiation in multi-dimension space. Elements identified as important contributors were Al, As, B, Ca, Co, Cs, Sr, and V. This study shows that shrimp from Ecuador tend to be more mineralized than shrimp from Asia, and classification models can discern samples from these countries successfully.

3.2 Introduction

Farmed penaeid shrimp are one of the world's most valuable aquaculture products, of which the value far exceeds the proportion of tonnage produced (FAO 2018). The whiteleg shrimp, *Litopenaeus vannamei*, is the most commonly cultured penaeid species globally, accounting for 83% of all penaeid shrimp culture (FAO 2019). Most of the production is centered in a few countries in Latin America and Southeast and South Asia, including Ecuador, Thailand, Vietnam, India, Indonesia, and China (FAO 2019). While China is the world's leading producer of whiteleg shrimp, most of its production is for domestic consumption (Zhang et al. 2017), while the rest of the countries listed above largely produce shrimp for international trade. The largest destination markets are Japan, USA, EU, Korea, and somewhat surprisingly because of its high domestic production, China (UN 2020).

Despite the importance of seafood to the human food supply chain, seafood products are plagued by fraudulent labeling which has been well documented in the United States and the European Union. Mislabeling occurs to manipulate prices (Jacquet and Pauly 2008), improve marketing (i.e., "sustainability") (Miller et al. 2012), substitute farmed species for wild species (Korzik et al. 2020), and meet processor quota demands. In aquaculture shrimp products, transshipping has been an issue in the past, resulting in incorrectly labeled country of origin among retail products (US Customs and Border Patrol 2010). Claims-making such as sustainability related claims in certifications also rely on traceability and accurately accounting for the origins of products.

Elemental profiling has been identified as a tool to increase traceability in seafood products (Gopi et al. 2019a, Li et al. 2016, Leal et al. 2015, Davis et al. 2021). Elemental profiling (or elemental fingerprinting, trace element analysis, etc.) involves analyzing samples

from predetermined groupings for element contents to determine if differences in the element concentrations can be used in conjunction with discriminant analyses to discern group membership. Elemental profiling has been used to delineate geographic variation (Albuquerque et al. 2016, Gopi et al. 2019b, Li et al. 2017), production origin (Anderson et al. 2010, Chaguri et al. 2017), species from similar geographies (Bouchoucha et al. 2018), and salinity of shrimp ponds (Li et al. 2019) with a high degree of success.

Elemental profiling has shown promise as a tool for discriminating geographic origins in shrimp. The first attempts to discriminate geographic origins with shrimp were by Courtney et al. (1994), which used elemental profiling to separate stocks of wild Eastern King Prawn *Penaeus plebejus* . Smith and Watts (2009) published their findings after elemental profiling was used in the case with CP Prima (US Customs and Border Patrol 2010), in which they collected a small number of samples from eight different countries. Whiteleg shrimp have also been successfully discriminated from farms in the USA (Li et al. 2014) and Southeast Asia (Li et al. 2017), and different geographies and production methods have been differentiated in black tiger shrimp *Penaeus monodon* (Gopi et al. 2019b). Altogether, these studies suggest there is a strong potential for elemental profiling in shrimp.

Ecuador has been an important producer of shrimp, since the 1970's (Hirono and Leslie 1992). Stricken with disease problems in the 1990's, production struggled to increase with major crashes in production between 1990 and 2000 (FAO 2020a). More recently, production has continually risen, with over 500,000 metric tons of shrimp being produced in 2018 (FAO 2020). Shrimp producers in Ecuador hold notions that their shrimp tends to be considered of a higher quality than shrimp from other major production countries, and therefore there are concerns over counterfeiting of Ecuadorian shrimp in global markets. To date, only one study evaluating

elemental profiling as a traceability tool has included Ecuador (Smith and Watts 2009), so the potential to differentiate shrimp from Ecuador using this method is relatively unexplored. The objective of this study was to understand the potential for elemental profiling to discriminate between major shrimp producing countries in Asia and Ecuador.

3.3 Methods

3.3.1 Shrimp Collection and Preparation

Whiteleg shrimp were collected from five countries for the purpose of this study; Ecuador, India, Indonesia, Vietnam, and Thailand. Utilizing the knowledge of the authors of this study who are experts in the aquaculture industries in their respective countries, collection sites were chosen in each country to be representative of the major production areas of shrimp (see figure 1). In Asian countries, one sample was collected from each farm, which consisted of shrimp from one pond on the farm. In Ecuador, up to three samples were collected on large farms (> 400 hectare), with a sample consisting of a collection event from one pond. In the case of farms where multiple samples were collected, the ponds that were chosen for sampling were spread out throughout the farm to capture spatial heterogeneity. Samples were collected from farms in the same manner as Li et al. (2017). Briefly, shrimp were caught with a cast net and 30 shrimp were put into a plastic bag on ice. Shrimp were then transported to a laboratory where they were dried within 24 hours. If the sample could not be dried within 24 hours, the sample was frozen until drying. Shrimp between 12-18 g (target of 15g) were selected for sampling. As per the sampling protocol in Li et al. (2017), care was taken to ensure that the shrimp samples did not come in contact with metal surfaces during sampling. Altogether, there were 123 samples collected from Ecuador, 68 from India, 37 from Indonesia, 48 from Thailand, and 53 from Vietnam.

Shrimp were prepared for storage until element analysis in the following manner. Shrimp were brought to a laboratory in the country of sampling and deheaded and peeled. The peeled tails were then dried in commercial food dehydrators at 50° C until the tissue reached constant mass, at least 12 hours. Upon drying, the samples were stored frozen until shipment for analysis.

3.3.2 Digestions

In preparation for digestion, samples were freeze dried overnight to remove any residual moisture. The digestion of the shrimp tissue was done following an adaptation of EPA method 200.8 (US EPA 1994) for solid materials (Environmental Express 2018). Briefly, 0.5 g of dried sample was digested with 2.0 ml of 1:1 nitric acid and 5.0 ml of 1:4 hydrochloric acid and refluxed in an Environmental Express Hotblock ™ (HotBlock 200, Environmental Express, Charleston SC USA) for 30 minutes at 85 C. Upon cooling, the samples were quantitively transferred and brought to volume in 50-ml volumetric flasks. Samples were then centrifuged at ambient temperature for 5 minutes and decanted to remove any insoluble material in the solution. The digestion method was verified by validating recovery of a spike quality control standard (recovery between 80-120%), determining the limits of detection, and repeatability of measurements with 15 replicants of a quality control standard (relative standard deviation < 20%).

3.3.3 Elemental Analysis

A NexION 350d ICP-MS (PerkinElmer Inc., Waltham MA USA) was used to conduct the elemental analysis for this study. Forty-two elements were analyzed for this study: Li, B, Na, Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Y, Mo, Ag, Cd, Sn, Sb, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Gd, Dy, Ho, Er, Tm, Yb, Lu, Pb, U. Several steps were taken to ensure consistency between runs and within runs. The instrument used for the analysis was

recalibrated with a two-point calibration each day. Each run on the instrument consisted of 40 unique samples, three blanks, a lab-generated matrix matched quality control sample run in triplicate, and three other quality control materials including one replicate of a certified reference sample oyster tissue (tissue NIST1566B, MilliporeSigma, St. Louis MO USA). Two of the three quality control replicates were aqueous solutions with known quantities of each element- in the appropriate range, and the third was a salmon flesh quality control matrix-matched sample that was previously validated with recoveries between 80-120%, and a relative standard deviation of <20% for all elements. A small number of samples were duplicated across runs to ensure consistency between runs on the instrument. Parameters of performance in relation to the limits of detection (LoD) for each element were calculated as a blank average plus three times the standard deviation of the blanks.

3.3.4 Statistical Analysis

Elements were removed from the analysis if more than 20% of the samples were below detection limits. Therefore, only the results of Al, As, B, Ba, Ca, Ce, Co, Cr, Cs, Cu, Dy, Er, Fe, Gd, K, La, Li, Mg, Mn, Mo, Na, Nd, Ni, Pb, Pr, Rb, Se, Sm, Sr, V, Y, Yb, and Zn are reported. In elements where >80% of samples were above detection limits, values below detection limits were replaced with half of the detection limit. The mean and standard deviation of element concentrations are reported by country of origin. A one-way MANOVA was conducted utilizing a test statistic in Friedrich and Pauly (2018), which is robust to heteroscedasticity and can be used with high dimensional data. The reported statistic is described by Friedrich and Pauly (2018) as a “modified ANOVA type statistic” (MATS), and the p value is derived from a parametric bootstrap procedure. Following the results of the MANOVA, the mean concentrations were subsequently compared using one-way Welch’s analysis of variance with a Bonferroni

corrected p value ($\alpha = 0.05/33$ comparisons for a significance level of $\alpha = 0.0015$). Welch's ANOVA is more robust than a traditional ANOVA to heterogeneity in variation (Delacre et al. 2019). In cases where the result of the ANOVA was significant, a Games Howell pairwise comparison procedure was used to compare individual means. Games Howell pairwise comparisons are likewise more robust to heterogeneity in variation than other pairwise comparison procedures (Lee and Lee 2018). A significance level of $\alpha = 0.05$ was used to determine significance in the post-hoc pairwise comparisons. Data were log transformed and subsequently centered and scaled to improve normality of the data before analysis, and results are reported on the original scale.

Following exploration of the data with univariate tests, classification of the country of origin of the retail samples was conducted with a Random Forest classification in the "caret" package in R using the method native to the "ranger" package (Wright and Ziegler 2017). Recursive feature selection was used to determine the best combination of variables for the random forest. Recursive feature selection ranks the importance of variables based on their contribution to classification models and subsequently eliminates less important variables to find the most informative subsets of variables (Guyon et al. 2002). The random forest was conducted with the following subset of elements after recursive feature selection; Al, As, B, Ba, Ca, Co, Cr, Cs, Li, Ni, Pb, Rb, Se, Sr, V, and Zn. Data were centered and scaled before classification to remove any effects of magnitude in the element concentrations. The expected accuracy based on the formula in Poulin and Kamiya (2015) is reported for reference for the results of the classification procedure. The accuracy of the model was assessed with k-fold cross validation where k =10. The random forest classification models were used because of the lack of distributional requirements for the technique, robustness to overfitting high dimensional

data, and the power of the model to obtain good fits when there are no strong predictor variables (Breiman 2001). Other models that were explored were linear discriminant analysis and K-nearest neighbors' classification. Following the classification procedure with the country of origin, a canonical discriminant analysis (CDA) was utilized to visualize the multivariate variation in the data. CDA is a dimension reduction/ordination technique similar to a principal component analysis, however a CDA maximizes the differences in variation in specified groupings (Matthew et al. 1994), which in this case will be country of origin. The elements selected by the recursive feature selection in the classification analysis were included in the CDA. All statistical analysis were conducted in R version 4.0.3 (R Core Team 2020).

3.4 Results

The MANOVA style test found a statistical difference among the elemental profiles of the five countries in this study (MATS = 947.047, $p < 0.001$). A summary of the elemental concentrations in shrimp tail muscle tissue and the univariate statistical tests that followed the MANOVA are presented (Table 1). Overall, significant differences were detected in 28 out of the 33 elements reported here. In 15 of the 28 elements where statistical differences were noted, and in 17 elements overall, shrimp from Ecuador had the highest concentrations on average (e.g., Al, Fe, Li, Sr). Out of the 28 elements with significant differences, Ecuador had unique group membership in seven of the elements. Vietnam and Thailand tended to belong to the same post-hoc groupings, being in the same group in 25 out of the 28 elements where significant differences were detected.

Overall, the random forest classification model obtained a cross validated accuracy of 91% (Table 2). The most accurate country was Ecuador (98%), while the least accurate results were for Indonesia (76%), which had samples misclassified as Ecuador, Thailand, and Vietnam.

While results for Thailand and Vietnam had lower accuracies than those for Ecuador, they were most frequently misidentified as one another, with 5/48 samples from Thailand being identified as being from Vietnam and 4/53 samples being identified from Vietnam being identified as Thailand. Indian shrimp were most commonly misidentified as Ecuadorian (4/61), but there were samples misclassified as Thailand and Vietnam as well. The expected accuracy based on random sampling for this dataset was 24.16%. Cohen's Kappa for this sample was 0.874 ± 0.079 sd.

The canonical discriminant analysis reduced the dataset to four canonical variables. In the first two dimensions, which account for ~78% of the total variation, Ecuador and India generally separate from the three countries in Southeast Asia (see Figure 2). Indian shrimp separate from shrimp of the other countries along the second canonical variable, while Ecuadorian shrimp separate along the first canonical variable. Elements with strong factor loadings in the first canonical variable include Al, As, Sr, while variables highly correlated with canonical variable 2 are Al, Co, and V (Table 3). While Thailand, Vietnam, and Indonesia overlap in the first two dimensions of the CDA, Indonesia separates from Thailand and Vietnam in the first and third dimension, which is highly associated with the elements Ca, Cs, and V (Figure 2b).

3.5 Discussion

Whiteleg shrimp are an important aquaculture species, as they have a relatively high value and are internationally traded (FAO 2020). Considering the history of poor production practices (Naylor et al. 2000, Naylor et al. 1998, Holmstrom et al. 2003) and socio-environmental issues (Bailey 1988, Ha et al. 2012) in shrimp aquaculture, there has been an increased push for accountability and consequently an increased demand for traceability in globally traded shrimp products. One tool that has been proposed for improving the traceability

of shrimp products is elemental profiling (Hassoun et al. 2020, Li et al. 2016), which could be used to identify country of origin based on tissue element concentrations (Davis et al. 2021). Here, we conduct a discriminant analysis with a random forest model with farmed whiteleg shrimp from major production countries with element concentrations from shrimp muscle tissue.

The basis for elemental profiling is that patterns of heterogeneity exist in samples from different predefined groupings (e.g., species, production method, geographic origins). In this dataset 28 out of 33 elements reported showed statistical difference among groupings. In general, samples from Ecuador were more mineralized than samples from other countries. This may be because of the freshwater runoff that comes from the Andean mountains in Ecuador being higher in minerals than the runoff of the lowland regions of Southeast Asia. Previous research has shown that mining in the Andes has also elevated surface water mineral contents (Niane et al. 2014, Appleton et al. 2001), however it is not necessarily known if mining occurs in the watersheds from which the farms in this study where samples were collected. Nevertheless, previous attempts to link surface water mineral concentrations or sediment concentrations to shrimp muscle tissue element concentrations from shrimp ponds has been relatively unsuccessful (Li et al. 2014), and deserves further exploration in future studies. The basis of heterogeneity in elemental profiling is relatively unstudied and cultured organisms may present an ideal specimen because they are contained in discrete enclosures and fed rationed food with discernable quantities of elements. Besides environmental exposure through contact with water and sediment, other sources of minerals include pelleted food and natural productivity in the ponds where the shrimp are grown.

Overall, the accuracy of this discrimination procedure with 322 samples was 91%. This compares favorably to other studies that have been done with regards to identifying geographic

location in cultured shrimp. Li et al (2017) covered a subset of the countries in this study, and had an overall accuracy of 97%, but with less samples and a more limited scope. Gopi et al. (2019b) was able to identify black tiger shrimp *Penaeus monodon* to regions in Australia and Southeast Asia with 98% accuracy, however the results of that study may be confounded with the mixture of wild capture and cultured shrimp in their sample, as culture vs. wild capture has been successfully delineated by elemental profiling in other cases (Anderson et al. 2010, Varra *et al.* 2019). Similarly, Ortea and Gallardo (2015) were able to achieve >90% classification success with geographic origin, but their study contained both multiple species and cultured and wild caught shrimp. Smith and Watts (2009) had classification success with >90%, however this study had mixed species in their sample, which could have confounded their results as well, as elemental profiling has been used to identify species instead of geographic origin in some cases (Bouchoucha et al. 2018). Davis et al. (2021) found that the average classification success for seafood products was Cohen's Kappa = 0.83. In this study, the Kappa, which is a measurement of interrater agreement (Cohen 1960), was 0.87 ± 0.08, which suggests that overall, this is a strong classification model when compared to other seafood items. Furthermore, the findings of this study are not confounded by some of the factors identified above, as the samples are all whiteleg shrimp from aquaculture ponds, unlike some of the studies described above.

Seafood traceability is a growing concern for producers, retailers, and consumers. Consumers are becoming more conscious of the effects of their buying power, and therefore are willing to purchase products that have a perceived "sustainability", even if they are a higher price (Roheim et al. 2011). In many cases, retailers use certification schemes as a proxy for sustainability and claims making, as it removes them from the process of validating the claims about the product but allows them to project environmental consciousness. Both the Aquaculture

Stewardship Council and the Global Aquaculture Alliance Best Aquaculture Practice (BAP) shrimp standards have chain of custody and traceability requirements. This study adds evidence towards the possibility of using elemental profiling as a traceability tool. Here, shrimp samples from five countries that are the world's leaders in shrimp exports were successfully discriminated based on country of origin. This is also a considerably larger sample than other similar studies (in the examples described above, Li et al. (2017) is the largest sample with n =120) and is not confounded by any species or cultured vs. wild production artifacts in the data. Moreover, this study shows that shrimp sampled from Ecuador likely have unique element profiles compared to shrimp in Asia, which has been the focus of previous efforts in elemental profiling in shrimp (e.g., Li et al. 2017, Gopi et al. 2019b, Ortea and Gallardo 2015). Overall, a large suite of shrimp samples such as this one from multiple production countries could serve as a database from which to identify potential unknown samples in traceability applications.

3.6 References

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Table 1. A summary of the element concentrations by country of origin in shrimp tail muscle tissue for this study. The elements in bold text are considered significant by a Welch's ANOVA with an adjusted p value of $\alpha = 0.0015$. Superscripts represent different groupings by pairwise post hoc tests. The limit of detection for the instrument is in parentheses next to the element.

