Sensory Perception, Development, and Preservation of Undersized American Freshwater Eel (*Anguilla rostrata*) as a Secondary Functional Food Product

By

Melissa Hamilton

A thesis submitted to the School of Graduate Studies

In partial fulfillment of the requirements for the degree of

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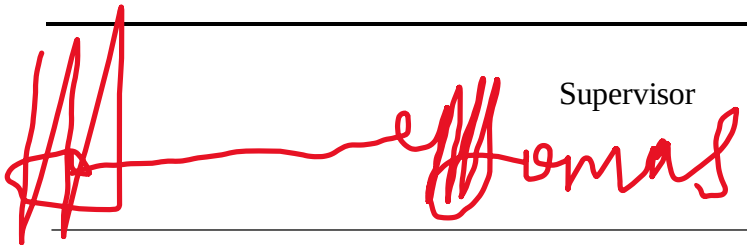
Dean of the Graduate School



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Supervisor

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Dr. Karen Doody and Dr. Peter Stewart

Abstract

Eels are well-known for being high in dietary lipids, although it is unclear how nutritional quality varies with eel size and ecological location. The nutritional quality of Newfoundland freshwater American eels (*Anguilla rostrata*) was defined and examined in this study to determine the potential of undersized eels in the manufacture of high-value secondary food products. The findings show that only the habitat location influenced the dietary fatty acid level and undersized eels from Gander, Robinson, and Flat Bay sites had exceptional fatty acid content. These findings suggest that habitat, rather than eel size, is a significant driver in the generation of high-quality dietary lipids in freshwater eels. Highly unsaturated fatty acids are vulnerable to oxidation and generation of toxic compounds when exposed to high temperatures. The use of sous-vide cooking before grilling eel reduced the formation of harmful HCAs, MRCs, and VOCs in Kabayaki. Sous-vide before grilling preserved nutritional value, improved quality and safety, and raised customer appeal. Berry Infused and Regular Kabayaki prepared using the optimized method can be marketed as a ready-to-eat functional food product that offers high overall lipid content and essential fatty acids, reduced toxic MRC, VOCs, and no HCAs, and a wide range of consumer acceptance.

General Summary

Eels are well-known for being high in dietary lipids, although it is unclear how nutritional quality varies with size and ecological location. The nutritional quality of Newfoundland freshwater American eels (*Anguilla rostrata*) was ~~defined and~~ examined in this study to determine the potential of undersized eels in the manufacture of high-value secondary food products. The findings show that the difference in size and colour between the larger and smaller eel population did not affect the dietary fatty acid level. These findings suggest that habitat, rather than eel size, is a significant driver in the generation of high-quality dietary lipids in freshwater eels. Incorporating sous-vide before grilling eel maintained the nutrition and reduced the formation of harmful compounds in Kabayaki. Infusing Newfoundland cranberries into the Kabayaki marinade also introduces more beneficial compounds. Kabayaki and Berry Infused Kabayaki products can be marketed as a ready- to-eat high-quality functional food product.

Dedication

All glory and honour belong to God!

This degree is a testament to God's faithfulness and love for me, and for this I am eternally grateful.

I would like to dedicate this degree to my parents, Donald and Paulette Hamilton. My dad sparked my interest in science as he taught my brothers and me how to connect circuits as toddlers, amongst other things. We quickly became very handy with the soldering iron and began creating our own "inventions" as we grew. After the tragic loss of my father, my mother filled the gap, and in doing so, she taught us resilience, strength, and determination as she supported her three young children alone in a foreign country. My parents are my inspiration, and I pray that this degree brings me one step farther in my journey to make them proud and continue their legacy.

Acknowledgment

It goes without saying that God is the reason for my success, and I am grateful to all those who He chose to be apart of my journey.

I am grateful to my brothers, close family, and friends for their unwavering support and encouragement. Special thanks to my godmother, Auntie Diana Shaw, who has consistently supported me and believed in my ability to succeed.

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Melissa Hamilton

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List of Abbreviations

AA – Arachidonic Acid

ALA – Alpha-linolenic Acid

ANOVA- Analysis of Variance

DG- Diglyceride

DHA – Docosahexaenoic Acid

4,8-DiMeIQ- the sum of 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline and 2-amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline

DPA – Docosapentaenoic Acid

EPA – Eicosapentaenoic Acid

FAME – Fatty Acid Methyl Ester

FFA – Free Fatty Acid

Fisher's LSD- Fisher's least significant difference

FTIR- Fourier Transform Infrared Spectroscopy

GC-FID – Gas chromatography– flame ionization detector

GCMS – Gas Chromatography Mass Spectrometer

HCA- Heterocyclic Amines

HILIC- Hydrophilic Interaction Liquid Chromatography

HUFA- Highly Unsaturated Fatty Acids

IQ: 2-amino-3-methylimidazo[4,5-f]quinoline

LA – Linoleic Acid

LO- Lipid Oxidation

LPC- Lysophosphatidylcholine

LPE- Lysophosphatidylethanolamine

MeIQx: 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline

MeIQ: 2-amino-3,4-dimethylimidazo[4,5-f]quinoline

MRC/ P: Maillard Reaction Compounds/ Products

MUFA: Monounsaturated Fatty Acids

NL- Newfoundland and Labrador

PCA- Principal component analysis

PC- Phosphatidylcholine

PE- Phosphatidylethanolamine

PG- Phosphatidylglycerol

PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

PI- Phosphatidylinositol

PL- Phospholipid

PS- Phosphatidylserine

PUFA – Polyunsaturated Fatty Acids

RET: Ready to Eat

SFA: Saturated Fatty Acids

SFE – Supercritical Fluid Extraction

SM- Sphingomyelin

TG- Triglycerides

UHPLC-HESI-HRMS/MS- Ultra-High-Performance Liquid Chromatography (LC) Coupled with Heated Electrospray Ionization High-Resolution Accurate Mass Tandem Mass Spectrometry

VOC- Volatile Oxidation Compounds

Chapter one

Introduction and overview

1. Introduction and overview

1.1. Introduction

Eels are highly nutrient-dense and contain a variety of minerals, vitamins (A, B12, B1, B2, D, E, and K), amino acids, and fatty acids. (Harlioğlu & Yilmaz, 2011; Islam et al., 2020). Eels are particularly high in omega-3 fatty acids and have a balanced amount of omega-6 fatty acids (Kontostathi et al., 2021; Kusharto et al., 2014). Freshwater American eels are in high demand at almost every life stage from East Asian, North American, and European markets (MacGregor et al., 2009). Undersized American 'yellow' eels, on the other hand, are severely underutilized and are primarily sold as bait for striped bass and other larger fish (Atlantic States Marine Fisheries Commission, 2017). There is not much information available concerning the nutritional value of American eels and the impact of morphological changes and environmental factors affect the lipid composition of subpopulations. The following literature review evaluates the potential of undersized freshwater American eels to be developed as high-quality functional food.

Eels are high in omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which aid in immune regulation and inflammation (Patterson et al., 2012). The western diet includes a disproportionate number of foods richer in omega-6s than omega-3s (approximately 15:1) which have been correlated with a higher rate of inflammatory or disease conditions (Patterson et al., 2012; Kris-Etherton et al., 2000). Several studies concluded that an increase in omega-3s coupled with a decrease in omega- 6s reduces inflammation and vasospasm, vasoconstriction, blood viscosity, and related conditions (Patterson et al., 2012; Delavar M et al., 2009; Ruidavets et al., 2007). Therefore, a good strategy to increase omega-3s intake would be to increase the consumption of fatty fish and omega-3-rich functional foods (Broadhurst et al., 2002; Din

et at., 2004). Regular consumption of eel and other oily fish also raises high-density lipoproteins (HDLs) and lowers the overall risk of cardiovascular disease (Rimm e al., 2018). True freshwater and saltwater eels (order: Anguilliformes, family: Anguillidae) provide similar nutritional value; however, freshwater eels have greater economic value and consumer acceptance mainly for their flavour and high flesh yield.

American eels are facultatively catadromous; hence, adults migrate from freshwater systems to the Sargasso Sea between February and April, where they breed and die (Jessop et al., 2009). Eggs drift for 1-2 days before hatching into Leptocephalus larvae. As the newly developed leptocephali migrate back to freshwater, they feed on plankton and, after 8–12 months, undergo metamorphoses to become glass eels. At the mouths of North American estuaries, eels become deeply pigmented elvers and begin feeding aquatic insects, small crustaceans, and small fish. After several years of feeding on worms, clams, frogs, fish, and animal carcasses, eels grow to become yellow eels (Jessop et al., 2006). This stage lasts an average of 12 years among Newfoundland populations, depending on food availability and weather. Undersized "yellow" eels return to their respective intercontinental water systems, where they continue to mature into larger migratory "silver" eels until it is time for spawning (Cairns et al., 2013).

Currently, Newfoundland and Labrador (NL), Canada has an American eel exportation industry for large adult American eels (Jessop et al., 2009). The American eel population, like its counterparts, has been declining, possibly due to anthropogenic interferences (Jessop et al., 2009; William et al., 2014). The Committee on the Status of Endangered Wildlife in Canada reassessed and designated the American eel from a species of ‘special concern’ to ‘threatened’ (COSEWIC 2012). As a result, the government of Newfoundland and Labrador implemented sustainable fishing practices, shortened the

fishing season, and limitation on license distribution for commercial, recreational, and aboriginal fisheries of juvenile and adult eels (Chaput et al., 2014). Despite the sustainable harvest of 'yellow' eels (length: >10 cm) (Kaifu et al., 2019), fishers have encountered the issue of utilizing these undersized freshwater eels. Currently, 'Yellow' eels are primarily sold as bait for striped bass and other larger fish (Atlantic States Marine Fisheries Commission, 2017). They have a lower market value in the live eel market compared to large eels and elvers (Magnusson & Dekker, 2020). however, there is an expanding market for freshwater eel secondary products.

The eel product market is expanding as consumers become more aware of the high nutrient content of eel. About 30 to 45% of the world's eel consumption comes from Japan alone (Shiraishi & Crook, 2015). Japanese people have a long-standing history of eating freshwater eels "unagi" in the summer months because they believe it can strengthen the body and provide functional benefits. Today, eel is a delicacy eaten year-round in the form of Kabayaki. Kabayaki is a popular Japanese method of grilling butterflied fillets of eel marinated in a sweet soy sauce base. Kabayaki has gained consumer acceptance across the world as sushi or served with rice "unadon" (Kaifu et al., 2019). The average price of Kabayaki was USD$ 26 per kg in 2011 and by 2013 it had reached USD $ 36 per kg. In 2018, the Japanese market declined; however, the demand was compensated for by the growing market mainly in China and to a lesser extent Taiwan, South Korea, Europe, and the west (Shiraishi & Crook, 2015; Kaifu et al., 2019). The Kabayaki market is expanding, but the products are mostly limited to frozen, traditional-style grilled fillets. As a result, there is room for product quality innovation and improvement.

To the best of my knowledge, there is not much information available concerning the lipid profile of American eels and the impact of morphological changes on fatty acid

composition are topics that receive very little research attention. It is also unclear how environmental factors, food, and other site-specific external factors affect the lipid composition of subpopulations. In this study, I investigated the influence of the colour, different habitat locations, and size on the nutritional value of undersized 'yellow' and large 'silver' freshwater American eels sourced from Gander, Robinson, and Flat Bay locations in Newfoundland. I compared the nutritional values of undersized and large eel samples and determine the potential of undersized eels for use as a functional secondary food product. Furthermore, I optimized the Kabayaki preparation and processing technique to develop four high-quality functional food products with improved nutritional quality, safety, and sensory perception.

1.2. Hypotheses

a) Size, colour, and growth habitat should affect the nutritional quality and potential of undersized freshwater American eels sourced from Newfoundland and their possible utility in the production of high-value eel-based secondary food products.

b) Nutritional quality, safety, and sensory perception of undersized freshwater American eel that has been marinated and then cooked at reduced heat and oxygen exposure should have less formation of toxic lipid oxidation and Maillard reaction compounds and carcinogens generated in eel during grilling. Incorporating Newfoundland cranberries, rich in antioxidants including polyphenols, in marinade should delay or inhibit lipid oxidation and the formation of toxic compounds, as well as increase the safety, sensory, and nutritional quality of Kabayaki.

1.3. Purpose of the thesis and objectives

The purpose of this project was ultimately to develop high-quality, ready-to-eat Kabayaki as functional food products using underutilized, locally harvested freshwater American eel.

The following objectives were investigated to test the proposed hypotheses:

i. To investigate the influence of the colour, different habitat locations, and size on the nutritional value of Freshwater American eels sourced from Newfoundland.
ii. To determine the potential of undersize juvenile eels to be used in the production of high-value eel-based secondary food products.
iii. To develop a high-quality Kabayaki eel product utilizing undersized Newfoundland freshwater American eel.

iv. To investigate the utility of sous- vide cooking technique as an innovative approach to maintaining the nutritional quality and enhancing the safety and sensory appeal of Kabayaki.

v. To develop Kabayaki as a functional food product by incorporating polyphenol-rich Newfoundland Cranberries.

1.4. Thesis organization

This thesis contains four chapters:

Chapter 1 provided an overview of the literature and concepts used to formulate the hypothesis and overall design of the experiment

Chapter 2 detailed the evaluation of the fatty acid and intact lipid content of freshwater American eels from three different locations and compared the undersized and larger eel populations from each to determine if the underutilized undersized population offered the same nutritional quality.

Chapter 3 described the utilization of undersized freshwater American eel in the development of Kabayaki eel by initially optimizing the cooking techniques required to maintain the nutritional quality and improve the sensory appeal and safety of the finished products. Furthermore, this chapter demonstrated the application of locally produced Newfoundland Cranberries infused into the Kabayaki marinade to develop a variant of Kabayaki with improved functional properties, while simultaneously maintaining the safety, nutritional, and sensory quality.

Chapter 4 includes a general discussion of the study findings, conclusions, and recommendations for future studies.

Chapter 5 includes pictures demonstrating achievements during product development

1.5. Definitions

All the possible definitions are given below (Augspurger et al., 2018; Shiraishi & Crook, 2015; Bogen & Keating, 2002; Hasler, 2002; Zamora and Hidalgo, 2005; Kim et al., 2019; Mozuraityte et al., 2016; Rod-In et al., 2020; Turkish & Sturley, 2009; Chew & Nyam, 2020)

a) Catadromous- describes the migratory behaviour of marine larvae to travel from marine to freshwater environments, where most of the growth phase occurs before adults migrate back to the marine environment to reproduce.

b) Free Fatty Acids- are the fatty acid tails formed from triacylglycerol via ester bond cleavage.

c) Functional Foods- describes fortified, enriched, or enhanced foods that provide health benefits beyond nutrition and energy

d) Heterocyclic Amines (HCA)- are potent mutagens generated during the thermal processing of muscle meats at high temperatures

e) Kabayaki- grilling filleted eels marinated in sweet soy sauce

f) Lipid oxidation- describes reactions between fatty acids and oxygen that result in the oxidative degradation of lipids

g) Malliard Reaction- describes a series of reactions that occur when the carbonyl moiety of reducing sugars and the free amino groups on the food's surface combine to produce volatile flavour and aroma compounds, as well as the brown colour (melanoidins pigment) seen in grilled foods.; non-enzymatic browning of food.

h) Neutral lipids- are energy reserves that result from the dehydration synthesis of fatty acid(s) with an alcohol

i) Polar lipids- are the main structural components of the cell membrane consisting of a hydrophilic head and hydrophobic tail

j) Sous-vide- cooking technique involves cooking food in a vacuum sealing bag by suspending it in a water bath at a controlled, low temperature for a prolonged time

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Chapter 2

2. Assessment of the Nutritional Quality of Newfoundland Freshwater Eels (*Anguilla rostrata*) as Influenced by Size and Growth Habitats: Implications in the Production of High-Quality Value Eel-Based Food Products

2.1 Abstract

Eels are excellent sources of dietary lipids, but how the nutritional quality varies with eel size, color, and habitat location is unknown. Eels were obtained from three locations in Newfoundland: Robinson, Gander, and Flat Bay and separated based on size (large and small in length and mass) to subgroups for dietary lipid analysis. Advanced lipidomics was done applying both high resolution accurate mass tandem mass spectrometry (HRAM-MS/MS) and FAME analysis using Gas chromatography coupled with tandem mass spectrometry and flame ionization detection (GC-MS/MS-FID) to assess the lipidome of the eels as differentiated by habitat location and size. The results demonstrate the difference in size and colour between the larger "silver" eel and the smaller "yellow" eel population did not influence the fatty acid content. The difference in ecology and overall nutrient availability in Robinson, Gander, and Flat Bay sites impacted the respective American eel populations' lipidome and dietary fatty acid content. Eels from Flat Bay was a superior source of PUFA, particularly C18:2n6 and EPA, while eels from Robinson were superior sources of omega- 3 fatty acids (DHA and LN). MUFA-enriched eel products could be produced from samples obtained from either Gander or Robinson. Furthermore, MUFA enriched PE molecular species were higher in samples from Flat Bay, while samples from Robinson were a superior source of ARA and DHA enriched PE. Similarly, samples from Gander and Robinson would be suitable for eel-based products enriched with TG molecular species containing C18 PUFA and MUFA. Flat Bay, on the other hand, would be a superior source of freshwater eels containing TG molecular species enriched with DHA, ARA, EPA, and DPA as well as oleic acids. These results demonstrated habitat location significantly influenced the dietary lipid composition of NL freshwater eels, but the size

and color was unremarkable. The possibility exists that unique eel based products with superior functional or dietary lipids could be sourced from NL freshwater eels as influenced by the habitat location.

Keywords: Freshwater American eel, free fatty acids, polar lipids, neutral lipids, Flat Bay, Gander Bay, Robinson Bay

2.2 Introduction

The American eel, *Anguilla rostrata* (Lesueur, 1817), is a native fish species to Newfoundland and Labrador (NL) that can be found in various freshwater ecosystems across the province including the southern part of Labrador from which they are known to migrate as far as South America (Department of Fisheries and Oceans Canada (DFO), 2010; COSEWIC, 2012). Wild American eels are a source of nutrition to local indigenous communities and were once prevalent in traditional Newfoundland cuisine (Atkinson, 2020; COSEWIC, 2012). According to the U.S. Department of Agriculture (2018), eels serve as a good source of essential fatty acids, proteins, minerals, and vitamins A, B12, and D. However, very little is known about the lipid and fatty acid composition of American eel and the effect morphological changes, in terms of color and size, and growth habitat have on the their lipid profile. This information will aid in determining the nutritional quality of Newfoundland freshwater American eels and the potential for "yellow" undersized eels to be utilized as a functional food product.

Anguilla rostrata is a catadromous species (fish that migrate from freshwater to the ocean to spawn) (U.S. Fish and Wildlife Service, 2015; COSEWIC, 2012). In the spring, eel

larvae, known as leptocephalus, are transported passively via the Gulf Stream from saltwater in the Atlantic to freshwater along the coast of North and South America where they develop as glass eels (COSEWIC, 2012). Anguilla species enter continental, freshwater bodies as transparent 'glass' eels (McCleave, 2001) and metamorphose into elvers as they travel further inland. At this stage, elvers start to gain pigment (COSEWIC, 2012) and become darker (typically ranging between brown, olive, and yellow on the colour gradient) and eventually yellow juveniles (COSEWIC, 2012; McCleave, 2001). American eels spend up to 25 years or more as 'yellow' eels (COSEWIC, 2012) that mainly live in benthic environments and feed on aquatic insects, small crustaceans, fish, etc. (McCleave, 2001).

After reaching over 20% of fat in muscle tissues, 'yellow' eels undergo drastic morphological changes to equip themselves for migration back to their spawning site (Greene et al., 2009). This process is described as 'silvering' which denotes the obvious change in colour of the eel. Silvering also indicates sexual maturation and involves changes such as the enlargement of eyes to adjust to seeing in the deep ocean, inflation of the swim bladder, lightening of the underside, and increase in skin thickness (Greene et al., 2009; COSEWIC, 2012; McCleave, 2001). In late summer/autumn, these eels no longer feed. Rather, the digestive tract of the adult eel degenerates and the percentage of somatic lipids increases to supply energy for migrating to the Sargasso Sea where adult eels spawn and then die (Greene et al., 2009; COSEWIC, 2012; McCleave, 2001).

According to the literature, American eel tissue contains 19% lipids (Wills and Hapkirk, 1976). There has been little recent research dedicated to the nutritional value of American eels, and little is known about the identity of these lipids and their fatty acid components.

Human health relies on lipids as its primary source of energy; however, lipids also function as structural support, a signalling molecule, and as a fundamental part of the development and function of the central nervous system (Shahidi, 2005). Certain dietary fatty acid constituents cannot be produced by the body due to the unavailability of the enzyme required for its biosynthesis; nevertheless, they are crucial for normal body function and survival. These fatty acids are recognized as 'essential' and tend to have bioactive functions, as well as act as a substrate to produce other important dietary fatty acids (Shahidi, 2005).

Saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids are distinguished by their chain length and degree of saturation. Polyunsaturated Fatty Acids (PUFAs) have more than one double bond and are particularly important because it includes the essential fatty acids. Linoleic acid (LA) - an omega-6 fatty acid and alpha-linolenic acid (ALA) - an omega-3 fatty acid are essential fatty acids that function as vital components of cell membranes and are precursors to compounds that regulate blood pressure and inflammatory responses (Sokoła-Wysoczańska et al., 2018; Shahidi, 2005). Monounsaturated Fatty Acids (MUFAs) only contain a single double bond along their hydrocarbon chain (Lunn and Theobald, 2006). The American Heart Association (2015) recommends incorporating foods containing copious amounts of MUFA, such as oil, avocado, nuts and seeds, and fatty fish, to aid in decreasing low-density lipoprotein (LDL; 'bad cholesterol), and in turn, lower the risk of atherosclerosis which can lead to cardiovascular diseases. Conversely, *trans* fatty acids and saturated fatty acids (SFAs), lack double bonds and increase LDL while decreasing high-density lipoproteins (HDL; 'good cholesterol) thus increasing the risk of cardiovascular diseases (American Heart Association, 2015).