	Country										
Element	Ecuador	sd	India	sd	Indonesia	sd	Thailand	sd	Vietnam	sd	p value
Al (0.4)	**121.18^{b}**	**102.44**	**49.47^{a}**	**55.74**	**54.18^{a}**	**71.40**	**30.53^{a}**	**40.41**	**40.72^{a}**	**67.79**	**< 0.00001**
As (0.05)	**1.55^{a}**	**1.12**	**2.33^{b}**	**1.14**	**8.24^{d}**	**7.69**	**4.88^{c}**	**2.40**	**7.05^{d}**	**4.19**	**< 0.00001**
B (0.4)	**1.77^{b}**	**2.90**	**1.11^{a}**	**1.08**	**2.63^{c}**	**1.71**	**1.86^{bc}**	**1.20**	**1.96^{c}**	**0.98**	**< 0.00001**
Ba (0.01)	**1.34^{b}**	**1.31**	**1.19^{b}**	**0.95**	**0.40^{a}**	**0.35**	**0.35^{a}**	**0.44**	**0.36^{a}**	**0.23**	**< 0.00001**
Ca (10)	**2596.2^{c}**	**1278.3**	**2329.0^{c}**	**1417.4**	**1228.7^{a}**	**415.4**	**2079.3^{c}**	**700.7**	**1508.5^{b}**	**563.0**	**< 0.00001**
Ce (0.0004)	0.088	0.083	0.080	0.096	0.085	0.124	0.081	0.132	0.070	0.123	0.002
Co (0.01)	**0.066^{cd}**	**0.050**	**0.087^{d}**	**0.072**	**0.051^{bc}**	**0.035**	**0.029^{a}**	**0.025**	**0.043^{ab}**	**0.045**	**< 0.00001**
Cr (0.05)	**0.220^{b}**	**0.209**	**0.288^{b}**	**0.504**	**0.146^{ab}**	**0.138**	**0.095^{a}**	**0.089**	**0.101^{a}**	**0.124**	**< 0.00001**
Cs (0.002)	**0.023^{b}**	**0.012**	**0.016^{a}**	**0.023**	**0.044^{c}**	**0.020**	**0.079^{d}**	**0.067**	**0.039^{c}**	**0.015**	**< 0.00001**
Cu (0.02)	27.20	6.77	24.93	8.81	23.71	7.18	31.18	13.83	26.82	11.36	0.011
Dy (0.0004)	**0.0085^{b}**	**0.0075**	**0.0058^{ab}**	**0.0069**	**0.0056^{ab}**	**0.0075**	**0.0038^{a}**	**0.0065**	**0.0063^{ab}**	**0.0109**	**< 0.00001**
Er (0.0004)	**0.0047^{b}**	**0.0040**	**0.0030^{a}**	**0.0036**	**0.0029^{a}**	**0.0038**	**0.0018^{a}**	**0.0030**	**0.0030^{a}**	**0.0050**	**< 0.00001**
Fe (0.4)	**141.59^{b}**	**125.46**	**76.97^{a}**	**85.84**	**70.59^{a}**	**81.35**	**48.95^{a}**	**73.89**	**55.14^{a}**	**81.49**	**< 0.00001**
Gd (0.0004)	**0.0102^{b}**	**0.0091**	**0.0070^{a}**	**0.0084**	**0.0076^{a}**	**0.0103**	**0.0054^{a}**	**0.0092**	**0.0080^{a}**	**0.0141**	**0.00001**
K (10.0)	**15511.7^{b}**	**1719.2**	**14448.4^{a}**	**2263.1**	**16316.1^{bc}**	**1673.5**	**15528.8^{bc}**	**1691.3**	**16344.8^{c}**	**1313.8**	**< 0.00001**
La (0.002)	**0.0368^{b}**	**0.0341**	**0.0349^{b}**	**0.0420**	**0.0377^{b}**	**0.0572**	**0.0315^{ab}**	**0.0502**	**0.0276^{a}**	**0.0476**	**0.000819**
Li (0.002)	**0.1512^{c}**	**0.0973**	**0.0558^{a}**	**0.0375**	**0.1176^{c}**	**0.0678**	**0.0876^{b}**	**0.0469**	**0.0875^{b}**	**0.0784**	**< 0.00001**
Mg (2.0)	1765.5	185.7	1766.5	151.3	1791.6	176.7	1747.7	154.6	1708.0	108.4	0.048
Mn (0.02)	7.30	16.04	2.97	2.50	6.21	10.82	4.06	4.05	4.48	6.88	0.052
Mo (0.01)	0.0490	0.0218	0.0411	0.0250	0.0422	0.0216	0.0516	0.0222	0.0449	0.0162	0.003
Na (10.0)	**6943.4^{b}**	**1954.9**	**6005.7^{a}**	**938.5**	**6326.9^{ab}**	**1137.4**	**6437.8^{ab}**	**1103.2**	**6158.0^{a}**	**966.0**	**0.000137**
Nd (0.0004)	**0.0477^{b}**	**0.0433**	**0.0348^{ab}**	**0.0411**	**0.0384^{ab}**	**0.0512**	**0.0323^{a}**	**0.0519**	**0.0343^{a}**	**0.0611**	**0.00003**
Ni (0.02)	**0.157^{b}**	**0.139**	**0.165^{b}**	**0.136**	**0.108^{b}**	**0.089**	**0.048^{a}**	**0.042**	**0.095^{a}**	**0.103**	**< 0.00001**
Pb (0.01)	**0.082^{b}**	**0.097**	**0.042^{a}**	**0.052**	**0.166^{b}**	**0.306**	**0.058^{a}**	**0.084**	**0.055^{a}**	**0.080**	**< 0.00001**
Pr (0.0004)	**0.0110^{b}**	**0.0102**	**0.0086^{b}**	**0.0103**	**0.0096^{b}**	**0.0141**	**0.0085^{ab}**	**0.0139**	**0.0082^{a}**	**0.0146**	**0.000114**
Rb (0.005)	**4.25^{a}**	**1.01**	**4.77^{ab}**	**2.79**	**5.49^{c}**	**1.74**	**4.81^{bc}**	**1.14**	**4.01^{a}**	**0.84**	**< 0.00001**
Se (0.05)	**1.203^{b}**	**0.369**	**1.232^{b}**	**0.530**	**0.841^{a}**	**0.365**	**1.155^{b}**	**0.148**	**1.251^{b}**	**0.238**	**< 0.00001**
Sm (0.0004)	**0.0104^{b}**	**0.0095**	**0.0070^{a}**	**0.0084**	**0.0078^{ab}**	**0.0108**	**0.0063^{a}**	**0.0107**	**0.0079^{ab}**	**0.0142**	**0.00001**
Sr (0.004)	**28.08^{d}**	**18.35**	**20.85^{c}**	**16.09**	**11.20^{a}**	**5.19**	**17.82^{bc}**	**7.54**	**13.98^{b}**	**6.42**	**< 0.00001**
V (0.04)	**0.282^{b}**	**0.219**	**0.238^{b}**	**0.239**	**0.195^{b}**	**0.177**	**0.078^{a}**	**0.057**	**0.102^{a}**	**0.119**	**< 0.00001**
Y (0.001)	**0.0424^{b}**	**0.0364**	**0.0319^{ab}**	**0.0354**	**0.0301^{ab}**	**0.0361**	**0.0197^{a}**	**0.0263**	**0.0348^{ab}**	**0.0558**	**0.00027**
Yb (0.0004)	**0.00398^{b}**	**0.00337**	**0.00259^{ab}**	**0.00297**	**0.00248^{ab}**	**0.00309**	**0.00152^{a}**	**0.00247**	**0.00242^{a}**	**0.00389**	**< 0.00001**
Zn (0.4)	**54.86^{a}**	**3.66**	**59.15^{b}**	**4.22**	**54.99^{a}**	**3.67**	**58.08^{b}**	**4.73**	**58.00^{b}**	**3.66**	**< 0.00001**

Table 2. A confusion matrix from the resulting random forest model after k-fold cross validation.

	Reference					
Prediction	Ecuador	India	Indonesia	Thailand	Vietnam	
Ecuador	119	4	5	2	0	
India	2	61	0	0	0	
Indonesia	0	0	28	0	1	
Thailand	0	2	2	41	4	
Vietnam	1	1	2	5	48	
Accuracy	98%	90%	76%	85%	91%	
Overall						91%

Table 3. The canonical variables of the of the canonical discriminant analysis with the elements used in the classification procedure.

Element	Can1	Can2	Can3	Can4
Al	0.611	-1.185	-0.614	-1.659
As	-0.678	0.049	0.404	-0.336
B	0.042	0.168	-0.092	0.025
Ba	0.229	0.123	0.126	0.054
Ca	-0.240	-0.395	-0.763	0.676
Co	-0.174	0.974	-0.202	-0.168
Cr	0.013	0.176	-0.153	-0.054
Cs	-0.434	-0.339	-0.619	0.311
Li	0.013	-0.559	0.231	0.592
Ni	0.083	0.116	0.071	-0.367
Pb	-0.186	-0.072	0.509	0.217
Rb	-0.010	0.084	0.264	0.410
Se	0.190	-0.061	-0.199	-0.225
Sr	0.560	0.407	0.525	-0.399
V	-0.097	0.589	0.752	1.586
Zn	-0.087	0.360	-0.393	-0.057
Eigenvalue	1.50	0.95	0.51	0.19
Percent Variation	47.54	30.11	16.26	6.09
Cumulative Variation	47.54	77.65	93.91	100

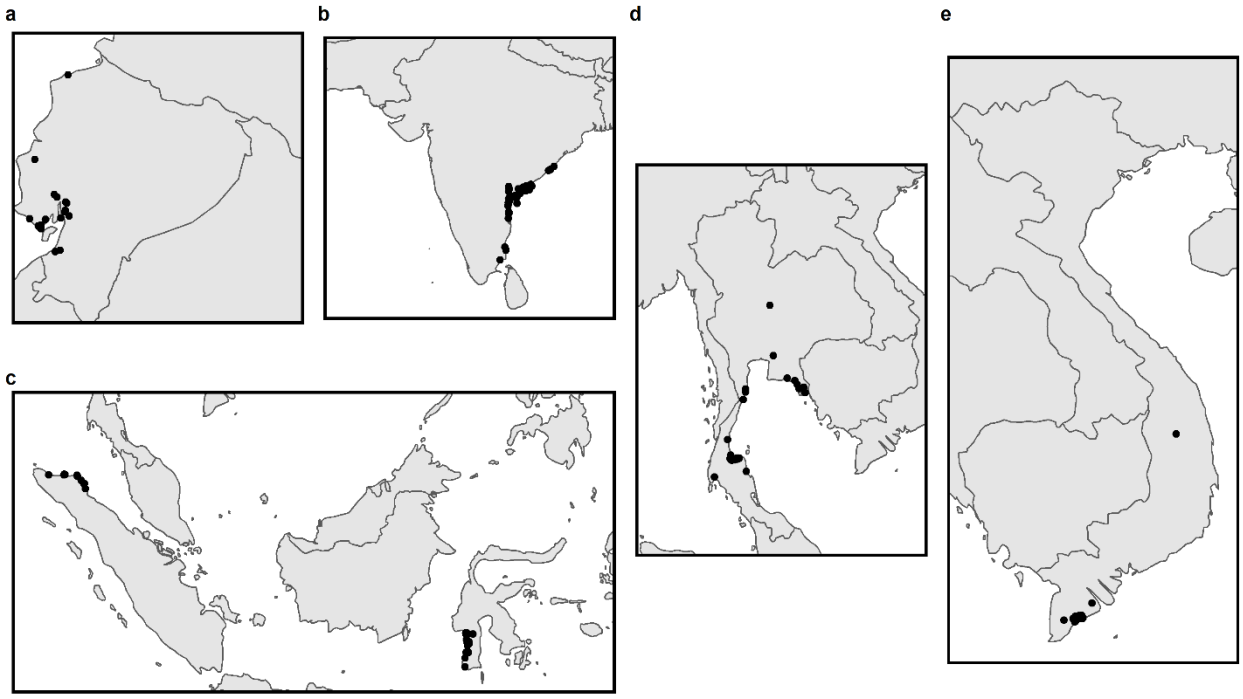

Figure 1. A map of the sampling locations in each country. The countries are as follows; a) Ecuador, b) India, c) Indonesia, d) Thailand, and e) Vietnam.

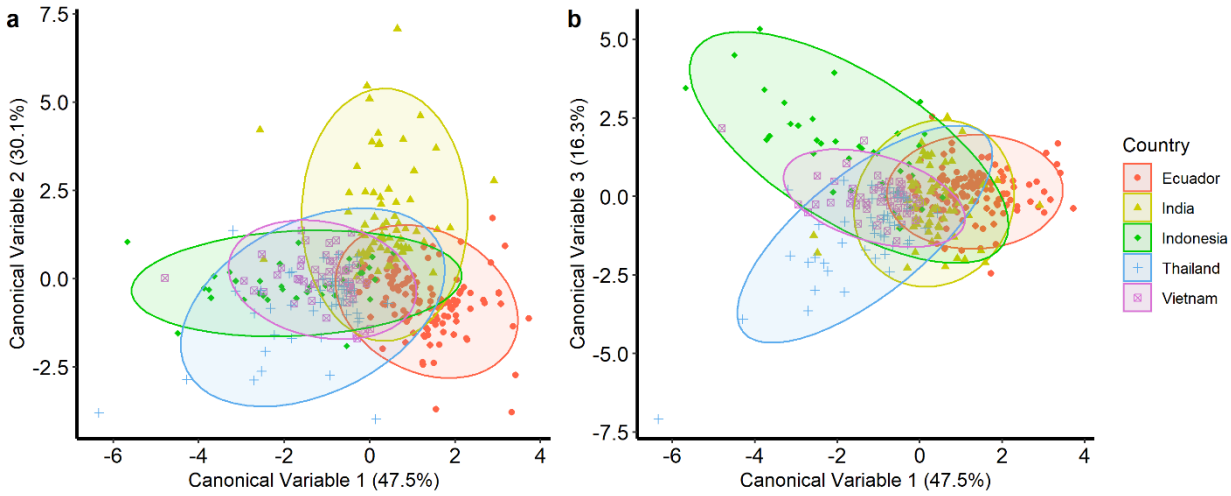

Figure 2. Bi-plots of a) the first and second and b) the first and third canonical variables in the canonical discriminant analysis. Samples from different countries are represented by different shape color/combinations. The combinations are; i.red circles =Ecuador, ii. yellow triangles = India, iii. Green diamonds = Indonesia, iv. Blue crosses = Thailand, and v. purple boxes = Vietnam. Ellipses represent normalized group ellipses in two-dimensional space.

CHAPTER 4

TRACE ELEMENT CONCENTRATIONS IN WHITE LEG SHRIMP *Litopenaeus vannamei* FROM RETAIL STORES IN THE EU, UK, AND USA AND THE ABILITY TO DISCERN COUNTRY OF ORIGIN WITH CLASSIFICATION MODELS

4.1 Abstract

Shrimp are a globally traded aquaculture commodity that accounts for a large proportion of the monetary value of aquaculture. There are concerns among consumers about seafood labeling fraud and environmental sustainability. Therefore, the geographic origin of shrimp from retail stores was investigated with trace element profiling. Ninety-four shrimp samples were collected from grocery stores across the USA, UK, and EU in 70 different grocery stores. The results of 24 elements are reported. Shrimp samples were from Thailand, India, Vietnam, Indonesia, and Ecuador were shown to have 15 elements that were statistically different across labeled country of origin, with Ecuador having unique post hoc group membership in 5 of the elements. Based on a classification procedure, shrimp were classified to labeled country of origin with an overall accuracy of 71.3%. Overall, the results suggest that elemental profiling could be a traceability tool in for classifying samples of shrimp from retail stores.

4.2 Introduction

The world's population is expected to grow to 9-10 billion by 2050 (FAO 2017). The need for animal proteins is expected to grow at an even higher rate than the population because of the growing global middle class, which will consume more meat (FAO 2009). Seafood products are an important source of nutrition for many people, and provide a source of protein, minerals, and healthy fats like Omega 3s. Currently about half of the world's seafood comes from aquaculture, and seafood accounts for about 23% of the world's meat supply (Edwards et al. 2019). Even more so than other meat products, aquaculture seafood products are global commodities where the global supply is consolidated in a few countries that send exports to international markets.

While shrimp only account for about ~5 % of the world's aquaculture production, they are disproportionally valuable as a commercial species, accounting for about 20% of the monetary value of aquaculture (FAO 2018). Shrimp production has grown dramatically in the last 15 years as the advent of specific pathogen free larvae (SPF) and better production practices have allowed for an increasingly steady supply of post larvae and higher densities in production ponds at farms. Currently, Southeast Asia is the hub of shrimp production for the world, but Ecuador is also an important source (FAO 2019). Whiteleg shrimp *Litopenaeus vannamei* is the predominant species produced, accounting for ~83 % of all penaeid shrimp aquaculture (FAO 2019). While some other shrimp species are consumed in domestic markets, whiteleg shrimp are produced almost exclusively for export, especially to developed nations like the USA and countries in the EU.

Aquatic invertebrates are well known sinks of metals, whether it be from food sources or environmental exposure (Rainbow 2002). Several factors that influence metal concentrations in

shrimp tissues have been examined, including geographical variations (Li et al. 2017, Smith and Watts 2009), source (i.e., farmed vs. wild) (Gopi et al. 2019b), and different tissues in the body (Li et al. 2014). Given that this natural variability exists in shrimp raised in differential locales, this makes shrimp from retail stores candidates for elemental profiling, which has been successful with shrimp from shrimp farms for identifying geographic origins (Li et al. 2017, Li et al. 2014, Gopi et al. 2019b)

Metal concentrations in shrimp in retail markets have not been documented, and no attempts have been made with regards to identifying geographic origins in shrimp retail products based on elemental concentrations. Traceability has been a growing concern in aquaculture in recent years, and techniques to verify geographic origins have been increasingly explored in a variety of seafood items (Hassoun et al. 2020, Gopi et al. 2019a). Fraudulent labeling has been a prevalent issue in the past (Jacquet and Pauly 2008), and is likely to be a concern going forward. Therefore, the objective of this study was to document element and trace metal concentrations in whiteleg shrimp from retail stores in the USA and EU and explore the use of element concentrations in shrimp muscle tissues to verify the country of origin in whiteleg shrimp.

4.3 Methods

4.3.1 Collections

Shrimp were collected from stores in the US and the EU between January and August of 2019. Stores were selected to cover a broad range of stores owned by unique parent companies, sampled from a range of store types (e.g., high-end organic markets, budget markets, private membership clubs, regional store chains, and national store chains). In total, 70 locations, 25 in the EU and 55 in the United States were selected as retail stores where samples were obtained (Figure 1). Altogether, 94 samples were collected from Belgium (n=2), France (8), Germany (6),

The Netherlands (3), the United Kingdom (12), and the USA (63). Shrimp collected were from five countries of origin: Ecuador (n=12), India (30), Indonesia (21), Thailand (12), and Vietnam (19). Due to the difficulty of traveling and collecting samples, sampling locations were chosen based on a parsimonious mix of minimizing travel while maximizing the number of unique stores covered. The distribution of country of origin among samples is an artifact of the availability of shrimp from each country in the stores chosen. At each location, bags of private label and store label frozen shrimp from the supermarket's freezer section were purchased. In the USA, At least 450 g (about 1 lb) of shrimp from brand or store brands were purchased and kept frozen until processed. Samples were chosen to be as consistent as possible with regards to size and roughly 30 count-sized shrimp were targeted. However, not all stores sold shrimp in this size so smaller and larger sizes were obtained based on availability. Shrimp were purchased in different stages of processing (whole shrimp to peeled deveined tails) but were subsequently standardized as peeled deveined tails prior to drying to maintain consistency between samples. The distribution of country of origin in the resulting sample was therefore constrained by the availability in the selected stores.

Shrimp were dried at 80° C to a constant mass in the laboratory in a drying oven. In the EU, at least 1 package of shrimp (ranging from 100g to 500g) was purchased and dried with a food dehydrator (Excalibur model no. 4400220G, Excalibur Products, Sacramento CA USA) within 12 hrs of purchase to facilitate travel and subsequent shipping. Shrimp were dried for at least 12 hrs at 70° C and stored in plastic bags dried until further processing. They were then further dehydrated to a constant mass at 80° C in the laboratory, consistent with samples in the collected in the United States. Once shrimp were dried, a sub sample of three to six shrimp were ground in a IKA Economical Analytical Mill with a carbide blade (Cole-Palmer, Vernon Hills,

Illinois USA) to avoid metal contamination. Dried tissue was then stored in sealed containers until digested.

4.3.2 Digestions

In preparation for digestion, samples were freeze dried overnight to remove any residual moisture. The digestion of the shrimp tissue was done following an adaptation of EPA method 200.8 (US EPA 1994) for solid materials (Environmental Express 2018). Briefly, 0.5 g of dried sample was digested with 2.0 ml of 1:1 nitric acid and 5.0 ml of 1:4 hydrochloric acid and refluxed in an Environmental Express Hotblock (TM) (HotBlock 200, Environmental Express, Charleston SC USA) for 30 minutes at 85 C. Upon cooling, the samples were quantitively transferred and brought to volume in 50-ml volumetric flasks. Samples were then centrifuged at ambient temperature for 5 minutes and decanted to remove any insoluble material in the solution. The digestion method was verified by validating recovery of a spike quality control standard (recovery between 80-120%), determining the limits of detection, and repeatability of measurements with 15 replicants of a quality control standard (relative standard deviation < 20%).

4.3.3 Elemental Analysis

A NexION 350d ICP-MS (PerkinElmer Inc., Waltham MA USA) was used to conduct the elemental analysis for this study. Forty-two elements were analyzed for this study: Li, B, Na, Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Y, Mo, Ag, Cd, Sn, Sb, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Gd, Dy, Ho, Er, Tm, Yb, Lu, Pb, U. Several steps were taken to ensure consistency between runs and within runs. The instrument used for the analysis was recalibrated with a two point calibration each day. Each run on the instrument consisted of 40 unique samples, three blanks, a lab-generated matrix matched quality control sample run in triplicate,

and three other quality control materials including one replicate of a certified reference sample oyster tissue (tissue NIST1566B, MilliporeSigma, St. Louis MO USA). Two of the three quality control replicates were aqueous solutions with known quantities of each element-in the appropriate range, and the third was a salmon flesh quality control matrix-matched sample that was previously validated with recoveries between 80-120%, and a relative standard deviation of <20% for all elements. A small number of samples were duplicated across runs to ensure consistency between runs on the instrument. Parameters of performance in relation to the limits of detection (LoD) for each element were calculated as a blank average plus three times the standard deviation of the blanks.

4.3.4 Statistical Analysis

Elements were removed from the analysis if more than 20% of the samples were below detection limits. Therefore, only the results of Al, As, B, Ba, Ca, Ce, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Nd, Ni, Rb, Se, Sr, Y, Zn are reported for further statistical analysis. Samples in reported elements that were below detection limits for a given element were replaced with a value at one half of the detection limit. The mean and standard deviation of element concentrations by labeled country of origin are reported. A one-way MANOVA was conducted utilizing a test statistic in Friedrich and Pauly (2018), which is robust to heteroscedasticity and can be used with high dimensional data. The reported statistic is described by Friedrich and Pauly (2018) as a "modified ANOVA type statistic" (MATS), and the p value is derived through a parametric bootstrap procedure. Following the results of the MANOVA, the mean concentrations were subsequently compared using one-way Welch's analysis of variance with a Bonferroni corrected p value ($\alpha = 0.05/24$ comparisons for a significance level of $\alpha = 0.0021$). Welch's ANOVA is more robust than a traditional ANOVA to heterogeneity in variation

(Delacre et al. 2019). In cases where the result of the ANOVA was significant, a Games Howell pairwise comparison procedure was used to compare individual means. Games Howell pairwise comparisons are likewise more robust to heterogeneity in variation than other pairwise comparison procedures (Lee and Lee 2018). A significance level of $\alpha = 0.05$ was used to determine significance in the post-hoc pairwise comparisons. Data was log transformed and subsequently centered and scaled to improve normality of the data prior to the analysis. Additionally, a principal components analysis (PCA) was conducted with elements to visualize any patterns in the underlying multivariate data structure.

Following exploration of the data with univariate tests and a PCA, two separate classification procedures were done to assess the ability to discern country of origin in samples collected from retail stores. In the first, country of origin of the retail samples was conducted with a Random Forest classification tree in the "caret" package in R using the method native to the "ranger" package. Recursive feature selection was used to determine the best combination of variables for the random forest. Recursive feature selection ranks the importance of variables based on their contribution to classification models and subsequently eliminates less important variables to find the most informative subsets of variables (Guyon et al. 2002). The random forest was conducted with all 24 elements following the feature selection procedure. Data was centered and scaled prior to classification to remove any effects of magnitude in the element concentrations. The expected accuracy based on the formula in Poulin and Kamiya (2015) is reported for reference for the results of the classification procedure. The accuracy of the model was assessed with k-fold cross validation where k =5. A second random forest classification procedure was conducted where the samples were grouped based on region of origin (Latin America vs. Asia) instead of country of origin. The recursive feature selection for the

classification procedure selected seven elements (Ce, Cu, Fe, K, Mo, Nd, Y) for inclusion in the model.

To move towards the goal of country-of-origin verification, a second discrimination procedure was conducted with samples from farms collected in the countries of origin included in the retail shrimp data, which will be called the "farm data" for the purpose of this study.They will be the subject of a separate study and therefore a complete set of information about these samples will not be presented here, and they are being used to provide context for the data that is the focus of this study (i.e., the retail samples). These samples were collected following the procedure in Li et al. (2017), and subsequently classified with the same procedure as described above. There are 122, 68, 37, 48, and 53 samples from Ecuador, India, Indonesia, Thailand, and Vietnam, respectively, in the farm dataset. In this discrimination procedure, a training model was built with the farm data, and the retail samples were used a naïve test data set. The recursive feature selection for the classification procedure for the farm data training model selected 11 elements (Al, As, Ba, Ca, Co, Cs, Li, Ni, Rb, Se, Sr) for inclusion in the model. The model Random forest classification models were used because of the lack of distributional requirements for the technique, robustness to overfitting high dimensional data, the power of the model to obtain good fits when there are no strong predictor variables (Breiman 2001).

Following the classification procedure with the country of origin, Fisher's exact test were used to determine if any relationship was apparent with regards to the likelihood of correct classification with country of origin, continent where the sample was collected, and certification via aquaculture certification standards (either best aquaculture practice or aquaculture stewardship council). These factors were chosen as potential factors of interest (e.g.,

certification), or possible influence over the classification model (country of origin and continent from which samples were collected).

4.4 Results

4.4.1 Elemental Compositions

Concentrations of individual elements in shrimp muscle tissues varied by orders of magnitude. The elements with the highest concentrations in shrimp muscle tissues from retail stores included Ca, K, Mg, and Na, which all averaged > 1000 mg/kg in at least one country with Na having the highest overall averages (Table 1). The elements with the lowest average concentrations out of the elements evaluated were Co, Cs, Mo, and Y with Yttrium having the lowest concentrations observed across all five countries. The global MANOVA test revealed that there were differences in the shrimp muscle tissue element concentrations from different countries (MATS = 248.164, p = <0.001). After correcting for multiple comparisons, 15 elements were statistically different among the five countries: As, As, Ce, Co, Cs, Cu, Fe, K, Li, Mg, Mo, Nd, Ni, Rb, and Y. In the case of several of the elements (e.g., Al, Fe, K), shrimp muscle tissue that had a country of origin as Ecuador tended to have higher element concentrations than shrimp from Asian countries. In a majority of the elements where differences were detected, Vietnam and Thailand tended belong to the same pairwise comparison groups.

A PCA was conducted to help discern any underlying patterns in the data that may be useful in terms of classification. The first two principal components are plotted in Figure 2, which capture 26.23 % and 19.33 % of the variation in the data, respectively. The shrimp samples from Ecuador appear to separating across the first principal component, which is most strongly associated with Ce, Co, Cu, Fe, and Y (see Table 2). The four Asian countries in the

dataset do not appear to have distinct groups but are primarily spread across the second principal component which is most strongly associated with Al, As, K, Mg, and Mn.

4.4.2 Classification

Overall, the random forest classification of the retail samples successfully classified 71.3% of the samples in into their respective country of origin based on K-fold cross validation (Table 3). The expected accuracy based on random chance for this dataset was 22.5 % and the Kappa was 0.62. The country with the highest rate of success was India, where 93% of the samples were correctly identified as being from India by the model. Conversely, the lowest country level classification was Vietnam, which was also frequently classified as being from India. When the Asian countries are collapsed into a single region classifier, the resulting classifications would be accurate 94.7% of the time (8/12 in Ecuador and 81/82 in Asia). In both cases, four samples from Ecuador were incorrectly classified as being from Asia, specifically India (3) and Indonesia (1). A second procedure was conducted where samples collected from shrimp farms were used as a training set and the samples of interest here (the "retail samples"), were used as a naïve test set. The results are presented in table 4. The baseline model with the farm samples achieved an accuracy of 90% based on k-fold cross validation (k=10), while the classification of the identity of the retail samples with the farm data model only achieved an overall accuracy of 40%. The country that performed most poorly in the retail samples as a naïve test set was Indonesia, where only about 10% of the samples were correctly classified. The country with the best performance in the naïve test set was Ecuador, where 67% of the samples were correctly classified. Cohens Kappa for the naïve test was 0.259 (std. error = 0.0258).

The relationship between external factors and classification success were subsequently examined with Fisher exact tests to determine if important factors (e.g., certification) were

related to the success of sample classification. Altogether, 48 out of the 94 samples had certifications with the most common being BAP (n= 30) and ASC (n=13), with the rest of the assorted certified samples being others. There was no relationship detected between classification success and the location the samples were obtained, be it the USA or Europe (p = 0.063). No relationship was detected between certification status of the samples and classification success (p = 0.131). A relationship was determined to exist between the country of origin of the sample and the success of classification (p = 0.0087).

4.5 Discussion

Shrimp are an important seafood commodity on the global market, accounting for approximately 20% of the total value of aquaculture globally (FAO 2018). However, production is consolidated into a few countries, especially that of the most widely traded species whiteleg shrimp, and therefore a robust international trade exists for aquaculture shrimp. Seafood labeling fraud has been widely documented in importing markets such as Europe (Christiansen et al. 2018, Jacquet and Pauly 2008) and the USA (Lagasse et al. 2014, Korzik et al. 2020), and given environmental problems of the past in shrimp aquaculture (Naylor et al. 2000, Bailey 1988, Richards and Friess 2016, Holmstrom et al. 2003) and recent allegations of human rights violations (Hodal et al. 2014), there is a growing interest in improving the traceability of seafood products. Elemental profiling is a tool that has been proposed to delineate a pre-determined groups (Hassoun et al. 2020, Gopi et al. 2019a, Davis et al. 2021) and therefore improve traceability. Here, we explore the potential to delineate the country of origin of retail shrimp products based on the element concentrations in the tail muscle tissues, the first attempt of its kind in retail shrimp products.

Elemental profiling has proliferated as a method to delineate geographic origins in shrimp over the last two decades and has been highly successful. Smith and Watts (2009) were able to discern shrimp samples from 8 different countries with >70 % accuracy in their discrimination procedure. This dataset and methodology was developed as part of a customs and border patrol case where several shipments of shrimp products from Charoen Pokphand's CP PRIMA in Indonesia were seized out of Indonesia by the US Customs and Border Patrol (US Customs and Border Patrol 2010). These samples were believed to be transshipped, meaning they were grown in a different country and sent to Indonesia for packaging (Kohn Ross 2005), however the samples were later released. Li et al. (2017) were able to classify shrimp to three Asian countries with over a 97% overall accuracy, and Gopi et al. (2019b) were able to obtain >82 % accuracy with shrimp from five countries. Here, the random forest obtained a 71.2% overall accuracy, which is the same or lower than the examples discussed above, and lower than several other examples with shrimp (Davis et al. 2021).

Extenuating factors may have played a role in the relatively low classification accuracy of 71.2% in this study. Several factors have been shown to affect element concentrations in shrimp tissue. Besides geography, the overall size of the shrimp plays a role in the elemental concentrations in the tissues (Boyd and Teichert-Coddington 1995). Boyd and Teichert-Coddington (1995) show that whiteleg shrimp across a range of sizes from (1.7 g- 24.2 g) have slightly different mineral compositions, which could be enough to affect elemental profiling. In this study, attempts were made to obtain shrimp of similar sizes, however this was based upon availability of shrimp at the stores where shrimp were purchased and did vary to some degree. Additionally, because of the role in metallothionein with metal movement in shrimp tissues when frozen (Pourang et al. 2004, Pourang et al. 2005), and chemical treatments at the pond and in

post-harvest processing with chemicals like sodium metabisulfite, sodium chloride, and polyphosphates (Boyd and McNevin 2014), there is reason to suspect that metal concentrations in shrimps post-harvest could be different than shrimp obtained from farms in countries where they are grown. As an example, the Na concentrations in this study are approximately five times what is reported in Li et al. (2017) and in the farm data in this study in shrimp from the same countries. This may present a challenge in any future attempts to use shrimp captured from farms as a validated database from which to identify country of origin in shrimp retail products. The validation of country of origin with the farm samples with the retail samples as a naïve test set here was basically unsuccessful and past attempts by the authors (unpublished data) have likewise yielded middling results. Another farm level factor that could play a role is the salinity of the ponds, which has been demonstrated to be a factor that can be distinguished via elemental profiling (Li et al. 2019). Due to the nature of the sampling here, it is unknown what the salinity of the water in which the shrimp were reared and to what extent that is affecting the results. However, Li et al. (2014) was unsuccessful in correlating water elemental concentrations with tissue levels and past attempts of elemental profiling that were highly successful (Li et al. 2017) would have captured variation in salinity levels in ponds.

Analytical differences in the data could potentially cause small differences, however the elemental concentrations here for retail shrimp are in good agreement with the USDA's food data for shrimp (USDA 2020), so it is unlikely that the analytical procedure is playing a significant role in the differences. A final confounding factor is the possibility that some samples in the data were transshipped, and therefore not labeled with the correct country of origin. It is difficult to ascertain the extent this could be a factor in the accuracy of the classification model.

The patterns in the elemental concentrations suggest that there are distinct differences in some of the countries in this study that will lend themselves to accurate elemental profiles in future efforts. Some elements were shown to have distinct group membership via pairwise comparisons for certain countries (e.g., Ecuador in Fe and Nd, Indonesia in K). Ecuador specifically was different with at least 3 of the 4 Asian countries in 5 out of the 15 elements where statistical differences were detected, suggesting that samples from Ecuador have a unique profile. With a greater sample size, it is likely this would lead to higher levels of correct classification in the discriminant models; however, it was difficult to obtain samples of Ecuadorian shrimp in retail markets. All twelve of the samples in this study that had Ecuador as a country of origin were from the EU. On the contrary, the data in this study supports previous research that shows samples from Thailand and Vietnam are more difficult to discriminate from each other than they are with other countries. In Li et al. (2017) attempts to classify samples to regions within Vietnam and Thailand were not as successful as attempts to discriminate these countries from other countries. In this study, Thailand and Vietnam were only statistically different from one another in the element Co, while they were statistically the same in the 11 other elements where there was statistical difference.

4.6 Conclusions

Elemental profiling is a well-researched tool that has been proposed as a means to identify the geographic origins of seafood products. This is the first attempt to identify retail shrimp products to country of origin based on elemental profiling, and this study highlights some of the challenges that profiling retail samples presents. These results are preliminary and suggest that with a large robust sample, it may be possible to identify the country of origin in retail products independent of their labeling. Based on the observation that some elements may be

different in post-processed shrimp obtained from retail stores when compared to samples collected from farms, a database based on post-processing plant samples may be more desirable for the validation of retail samples when compared to samples obtained from farm ponds, which could overcome some of the difficulties observed with profiling samples from retail stores. Additionally, shrimp shells may be more resilient to industrial processing, and should be explored as an alternative tissue to shrimp muscle tissue. Overall, there is potential to apply this technique to seafood retail products.

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Table 1. A summary of mean elemental concentrations in mg/kg dry weight in shrimp muscle tissues from retail stores by the labeled country of origin. The sample size from each country is listed in parentheses next to the country. The letter next the mean concentration denotes differences detected by pairwise multiple comparisons, and sd = standard deviation. The limit of detection (LOD) is listed next to each element in parentheses.

	Country of Origin										
Element (LOD)	Ecuador (12)	sd	India (30)	sd	Indonesia (21)	sd	Thailand (12)	sd	Vietnam (19)	sd	p value
Al (0.4)	**93.03^{c}**	**68.855**	**28.93^{a}**	**53.993**	**72.67^{bc}**	**72.553**	**10.53^{a}**	**13.277**	**37.44^{ab}**	**60.325**	**1.08E-05**
As (0.05)	**1.15^{a}**	**0.618**	**1.74^{ab}**	**0.990**	**1.21^{a}**	**0.547**	**2.14^{b}**	**0.763**	**1.91^{ab}**	**1.426**	**0.000687**
B (0.4)	1.43	0.899	1.57	1.132	2.76	2.566	1.30	0.787	1.55	1.407	0.147
Ba (0.01)	1.42	0.871	1.63	1.582	1.44	1.393	0.75	0.998	1.31	1.026	0.126
Ca (10.0)	3612.0	1707.45	2959.6	1404.05	3744.6	1284.76	2579.5	777.47	2975.5	1254.63	0.097
Ce (0.0004)	**0.0557^{a}**	**0.04626**	**0.0169^{c}**	**0.01570**	**0.0055^{b}**	**0.00497**	**0.0061^{b}**	**0.00599**	**0.0125^{bc}**	**0.01529**	**3.44E-05**
Co (0.01)	**0.040^{c}**	**0.0294**	**0.031^{c}**	**0.0265**	**0.018^{ab}**	**0.0106**	**0.009^{a}**	**0.0056**	**0.019^{bc}**	**0.0159**	**2.78E-05**
Cr (0.05)	0.220	0.1188	0.450	0.5954	0.513	0.7490	0.145	0.0990	0.139	0.1384	0.002
Cs (0.0002)	**0.014^{c}**	**0.0090**	**0.005^{a}**	**0.0031**	**0.009^{ab}**	**0.0073**	**0.026^{bc}**	**0.0125**	**0.010^{b}**	**0.0074**	**7.48E-08**
Cu (0.02)	**18.57**	**8.258**	**8.08**	**2.217**	**7.16**	**1.416**	**7.88**	**2.201**	**7.47**	**4.508**	**0.001**
Fe (0.4)	**76.61^{b}**	**65.990**	**19.78^{a}**	**14.904**	**12.33^{a}**	**5.480**	**12.22^{a}**	**9.955**	**17.19^{a}**	**19.254**	**0.000247**
K (10.0)	**9824.1^{b}**	**3876.10**	**6098.4^{b}**	**2164.32**	**3091.4^{a}**	**1567.24**	**5150.3^{b}**	**2217.21**	**5189.0^{b}**	**4196.79**	**1.61E-05**
Li (0.002)	**0.112^{c}**	**0.0815**	**0.037^{a}**	**0.0182**	**0.110^{bc}**	**0.1885**	**0.054^{ab}**	**0.0636**	**0.074^{bc}**	**0.0329**	**1.28E-05**
Mg (2.0)	**1400.7^{b}**	**225.27**	**1261.5^{b}**	**209.84**	**951.8^{a}**	**173.92**	**1124.4^{ab}**	**219.55**	**1156.9^{ab}**	**275.53**	**2.15E-05**
Mn (0.02)	1.99	0.995	1.80	1.064	2.12	1.527	0.94	0.476	1.81	0.924	0.004
Mo (0.01)	**0.036^{a}**	**0.0180**	**0.116^{b}**	**0.0964**	**0.215^{b}**	**0.5369**	**0.103^{ab}**	**0.1007**	**0.061^{ab}**	**0.0605**	**4.76E-05**
Na (10.0)	22798.0	9357.5	27271.3	13761.9	40602.5	12577.7	30333.2	16235.3	26788.6	17186.0	0.002
Nd (0.0004)	**0.0307^{d}**	**0.02637**	**0.0076^{c}**	**0.00723**	**0.0025^{a}**	**0.00244**	**0.0025^{ab}**	**0.00234**	**0.0065^{bc}**	**0.00776**	**1.18E-05**
Ni (0.02)	**0.130^{ab}**	**0.0668**	**0.326^{b}**	**0.7033**	**0.201^{ab}**	**0.2970**	**0.067^{a}**	**0.0438**	**0.084^{a}**	**0.0771**	**0.00071**
Rb (0.005)	**2.76^{b}**	**1.308**	**1.75^{b}**	**0.820**	**0.88^{a}**	**0.377**	**1.80^{b}**	**0.723**	**1.45^{ab}**	**0.991**	**1.12E-05**
Se (0.05)	1.22	0.250	1.28	0.377	1.17	0.262	1.20	0.372	1.15	0.163	0.765
Sr (0.004)	42.63	21.3117	28.70	15.0207	45.38	17.3434	25.44	9.5675	28.49	13.6779	0.002
Y (0.001)	**0.031^{b}**	**0.0242**	**0.008^{b}**	**0.0069**	**0.009^{b}**	**0.0230**	**0.003^{a}**	**0.0025**	**0.008^{b}**	**0.0095**	**0.000876**
Zn (0.4)	48.95	2.7324	48.83	4.8461	45.57	4.1944	47.15	3.4685	47.28	5.7204	0.07

Table 2. Eigenvectors, eigenvalues, and variation explained of the data in this study. Principal components with an eigenvalue >1 are presented.