The human body is inefficient in converting ALA to other essential omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are necessary for fetal development and cardiovascular health (Ebm et al. 2021; Burdge and Calder, 2005; Sokoła-Wysoczańska et al., 2018). As a result, humans rely on dietary sources of EPA and DHA to function.

Many fish are rich in EPA and DHA because of direct or indirect (through bioaccumulation) consumption of microalgae, such as diatoms and dinoflagellates (phytoplankton) (Maltsev & Maltseva, 2021; Barkia et al. 2019; Sathasivam et al. 2019; Chalima et al. 2019, 2020). Microalgae are unicellular eukaryotic organisms that play several vital roles in the ecosystem; however, in the marine food web, photosynthetic microalgae serve as the primary producers (Chauton et al., 2015). Microalgae are efficient producers of omega-3s with certain species producing mainly EPA or DHA or both (Chauton et al., 2015; Adarme-Vega et al., 2012; Guedes et al., 2011). Sufficient consumption of DHA and EPA allows for efficient desaturation and chain elongation required for proper body function. (Kaur et al., 2014).

Fish lipid and fatty acid content can differ between species and among members of the same species because fat content is heavily influenced by environmental factors (Zhang et al., 2014). Fish obtain neutral lipids from diet and alter polar lipids composition during evolutionary development (Sushchik et al., 2020). Identification of polar and neutral lipid content helps characterize the molecular species composition of specific lipid classes and determine the nutritional value of fish (Mika et al., 2016). Neutral lipids, such as triacylglycerol (TG), and diacylglycerol (DG), consist of fatty acids (tri- indicates 3, di- indicates 2 fatty acids) esterified to a glycerol backbone (Rod-In et al., 2020). In humans

neutral lipids are the primary energy reserve of the body (Rod-In et al., 2020); however, in excess, they can accumulate around vital organs and become detrimental to health. Polar lipids include phospholipids, such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC), and sphingolipids. Phospholipids consist of a glycerol backbone (refer to as glycerophospholipid) or alcohol esterified to two fatty acids tails in the sn-1 and sn-2 positions, and a phosphate group attached to a polar head group at the sn-3 position (Blanco & Blanco, 2017). Phospholipids facilitate the formation of the lipid bilayer which maintains the structure of the cell membrane (Cooper, 2000). Food high in phospholipids supply essential LC-PUFA required for the proper development and function of the human body and have cardiovascular benefits (Abedi and Sahari, 2014). Sphingomyelins consist of sphingoid backbone connected to a fatty acid, an alcohol attached by an amide bond and a head group. Sphingomyelin is the second most lipid in cell membrane that is found mainly in brain and nerve tissue.

The western diet includes a disproportionate amount of foods that are richer in omega-6s than omega-3s (approximately 15:1). The imbalance of omega-6:omega-3 in diet has been correlated with a higher rate of inflammatory or disease conditions (Patterson et al., 2012). Furthermore, dietary fatty acids alter the lipid profile of the liver and subsequent fatty acids produced by the body (Ranković et al., 2017). Ranković et al., (2017) observed that female rats exclusively fed fish had greater levels of essential fatty acids EPA, DHA, and DPA (docosapentaenoic acid) and lower levels of AA (arachidonic acid). Research has also shown that reducing dietary linoleic acid (LA), an omega-6 fatty acid, in the plasma of patients suffering from chronic headaches increased the bioavailability of EPA and DHA (Taha et al., 2014).

Consumers are seeking *ready-to-eat* and healthy products. Functional food products maximize the health benefits provided by foods as they contain bioactive compounds which provide health benefits in addition to basic nutrition (Hasler, 2002). Eels have an immense potential to develop high-quality-value functional products because of their significant lipid content. The health benefits associated with the regular consumption of bioactive lipids include the prevention, delay, or treatment of chronic and acute diseases including cancer, cardiovascular disease (CVD), and inflammatory conditions (Patterson et al., 2012; Delavar M et al., 2009; Ruidavets et al., 2007). There is a growing interest among consumers in the impact of diet on improving overall health. As a result, more consumers are seeking functional food products that will provide nutrition as well as aid in reducing their disease risks.

This research will investigate the influence of colour, different habitat locations, and size on the nutritional value of local freshwater American eels. We hypothesize that size, colour, and growth habitat should affect the nutritional quality and potential of undersized freshwater American eels sourced from Newfoundland and their possible utility in the production of high-value eel-based secondary food products.

2.3 Materials and Methods

Chemicals

Ammonium acetate, LC-grade chloroform, acetonitrile, methanol, formic acid, and acetic acid were sourced from Fisher Scientific (Ontario, Canada). Phospholipid standards were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Deionized water from PURELAB Purification System, ELGA Labwater (Ontario, Canada) was used to prepare solutions for this study. Lipid standards were purchased from Avanti Polar Lipids (Alabama, USA) and used for the lipid class analyses. The prepared standard mixture was to develop external standard curves (1− 10μg/mL for the low concentration range and 10− 100 μg/mL for the high concentration range) for the quantification of the lipids in the samples.

Experimental Design

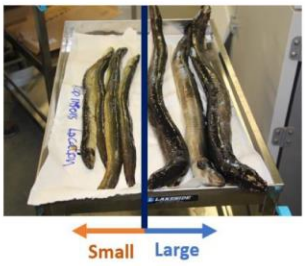
Small and large sized American eels (*Anguilla rostrata*) were obtained from local fishermen and industry producers for the use of this study. The samples were obtained from the following three local sites in Newfoundland: Gander Bay (Lat:49.2681827, Long: -54.5086546), Robinson Bay (Lat: 48.2551935, Long: -58.7951296), and Flat Bay (Lat: 48.4227555, Long: -58.5535431) (Figure 1. A). Flat Bay has a peculiar geographical feature in which it is partially enclosed by the former peninsula, Sandy Beach, and runs out into the Gulf of St. Lawrence where the barrier ends. Robinson and Flat Bay sites are relatively close (approximately 43.9km apart) along the west coast of Newfoundland.

Robinson Bay, unlike Flat Bay, is fed by Robinson River; however, both bodies of water flow directly into the Gulf of St. Lawrence. Gander Bay in central Newfoundland is fed

by the Gander River, which connects a series of ponds and Gander Lake to the bay, which runs directly into the Labrador Sea. Both large and undersized samples were collected from Robinsons and Flat Bay locations. Only large eels were harvested from Gander Bay.

A)

B)



Robinson bay (R)	Flat bay (F)	Gander bay (G)
Lat: 48.2551935	Lat: 48.4227555	Lat:49.2681827
Long: -58.7951296	Long: -58.5535431	Long: -54.5086546

Figure 2.1: A) Gander Bay (Lat: 48.2551935, Long: -58.7951296), Flat Bay (Lat: 48.4227555, Long: -58.5535431), Robinson Bay (Lat: 48.2551935, Long: -58.7951296) location sites for the sampling of undersized freshwater eel (Anguilla Rostrate) on the island of Newfoundland, NL. B) Image showing the eel samples according to size obtained from each habitat location

Colour Analysis

The CR-400 Chroma Meter (Konica Minolta Sensing Americas Inc., NJ, USA) was used to measure the skin colour of the eels harvested. The distance between two colours is known as the Delta-E (ΔE* = Total colour difference), which is the industry standard that is overseen by the International Commission on Illumination. The colorimetric analysis was conducted to quantify and differentiate the colour of each sample to determine the influence of the size and location. Readings were based on colour-opponent theory which dictates that two colours cannot simultaneously be red and green or yellow and blue. L* indicates lightness, a* is the red/green coordinate, and b* is the yellow/blue coordinate each of which can have a positive (+) or negative (-) delta. The equation $\Delta E_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ was used to measure the total colour change (ΔE)(Ly et al., 2020). A standard white plate was used as a reference.

Lipid Analysis:

Lipid extraction

The modified Bligh and Dyer method was used to extract the total lipids from each sample (Bligh and Dyer, 1959; Pham et al., 2019a, b). Four replications of each treatment was conducted. A vial containing 100 mg of homogenised eel sample was filled with 1 mL of 1 methanol containing 0.01 percent butylated hydroxytoluene and 1 mL of chloroform. After the mixture was homogenised, 0.8 g of water was added, and the mixture was centrifuged at 5000 x g for 15 minutes. The aqueous top layer of was removed, leaving behind the organic

bottom layer. The organic layer was then transferred to a pre-weighed 2 mL vial with a PTFE coated top (VWR), where it was dried under a constant flow of nitrogen. To determine the amount of recovered lipid, the vial was reweighed. After that, dried lipid samples were reconstituted in 1 mL of a 1:1 v/v solution of chloroform and methanol. The extracted eel samples were then analyzed using either gas chromatography-mass spectrometry/flame ionization detection (GC-FID/MS), ultra-high performance liquid chromatography coupled to heated electrospray ionization tandem high resolution mass spectrometry (UHPLC-HESI-HRMS/MS), and Fourier-transform infrared spectroscopy (FTIR).

Lipids analysis by Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) was performed on extracted eel oil without derivatization. This analysis was used to obtain the infrared spectrum of absorption, emission, and photoconductivity of eel samples to detect different functional groups of intact lipids. FTIR spectrum was recorded between 4000 and 400 cm^{-1} in the absorbance mode with a resolution of 2 cm^{-1}. The spectrum of eel lipids showed peaks that correspond to specific rotations around carbon atoms revealing functional group and degree of unsaturation.

Conversion of lipid extract to Fatty Acid Methyl Esters (FAMEs)

To assess the amount of free fatty acids present, the eel lipid extracts were converted into fatty acid methyl esters (FAMEs). 100 microlitres of the extracted lipids were mixed with 100 microlitres of internal standards (C19:0 FA: 1 mg/mL in chloroform: methanol (2:1

v/v)), and the samples were then dried under nitrogen. By adding 400 microliters of methanolic-HCl 3 N (Sigma-Aldrich, ON, Canada), vortexing, and incubating (80 °C) for 30 minutes, the fatty acids in the samples were methylated. Following incubation, 0.8 mL of distilled water was added to the samples, and 0.5 mL of hexane aliquots were used to extract the FAMEs (x3). After being combined, the fractions were mixed, dried under nitrogen gas, and then redissolved in 0.5 mL of hexane. GC/MS and GC/FID were used to evaluate the FAMES in each sample.