Element	PC1	PC2	PC3	PC4	PC5	PC6
Al	-0.14	0.24	-0.21	-0.22	0.15	0.23
As	-0.02	-0.30	0.03	0.14	0.10	0.03
B	0.03	0.12	-0.34	0.27	0.18	-0.35
Ba	-0.12	0.22	0.30	0.00	-0.13	0.00
Ca	-0.16	0.22	0.09	0.34	-0.47	0.15
Ce	-0.37	0.04	-0.11	-0.17	-0.01	-0.08
Co	-0.31	0.15	0.17	0.02	0.23	-0.11
Cr	-0.08	0.23	0.30	0.23	0.37	0.15
Cs	-0.10	-0.20	-0.24	0.32	0.06	0.11
Cu	-0.31	-0.14	-0.09	0.05	0.01	0.13
Fe	-0.36	0.05	-0.16	-0.13	0.05	-0.04
K	-0.23	-0.29	-0.02	0.06	0.09	0.17
Li	-0.08	0.06	-0.30	0.22	-0.03	-0.43
Mg	-0.21	-0.30	0.13	0.16	-0.10	-0.05
Mn	-0.17	0.28	0.12	-0.10	0.05	-0.08
Mo	0.02	0.04	-0.13	0.45	0.18	-0.21
Na	0.10	0.31	-0.26	0.03	0.07	0.25
Nd	-0.37	0.03	-0.14	-0.17	-0.01	-0.06
Ni	-0.11	0.20	0.33	0.23	0.41	0.05
Rb	-0.23	-0.31	0.01	0.07	0.14	0.19
Se	-0.02	0.03	0.22	-0.18	-0.03	-0.55
Sr	-0.14	0.23	0.03	0.34	-0.47	0.11
Y	-0.31	0.11	-0.14	-0.15	-0.12	-0.05
Zn	-0.12	-0.24	0.33	0.05	-0.13	-0.22
Eigenvalue	2.51	2.15	1.64	1.26	1.26	1.14
Percent Variation	26.23	19.33	11.16	6.652	6.582	5.424
Cumulative	26.23	45.56	56.72	63.372	69.954	75.378

Table 3. The classification of samples in this study to country of origin with a random forest model. The samples are assigned a classification-based k-fold cross validation.

	Reference				
Prediction	Ecuador	India	Indonesia	Thailand	Vietnam
Ecuador	8	0	0	0	1
India	3	28	2	0	6
Indonesia	1	2	16	2	3
Thailand	0	0	2	8	2
Vietnam	0	0	1	2	7
Accuracy	67%	93%	76%	75%	42%
Overall					71.28 %

Table 4. The results of the retail samples as naïve testing data when classified against the farm samples as training data.

	Reference				
Prediction	Ecuador	India	Indonesia	Thailand	Vietnam
Ecuador	8	7	14	1	7
India	0	18	3	1	3
Indonesia	3	4	2	2	1
Thailand	1	0	2	6	4
Vietnam	0	1	0	2	4
Accuracy	67%	60%	9.5%	50%	21%
Overall					40.4%

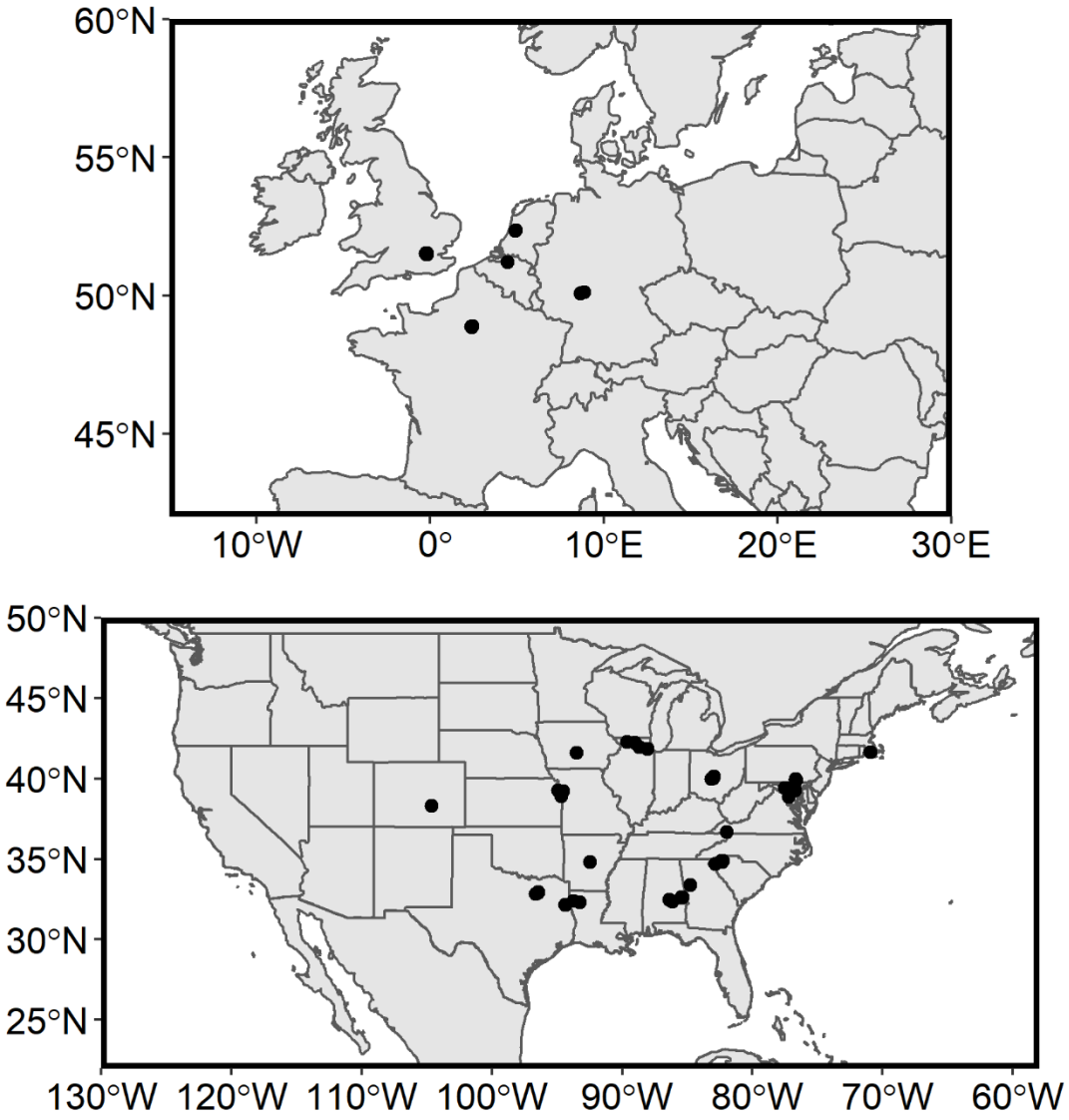

Figure 1. A map of sampling locations throughout the USA and Western Europe.

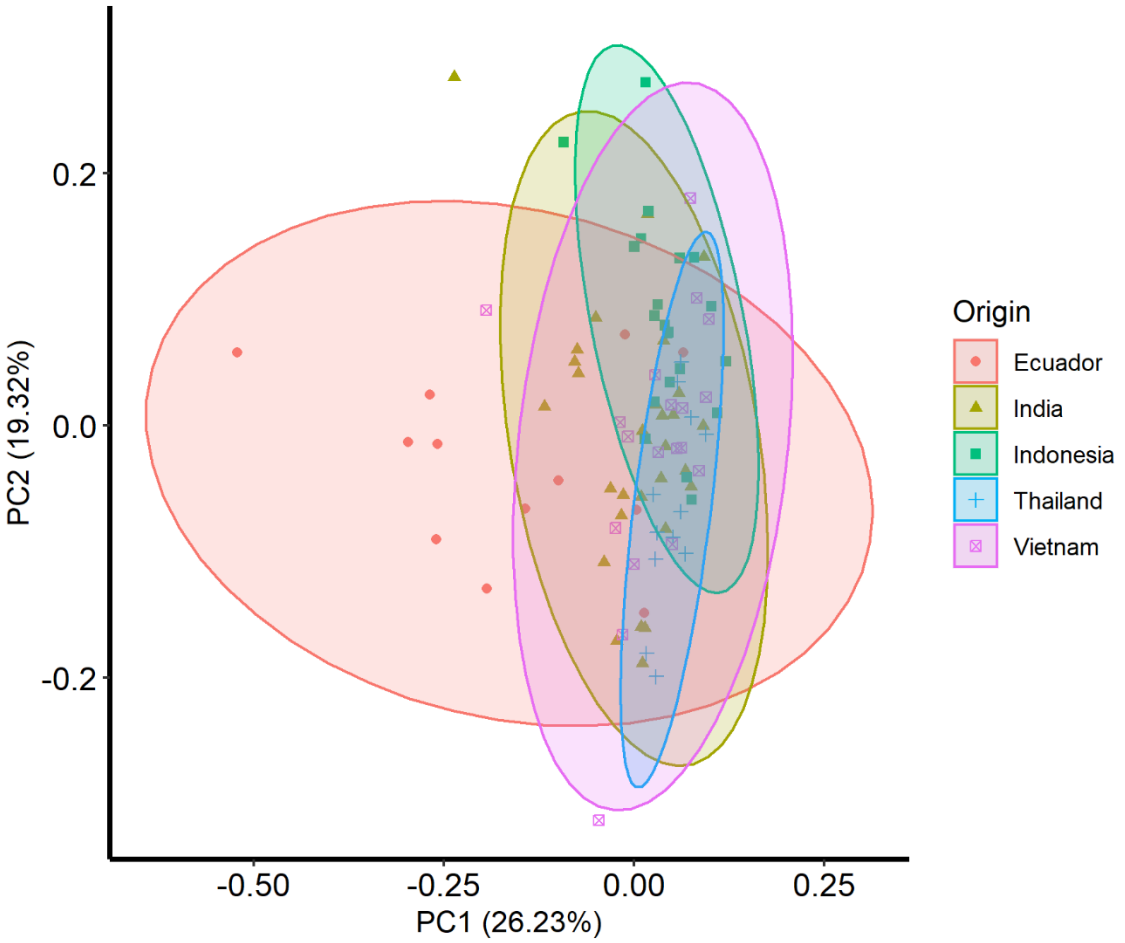

Figure 2. A bi-plot of the first and second component of the principal components analysis. The variation explained in the data is in parentheses. Samples from different countries are represented by different shape color/combinations. The combinations are; i.red circles =Ecuador, ii. yellow triangles = India, iii. Green diamonds = Indonesia, iv. Blue crosses = Thailand, and v. purple boxes = Vietnam. Ellipses represent normalized group ellipses in two-dimensional space.

CHAPTER 5

THE EFFECT OF COMMERCIAL SCALE PROCESSING ON TRACE ELEMENT CONCENTRATIONS IN SHRIMP MUSCLE TISSUE – A PRELIMINARY STUDY FROM TWO PROCESSORS IN THAILAND AND ECUADOR

5.1 Abstract

Elemental profiling has been explored as a traceability tool for seafood items with some success in the literature. Commercial processing, including the chemical treatments that shrimp are subjected to, has been identified as a confounding factor in the use of elemental profiling in shrimp. Here, two sets of samples were collected pre/post processing: one from Thailand (n=15) and one from Ecuador (n=19). The samples from Ecuador were subjected to a meta-bisulfite bath as part of the processing regimen while the samples from Thailand were subjected to a salt bath. Samples were analyzed via ICP-MS for trace elements. Overall, elements from samples in Ecuador were unaffected by the processing regimen (MATS = 15.47, p = 0.3), but samples from Thailand (MATS = 766.29, p = < 0.001) were. Eleven out of 23 elements above detection limits were significantly different (Ca, Co, Cu, K, Li, Mg, Mn, Na, Ni, Sr, and Z). The pre-post processing samples were classified to country of origin with a training model developed from samples collected from shrimp farms in five countries (overall accuracy = 92 %). The samples post processing were less accurately classified (50% overall accuracy vs. 67% accuracy) compared to unprocessed samples, with the samples from Ecuador showing high fidelity regardless of processing while the samples from Thailand were less accurately classified. Overall, this study shows that processing can alter the profile of trace elements in shrimp muscle

tissue and ultimately impact the accuracy of classification. Implications for practical implementation are discussed.

5.2 Introduction

Seafood is an important source of protein for humankind, accounting for slightly less than 20% of meat for food consumption (Edwards et al. 2019). Because of to the highly globalized nature of seafood production, seafood products are plagued with fraudulent labeling which has been well documented in the United States and the European Union. Mislabeling occurs to manipulate prices (Jacquet and Pauly 2008), improve marketing (i.e., "sustainability") (Miller et al. 2012), substitute farmed species for wild species (Korzik et al. 2020), and meet processor quota demands. In aquaculture shrimp products, transshipping has been an issue in the past, resulting in incorrectly labeled country of origin among retail products. Mislabeling, and by extension traceability, poses a distinct problem for those who wish to improve seafood sustainability, as fraudulent labeling enables a lack of accountability for producers, processors, and buyers. There is wide interest in diminishing the amount of fraudulent labeling in seafood, especially in highly valuable, internationally traded products such as penaeid shrimp.

Elemental profiling has been identified as a tool to increase traceability in seafood products (Gopi et al. 2019a, Leal et al. 2015, Li et al. 2016). Elemental profiling (or elemental fingerprinting, trace element analysis, etc.) involves analyzing samples from pre-determined groupings for element contents to determine if differences in the element concentrations can be used in conjunction with discriminant analyses to discern group membership. Elemental profiling has been used to determine geographic origins (Albuquerque et al. 2016, Gopi et al. 2019c, Li et al. 2017) production origin (Anderson et al. 2010, Chaguri et al. 2017), and salinity of shrimp ponds (Li et al. 2019) with a high degree of success. It has been successful in a wide variety of taxa as well, such as crustaceans (Luo et al. 2019), molluscs (Zhao and Zhang 2016, Bennion et al. 2019), fish (Flem et al. 2017, Avigliano et al. 2019), and marine algae (Hattori et al. 2009).

Elemental profiling has shown promise as a tool for discriminating geographic origins in shrimp. The first attempts to discriminate geographical origin with shrimp were by Courtney et al. (1994), which used elemental profiling to separate stocks of wild Eastern King Prawn *Penaeus plebejus*. Smith and Watts (2009) published their findings after elemental profiling was used in the case with CP Prima (US Customs and Border Patrol 2010), in which they collected a small number of samples from eight different countries. Whiteleg shrimp have also been successfully discriminated from farms in the USA (Li et al. 2014) and Southeast Asia (Li et al. 2017), and geographic origin and production methods have differentiated in black tiger shrimp *Penaeus monodon* (Gopi et al. 2019b). Altogether, these studies suggest there is a strong potential for elemental profiling in shrimp.

One challenge that has been identified in the use of elemental profiling as a widespread traceability tool is the confoundment of element concentrations in tissues that have undergone commercial processing (Leal et al. 2015). Indeed, there are relatively few examples of practical applications. The previously mentioned case with CP Prima and the US Customs and Border Control is perhaps the only instance where elemental profiling was used as a tool in meaningful way to determine the origins of seafood. The underlying issue identified by Leal et al (2015) hinges on the assumption that commercial processing for packaging and distribution affects the elemental profiles of the tissues or that the soft tissues in the products (i.e., muscle) do not retain enough elements to strongly discriminate. In shrimp processing, there is a history of using chemicals such as sodium metabisulfite (preservative), polyphosphates (water retention), and salts such as sodium chloride during commercial processing (Boyd and McNevin 2015).

Preliminary attempts to use elemental profiling with retail shrimp products as test data and the farmed shrimp collected before processing as the training data for classification models

were relatively unsuccessful, and significant differences were observed in elements from shrimp tissues in the same geographic origins between the two groups (authors' unpublished data). This led to the conclusion there is a possibility that commercial harvesting and processing likely affects the element profile of shrimp. Therefore, the broad objective of this study was to understand the effect of commercial processing on shrimp muscle tissue and the implications for trace elemental profiling. This was accomplished by obtaining paired samples from shrimp processors and shrimp farms in Ecuador and Thailand to analyze the effect of processing on shrimp muscle tissue element concentrations.

5.3 Methods

5.3.1 Sample Procurement

Two sets of paired samples were procured for this study: one from Thailand and one from Ecuador. The samples were paired with each pair containing one sample obtained from a farm pond and another after some processing in a commercial processing plant (see Figure 1 for locations of the farms). Samples were obtained in coordination with a commercial processor in each country as part of the regular harvesting and processing operations. No changes were made to the processing of the shrimp, as the study was meant to reflect what occurs during the normal course of processing. Shrimp collected at the farm pond (not subjected to any chemical treatments or processing) were collected in the same manner as Li et al. (2017). A total of 15 paired samples were obtained in Thailand and a total of 19 paired samples were obtained in Ecuador. The samples from each country were subjected to different processing regimes. Shrimp from ponds in Ecuador were captured for harvest and treated in a sodium metabisulfite bath pondside, which is a preservative meant to prevent black spots in shrimp flesh (Boyd and McNevin 2015). After the metabisulfite bath, the shrimp were transferred to ice totes until

transport to the processing facility. At the processing facility, shrimp were removed from ice, graded, and washed with water and subsequently collected. In Thailand shrimp were collected from ponds for commercial harvest, put on ice and taken to the processing facility. At the processing facility, they were subjected to a freshwater rinse for washing, followed by deheading, peeling, deveining, brining in a sodium chloride solution, and were subsequently glazed and frozen via individual quick freezing (IQF) for packaging. Shrimp from the processor in Thailand were obtained after packaging for analysis.

Shrimp were prepared for storage until element analysis in the following manner. Shrimp obtained from ponds were brought to a laboratory in the country of sampling and deheaded, peeled, and deveined. The peeled tails were then dried in commercially available food dehydrators at 50° C until the tissue reached constant mass, at least 12 hours. Upon drying, the samples were stored frozen until shipment for analysis. Processed shrimp were prepared in the same manner with the exception that that they were deheaded, peeled, and deveined as part of processing in Thailand. Upon drying shrimp were stored frozen until shipment for laboratory analysis.

5.3.2 Digestions

In preparation for digestion, samples were freeze dried overnight to remove any residual moisture. The digestion of the shrimp tissue was done following an adaption of EPA method 200.8 (US EPA 1994) for solid materials (Environmental Express 2018). Briefly, 0.5g of dried sample was digested with 2.0 ml of 1:1 nitric acid and 5.0 ml of 1:4 hydrochloric acid and refluxed in an Environmental Express Hotblock (TM) (HotBlock 200, Environmental Express, Charleston SC USA) for 30 minutes at 85 C. Upon cooling, the samples were quantitively transferred and brought to volume in 50-ml volumetric flasks. Samples were then

centrifuged at ambient temperature for 5 minutes and decanted to remove any insoluble material in the solution. The digestion method was verified by validating recovery of a spiked quality control standard (recovery between 80-120%), determining the limits of detection, and repeatability of measurements with 15 replicants of a quality control standard (relative standard deviation <20%).