Analysis of fatty acids by GC-MS

FAMEs in the eel samples were analyzed using a Gas Chromatography coupled with mass spectrometry in accordance with our previous publications (Pham et al., 2019a, b; Vidal et al., 2018). Helium was used as the carrier gas at a flow rate of 1 mL/min to separate methylated fatty acids using a DB23 high-resolution column (60 m × 0.25 mm × 0.2 µm; Agilent Technology, Mississauga, ON, Canada). A Tri-plus auto-sampler was used to apply one microliter (1µL) of each sample using the injection system in split mode (20:1) (Thermo Scientific, Burlington, ON, Canada). The oven temperature of 80 ◦C was set to raise by 4°Celsius per minute to 220°C, where it was held for 5 minutes. It was then increased by 4 degrees Celsius per minute to 240 degrees, where it was held for 10 minutes. The methylated fatty acids were determined based on a comparison of the retention times and mass spectra obtained from commercial standards (Supelco 37 component mix, Supelco PUFA No. 3, Supelco FAME mix C8-C24, C16 DMA, and C10 DMA; Sigma Aldrich, Oakville, ON, Canada) and the NIST database (Thermo Scientific, Burlington, ON, Canada). Values were provided as nmol percent, and the quantities of distinct fatty acids were determined using standard curves created from the standard mixtures.

Analysis of fatty acids by GC-FID

FAMEs in the eel samples were analyzed using a combination of Gas Chromatography coupled to a Flame Ionization Detector (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with our previous publications (Pham et al., 2019a, b; Vidal et al., 2018). Four replications of each treatment was conducted. Helium was used as the carrier gas at a flow rate of 1 mL/min to separate methylated fatty acids using a DB23 high-resolution column (30 m × 0.25 mm × 0.25 µm; Agilent Technology, Mississauga, ON, Canada). A Tri-plus auto-sampler was used to apply one microliter (1µL) of each sample using the injection system in splitless mode (Thermo Scientific, Burlington, ON, Canada). The oven temperature of 50 ◦C was set to raise by 20 degrees Celsius per minute to 175 degrees, where it was held for 1 minute. It was then increased by 4 degrees Celsius per minute to 230 degrees, where it was held for 5 minutes. The methylated fatty acids were determined based on a comparison of the retention times and mass spectra obtained from commercial standards (Supelco 37 component mix, Supelco PUFA No. 3, Supelco FAME mix C8-C24, C16 DMA, and C10 DMA; Sigma Aldrich, Oakville, ON, Canada) and the NIST database (Thermo Scientific, Burlington, ON, Canada). Values were provided as nmol percent, and the quantities of distinct fatty acids were determined using standard curves created from the standard mixtures.

Analysis of lipids by UHPLC- HESI-HRAM-MS/MS

The complex lipids in the eel samples were determined using ultra-high-performance liquid chromatography (LC) coupled with heated electrospray ionization high-resolution accurate mass tandem mass spectrometry (UHPLC- HESI-HRAM-MS/MS) according to our

previous publication (Pham et al., 2019a, b). The lipids extracted from undersized and large eel samples were separated using both hydrophilic interaction liquid chromatography (HILIC) using a Luna 3µm, particle size: 3 µm, pore diameter: 200 Å, 100 × 2 mm diameter column (Phenomenex, Torrance, CA, USA) and reverse phase chromatography using an Accucore C30 column [150 × 2 mm I.D., particle size: 2.6 µm, pore diameter:150 Å] (Fisher Scientific, ON, Canada). HILIC separation was conducted using a solvent system of acetonitrile and water (97:3v/v) containing 10 mM ammonium acetate buffer (solvent A) and 10 mM ammonium acetate in pure water (solvent B). The mobile phase system gradient was as follows: 100% solvent A for 2 min; solvent B increased to 10% for 23 min, then increased from 10–15% for 10 min, and finally kept at 15% for 5 min. The column was re-equilibrated to starting conditions (100% solvent A) for 10 minutes prior to each new injection. Column temperature during HILIC separation was maintained at 25 °C and the flow rate at 0.2 mL/min. Ten microliters of the lipid extract was suspended in chloroform: methanol (1:1 v/v) and then injected into the machine.

Separation using C30 reverse-phase liquid chromatography (C30RPLC) was conducted with a gradient mixture of solvent B consisting of isopropanol: acetonitrile: water at 90:10:1v/v/v containing 10 mM ammonium formate in formic acid (0.1%). Solvent A consisted of acetonitrile: water at 60:40 v/v with 10 mM ammonium formate and 0.1% formic acid. The gradient system used was as follows: 30% solvent B for 3 min; increased solvent B to 43% over 5 min, then to 50% B in 1 min, then to 90% B for 9 min, then to 99% B for 8 min and concluded at 99% B for 4 min. The column was re-equilibrated to starting conditions (70% solvent A) for 5 min prior to each new injection. Column temperature during C30 reverse phase chromatography was maintained at 30°C with a flow rate of

0.2mL/min, and a 10µL sample was injected into the machine.

Data analysis (UHPLC-C30RP-HESI-HRMS/MS)

All lipid analysis was done using a high-resolution accurate mass tandem mass spectrometer (Q-Exactive Orbitrap) coupled to an automated Dionex ultimate 3000 UHPLC system (ThermoScientific, MO, USA) operated with the Chromeleon software. The mass spectrometer was operated in both positive and negative ion modes using the following parameters: sheath gas: 40, auxiliary gas: 2, ion spray voltage: 3.5 kV, capillary temperature: 300 ◦C; S-lens RF: 35 V; mass range: 200–2000 m/z; full scan mode at a resolution of 70,000 m/z; top-20 data-dependent MS/MS at a resolution of 35,000 m/z and step collision energy of 35 and 40 (arbitrary unit); injection time 50 min; isolation window: 1m/z; automatic gain control target: 1e5 with dynamic exclusion setting of 5.0 s. The instrument was externally calibrated to 1ppm using ESI negative and positive calibration solutions.

Statistical Analysis

Four replicates were made for each experiment. A supervised multivariate analysis approach using XLSTAT (Addinsoft, New York, USA) was applied to the eel fatty acids, complex lipid classes and molecular species identified using the Lipid Search version 4.1 (Mitsui Knowledge Industry, Tokyo, Japan) and manually confirmed with XCalibur 4.0 software (ThermoScientific, MO, USA). Principal component analysis (PCA) was used to ordinate the samples based on similarity, and analysis of variance (ANOVA) was applied to determine the differences in lipid composition and content as influenced by morphology and habitat locations. Fisher's least significant difference (LSD) was used to separate the

means whenever significant effects were observed at $\alpha = 0.05$. Figures were created using a combination of the XLSTATs premium version (Addinsoft, Long Island, NY, USA) and Sigma plot 13.0 (Systat Software Inc., San Jose, CA).

2.4 Results

Changes in colour as influenced by the size and habitat location

Colorimetric analyses were conducted on all the samples across locations to confirm and quantify the difference in colour among samples and to determine if this factor was associated with the size of the fish and/ or the location from which it was harvested. Principal component analysis (PCA) explaining 91.35% of the total variance in the data (F1 80.51% and F2 10.84%) showed the eel samples cluster according to size (Figure 2.2B). The smaller eel samples almost exclusively clustered in quadrants 1 and 2 while the larger samplers cluster in quadrants 3 and 4 regardless of harvesting site. The adjacent biplot showed vectors L* (white colour) and b* (yellow colour) farthest from the origin with a large angle between the two suggesting that these colours have more influence on grouping the samples according to size compared to ΔE* (total colour difference) and a* (redness). Further analysis showed that smaller eel samples had significantly higher b* values (yellow colour) compared to the larger samples across locations (Figure. 2.2C).

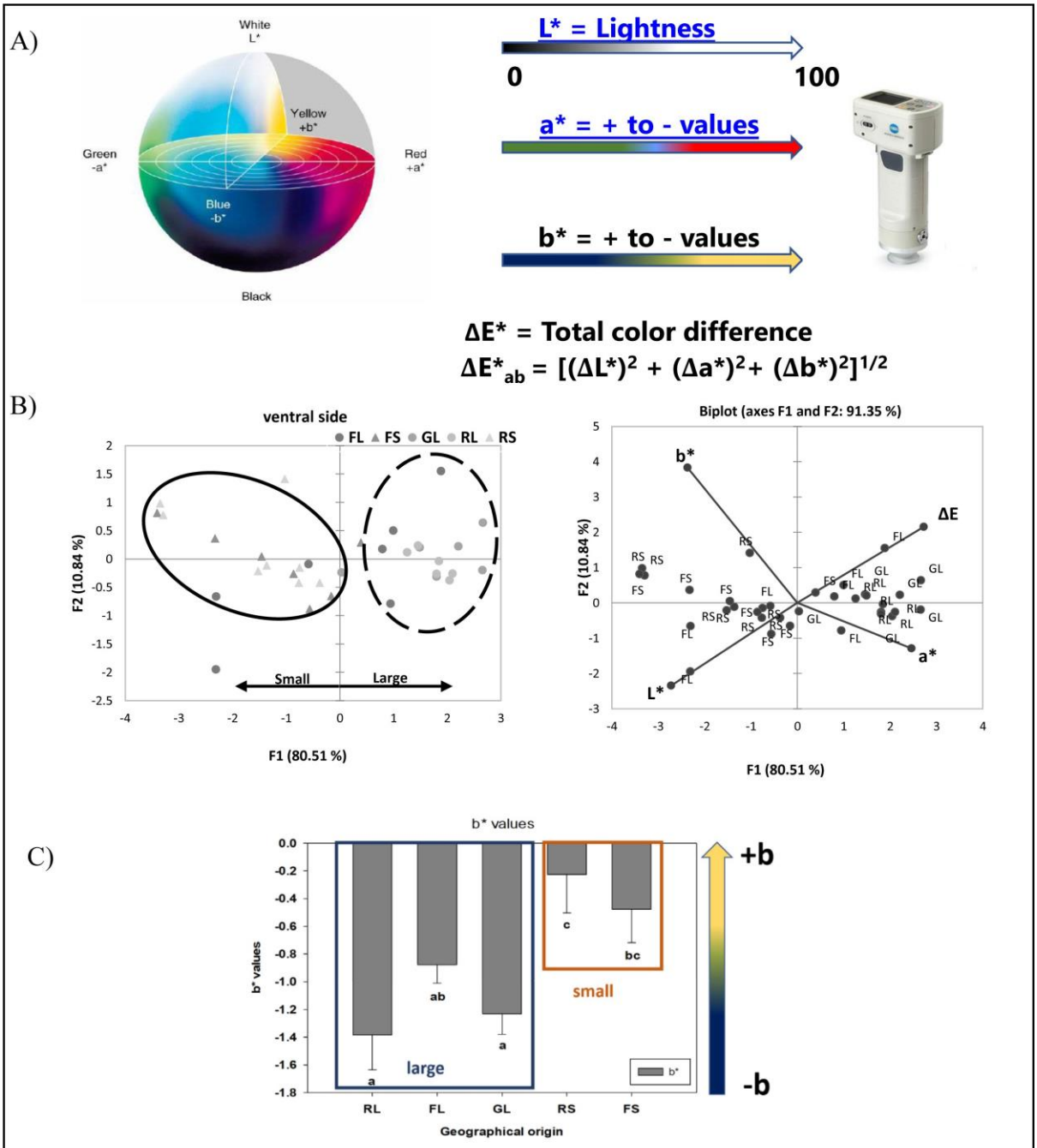


Figure 2.2: A) Color measurements of freshwater eels collected from different habitats in Newfoundland. A) CIE LAB color space: L* axis: gray, 0-100 (black to white respectively); a* axis: red/green, +a*: red values and –a*: green values; b* axis: yellow/blue values, +b*: yellow and –b*: blue values. Color difference $\Delta E* = [(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2]^{1/2}$. B) Principal Component Analysis (PCA) showing association between size, location, and color of eel. C) Bar chart showing the difference in total color for large and small eels in each location. Values in bar charts represent b* value, where b* value accompanied by different superscripts are significantly different at LSD $\propto = 0.05, n = 15$.

Assessment of Lipid content using FTIR

The Fourier Transform Infrared (FTIR) was used to determine the lipid content of American eel samples using the association of absorbance peaks to bonds present in eel sample. On the high-frequency end of the spectrum (above 1,500cm^{-1}) the bond absorption at 3011cm^{-1} is associated with C-H stretching of cis double bonds, the peak at 2854cm^{-1} is associated with methylene C-H symmetrical stretching, the peak at 2925 cm^{-1} is associated with methylene C-H asymmetrical stretching (Figure. 2.3A). On the low-frequency end of the spectrum (below 1,500cm^{-1}), the peak at 1160 cm^{-1} is associated with methyl C-H symmetrical bending and the peak at 721 cm^{-1} is associated with C-H rocking (Forfang et al., 2017).

A PCA was conducted to investigate the association between the habitat location and the absorbance peaks corresponding to the fatty acids present in eel sample Figure. 2.3B). The PCA explained 79.57% of the total variance in the data (F1 48.91% and F2 30.66%). The biplot suggested an association between samples from Flat Bay and the absorption band =CH stretch at 3011cm^{-1}. Samples from Gander Bay and Robinsons were more associated with peaks C-H stretch at 2854cm^{-1} and 2925 cm^{-1}, and C-H bend (mono) at 721 cm^{-1}. A follow-up PCA was conducted to determine the potential of a correlation between habitat location and the fatty acids identified in the previous section (see Figure. 2.3C). There was no obvious clustering of sample points according to location.

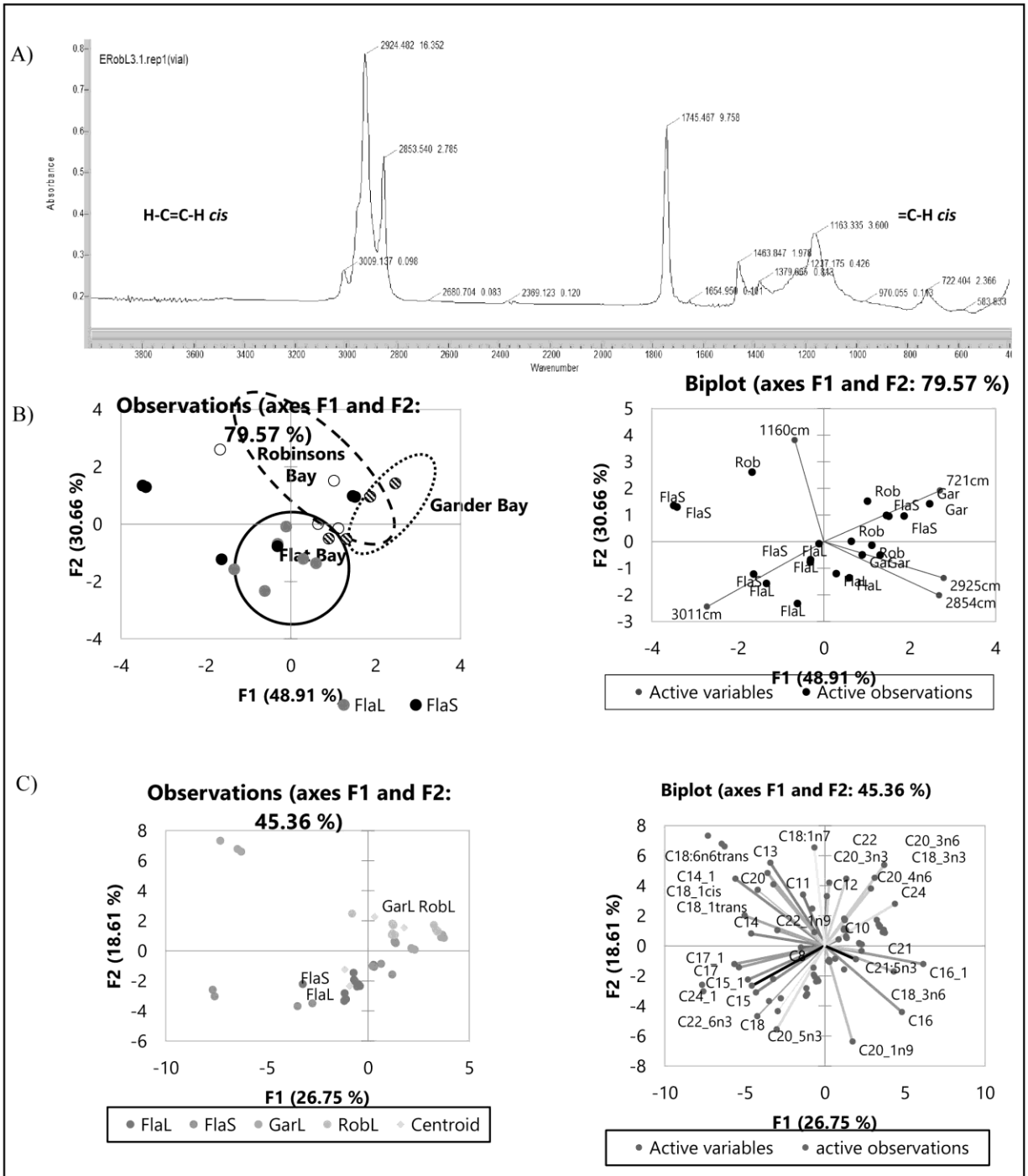


Figure 2.3: A) Fourier Transform Infrared (FTIR) spectrum showing the profile of fatty acids detected in American eel (*Anguilla rostrata*). Principal component analysis (PCA) showing B) the association between fishing site and the absorption signals related to fatty acids detected in eel samples (1160, 721, 2925, 2854, 3011cm-1). C) The association between the fishing site and fatty acids detected in eel samples.

Influence of Size on eel lipid composition

In the present study, undersized freshwater eels were assessed for their potential use as raw materials for the development of functional food products. The characteristics and nutritional value of both large and undersized freshwater eels were assessed and compared to determine whether undersized eels could offer the same nutritional value as their larger counterpart based on the quality and content of important dietary lipids.

FAMEs analysis using GC-FID showed the presence of essential fatty acids including EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) at retention times of 11.93 and 13.77 (Figure 2.4C). The abundance of each fatty acid identified from the GC-FID output was used to calculate to the percent total fatty acid for each sample. The large and small showed significant difference according to fatty acid class (Figure 2.4B). Larger eels contained significantly higher saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA). Amongst PUFA species, larger eels possessed significant greater omega-6s as well as omega-3s. There was no difference in MUFA content between sizes. was used PCA, we observed no clear separation of the lipids in the samples across locations regardless of size (Figure 2.4E). A follow-up PCA accounting for 86.18% of the total variance in the data (F1 56.66% and F2 29.52%) was conducted to determine the association between eel size and fatty acid classes (see Figure. 2.3C). The output showed that smaller eels were associated with MUFA while larger eels were associated with SFA and PUFA, omega-6s and omega-3s.

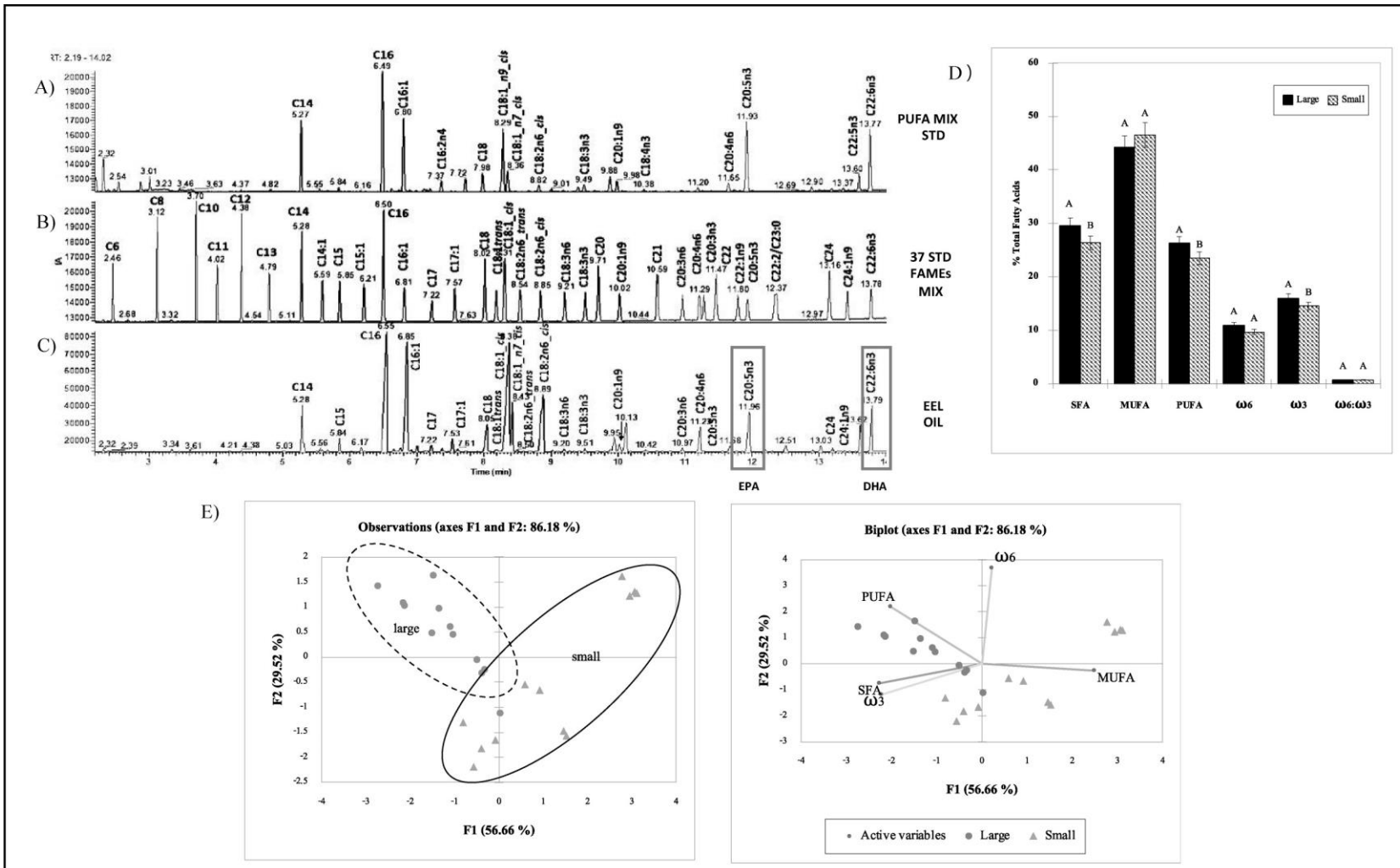


Figure 2.4: Chromatograph of the resolved fatty acids obtained from A) Polyunsaturated standard mix B) 37 components fatty acid methyl esters (FAMEs) mix and C) Eel oil, a source of EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid). D) Fatty acid composition of large and small American eels (*Anguilla rostrata)*. Values in bar chart represent mol $\pm$ standard of errors of the scores. % Total fatty acids accompanied by different superscripts are significantly different at LSD $\propto = 0.05, n = 10$. E) Principal component analysis (PCA) showing the association of eel size with fatty acid classes.