5.3.3 Elemental Analysis

A NexION 350d ICP-MS (PerkinElmer Inc., Waltham MA USA) was used to conduct the elemental analysis for this study. Forty-two elements were analyzed for this study: Ag, Al, As, B, Ba, Ca, Ce, Cd, Co, Cr, Cs, Cu, Dy, Eu, Er, Fe, Gd, Ho, K, La, Li, Lu, Mg, Mn, Mo, Na, Nd, Ni, Pb, Pr, Rb, Sb, Se, Sm, Sn, Sr, Tm, U, V, Y, Yb, and Zn. Several steps were taken to ensure consistency between runs and within runs. The instrument used for the analysis was recalibrated with a two-point calibration each day. Each run on the instrument consisted of 40 unique samples, three blanks, a lab-generated matrix matched quality control sample run in triplicate, and three other quality control materials including one replicate of a certified reference sample oyster tissue (tissue NIST1566B, MilliporeSigma, St. Louis MO USA). Two of the three quality control replicates were aqueous solutions with known quantities of each element-in the appropriate range, and the third was a salmon flesh quality control matrix-matched sample that was previously validated with recoveries between 80-120%, and a relative standard deviation of <20% for all elements. A small number of samples were duplicated across runs to ensure consistency between runs on the instrument. Parameters of performance in relation to the limits of detection (LoD) for each element were calculated as blank average plus three times the standard deviation of the blanks.

5.3.4 Statistical Analysis

Elements were removed from the analysis if more than 20% of the samples were below detection limits. Samples in reported elements that were below detection limits for a given element were replaced with a value at one half of the detection limit. Therefore, only the results of Al, As, Ba, Ca, Ce, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Nd, Ni, Rb, Se, Sr, Y, and Zn are reported. The mean and standard deviation of element concentrations by country-of-origin x processing status. A principal components analysis was used to visualize and understand the data structure in reduced dimensions. The data were centered and scaled before the PCA analysis.

A repeated measure multivariate comparison was made with a test statistic developed by Friedrich and Pauly (2018), which is robust to heteroscedasticity and can be used with high dimensional data. The reported statistic is described by Friedrich and Pauly (2018) as a "modified ANOVA type statistic" (MATS), and the p value is derived by a parametric bootstrap procedure. The multivariate comparison was conducted within each country (Ecuador and Thailand), because of the different types of processing used at the respective processing facilities. Conceptually, these are equivalent to two repeated measures t-test testing for the effect of processing, but in a multivariate context. A factorial concept was not utilized because we were not interested in understanding any differences between countries, simply processing status. Following the results of the multivariate comparison, mean concentrations were subsequently compared using repeated measures t-test a Bonferroni corrected p value (α = 0.05/23 comparisons for a significance level of $\alpha = 0.0022$). Data were centered and scaled to improve normality of the data before the univariate analysis, and results are reported on the original scale. Any relationship between pre/post processing samples from the same countries were examined by evaluating the correlation coefficient between the same elements in the paired samples. Conceptually, if the elements are unchanged by processing, the correlations between

pre/post sample concentrations should be close to one, although some variation is expected from the analytical procedure.

Following exploration of the data with univariate tests, the pre/post processed datasets were used in a classification exercise as "testing" data. For "training" data, samples collected from farms in five major shrimp production countries processed and analyzed in the same way as the pre/post processing samples described above. In total, 103 samples from Ecuador, 68 from India, 37 from Indonesia, 30 from Thailand, and 53 samples from Vietnam were included in the training data. Classification of the country of origin of the training data was conducted with a Random Forest classification in the "caret" package in R using the method native to the "ranger" package (Wright and Ziegler 2017). Recursive feature selection was used to determine the best combination of variables for the random forest. Recursive feature selection ranks the importance of variables based on their contribution to classification models and subsequently eliminates less important variables to find the most informative subsets of variables (Guyon et al. 2002). The random forest was conducted with the following subset of elements after recursive feature selection; Al, As, Ba, Ca, Co, Cu, Cr, Cs, Fe, K, Li, Mo, Ni, Rb, Se, Sr, and Zn. Data was centered and scaled before classification to remove any effects of magnitude in the element concentrations. The accuracy of the model was assessed with k-fold cross validation where k =10. The model random forest classification models were used because of the lack of distributional requirements for the technique, robustness to overfitting high dimensional data, the power of the model to obtain good fits when there are no strong predictor variables (Breiman 2001). Other models that were explored were linear discriminant analysis and K-nearest neighbors' classification. Following construction of the discrimination model, pre and post processed samples from Ecuador and Thailand were classified to demonstrate any effect of

processing on the observed accuracy of the classification. In all cases except where specified above, a p value of $\alpha = 0.05$ was used to determine significance. All statistical analysis were conducted in R version 4.0.3 (R Core Team 2020).

5.4 Results

The repeated measures multivariate comparison was not significant for the effect of processing in Ecuador (MATS = 15.47, p = 0.3), but was significant in Thailand (MATS = 766.29, p = < 0.001). The concentrations of elements in each set of samples are presented in Table 1. In total, 11 elements were statistically different after the Bonferroni correction in the samples from Thailand, no univariate comparisons were conducted on the samples from Ecuador. The largest relative differences among elements that were considered statistically different were Co, Na, Mn, and Ni. The relative ratios of means between pre/post processing samples in these elements were 4.0, 0.27, 3.5, and 3.5, respectively. Other elements that were not significantly different also exhibited high relative ratios. The elements Al, Ce, Fe, and Nd had relative ratios of pre/post processing means of 14.9, 16.4, 12.0, and 20.3. For comparison, the relative ratios of means of elements in the samples from Ecuador ranged between 0.66 and 0.99. The greatest difference in magnitude between pre/post samples was Na, which had a mean of 5589 mg/kg in preprocessed samples in Thailand and a mean of 20497 mg/kg in post processed samples.

Dimension reduction via a PCA showed that the processed samples from Thailand are distinct from the other subsets of data (Figure 2). A summary of the principal components analysis is presented in Table 2. The processed samples from Thailand are separating from the unprocessed samples across principal component 1, which is most strongly associated with Al, Co, Cr, and Fe. Thailand and Ecuador are showing a slight separation across principal

component two, which is most strongly associated As, Ca, Cs, and Sr. Pre and post processing samples from Ecuador do not seem to be very different in reduced dimensional space, occupying roughly the same area in the first two principal components.

The correlation between element concentrations in pre/post pairs was examined to examine the fidelity of the elements after exposure to processing. In Ecuador, 11 of the 23 elements had a significant correlation in pre/post processing samples (Table 3). In contrast, only six elements in the samples from Thailand had a significant correlation pre/post processing. Overall, the Pearson coefficient for elements were higher in the Ecuador subset (range: 0.10 – 0.88) than the Thailand subset (range: 0.04 – 0.91), although the two strongest correlations were in the Thailand subset, with Rb at 0.91 and As at 0.90. On average the absolute magnitude of the correlations was stronger in Ecuador than Thailand ($\bar{x}$ = 0.48 vs 0.33).

The pre/post processing subsets from Thailand and Ecuador were classified based on a model developed with samples from shrimp farms in major production countries. The overall accuracy of the training model is 92% (see Table 4). Overall, 67% of the unprocessed samples from Thailand and Ecuador were correctly classified to country of origin with the training model (Table 6). The samples from Ecuador were classified with high fidelity (18/19 samples correctly classified) while the samples from Thailand were less accurately classified, with only 5/15 samples being classified to Thailand. The majority of the incorrect classifications were classified as Vietnam (7/15), with the remainder being classified as Ecuador (2/15) and India (1/15). Classification was less successful in post processed samples, with the overall classification success only being 50% (Table 6). Samples from Ecuador still had high fidelity (17/19 samples correctly classified), but samples from Thailand were correctly classified 0/15 times. The

majority of the samples were classified as Vietnam (14/15), while one sample was classified as Ecuador.

5.5 Discussion

Traceability solutions are being sought for shrimp, and more broadly, seafood supply chains. Elemental profiling has been proposed as a tool to improve the traceability of agricultural, livestock, and seafood products (Gopi et al. 2019a, Li et al. 2016). Here, we investigated the effect of commercial processing on shrimp muscle tissue after preliminary attempts to classify retail samples to country of origin revealed differences in the elemental profiles of shrimp from retail stores and shrimp from farm ponds (author's unpublished data).

The role processing plays in the final composition of raw food items is relatively unexplored. Bekhit et al. (2008) found that salting of fish roe generally increased the trace metal content in the roe when compared to untreated roe. Processing was identified by the authors as a potential source of variation in elemental profiles because of the increase in sodium concentrations, a proverbial "canary in the coal mine". Given that shrimp processing can involve treatments of either salt baths or the use of sodium polyphosphates (Boyd and McNevin 2015), this was a logical factor that could be playing a role. In fact, in the shrimp from Ecuador, the samples were relatively the same pre and post processing (see Table 1 and Figure 2). However, in the samples from Thailand, there are drastic differences. In fact, all elements except for sodium (and selenium which is essentially the same) either marginally decrease or drastically decrease. Sodium, on the other hand, increases by a factor of about 3.66 on average.

This difference is more apparent when expressed on a molar basis. On average (mean + St. Dev), the tails in the pre-processed Thai samples contain 0.755 ± 0.057 moles of elements. Post processing, the average number of moles of elements increases to 1.283 ± 0.111. However,

this difference is driven entirely by an increase of the sodium in the tissues. The number of moles of elements, without sodium, decreases in the samples post-processing from an average of 0.511 to 0.391 moles of elements. This pattern is not seen in the samples from Ecuador, where the number of moles of elements in the tissues is relatively unchanged ($\bar{x}$ 0.813 ± 0.095 vs 0.837 ± 0.072 pre-post processing) with little change without sodium (0.520 vs. 0.534 pre-post processing). Most likely, elements in the muscle tissues of shrimp from Thailand are being flushed out by sodium ions while sodium ions are being retained. This has not been explicitly demonstrated in shrimp muscle tissue, but the displacement of ions by sodium in cured animal meats and blue mussels *Mytilus edulis* has been documented previously (Cittadini et al. 2020, Slabyj and Carpenter 1977).

The use of elemental profiling as a traceability tool has been explored in great detail in the literature (Davis et al. 2021, Li et al. 2016), but has been limited in practical applications. In shrimp, it has been successfully used to delineate wild stocks (Courtney et al. 1994), shrimp from farms in southeast Asia (Li et al. 2017) and Oceania (Gopi et al. 2019b), as well as shrimp species from similar geographic origins (Ortea and Gallardo 2015). In 2009, the US customs and border patrol attempted to use elemental profiling to validate the geographic origin of some shipments seized from Indonesia (Seafood Source Staff 2009), and later published their methods (Smith and Watts 2009). However, there have been few other examples where this technique has been applied in a practical setting. In shrimp, the evidence in this study suggests that processing could be a potential problematic factor. The shrimp from Thailand had a different profile after being subjected to commercial scale processing, and it ultimately affected the ability to identify the samples when entered into a classification model (see tables 5 and 6). Additionally, the lack of strong correlations between elements pre-post processing in samples from Thailand suggest it

will be difficult to model the change or create correction factors to account for processing. Altogether, this suggests that shrimp samples that have been subjected to processing are likely best classified via elemental profiling with other samples that have been processed. Additionally, the effect of processing is likely processing-type dependent (i.e., some will affect the profile while others do not), confounding the ability to apply this technique to grab sampling from retailers for independent verification as not all retailers will request the same types of processing in their samples.

This study examined the role of commercial processing on shrimp muscle tissue and the implications of the effects of processing on the ability to successfully profile shrimp post-processing. While this work demonstrates the effect that a salt brine on the elemental concentrations of shrimp muscle tissue, it was from only one processor and therefore, future works could look to expand the scope by including multiple processors using different treatment combinations from the same country. Additionally, the authors found it difficult to find processors willing to cooperate with the researchers who were collecting the samples within the countries where the study took place. An expansion of this study, where there are enough samples to properly create a classification model with samples obtained from processors could improve the understanding of whether or not processing impacts the success of classification. Given that shrimp have been successfully classified from samples in farms (Li et al. 2017, Gopi et al. 2019b), collecting samples to classify post-processing would be a more definitive indicator of the ability of elemental profiling to be implemented as a practical traceability tool.

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Table 1. A summary of element concentrations in each country x processing subset. Means and standard deviations (sd) are presented. Elements that are bolded were statistically different by pre/post processing in the Thailand subset of samples. The limit of detection is in parentheses next to the name of the element. Limits of detection and means and sd are presented in µg/g dry weight of tissue.

	Ecuador (n=19)				Thailand (n=15)				
Element	Unprocessed	sd	Processed	sd	Unprocessed	sd	Processed	sd	p value
Al (0.4)	88.5	76.5	105.4	81.9	50.096	61.725	3.359	6.289	0.0070
As (0.05)	1.54	1.56	1.54	1.36	4.507	2.099	3.797	1.914	0.3420
Ba (0.4)	1.58	1.55	1.98	2.07	0.589	0.727	0.267	0.305	0.1250
Ca (10)	3022.2	1235.0	3023.2	1305.0	**2706.4**	**515.0**	**1246.7**	**581.6**	**< 0.0001**
Ce (0.004)	0.070	0.065	0.107	0.104	0.148	0.199	0.009	0.016	0.0110
Co (0.01)	0.052	0.034	0.067	0.047	**0.047**	**0.029**	**0.012**	**0.007**	**0.0001**
Cr (0.05)	0.132	0.101	0.161	0.114	0.129	0.093	0.052	0.02	0.0040
Cs (0.05)	0.016	0.007	0.017	0.005	0.093	0.089	0.058	0.062	0.2140
Cu (0.02)	30.0	4.3	31.3	7.3	**22.645**	**7.868**	**12.599**	**3.665**	**0.0001**
Fe (0.4)	86.0	73.5	104.5	83.9	94.184	114.119	7.836	6.88	0.0070
K (10)	14454.9	1031.9	14933.9	1512.5	**14365.4**	**1565.8**	**11625.1**	**1520.0**	**< 0.0001**
Li (0.002)	0.115	0.079	0.116	0.069	**0.09**	**0.066**	**0.031**	**0.012**	**0.0020**
Mg (2)	1662.2	192.7	1679.8	161.0	**1734.3**	**196.0**	**1489.0**	**89.9**	**0.0001**
Mn (0.02)	2.36	1.61	3.18	2.61	**3.641**	**2.732**	**1.026**	**0.593**	**0.0010**
Mo (0.01)	0.036	0.010	0.04	0.016	0.049	0.031	0.022	0.006	0.0030
Na (10)	6728.8	1593.1	6958.4	1323.9	**5589.3**	**602.1**	**20497.1**	**2565.7**	**< 0.0001**
Nd (0.0004)	0.037	0.033	0.056	0.049	0.061	0.081	0.003	0.005	0.0100
Ni (0.02)	0.108	0.073	0.132	0.092	**0.063**	**0.042**	**0.018**	**0.013**	**0.0004**
Rb (0.005)	3.49	0.87	3.54	0.82	4.641	1.356	3.583	1.067	0.0250
Se (0.05)	1.17	0.28	1.19	0.28	1.262	0.16	1.279	0.166	0.7740
Sr (0.004)	31.6	13.7	31.6	14.2	**23.246**	**4.965**	**10.736**	**8.266**	**< 0.0001**
Y (0.001)	0.032	0.028	0.049	0.044	0.033	0.044	0.002	0.002	0.0090
Zn (0.4)	52.8	2.8	53.6	3.0	**56.271**	**4.886**	**50.746**	**3.816**	**0.0020**

Table 2. A summary of the principal components analysis of the data.

Element	Principal Component				
	1	2	3	4	5
Al	0.303	-0.039	0.122	-0.037	0.082
As	-0.086	-0.389	-0.175	0.291	-0.132
Ba	0.155	0.244	0.109	-0.330	-0.069
Ca	0.171	0.334	-0.036	-0.043	-0.379
Ce	0.255	-0.257	0.037	0.033	-0.097
Co	0.298	-0.051	0.071	0.037	-0.069
Cr	0.292	-0.118	0.125	-0.023	0.027
Cs	-0.031	-0.352	-0.297	-0.280	-0.257
Cu	0.206	0.293	-0.138	-0.055	0.083
Fe	0.294	-0.156	0.081	-0.027	0.040
K	0.138	0.217	-0.375	0.208	0.319
Li	0.260	-0.127	0.008	0.214	0.218
Mg	0.123	-0.044	-0.431	0.169	0.217
Mn	0.238	-0.111	-0.059	0.169	-0.006
Mo	0.141	-0.032	-0.135	-0.388	-0.275
Na	-0.191	-0.236	0.332	0.063	0.012
Nd	0.276	-0.224	0.063	0.020	-0.070
Ni	0.268	0.081	0.145	-0.063	-0.017
Rb	0.011	-0.204	-0.385	-0.396	-0.061
Se	-0.016	0.026	0.072	0.432	-0.602
Sr	0.172	0.309	-0.009	0.133	-0.207
Y	0.292	-0.152	0.089	0.028	0.038
Zn	0.026	0.081	-0.401	0.228	-0.224
Eigenvalue	3.07	1.64	1.55	1.29	1.13
Percent Explained	41.0%	11.7%	10.4%	7.2%	5.6%
Cumulative Variation	41.0%	52.6%	63.1%	70.3%	75.8%

Table 3. A summary of the Pearson correlation and significance of the relationship between pre/post processed pairs within each country subset.

	Ecuador		Thailand	
Element	Correlation	p value	Correlation	p value
Al	0.38	0.108	-0.07	0.799
As	0.35	0.140	0.90	< 0.001
Ba	0.88	< 0.001	0.76	0.001
Ca	0.60	0.007	0.02	0.933
Ce	0.28	0.239	-0.04	0.880
Co	0.35	0.141	0.20	0.458
Cr	0.44	0.060	0.04	0.873
Cs	0.51	0.026	0.90	< 0.001
Cu	0.19	0.434	0.88	< 0.001
Fe	0.42	0.074	-0.18	0.496
K	0.67	0.002	0.43	0.100
Li	0.80	< 0.001	-0.19	0.488
Mg	0.59	0.007	0.18	0.512
Mn	0.71	0.001	0.05	0.841
Mo	0.50	0.028	0.08	0.759
Na	0.45	0.056	0.09	0.737
Nd	0.25	0.311	-0.09	0.747
Ni	0.46	0.050	-0.24	0.376
Rb	0.91	< 0.001	0.92	< 0.001
Se	0.86	< 0.001	0.85	< 0.001
Sr	0.41	0.085	0.23	0.400
Y	0.11	0.665	-0.11	0.679
Zn	0.15	0.536	0.17	0.526

Table 4. A summary of the results of the random forest classification model for the training data.

	Reference				
Prediction	Ecuador	India	Indonesia	Thailand	Vietnam
Ecuador	99	2	4	0	0
India	3	64	0	0	0
Indonesia	0	0	28	1	1
Thailand	0	1	2	24	0
Vietnam	1	1	3	5	52
Overall					92%

Table 5. A summary of the results of the classification of pre-processed samples from Thailand and Ecuador.

	Reference				
Prediction	Ecuador	India	Indonesia	Thailand	Vietnam
Ecuador	18	0	0	2	0
India	1	0	0	1	0
Indonesia	0	0	0	0	0
Thailand	0	0	0	5	0
Vietnam	0	0	0	7	0
Overall					67%

Table 6. A summary of the results of the classification of post-processed samples from Thailand and Ecuador.