Influence of location on eel lipid composition

Evaluating the influence of the growing habitat is important for determining the nutritional quality of American eel subpopulations. The distribution of fatty acid class content across location were as follows: A) Robinson: PUFA: 25±0.2%, MUFA: 47±0.2%, SFA: 28±0.2%, B) Flat Bay: PUFA: 26±0.4%, MUFA: 44±0.3%, SFA: 30±0.1%, and C) Gander Bay: PUFA: 21±0.5%, MUFA: 51±0.8%, SFA: 28±0.5%, NL (see Figure 2.5a). Analysis of variance distinguished the difference between fatty acid content of eel samples according to location. In terms of SFA, palmitic acid (C16) was the greatest in abundance. Flat Bay eels had significantly higher C16 content. Robinson and Gander populations also had very high levels as well. Notable percentages of stearic acid (C18) and myristic acid (C14) were also found during analysis. Flat Bay eels had more C18 content followed by the Gander population (see Figure 2.5b A). The MUFA content was highest in eels obtained from Robinson Bay followed by Gander. Oleic acid C18:1 (n-9) predominated with significantly higher levels in samples from Gander Bay, followed by Robinson Bay (Gander Bay >Robinson Bay >Flat Bay) (Figure. 2.5b B). Although significantly lower abundance, the fatty acid content of *cis*-Vaccenic acid (C18:1n7) was the second highest of the MUFAs with elevated levels in samples from Robinsons and Gander Bay (Figure. 2.5b B). Flat Bay eel population had the highest PUFA content. Flat Bay samples had the highest DPA (C22:5n3), DHA (C22:6n3), EPA (C20:5n3), stearidonic acid (SA, C18:4n3), heneicosapentaenoic acid (HPA, C21:5n3) levels. Robinson had the highest linoleic acid (C18:2n6cis), α-linolenic (C18:3n3), 11,14,17-eicosatrienoic acid C20:3n3cis) content, while Gander Bay had the highest arachidonic acid (C20:4n6) content (see Figure 2.5b C). Figure 2.5b D) showed the percentages of each fatty acid class as well as omega-3 and 6 fatty acids content and its ratio. Flat Bay eel population had the greatest percentage of

PUFA and SFA followed by Robinson and Gander Bay. MUFA content was the high across sample with Gander Bay eel population having the greatest percentage followed by Robinson. Robinson had significantly higher omega 6 content that Flat Bay; however, Flat Bay samples contained significantly more omega-3s than both Gander and Robinson. Omega-6: omega-3 ratio percent was low across sampling locations; however, Robinson and Gander had significantly higher abundances than Flat Bay (see Figure 2.5b D).

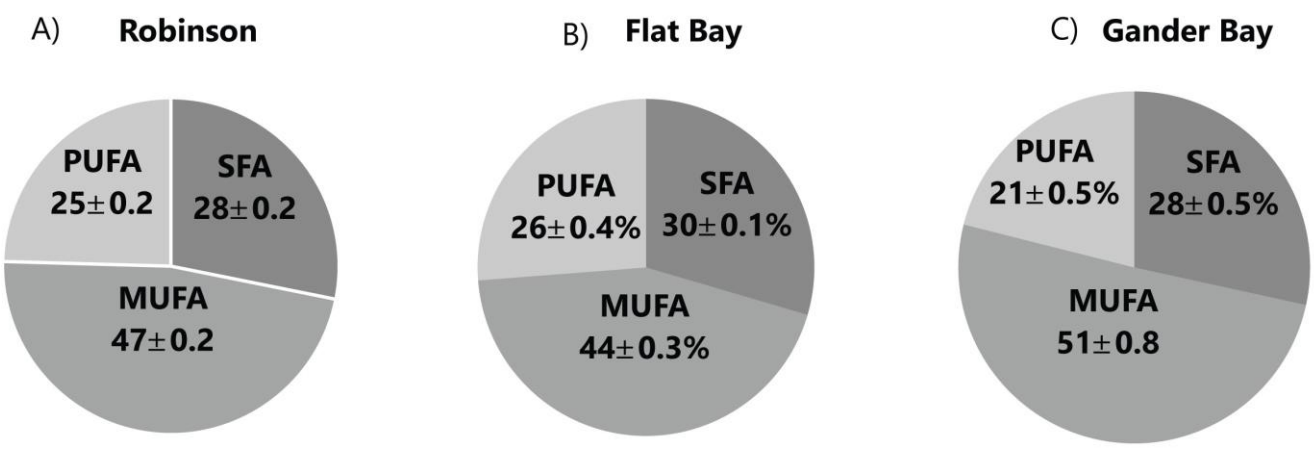


Figure 2.5a: Percent Total Fatty acid composition of freshwater American eels (*Anguilla rostrata*) obtained from A) Robinson, B) Flat Bay, and

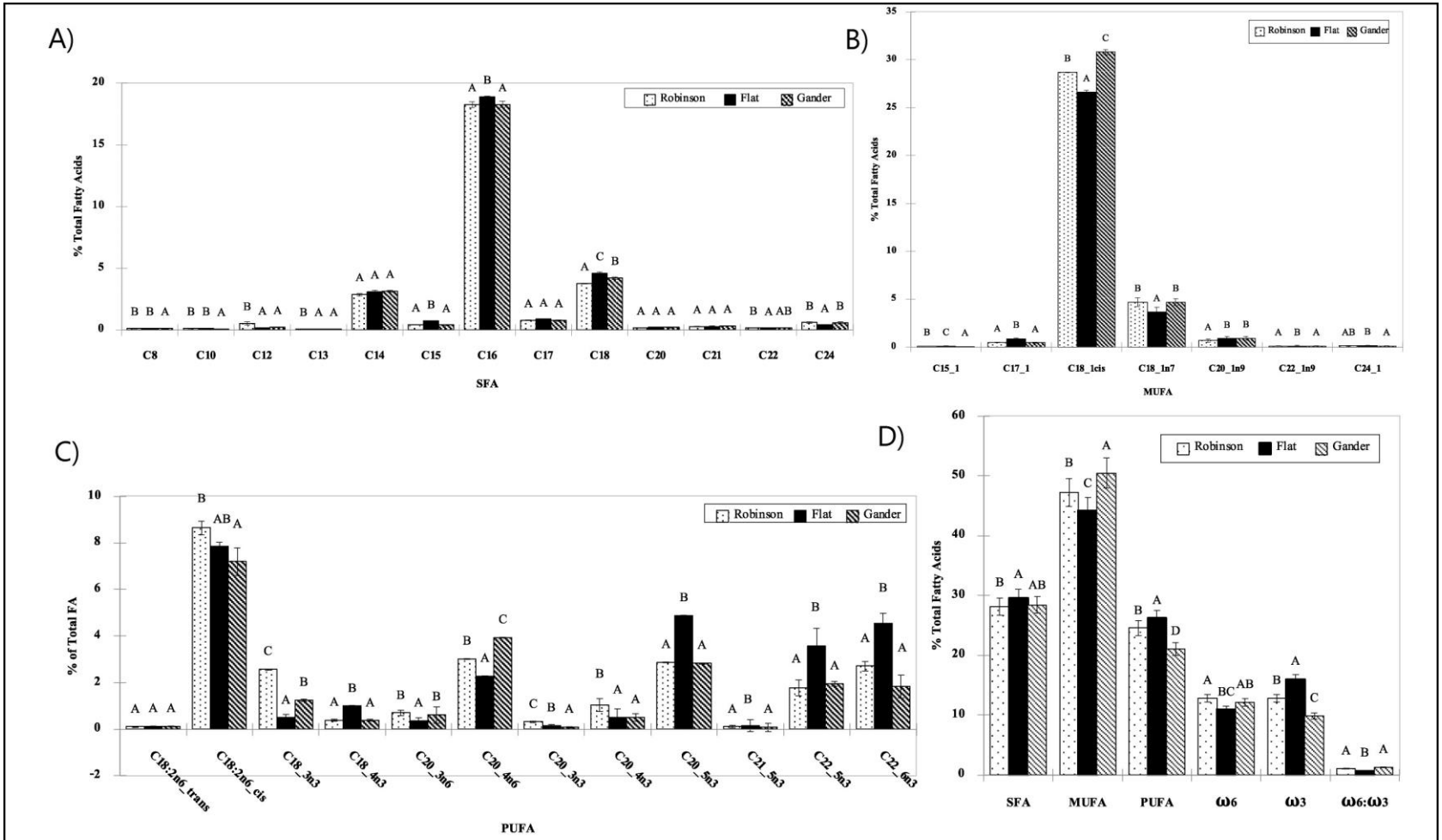


Figure 2.5b: A) Fatty acid composition of freshwater American eels (*Anguilla rostrata*) obtained from Gander, Robinson, and Flat Bay, NL. A) Monounsaturated (MUFAs), B) Saturated (SFAs), C) Polyunsaturated fatty acids (PUFAs), and D) Summary of total fatty acids for each level of saturation and Omega 3 & 6 content and ratio. Values in bar chart represent mol $\pm$standard of errors of the scores. Mol% accompanied by different superscripts are significantly different at LSD $\propto = 0.05, n = 15$.

Analysis of polar lipid content

The polar lipids were determined in the samples following analysis by UHPLC-HESI-HRAM/MS-MS. We observed the PL present in samples from each location were as follows: Robinsons Bay: 12% LPC, 3% LPE, 21% PC, 30% PE, 1% PG, 3% PI, 2% PS, 28% SM. Gander Bay: 21% LPC, 5% LPE, 20% PC, 26% PE, 2% PI, 1% PS, 25% SM. Flat Bay: 16% LPC, 1% LPE, 35% PC, 9% PE, 2% PI, 36% SM. Robinsons Bay had the largest percentage of PE as well as PG, PI, and PS (present in small amounts across locations), and the lowest LPC. Gander Bay had the largest percentage of LPC and LPE and the lowest SM and PC. Flat Bay had the largest percentage of SM and PC and the lowest LPE, PE, and PS (see Figure 2.6a).

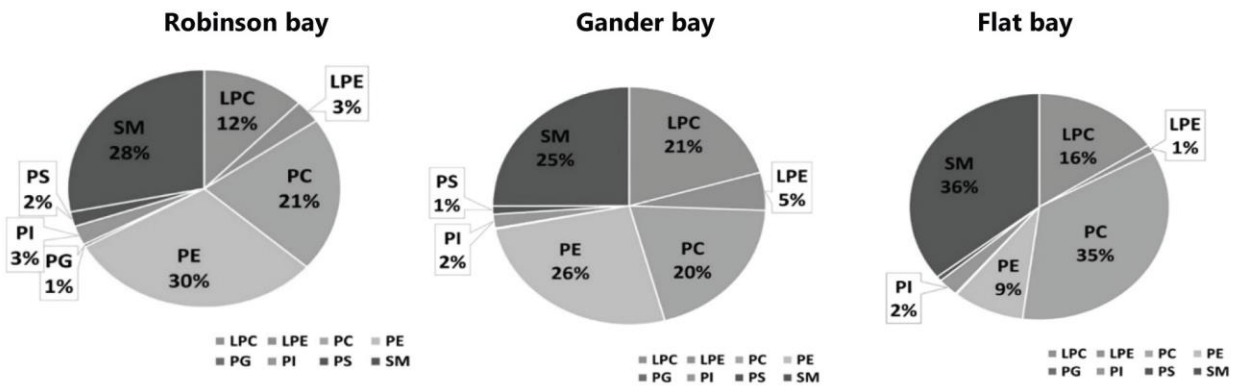


Figure. 2.6a. Polar lipid composition of American eels obtained from Robinson, Gander and Flat Bay, NL. Pie chart showing percent-composition of polar lipids extracted from eel oil.

The study of the molecular species in the different polar lipids determined the lipid composition of eels from Robinson, Gander, and Flat Bay (see Figure 2.6b A). The PCA explained 54.61% of the total variation in the data (F1 36.45% and F2 18.15%) and showed the distribution of PC molecular species clearly segregated the eel samples by location. There was a clear separation of MUFA enriched PC in eel samples from Flat Bay in quadrants 1 (Q1) and 2 (Q2). PUFA enriched PC were associated with samples from Robinsons and Gander Bay clustered in quadrants 3 (Q3) and 4 (Q4). Flat Bay had a significantly higher levels of PC16:0_16:1, PC17:1_16:0, PC16:0e_18:1, PC16:0_20:5 and PC16:1e_18:1 than Robinsons and Gander Bay. In Q3 and Q4, PUFA molecular species were higher in samples from Robinsons Bay and Gander Bay compared to Flat Bay. Particularly, ether linked PC molecular species (PC16:0e_20:4 and PC16:0e_22:5) were elevated in samples from Gander Bay.

PE had more diversity in molecular species distribution compared to PC and provided clear groupings of the eel samples based on habitat locations (Figure. 6C). Samples from Flat Bay almost exclusively clustered in Q1, while those from Gander clustered in Q3 and those from Robinsons Bay in both Q3 and Q4. Samples grouped in Q1 and Q2 showed Flat Bay had significantly higher levels of MUFA enriched PE molecular species especially 16:1e_20:5, 16:1e_18:1, 16:1e_17:1, and 16:1e_16:1. In contrast, eel samples from Robinson Bay clustered in Q3 and 4 had elevated levels of C20:4 enriched PE molecular species (Figure 2.6b B). Another interesting observation was that eel PE had high levels of ether linked molecular species regardless of the geography of habitat location.

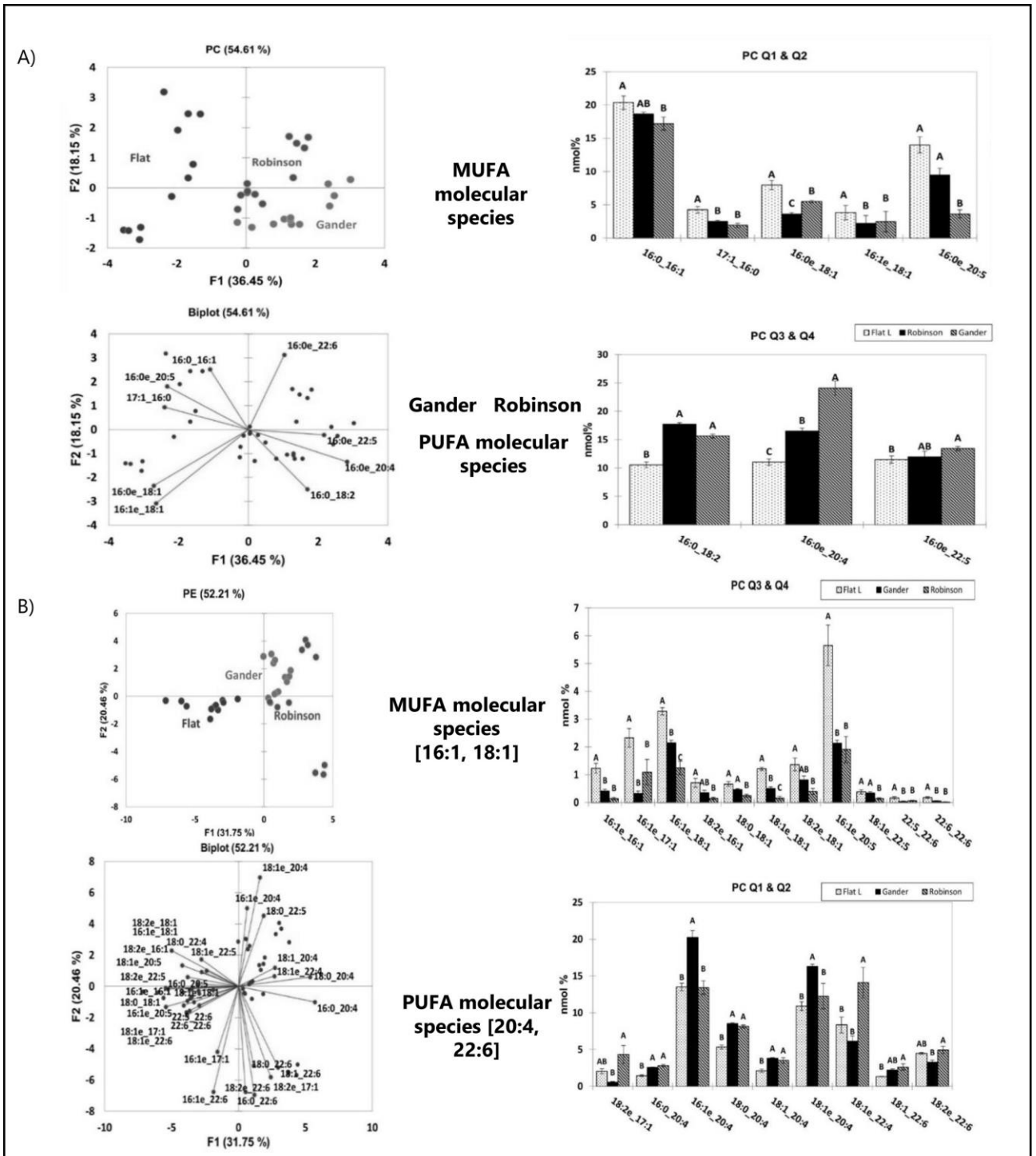


Figure 2.6b: Polar lipid composition of American eels obtained from Robinson, Gander and Flat Bay, NL. A). Principal Component Analysis (PCA) and bar chart showing the association between B) Phosphatidylcholine (PC) or B) Phosphatidylethanolamine (PE) and habitat location based on ordination (quadrant 1 & 2) and (quadrant 3 & 4)

Intact Lipid Analysis: Neutral lipids

As anticipated, triacylglycerides (TG) predominated (97-98%) the neutral lipid composition with diacylglycerides (DG) occurring as minor (2-3%) components in eel samples regardless of geography of habitat location (Figure. 2.7a). To discern the molecular species (> 220 molecular species) that best differentiated the samples based on habitat location, partial least squares-discriminant analysis (PLS-DA) was conducted (see Figure 2.7b). Triacylglycerides (TG) clustered the samples according to location with Flat Bay ordinated in Q3 & Q4, Robinson Bay mainly in Q2, and Gander Bay in Q1. The Cumulative Q2 quality index was Flat Bay- Comp1 0.0858, Comp2 0.880; Gander Bay- Comp1 0.090, Comp2 0.651; Robinsons Bay- Comp1 0.259, 0.615; Total Comp1 0.402, 0.721. TG molecular species with VIP scores >1 from the PLS-DA model were selected for further analysis using a heatmap (Figure 7). Output from the heat map showed that PUFA [22:5, 22:6, 20:4, 22:4] and MUFA [C18:1] enriched TG molecular species were higher in eel samples from Flat Bay (Figure 7B). Conversely, samples from Robinson and Gander Bay had higher levels of TG molecular species enriched with C8:2 and C18:3 fatty acids (see Figure 2.7b A). Similar analysis of DG showed that MUFA [18:1] & PUFA [20:5, 22:5, 22:6] enriched molecular species were higher in eels collected from Flat Bay and PUFA molecular species containing 18 carbons [18:2, 18:3] were higher in samples obtained from Gander and Robinson Bay (Figure 2.7b B).

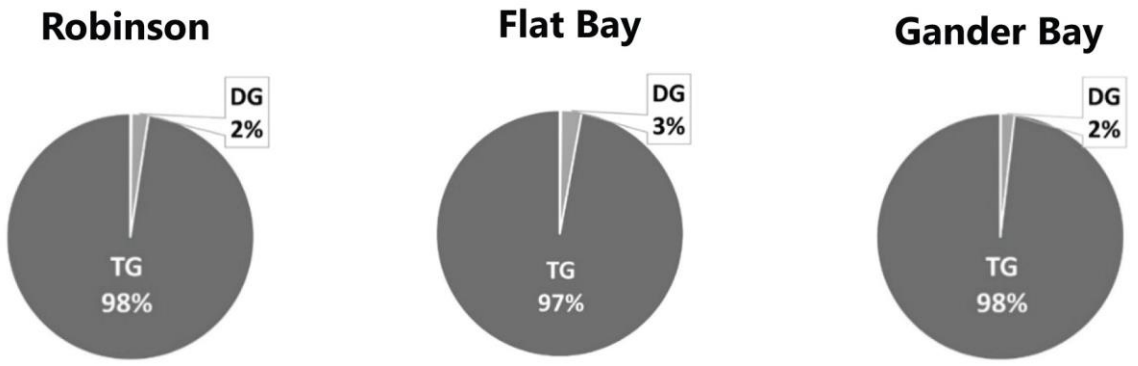


Figure 2.7a: Neutral lipid composition of American eels obtained from Gander, Robinson, and Flat Bay, NL. A) Pie chart showing percent composition of major neutral lipids: triacylglycerides (TG) and diacylglycerides (DG) present in eels oil extract.