	Reference				
Prediction	Ecuador	India	Indonesia	Thailand	Vietnam
Ecuador	17	0	0	1	0
India	2	0	0	0	0
Indonesia	0	0	0	0	0
Thailand	0	0	0	0	0
Vietnam	0	0	0	14	0
Overall					50%

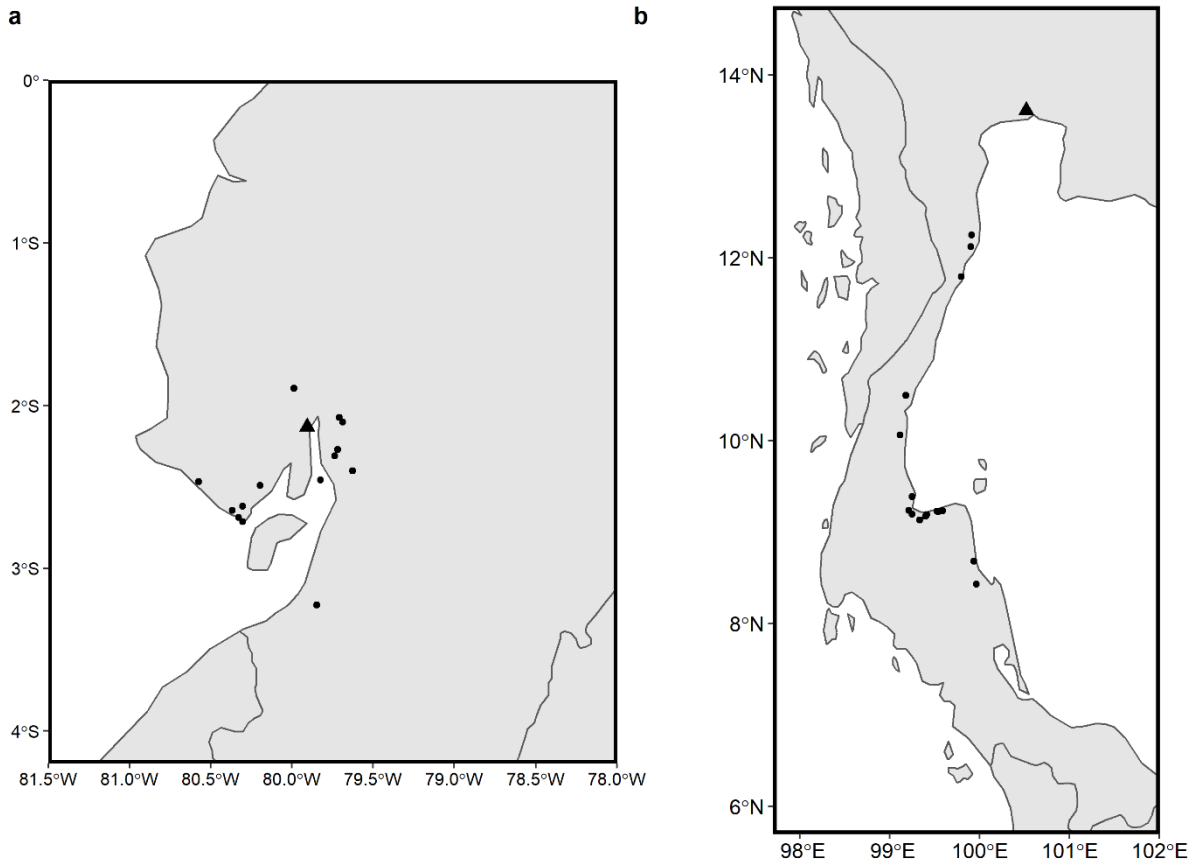

Figure 1. A map of sampling locations of the farms in Ecuador (a) and Thailand (b) from which paired pre/post processing samples were obtained. Triangles represent Guayaquil and Bangkok, on each map, respectively.

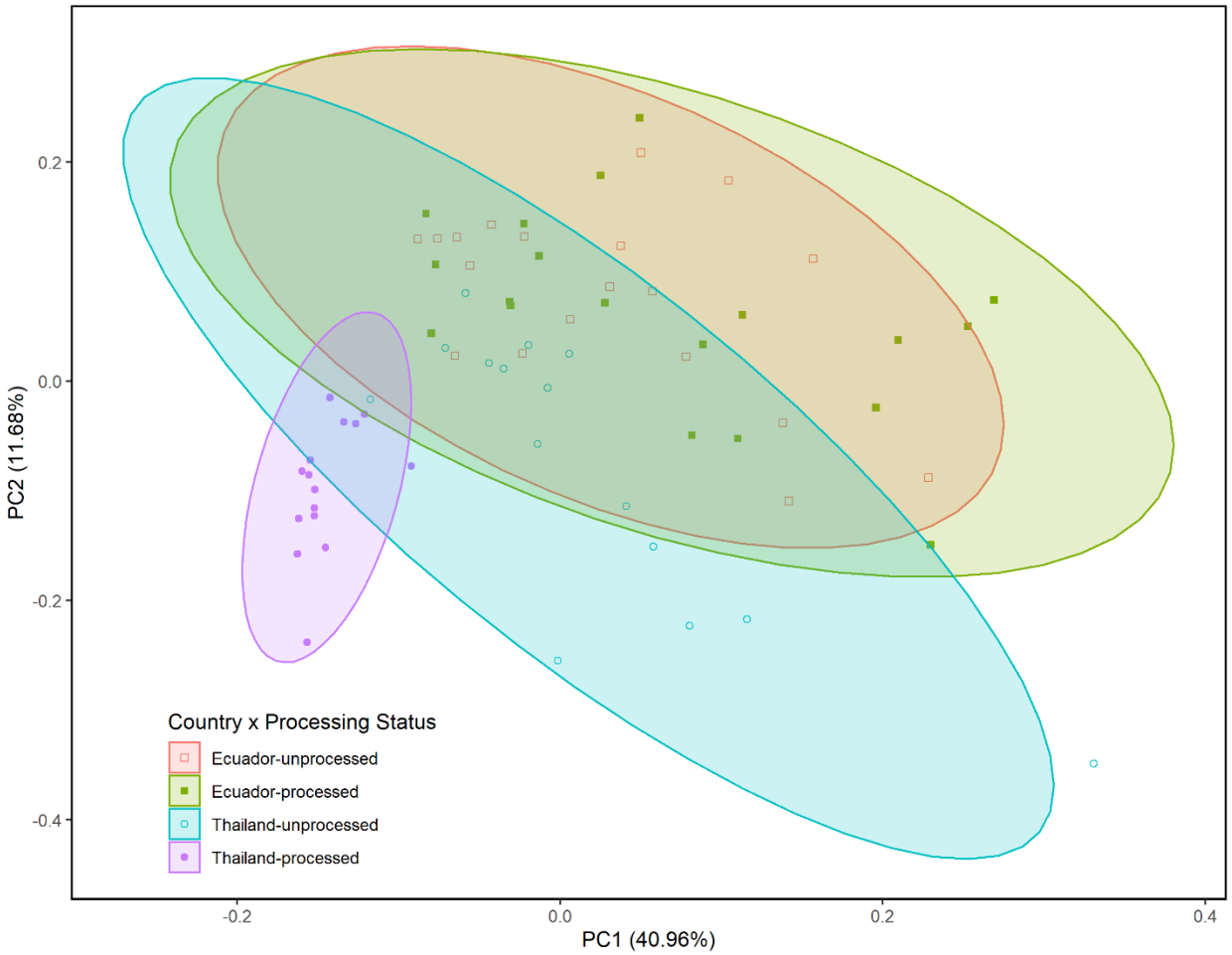

Figure 2. A biplot of the first two principal components of the principal components analysis of the pre/post processed paired samples. Unique country x processing subsets are represented by different color/shape combinations. Ellipses represent the normal ellipse in multivariate space. The percentage of variation explained by each principal is presented on each axis.

CHAPTER 6

SOURCES OF VARIATION IN ELEMENTAL PROFILES AND THEIR EFFECTS ON THE ACCURACY OF DISCRIMINANT ANALYSES

6.1 Abstract

Elemental profiling is a tool that has been proposed to improve the traceability of seafood products. Small sources of variation can affect the outcome of elemental profiling and therefore pose to lower the overall accuracy of analyses. Here, we investigate two potential sources of variation through three experiments: laboratory variation (intra-, interlaboratory variation, and tissue matrix) and tissue variation. Samples were obtained from 20 farms in Ecuador and 2 farms in Alabama to be analyzed. In the first experiment of the study, samples from Ecuador were analyzed at 3 different labs and compared. Two out of the five elements reported were statistically different across the three labs (Cu and Se). In the second experiment, the effect of tissue matrix (ground vs whole tissue during acid digestion) was investigated. Altogether, 5 out of 29 elements analyzed were statistically different. In the third experiment, samples from two farms in Alabama were analyzed to understand the variation in element concentrations in different tissues (head on shell on (HOSO), headless shell on (HLSO), headless peeled (PLD) and headless peeled and deveined (PLDV)). Elemental concentrations varied across tissues, and patterns in elemental concentrations were site specific. The samples from the two farms were analyzed with a random forest classification model to site x tissue groupings with 94 % accuracy. The implications of these results are discussed in the context of practical applications of elemental profiling, and recommendations for future attempts involving elemental profiling with shrimp are given.

6.2 Introduction

The world's population is projected to grow to 9 to 10 billion people by 2050 and the need for edible proteins is estimated to increase at an even higher rate (FAO 2017). Seafood is an important source of protein and healthy fats (e.g., Omega 3's) for many people and constitutes roughly 20% of current global animal meat production (Edwards et al. 2019). Seafood products are derived either from wild capture fisheries or aquaculture. Capture fisheries have plateaued (FAO 2020) whereas aquaculture continues to expand and has surpassed fisheries production in terms of volume for human consumption.

Despite the importance of seafood to the human food supply chain, seafood products are plagued by fraudulent labeling which has been well documented in the United States and the European Union. Mislabeling occurs to manipulate prices (Jacquet and Pauly 2008), improve marketing (i.e., "sustainability") (Miller et al. 2012), substitute farmed species for wild species (Korzik et al. 2020), and meet processor quota demands. In aquaculture shrimp products, transshipping has been an issue in the past, resulting in incorrectly labeled country of origin among retail products.

Elemental profiling has been identified as a tool to increase traceability in seafood products (Gopi et al. 2019a, Leal et al. 2015, Li et al. 2016). Elemental profiling (or elemental fingerprinting, trace element analysis, etc.) involves analyzing samples from pre-determined groupings for element contents to discern if differences in the element concentrations can be used in conjunction with discriminant analyses to assign group membership. Elemental profiling has been used to determine geographic origins (e.g., Albuquerque et al. 2016, Gopi et al. 2019b, Li et al. 2017), production origin (Anderson et al. 2010, Chaguri et al. 2017), and salinity of shrimp ponds (Li et al. 2019) with a high degree of success. It has been successful in a wide variety of

taxa as well, such as crustaceans (Luo et al. 2019), mollusks (Bennion et al. 2019), fish (Varra et al. 2019), and marine algae (Hattori et al. 2009).

Elemental profiling has been limited in scope by several practical constraints, even though a growing body of research suggests it has utility as a tool to improve traceability and discern the geographic origins of seafood products. The ability to use multiple labs in the same dataset is understood to be complicated by laboratory variation in the results of an analysis, and even within the same laboratory results may differ due small changes in operating procedures. Additionally, the sample matrix may play a role in the results of the laboratory analysis. Li et al. (2014) found that shrimp tissues concentrations varied based on what tissues of white leg shrimp *Litopenaeus vannamei* were included in the digestion, but made no effort to discern the effect of tissue on the ability to separate geographies.

Altogether, small sources of variation such as interlaboratory variation or tissue matrices may interfere with the ability to accurately discriminate samples via elemental profiling. Therefore, the objective of this study is to assess the influence of different external factors on the elemental concentrations of shrimp muscle tissue with the goal of informing practical applications of elemental profiling. The first objective (henceforth, experiment 1) is to perform a laboratory ring test (e.g., Popot et al. 2017), with shrimp muscle tissue to understand the effect of laboratory variation on the accuracy of a discriminant analysis. The second objective (experiment 2) will be to understand the effect of sample matrix condition (e.g., ground vs. whole) on the elemental profile. The third objective (experiment 3) is to discern the effect of tissue on the accuracy of a discriminant analysis, with samples from two locations and four unique tissue combinations. These three experiments are summarized in table 1 for clarity.

6.3 Methods

6.3.1 Experiment 1 – Laboratory Ring Test

Experiment one seeks to understand the underlying variability in analysis from different analytical labs and how these would undermine elemental profiling. Shrimp were sampled from 20 shrimp farms in close geographical proximity in Ecuador in the Guayas and Santa Elena provinces (figure 1). These shrimps were collected in a manner consistent with Li et al. (2017). Samples of ~ 30 shrimp were obtained at one pond each on the 20 selected farms via cast net, placed in labeled plastic bags, and transported to a laboratory for processing. At the laboratory, shrimp samples were deheaded and peeled. The peeled tail muscles were dried in a commercially available food dehydrator at 50 C° to constant mass, at least 12 hours. The samples were then stored frozen until analysis.

Three labs were chosen to be included in this ring test; an international commercial lab, a US based commercial lab, and a university lab. For each sample of shrimp, a sub-sample of 3-5 shrimp were ground with an analytical ball mill (Cole-Palmer, Vernon Hills, Illinois USA) with a carbide blade. From this pool, the required amount of sample from each lab was sent for analysis. All three labs used microwave acid digestions followed by Inductively coupled plasma spectrometry to measure concentrations of elements in the shrimp muscle tissue. In addition, 20 replicates of a ground homogenized sample of >50 shrimp culture at CPMC were analyzed at lab one to evaluate intralaboratory variation.

6.3.2 Experiment 2 – Tissue Digestions

The second experiment seeks to understand if the state of the tissue upon digestion affects the elemental profile. For this experiment, the 20 samples from the farms in Ecuador described in Experiment 1 were utilized again. Two sets of samples were sent to be analyzed; one where the

tissue was ground prior to acid digestion as described in experiment 1, and a second where the tissue was not mechanically ground prior to digestion. In preparation for digestion, samples were freeze dried overnight to remove any residual moisture. The digestion of the shrimp tissue was done following an adaptation EPA method 200.8 (US EPA 1994) for solid samples (Environmental Express 2018). Briefly, 0.5g of dried sample was digested with 2.0 ml of 1:1 Nitric Acid and 5.0 ml of 1:4 Hydrochloric Acid and refluxed in an Environmental Express Hotblock (TM) (HotBlock 200, Environmental Express, Charleston SC USA) for 30 minutes at 85 C. Upon cooling, the samples were quantitively transferred and brought to volume in 50 ml volumetric flasks. Samples were then centrifuged at ambient temperature for 5 minutes and decanted to remove any insoluble material in the solution. The digestion method was verified by validating recovery of a spiked quality control standard (recovery between 80-120%), determining the limits of detection, and repeatability of measurements with 15 replicants of a quality control standard (relative standard deviation <20%).

A NexION 350d ICP-MS (PerkinElmer Inc., Waltham MA USA) was used to conduct the elemental analysis for this study. Forty-two elements were analyzed for this study: Ag, Al, As, B, Ba, Ca, Ce, Cd, Co, Cr, Cs, Cu, Dy, Eu, Er, Fe, Gd, Ho, K, La, Li, Lu, Mg, Mn, Mo, Na, Nd, Ni, Pb, Pr, Rb, Sb, Se, Sm, Sn, Sr, Tm, U, V, Y, Yb, and Zn. Several steps were taken to ensure consistency between runs and within runs. The instrument used for the analysis was recalibrated with a two-point calibration each day. Each run on the instrument consisted of samples, three blanks, a lab-generated matrix matched quality control sample run in triplicate, and three other quality control materials including one replicate of a certified reference sample oyster tissue (tissue NIST1566B, MilliporeSigma, St. Louis MO USA). Two of the three quality control replicates were aqueous solutions with known quantities of each element-in the

appropriate range, and the third was a salmon flesh quality control matrix-matched sample that was previously validated with recoveries between 80-120%, and a relative standard deviation of <20% for all elements. A small number of samples were duplicated across runs to ensure consistency between runs on the instrument. Parameters of performance in relation to the limits of detection (LoD) for each element were calculated as blank average plus three times the standard deviation of the blanks.

6.3.3 Experiment 3 – Tissue Type

The third experiment included in this study was designed to understand the variation in elemental profiles across different tissues in shrimp. For this experiment, samples were obtained from two locations; Claude Peteet Mariculture Center in Gulf Shores Alabama and Green Prairie shrimp farms in the Black Prairie region of Alabama in the western part of the state, henceforth referred to as West Alabama (figure 2). This region has a saline aquifer which allows for shrimp culture at low salinities (Pine et al. 2018, Roy et al. 2010). Shrimp were taken from each pond at the two locations near the intended harvest date, with shrimp averaging over 20g at each location. Thus, there were 15 samples from Gulf shores and 20 samples from West Alabama. Samples were collected in the manner described in experiment 1. Upon collection and transportation to the laboratory for processing, a subset of three to five shrimp were dried as one of the following tissue groups; head-on shell-on (HOSO), headless shell-on (HLSO), headless peeled (PLD), and headless peeled and deveined (PLDV), similarly to Li et al. (2014). Upon drying to constant mass in a drying oven, samples were ground with an analytical mill (Cole-Palmer, Vernon Hills, Illinois USA) and subsequently digested and analyzed in the same manner as experiment 2.

6.3.4 Statistical Analysis

The resulting elemental concentrations from all laboratories and experiments described above were collected and screened for elements below detection limits. In each dataset, if more than 20% of the samples for a given element were below detection limits, the element was omitted from the analysis. As summarized in table 1, this means that for example, although the samples were analyzed for 12 elements, only the results of five are used in the statistical analysis and reported. In elements where >80% of the samples were above detection limits, any values that were not were replaced with a value at half of the detection limit. The detection limits for the three labs in experiment 1 are reported in table 2, and the detections limits for experiments 2 and 3 will be reported in tables that contain the results for those experiments.

Experiment 1

The results of five elements, As, Cu, Fe, Mn, and Se are reported with the means and standard deviations. The laboratories were compared using a repeated measure multivariate comparison. The test statistic used for this comparison was developed by Friedrich and Pauly (2018), and is robust to heteroscedasticity. The reported statistic is described by Friedrich and Pauly (2018) as a "modified ANOVA type statistic" (MATS), and the p value is derived by a parametric bootstrap procedure. Following the multivariate procedure, univariate repeated measures analysis of variance (ANOVAs) was used to determine which elements were different between labs. A Bonferroni corrected p value of $\alpha = 0.05/5 = 0.01$ was used for the comparison to determine significance. If elements were statistically significant, the ANOVA was followed with a post hoc comparison using a Games Howell test, which is robust to heterogeneity in variation in comparison to other pairwise comparison procedures (Lee and Lee 2018). A significance level of $\alpha = 0.05$ was used to determine significance in the post-hoc

pairwise comparisons. Data were scaled and centered prior to analysis. The results of the homogenized replicated samples analyzed at lab one was summarized with the mean, standard deviation, and coefficient of variation.

Experiment 2

A repeated measure multivariate comparison as described in experiment 1 was used to analyze the multivariate data in experiment 2. If a significant difference was determined by the MATS statistic, paired sample t-test were used to determine if univariate differences existed in elements in the ground vs. solid samples. A Bonferroni adjusted p-value was used to determine significance (0.05/29 comparisons = $\alpha = 0.0017$). Data were scaled and center prior to analysis.

Experiment 3

Data from the two farms in Alabama were summarized for each element via the mean and standard deviation. A Two-way repeated measures multivariate comparison (i.e., a MANOVA with the MATS statistic described above) was conducted with the main effects being location and tissue type. Based on the results, univariate repeated measures ANOVA tests were conducted for each element at the two separate farms to determine differences in tissue type because of the interaction between location and tissue type. A Bonferroni corrected p value of 0.05/32 = 0.0015 was used to determine significance of tissue type at each farm. Following the ANOVA tests, games Howell pairwise comparisons were used to determine any significant differences between tissue type in each element at each location.

Following exploration of the data with univariate tests, classification of the of the tissue x location groupings was conducted with a Random Forest classification in the “caret” package in R using the method native to the “ranger” package (Wright and Ziegler 2017). Recursive feature selection was used to determine the best combination of variables for the random forest.

Recursive feature selection ranks the importance of variables based on their contribution to classification models and subsequently eliminates less important variables to find the most informative subsets of variables (Guyon et al. 2002). The random forest was conducted with the following subset of elements after recursive feature selection; As, Ba, Ca, Cs, Cu, Mg, Mn, Na, Sm, Sr, and Y. Data was centered and scaled prior to classification to remove any effects of magnitude in the element concentrations. The expected accuracy based on the formula in Poulin and Kamiya (2015) is reported for reference for the results of the classification procedure. The accuracy of the model was assessed with k-fold cross validation where k =10.
The model Random forest classification models were used because of the lack of distributional requirements for the technique, robustness to overfitting high dimensional data, and the power of the model to obtain good fits when there are no strong predictor variables (Breiman 2001). Other models that were explored were linear discriminant analysis and K-
nearest neighbors classification.

Following the classification procedure with the location and tissue type, a canonical discriminant analysis (CDA) was utilized to visualize the multivariate variation in the data. CDA is a dimension reduction/ordination technique similar to a principal component analysis, however a CDA maximizes the differences in variation in specified groupings (Matthew et al. 1994), which in this case will be tissue x site combinations. The elements selected by the recursive feature selection in the classification analysis were included in the CDA. All statistical analysis were conducted in R version 4.0.3 (R Core Team 2020). When not specifically noted, a p value of $\alpha = 0.05$ was used to determine significance for all analysis across the three experiments.

6.4 Results

6.4.1 Experiment 1 – Interlaboratory variation

A statistical analysis showed that the element concentrations in shrimp muscle tissue from 20 farms in Ecuador, that were analyzed at three laboratories, were significantly different (MATS = 34.582, $p < 0.0001$). After univariate analysis, Lab 2's concentrations of Cu and Se were significantly higher than Lab 1 and Lab 3's (table 3). The concentrations of As, Fe, and Mn, were similar across all three labs. In all cases except Mn, Labs 1 and 3 had lower standard deviations and were similar, while Lab 2 had higher standard deviations for the same element. The mean concentrations of the homogenized shrimp samples that were replicated 20 times at Lab 1 are reported in table 4. The coefficient of variation ranged from 0.07 – 0.35 and averaged 0.16 across the five elements.

6.4.2 Experiment 2 – Tissue digestion

The multivariate analysis revealed that there were significant differences in the element concentrations between the ground and whole samples (MATS = 648.77, $p < 0.001$). Univariate tests revealed that 5 elements were statistically different between the two datasets; K, Mg, Na, Rb, and Zn (see table 5). In all cases, the concentrations in the whole tissues were statistically higher than the ground samples.

6.4.3 Experiment 3 – Tissue matrix

In experiment three, both location and tissue type were significantly different, as well as the interaction (see table 6). Univariate comparisons were done at each location separately, and the results are presented in table 7 and 8. Several patterns emerge at both locations. In Gulf Shores, the most common pattern of tissue concentrations among elements were

HOSO=HLSO=PLD>PLDV, which occurred in 13 elements (Al, Ce, Er, Fe, Gd, La, Li, Nd, Pr, Sm, V, Y, Yb). The next most common pattern was HOSO>HLSO>PLD=PLDV, which occurred in 4 elements (Ca, Cu, Mg, Sr). In West Alabama, the most common pattern among elements was HOSO>HLSO=PLD>PLDV, which occurred in 12 elements (Ba, Ce, Ca, Cr, Cu, Fe, Li, Ni, Sm, V, Y, Yb), while the second most common pattern was HOSO=HLSO=PLD>PLDV (6 elements, Cs, Er, Gd, La, Nd, Pr). Between the two sites only 10 out of the 32 elements above detection limits had the same pattern of distribution between the different tissue groupings based on post hoc comparisons. In general, elemental concentrations were higher in West Alabama than Gulf Shores, with 18 of the 32 elements being numerically higher in the same tissues. In contrast, 7 of the 32 elements were higher in Gulf Shores when compared to the same tissues as shrimp from West Alabama, while 7 of the elements were roughly the same between the two locations.

To understand the variation in the dataset in multivariate space, a CDA was conducted with the elements reported in the methods that were used for classification. In the first two canonical variables (CV), a separation of both location and tissue type occurs (see figure 3). The two locations mostly separate across the first CV, which is most highly associated with Y, Sm, Cs, and Ba (see table 9). Tissue type separates across the second CV which is most strongly associated with Ca, Y, Cu, and Cs.

A random forest classification model was used to discriminate location x tissue combinations between the two farms in Alabama. Overall, the model had an accuracy of 94%, with no samples being misclassified between the two locations (table 10). In all cases of misclassification, HOSO and HLSO or PLD and PLDV samples were misclassified as one another from the same location. The expected accuracy of the model based on random chance

was 10.5 %. The most misclassified sample was the Gulf Shores PLD samples, which were misclassified as Gulf Shores PLDV samples 20% of the time.

6.5 Discussion

Elemental profiling is receiving considerable attention as a tool for traceability in seafood supply chains and has been demonstrated to work in the literature with good to great success (Davis et al. 2021). However, little to no attention has been paid to the underlying assumptions of the technique, or practical considerations in implementing an elemental profiling scheme as part of a traceability program. Here, two sources of potential variation that could affect profiles, laboratory analyses and the elemental distribution among tissues are examined.

Many studies that analyze elemental profiles are highly controlled due to the complex nature of multivariate data analyses and sensitivity of the analysis to variation. However, applications in a practical context would be more complicated. Samples may not be analyzed all at once, introducing the potential for intralaboratory variation due to small minute differences between runs, even on the same instrument (Grothe and Kimerle 1985, Lengyel et al. 1996). Additionally, customs regulations may make it difficult to analyze the samples at the same laboratory in all cases, introducing the potential for interlaboratory variation. Here, we examined the potential for both interlaboratory and intralaboratory variation with samples from Ecuador being analyzed at three different labs and a homogenized sample being analyzed in 20 replicates at the same lab.

Challenges arose analyzing samples between labs. Limits of detection were different between all three labs, and even though 12 elements were quantified at all three labs, only a total of five were above detection limits 80% of the time across the labs. Among the five elements that were consistently quantifiable, two of them showed significant variation among the three labs,

while three were relatively similar. This lack of data availability limited the author's ability to attempt classification models with the data to understand if the variation impacted the accuracy of classification, and therefore a more detailed analysis was not undertaken. This presents an issue to any organization that would attempt to carry out an elemental profile "database" in a traceability application. One possible reason lab two was so different was that different digestion methods can produce different elemental concentrations in the same tissue samples (Ashoka et al. 2009), and the lab methodologies were not standardized here besides the overall goal of obtaining elemental tissue concentrations on the same samples. In the homogenized sample that was analyzed in 20 repeated replicates, there was still high coefficients of variation, suggesting that even a homogenized sample analyzed in the same laboratory can be variable when it comes to elemental profiles.

Another source of variation in the laboratory analysis of elemental profiles appears to be the state of the tissue sent for analysis. Five out of the 29 elements that were above detection limits were statistically different, which is about 17%. In other elements that were not statistically different, the means of the whole tissue samples tended to be numerically higher than the ground samples. Few studies exist examining the effect of the state of the tissue upon digestion. Zarcinas et al. (1987) found that ground wheat grains did not vary compared to whole wheat grains when digested in a nitric-perchloric acid digestion. While the profiles largely did not vary in the 20 samples analyzed here, this is a concern if laboratory methods are changed, or if different labs use different methods.

Some elemental profiling studies have investigated the use of different tissue groupings in the same animals (e.g.,Bennion et al. 2019, Clarke et al. 2007). Here, we investigated the change in elemental concentrations in four different subsets of shrimp tissue; whole shrimp

(HOSO), headless tails (HLSO), peeled tails (PLD), and peeled deveined tails (PLDV). Several patterns emerged from the two farms that were investigated. Overall, the PLDV shrimp tail muscles were the least mineralized tissue in the shrimp. This is not surprising to say the least and is consistent with another study that was done with shrimp from these two locations (Li et al. 2014), but does suggest that shrimp tail muscles may be the least useful tissue for elemental profiling. This is unfortunate for practical applications, as many shrimp sold in grocery stores in the domestic market in the United States are peeled and deveined. In other major markets such as the EU or Japan, this is likely less of an issue as shrimp are sold whole more often. Many of the elements did not exhibit the same patterns across both farms, which suggests that the distribution of micronutrients and trace elements, especially ones that are not regulated metabolically like Na or K, are site dependent. This partially validates one of the assumptions of elemental profiling; that the profiles are a reflection of inherent variation due to environmental factors. However, Li et al. (2014), which analyzed shrimp tissue from the two farms used in this study, was largely unable to correlate pond water element concentrations to shrimp tissue concentrations. While water was not taken as part of this study, a comparison of elemental concentrations that were higher in the water at a given site as measured in Li et al. (2014) and the shrimp tissue concentrations in this study that were higher at a site did not reveal any specific pattern (i.e., the patterns seen here in which elements were higher or lower in concentration at Gulf Shores vs. West Alabama did not correspond to the elements being higher or lower in the water at the same sites in the study by Li et al. in 2014). The other major source of minerals for shrimp in these ponds is natural productivity, which can be a significant portion of a shrimp's diet (Cardona et al. 2015, Gamboa-Delgado 2014), and it is less understood what role this plays in influencing elemental profiling.

The classification analysis of the shrimp samples from Gulf Shores and West Alabama had an overall accuracy of 94%, with the most common error being the PLD and PLDV being classified as the other (see table 10). The misclassification of PLD and PLDV samples for each other suggests that these are indeed similar, which is confirmed by the CDA showing that the center of the groups do not separate well in in reduced dimensions (figure 3). However, due to the differences in the elemental concentrations on a univariate basis, there are likely differences that may affect elemental profiling at a broader scale, although this could deserve more attention.

Based on the findings here, the authors have several recommendations for future investigations regarding elemental profiling. The simplest approach to overcoming the obstacle of laboratory error and variation would be to use the same laboratory for all analyses and use a laboratory that is familiar with elemental profiling and can safeguard against small variations in the profile that may affect the usefulness of the samples. This is likely not feasible in many situations due to logistical constraints. Therefore, if multiple labs are needed, correction factors could be used to correct the data to known values from the principal lab that is used for the analysis, or elements that are known to be deviant (in this case, Cu and Se), can be eliminated from the subsequent elemental profiling analysis. The standardization of digestion method can also improve laboratory analysis, as this has been shown to affect resulting element concentrations as well (Ashoka et al. 2009).

In terms of elemental profiling with shrimp, it is not recommended to mix different tissue subsets, as there can be major differences in the elemental tissue concentrations, and differences in the patterns among elements in different tissues. While the tail muscle deveined is the least mineralized tissue in shrimp, it may be the most practical, due to it being the least common denominator. As an example, shrimp that are HOSO can always be processed further to become

PLDV, but shrimp that are PLDV cannot be reassembled. Based on the data in this study, it is likely that small differences in PLD and PLDV tails would preclude them from being used in the same analysis, although this needs to be examined in more detail at a broad geographic scale. Altogether, these results show the utility of exploring basic underlying principles of elemental profiling that may ultimately affect the "bottom line" result of an analysis. Given that the mineral digestions tend to be relatively expensive, any ancillary variation in elemental profiles that creates more "noise' in the analysis warrant further investigation in the future to progress and refine the technique.

6.6 Conclusions

This study sought to understand potential sources of variation in elemental profiles that are not related to the natural variation that the analysis is attempting to capture and use to discriminate samples in pre-determined groupings. We found that both intralaboratory variation and intralaboratory variation are significant enough to affect practical applications of elemental profiling. Additionally, elements varied when the tissues were ground vs. whole, further complicating interlaboratory variation. Finally, we examined differences in tissues, and found that tissues at two sites did not exhibit the same patterns in element concentrations, suggesting that patterns in element concentrations are not only tissue dependent but also spatially variable. Altogether this work progresses the underlying understanding and basis of elemental profiling.

6.7 References

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Table 1. A summary of the three experiments in this study.

Experiment	Source of Variability	Sample Origin	n	Number of Elements Analyzed	Number of elements reported	Number of labs
one	laboratory	Ecuador, Alabama	20	12	5	3
two	tissue condition (ground vs. whole)	Ecuador	20	42	29	1
three	tissue type	Alabama	20	42	32	1

Table 2. The limit of detection for each laboratory expressed in mg/kg of dry tissue.

Element	Lab 1	Lab 2	Lab 3
As	0.05	0.001	0.10
Cu	0.02	0.02	0.20
Fe	0.4	0.05	5.00
Mn	0.02	0.01	0.50
Se	0.05	0.001	0.10

Table 3. Mineral concentrations of 20 samples from Ecuador analyzed at the three labs in experiment 1. Results are reported in mg/kg of dry tissue and sd= standard deviation. Elements that are statistically different are bolded.

Element	Lab 1	sd	Lab 2	sd	Lab 3	sd	p value
As	1.17	0.60	1.75	0.90	1.34	0.64	0.043
Cu	**26.12[b]**	**3.63**	**32.72[a]**	**7.55**	**27.01[b]**	**3.99**	**<0.001**
Fe	122.31	115.70	143.89	176.38	160.39	115.66	0.687
Mn	8.99	21.39	9.70	14.32	8.59	16.85	0.98
Se	**0.89[b]**	**0.23**	**1.22[a]**	**0.32**	**0.92[b]**	**0.23**	**<0.001**

Table 4. Elemental concentrations of a homogenized sample obtained from shrimp reared at CPMC in Gulf Shores, Alabama. The results are reported in mg/kg dry tissue and sd= standard deviation and CV = the coefficient of variation.

Element	Reference (n = 20)	sd	CV
As	1.30	0.14	0.10
Cu	11.61	1.39	0.12
Fe	48.57	17.21	0.35
Mn	0.97	0.18	0.19
Se	1.08	0.08	0.07

Table 5. The mean concentrations of elements of 20 shrimp samples obtained from Ecuador, analyzed ground and whole (solid). The limit of detection for each element is parentheses next to the element name. The results are reported in mg/kg dry tissue and sd = standard deviation. Elements that are statistically different are bolded.

Element	Ground		Solid		P value
	mean	sd	mean	sd	
Al (0.4)	120.1	101.3	150.5	124.9	0.403
As (0.05)	1.17	0.60	1.66	0.71	0.024
B (0.4)	2.03	1.02	2.08	1.36	0.879
Ca (10)	1154.0	502.6	1359.0	697.6	0.293
Ce (0.0004)	0.069	0.049	0.099	0.092	0.206
Co (0.01)	0.061	0.047	0.078	0.062	0.346
Cr (0.05)	0.318	0.216	0.267	0.218	0.464
Cs (0.002)	0.022	0.008	0.027	0.012	0.142
Cu (0.02)	26.12	3.63	27.55	5.65	0.347
Dy (0.0004)	0.008	0.006	0.011	0.009	0.223
Er (0.0004)	0.004	0.003	0.006	0.005	0.23
Fe (0.4)	122.3	115.7	178.3	152.5	0.199
Gd (0.0004)	0.009	0.006	0.012	0.01	0.231
K (10)	**12376.8**	**744.1**	**17466.2**	**1399.9**	**<0.0001**
La (0.002)	0.032	0.023	0.045	0.042	0.226
Li (0.002)	0.156	0.093	0.182	0.108	0.421
Mg (2)	**1363.0**	**109.9**	**1952.7**	**147.2**	**<0.0001**
Mn (0.02)	8.99	21.39	10.85	20.03	0.778
Mo (0.01)	0.052	0.023	0.040	0.015	0.067
Na (10)	**5145.3**	**809.5**	**7014.9**	**1241.7**	**<0.0001**
Nd (0.0004)	0.037	0.026	0.055	0.049	0.148
Ni (0.02)	0.205	0.139	0.163	0.129	0.321
Pr (0.0004)	0.009	0.006	0.012	0.011	0.19
Rb (0.005)	**3.27**	**0.62**	**4.52**	**0.88**	**<0.0001**
Se (0.05)	0.890	0.232	1.130	0.294	0.007
Sm (0.0004)	0.008	0.006	0.012	0.010	0.209
Sr (0.004)	11.65	6.67	13.34	9.13	0.507
V (0.04)	0.307	0.222	0.351	0.279	0.582
Zn (0.4)	**46.707**	**2.354**	**57.434**	**3.36**	**<0.0001**

Table 6. An ANOVA like table of the two-way repeated measures multivariate analysis of the site x tissue interactions of the shrimp samples from Gulf Shores and West Alabama.

Factor	MATS	P value
Location	5854.9	<0.001
Tissue	12705.0	<0.001
Interaction	2266.0	<0.001

Table 7. Elemental Concentrations of different shrimp tissues from shrimp harvested from ponds in Gulf Shores, Alabama. The limit of detection for each element are reported in parentheses next to the element. The lettering represents univariate statistical differences based on a post hoc test. The results are reported in mg/kg of dry tissue and sd = standard deviation.

Element	HOSO	sd	HLSO	sd	PLD	sd	PLDV	sd	P value
Al (0.4)	65.21[a]	33.89	65.45[a]	39.38	50.17[a]	42.15	11.12[b]	9.07	<0.0001
As (0.05)	2.69	0.77	2.49	0.67	2.37	0.67	2.40	0.58	0.582
B (0.4)	2.11[a]	0.44	1.60[a]	0.27	1.17[b]	0.29	1.06[c]	0.20	<0.0001
Ba (0.01)	3.48[a]	0.62	2.59[b]	0.58	0.20[c]	0.12	0.08[d]	0.03	<0.0001
Ca (10)	25915.1[a]	2446.8	17614.7[b]	2964.8	1571.9[c]	493.9	1563.0[c]	586.5	<0.0001
Ce (0.0004)	0.061[a]	0.032	0.062[a]	0.036	0.056[a]	0.046	0.009[b]	0.007	<0.0001
Co (0.01)	0.039	0.013	0.022	0.009	0.034	0.017	0.038	0.017	0.006
Cr (0.05)	1.11	0.74	0.72	0.60	0.79	0.98	0.56	0.68	0.261
Cs (0.002)	0.038[c]	0.004	0.043[b]	0.004	0.046[a]	0.005	0.041[bc]	0.003	<0.0001
Cu (0.02)	91.40[a]	9.55	43.84[b]	5.67	26.49[c]	3.53	23.56[c]	3.07	<0.0001
Dy (0.0004)	0.004[b]	0.002	0.004[b]	0.002	0.003[b]	0.003	0.0005[a]	0.0005	0.0001
Er (0.0004)	0.002[a]	0.001	0.002[a]	0.001	0.002[a]	0.001	0.0003[b]	0.0002	<0.0001
Fe (0.4)	79.85[a]	35.13	71.84[a]	43.51	67.94[a]	57.65	14.80[b]	7.57	0.0001
Gd (0.0004)	0.005[a]	0.002	0.005[a]	0.003	0.004[a]	0.004	0.0006[b]	0.0005	<0.0001
K (10)	13164.0[c]	693.7	15158.0[b]	825.5	16586.9[a]	969.1	16193.1[a]	776.1	<0.0001
La (0.002)	0.028[a]	0.014	0.028[a]	0.017	0.024[a]	0.019	0.004[b]	0.003	<0.0001
Li (0.002)	0.114[a]	0.037	0.106[a]	0.05	0.087[a]	0.052	0.041[b]	0.01	<0.0001
Mg (2)	3066.1[a]	270.0	2498.5[b]	198.7	1708.9[c]	123.7	1669.0[c]	94.0	<0.0001
Mn (0.02)	3.52[a]	0.64	2.33[b]	0.47	1.20[c]	0.48	0.72[d]	0.13	<0.0001
Mo (0.01)	0.21[a]	0.05	0.09[b]	0.04	0.09[b]	0.11	0.08[b]	0.09	<0.0001
Na (10)	7684.6[a]	439.5	6256.4[b]	521.9	5868.1[b]	731.9	5936.7[b]	509.1	<0.0001
Nd (0.0004)	0.026[a]	0.013	0.027[a]	0.016	0.025[a]	0.020	0.004[b]	0.003	<0.0001
Ni (0.02)	0.504	0.148	0.275	0.157	0.384	0.581	0.340	0.484	0.046
Pr (0.0004)	0.007[a]	0.003	0.007[a]	0.004	0.006[a]	0.005	0.001[b]	0.001	<0.0001
Rb (0.005)	4.03[c]	0.23	4.41[b]	0.30	4.77[a]	0.36	4.70[a]	0.28	<0.0001
Se (0.05)	1.17[a]	0.08	1.04[b]	0.10	1.08[ab]	0.11	1.07[b]	0.08	0.0001
Sm (0.0004)	0.005[a]	0.003	0.005[a]	0.003	0.005[a]	0.004	0.001[b]	0.001	<0.0001
Sr (0.004)	343.74[a]	33.23	241.63[b]	39.10	14.64[c]	5.01	14.61[c]	6.07	<0.0001
V (0.04)	0.183[a]	0.073	0.141[a]	0.072	0.126[a]	0.083	0.041[b]	0.023	<0.0001
Y (0.001)	0.024[a]	0.009	0.023[a]	0.011	0.019[a]	0.015	0.003[b]	0.002	<0.0001
Yb (0.0004)	0.002[a]	0.001	0.002[a]	0.001	0.002[a]	0.002	0.0003[b]	0.0002	<0.0001
Zn (0.4)	58.41[a]	2.89	52.97[b]	3.66	58.11[a]	3.93	56.69[a]	3.59	<0.0001

Table 8. Elemental Concentrations of different shrimp tissues from shrimp harvested from ponds in West Alabama. The limit of detection for each element are reported in parentheses next to the element. The lettering represents univariate statistical differences based on a post hoc test. The results are reported in mg/kg of dry tissue and sd = standard deviation.

Element	HOSO	sd	HLSO	sd	PLD	sd	PLDV	sd	P value
Al (0.4)	153.89[a]	55.46	107.95[b]	34.30	101.25[b]	58.67	16.68[c]	9.90	<0.0001
As (0.05)	1.10[a]	0.22	0.82[b]	0.18	0.80[b]	0.20	0.71[a]	0.17	<0.0001
B (0.4)	1.52	0.50	1.28	0.37	1.12	0.37	1.09	0.36	0.005
Ba (0.01)	98.97[a]	32.36	65.17[b]	21.53	3.01[c]	0.77	2.54[c]	0.69	<0.0001
Ca (10)	34169.0[a]	2992.3	21884.1[b]	4216.8	2592.8[c]	451.4	2003.3[d]	332.1	<0.0001
Ce (0.0004)	0.364[a]	0.137	0.254[b]	0.096	0.247[b]	0.138	0.045[c]	0.034	<0.0001
Co (0.01)	0.113[a]	0.044	0.043[b]	0.014	0.039[b]	0.016	0.020[c]	0.015	<0.0001
Cr (0.05)	0.805[a]	0.309	0.523[b]	0.300	0.422[b]	0.231	0.129[c]	0.091	<0.0001
Cs (0.002)	0.016[a]	0.004	0.013[a]	0.003	0.014[a]	0.005	0.007[b]	0.002	<0.0001
Cu (0.02)	77.43[a]	13.79	43.08[b]	8.01	24.23[b]	4.96	23.93[c]	4.98	<0.0001
Dy (0.0004)	0.020[a]	0.008	0.014[b]	0.006	0.014[ab]	0.008	0.002[c]	0.002	<0.0001
Er (0.0004)	0.010[a]	0.004	0.007[a]	0.003	0.007[a]	0.004	0.001[b]	0.001	<0.0001
Fe (0.4)	190.41[a]	62.06	129.10[b]	42.17	122.93[b]	66.62	22.49[c]	11.45	<0.0001
Gd (0.0004)	0.026[a]	0.010	0.018[a]	0.008	0.017[a]	0.010	0.003[b]	0.002	<0.0001
K (10)	12387.9[c]	625.1	13740.9[b]	803.2	15331.1[a]	745.0	15062.7[a]	968.0	<0.0001
La (0.002)	0.145[a]	0.063	0.100[a]	0.041	0.099[a]	0.053	0.019[b]	0.013	<0.0001
Li (0.002)	0.235[a]	0.077	0.167[b]	0.051	0.156[b]	0.087	0.041[c]	0.019	<0.0001
Mg (2)	1914.2[a]	146.8	1702.3[b]	153.2	1476.7[c]	115.1	1465.2[c]	110.8	<0.0001
Mn (0.02)	7.83[a]	2.72	4.56[b]	2.18	2.55[c]	0.99	1.24[d]	0.462	<0.0001
Mo (0.01)	0.162[a]	0.027	0.057[b]	0.035	0.045[b]	0.015	0.038[b]	0.013	<0.0001
Na (10)	7124.4[a]	598.8	5610.0[b]	446.5	5320.0[bc]	470.0	5198.3[c]	498.4	<0.0001
Nd (0.0004)	0.158[a]	0.064	0.113[a]	0.048	0.109[a]	0.064	0.021[b]	0.019	<0.0001
Ni (0.02)	0.503[a]	0.128	0.218[b]	0.103	0.187[b]	0.092	0.072[c]	0.054	<0.0001
Pr (0.0004)	0.041[a]	0.016	0.029[a]	0.013	0.028[a]	0.017	0.005[b]	0.004	<0.0001
Rb (0.005)	3.42	0.51	3.55	0.57	3.96	0.67	3.85	0.63	0.123
Se (0.05)	1.17	0.16	1.07	0.19	1.21	0.20	1.21	0.20	0.085
Sm (0.0004)	0.037[a]	0.013	0.025[b]	0.009	0.021[b]	0.012	0.004[c]	0.003	<0.0001
Sr (0.004)	1062.0[a]	256.7	700.8[b]	205.4	52.7[c]	13.9	47.2[d]	12.4	<0.0001
V (0.04)	0.514[a]	0.210	0.312[b]	0.118	0.322[b]	0.181	0.058[c]	0.037	<0.0001
Y (0.001)	0.126[a]	0.045	0.087[b]	0.035	0.078[b]	0.044	0.015[c]	0.010	<0.0001
Yb (0.0004)	0.009[a]	0.003	0.006[b]	0.002	0.006[b]	0.003	0.001[c]	0.001	<0.0001
Zn (0.4)	60.18[a]	7.20	54.34[b]	5.37	61.41[a]	2.05	61.38[a]	2.29	<0.0001

Table 9. Factor loadings for canonical variables as a result of the CDA analysis of the shrimp samples from experiment three.

Element	CV 1	CV 2	CV 3	CV 4	CV 5
Sr	-0.45	-0.25	-0.37	-0.44	-0.06
Ba	0.68	0.26	0.03	-0.02	-0.52
Ca	-0.51	-0.63	-0.53	0.74	0.03
Cs	1.18	-0.44	-0.76	-0.01	0.17
Cu	-0.15	-0.53	0.50	-0.70	-0.12
As	-0.01	-0.01	-0.10	-0.02	-0.21
Mg	0.27	-0.34	1.03	0.21	0.01
Na	0.09	0.13	-0.09	-0.53	0.11
Y	1.09	0.54	0.72	0.55	1.06
Sm	-2.07	0.18	-0.56	-0.39	-0.23
Mn	-0.19	0.05	-0.21	-0.35	0.34
Eigenvalue	53.6	35.58	7.97	1.25	0.54
Variation Explained (%)	54.13	35.93	8.05	1.27	0.54
Cumulative Variation	54.13	90.06	98.11	99.38	99.92

Table 10. The results of a random forest classification analysis of the shrimp samples from Gulf Shores and West Alabama.

	Reference							
Prediction	GS HOSO	GS HLSO	GS PLD	GS PLDV	WA HOSO	WA HLSO	WA PLD	WA PLDV
GS HOSO	15	0	0	0	0	0	0	0
GS HLSO	0	15	0	0	0	0	0	0
GS PLD	0	0	12	1	0	0	0	0
GS PLDV	0	0	3	14	0	0	0	0
WA HOSO	0	0	0	0	20	2	0	0
WA HLSO	0	0	0	0	0	18	0	0
WA PLD	0	0	0	0	0	0	19	1
WA PLDV	0	0	0	0	0	0	1	19
Accuracy	100	100	80	93	100	90	95	95
Overall								94%

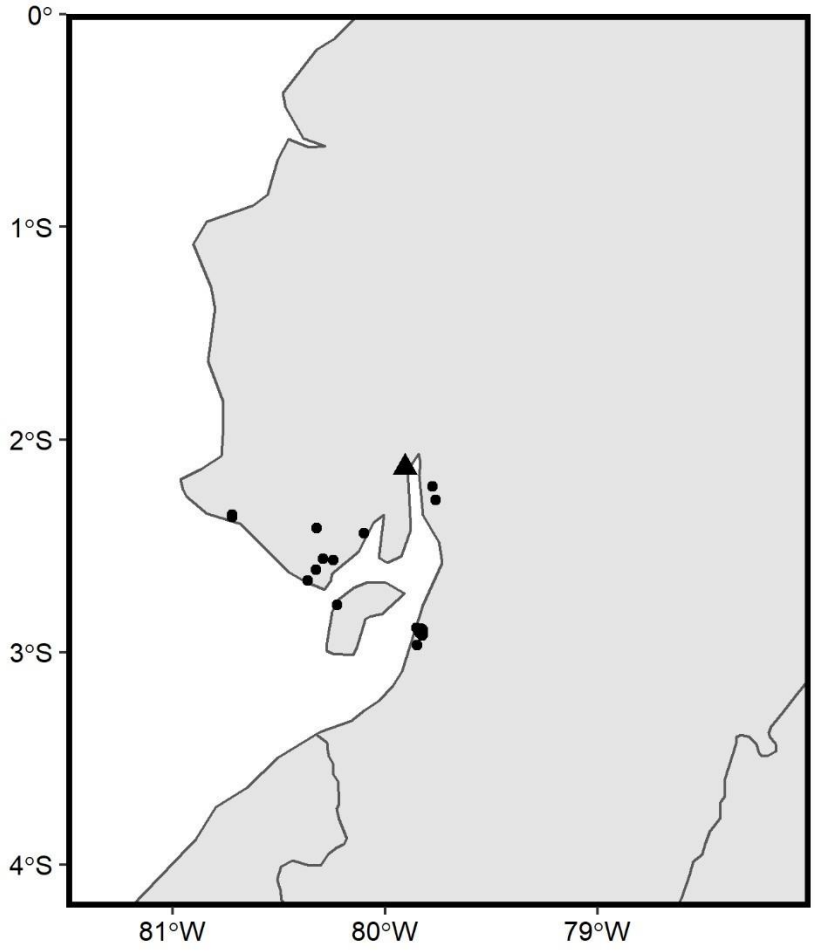

Figure 1. A map of the locations of the farms that were sampled from Ecuador in the Guayas and Santa Elena Province. The triangle represents the city of Guayaquil.

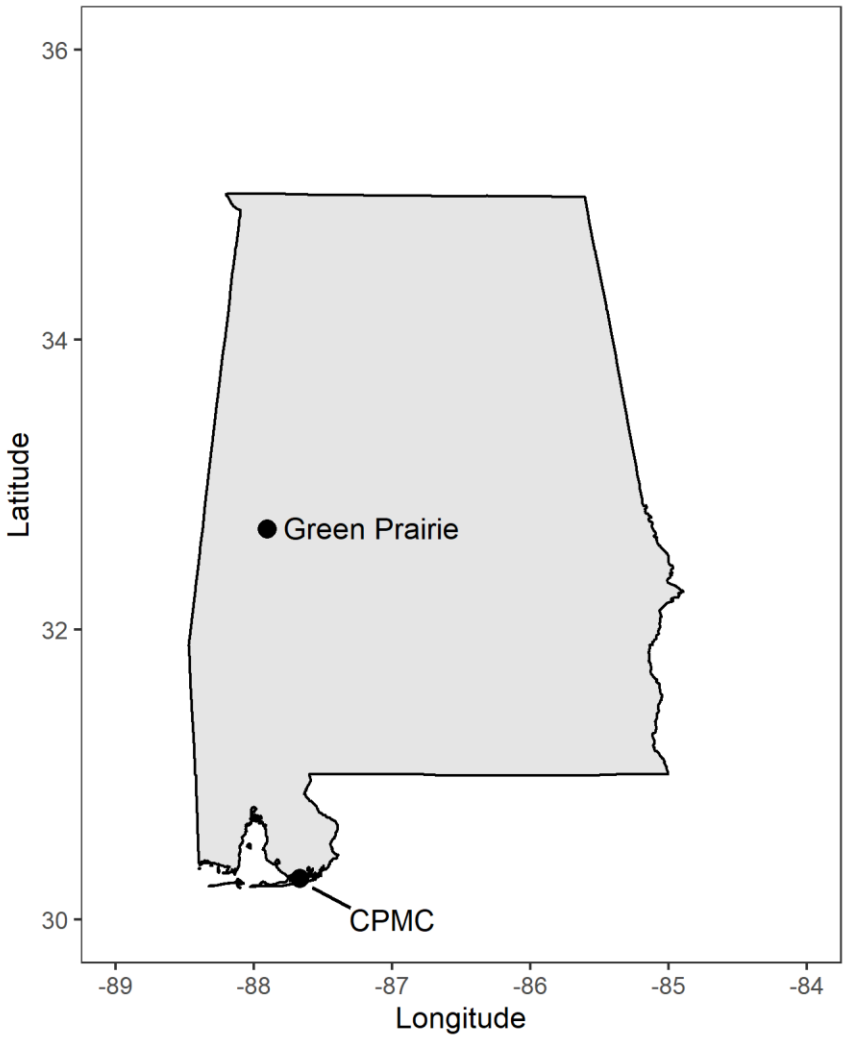

Figure 2. A map of the location of the two farms used in Experiment three from Alabama.

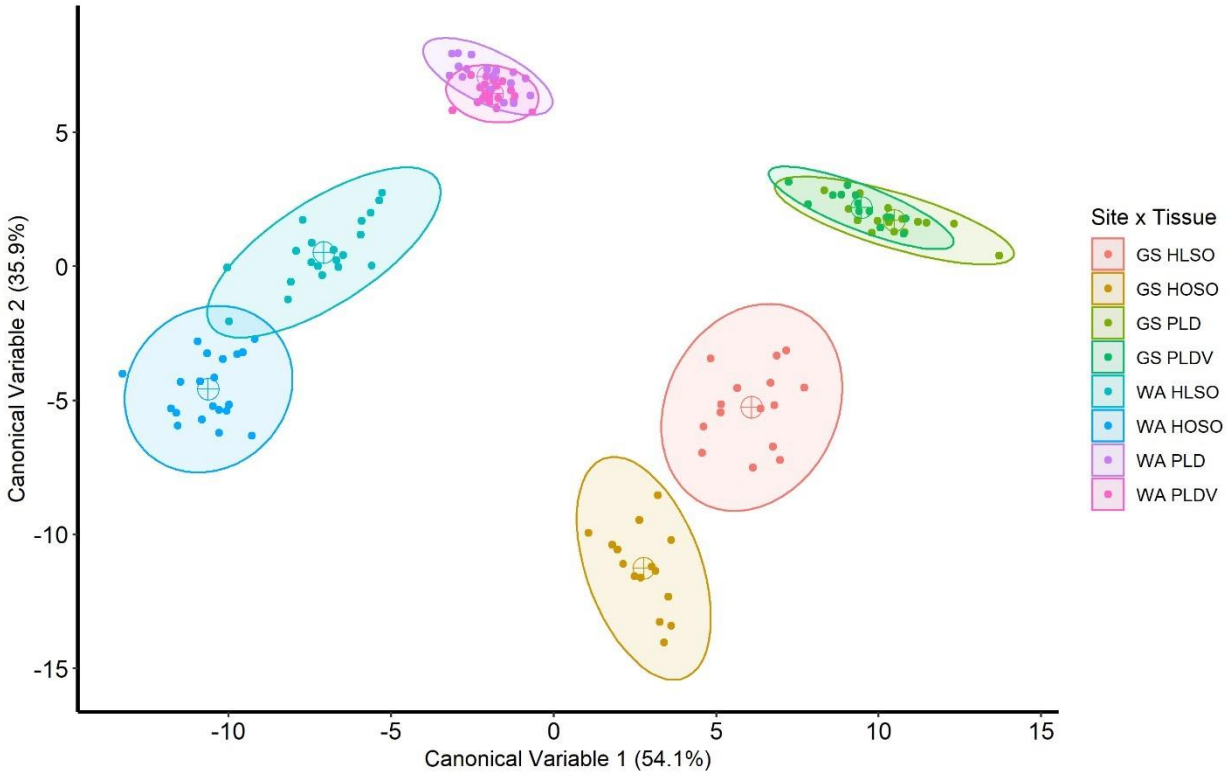

Figure 3. A biplot of the first two canonical variables resulting from the canonical discriminant analysis of the samples analyzed in experiment three. Colors represent site x tissue combinations. Locations are Gulf Shores (GS) and West Alabama (WA) and tissues are head on shell on (HOSO), headless shell on (HLSO), headless peeled (PLD) and headless, peeled and deveined (PLDV). Ellipses represent normalized group ellipses in two-dimensional space. Bullseyes represent group means in ordination space. The percentage of variation explained by each canonical variable is presented in parentheses.

CHAPTER 7

CONCLUDING REMARKS

Traceability is going to be a pressing issue in the future of a globalized seafood market. Labeling fraud in seafood is considered commonplace and has been documented in the USA and Europe. Labeling fraud, and therefore the loss of traceability, is a negative outcome not only for consumers, but producers and retailers as well. This research contributes to the goal of utilizing elemental profiling as a tool to improve traceability in seafood supply chains, especially shrimp. In Chapter 3, shrimp from five major production countries were separated with greater than 91% success laying the groundwork for elemental profiling efforts in the future. A first attempt was made to classify samples from the commercial retailers, although with less success, which is believed to be related to processing as explored in Chapter 5, and thus presents a challenge to a practical application of elemental profiling.

The research contained herein leaves several avenues for continuing to improve elemental profiling. The contribution of minerals (and which ones) by the local environment vs. food consumption is still not clear. Ingredients in commercial shrimp feeds could impact elemental profiles, although the extent of which has not been documented. Additionally, the relative contribution of feed and the environment has not been researched in a meaningful way and the scale at which the variation becomes enough to distinguish shrimp from different locations is unknown. In the meta-analysis (Chapter 2) contained herein of elemental profiling in seafood, scale was not a significant factor in determining the success of a classification model. In Chapter 6, we found that two locations in Alabama were discernable from another, although they have unique geographical/hydrological properties that could affect the profile. However, in a larger scale elemental profiling exercise, these two sites

could hypothetically be included in the same group (e.g., if one wanted to distinguish between two states, or two countries where the USA was one of the countries), even though they are quite different from each other. This phenomenon could be impacting the results of other elemental profiling studies.

The relative lack of success in the samples collected in grocery stores is also concerning. Chapter 5 highlights the effects of commercial processing on shrimp samples, and it is clear that processing can alter the profile. However, the effect was dependent on the type of chemicals used in processing, which is not consistent across processing plants and often done to clients (importers like retail stores) specifications. One possible avenue to overcome this problem would be to investigate the plasticity of the profile in the shells. Shells were not considered in the studies herein because many shrimp are sold peeled in the grocery store, but the shell on shrimp tail pieces, the telson, uropods, and the sixth abdominal segment, are often left on shrimp even when they are peeled. In Chapter 6, it was clear the shells are more mineralized than the muscle tissue in shrimp from Gulf Shores and West Alabama, so this is a promising avenue to potentially capture geographical differences in shrimp in a tissue that may be more robust to the chemical treatments of processing, although it is unknown.

Avenues to ensure the authenticity of retail products will continue to proliferate in the future as environmental concerns about food production increase, and the traceability of a given product becomes more important. This research lays the groundwork to solidify elemental profiling as a viable and meaningful traceability tool for seafood products.

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