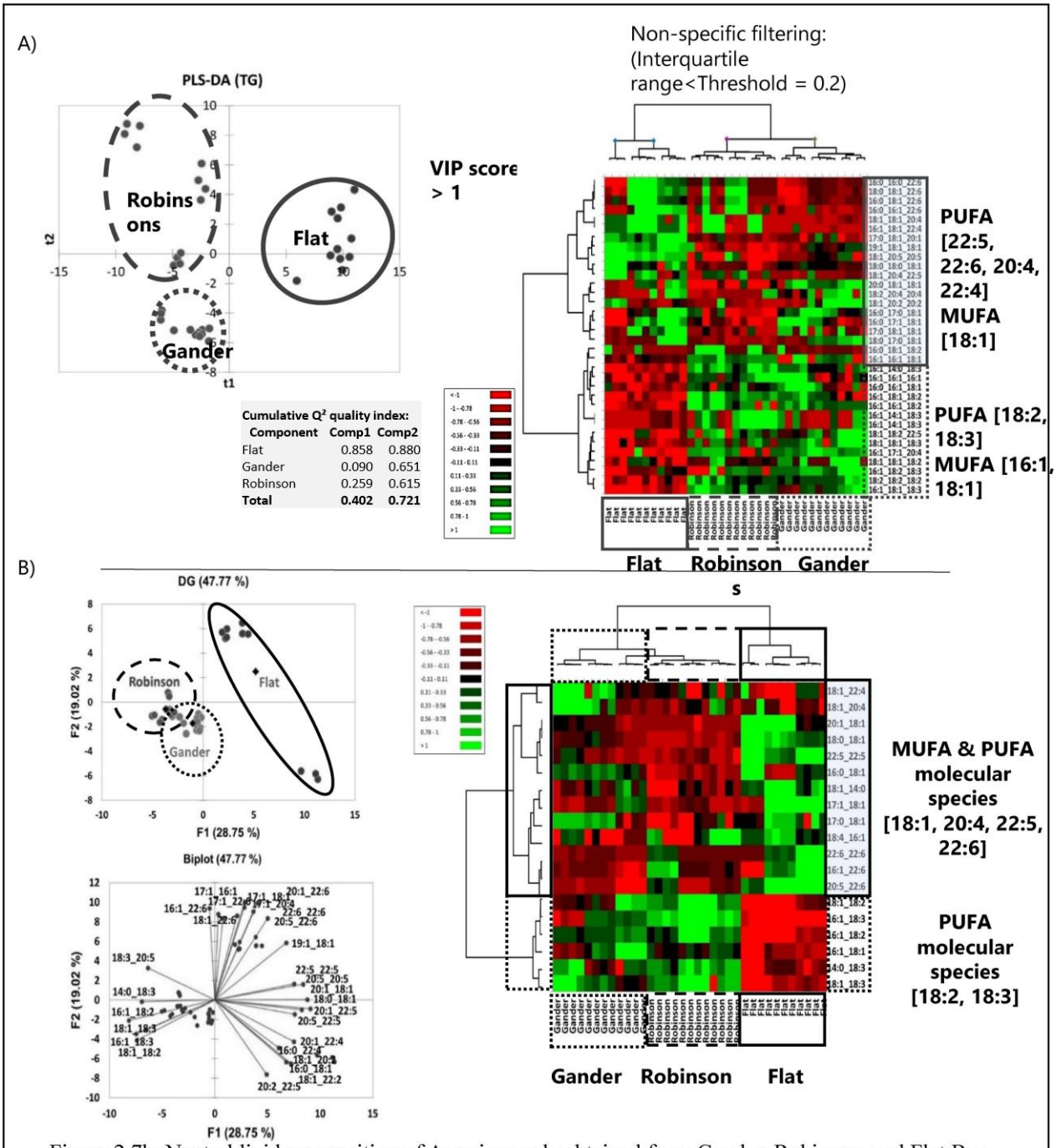


Figure 2.7b: Neutral lipid composition of American eels obtained from Gander, Robinson, and Flat Bay, NL. Partial least squares discriminant analysis (PLS-DA) score map and heatmap of select PUFA with VIP scores >1. A) TG and B) DG and location and heatmap of MUFA and PUFA molecular species. The light boxes indication and expression ratio that is less than the mean and the red boxes denote an expression ratio that is greater than the mean. The darker the color, the larger the difference there is from the mean value.

2.5 Discussion

The evaluation of American eel lipid content undertaken in this study provides insight into the nutritional value of undersize, yellow and large silver eels as well as identifies the factors that contribute to their degree of enrichment. The ability of undersized freshwater American eels from Newfoundland to be utilised in the development of high-value eel-based functional food products is highly dependent on the existence of essential fatty acid species and those with related bioactive qualities. This investigation revealed that the entire sample population of large eels were light/silver in colour, and therefore were in the migratory stage. The juvenile/sedentary phase of undersized eels were more yellow in colour (see Figure 2.2C). Additionally, migratory American eels have a greater amount of fatty acids than juvenile yellow eels (see Figure 2.4D). In addition to size, habitat also affects lipid content, with Flat Bay eels possessing the highest levels of PUFA, omega 3, and SFA, and Gander Bay eels possessing the highest levels of MUFA. In this investigation, all American eel samples contained elevated levels of MUFAs (see Figure 2.5a-b). The examination of polar and neutral lipids gave additional support for the observation that the total lipid classes of American eels are considerably influenced by the growing habitat (see Figure 2.6a-b, Figure2.7a-b). Despite having a somewhat lower lipid content, undersized American eel are nutrient-dense, high-quality ingredients for the production of secondary functional food products.

Influence of Size and Habitat Location on American Eel Color Analysis

A comprehensive examination of the association between the colour of the samples, size, and habitat location revealed that the colour difference amongst eels was correlated with their size (see Figure 2.2). Smaller eels showed higher b* values, indicating that they possessed a greater proportion of

yellow colours than their larger counterparts, and that the colour difference between the sizes was substantial. These findings are consistent with studies conducted on the life phases of the American eel, which characterise juvenile American eels as becoming darker and more yellow as they mature physically, and lighter/silver as they reach sexual maturity (Greene et al., 2009; COSEWIC, 2012; McCleave, 2001).

Evaluation of Lipid content utilising FTIR.

There has been minimal, if any, research on the lipid composition of American eels and the effect of morphological changess on the content. In this investigation, lipid signals confirmed the presence of unsaturated fatty acids in samples of American eel (Figure 2.3A). Therefore, it is expected that the sample should contain a variety of MUFA and PUFA species including essential omega-3s. Eels from the Flat Bay site were associated with -C=C- and methyl single bonds, respectively (Figure 2.3B). The presence of unsaturated fatty acids indicates nutritional and bioactive qualities that have the potential to promote human health (Marchioli et al., 2002).

Analysis of the lipids of eels of various sizes.

Further investigation revealed that American eels contain EPA and DHA, both of which are necessary fatty acids (see Figure 2.4A). In this study, lipid analysis revealed that as American eels progress from juvenile to adult size, their fatty acid concentration increases. This alteration is a result of juvenile American eels transitioning to silver after accumulating enough lipid storage in preparation for migration (COSEWIC, 2012).

Larger silver eels were associated with SFA, PUFA, omega-6s, and omega-3s, while smaller eels were associated with MUFA. Additional study revealed that smaller eels contained a somewhat lower proportion of the fatty acids seen in larger eels (see Figure 2.4

D-E). This result is consistent with the findings of Van Ginneken et al. (2007), who investigated the fatty acid content of European eels (near relatives of American eels) and found that the blood lipid content and body fat were lower in small yellow eels and higher in larger silvering eels. During its yellow pre-migratory phase, the European eel accumulated lipids in its muscle and liver in preparation for its transition to the migratory phase (Parzanini et al., 2021; Palstra & van den Thillart, 2010; Greene et al., 2009; COSEWIC, 2012; McCleave, 2001). These findings imply that yellow American eels utilise similar migration preparation strategies.

Analysis of eel lipids according to their habitat of growth.

In a recent study, Parzanini et al. (2021) found that eel lipid content can fluctuate significantly in response to changes in habit-specific environmental variables. This experiment proved the existence of interspecific variation in growth environments. Flat Bay eels exhibited the largest concentrations of PUFA, including DPA (C22:5n3), DHA (C22:6n3), EPA (C20:5n3), stearidonic acid (SA, C18:4n3), and heneicosapentaenoic acid (HPA, C21:5n3) (see Figure 2.5a), and SFA, which contained C18 and C16 (see Figure 2.5b). The EPA and DHA content of Flat Bay eels has the potential to regulate inflammatory cytokines, vasodilation and vasoconstriction, and many other important processes in the body (Wall et al., 2010), whereas the DHA content can function to reduce LDL and subsequent risks of coronary heart disease (Hu et al., 2002; Burdge and Calder, 2005; and Sokoa-Wysoczańska et al., 2018). The availability and abundance of essential fatty acids in Flat Bay eels indicate that the site is conducive for the nuturing of eels rich in polyunsaturated fatty acids (PUFA). Flat Bay may have a healthy population of microalgae to supply its much greater omega-3s content (see Figure 2b D) and total food

availability (Maltsev & Maltseva, 2021; Barkia et al. 2019; Sathasivam et al. 2019; Chalima et al. 2019, 2020). Gander eels had the largest proportion of MUFA (see Figure 2.5a), which was mostly composed of oleic acid C18:1 (n-9) followed by *cis*-vaccenic acid (C18:1n7) and arachidonic acid (PUFA) (C20:4n6). Robinson eels were supplemented with important -linolenic (C18:3n3) and linoleic (C18:2n6cis) acids, as well as 11,14,17-eicosatrienoic (C20:3n3cis) and C18:1n7 acids (see Figure 2.5b). It is believed that site-specific nutrition influenced the variation in fatty acids within a species (Dalsgaard et al., 2003). Osmoregulatory (salinity) and acclimation (temperature) may possibly have an impact on lipid content; however, measurements of these parameters are required to determine their contribution, if any (Parzanini et al., 2021). Despite variations in quantity and molecular species, the fatty acid composition of eel from each region includes bioactive fatty acids.

Polar and neutral lipid content analysis of American eels.

Long-chain polyunsaturated fatty acids (LC-PUFA) accumulate in polar lipids found in muscle tissue (Sushchik et al., 2020). This examination of polar lipids revealed that Robinson eels have the highest levels of PC (35%) and SM (36%) of any species (Figure 2.6a). Including rich sources of PC and SM which may positively affect or reduce the onset of degenerative diseases asssociated with the decline of theses phospholipids (Cooper, 2000; Abedi and Sahari, 2014). Gander Bay delivered eels with the greatest amount of LPC (16%). Flat Bay was the greatest source (30%) of PE. PC and PE molecular species enriched with MUFA were more abundant in Flat Bay samples, while PUFA-enriched molecular species were more abundant in Robinson and Gander Bay samples (Figures 2.6 B and C). Differences in lipid content can be accounted for by variations that exist between

locations. Polar lipids that are ether-linked are prevalent in American eels (see Figure 2.6b). This study discovered several PC and PE ether connections in American eels, with PE being the most prevalent. Regarding membrane fluidity and fusion, the presence of ether lipids has biological significance in humans (Dean & Lodh, 2018). In addition to regulating cell differentiation and signalling, ether-linked polar lipids also affect cell differentiation and signalling. These lipids are also capable of acting as antioxidants (Guang &Tong, 2010). Ether lipid shortage is linked to neurological illnesses, cancer, and metabolic conditions (Eisinger et al., 2014; Chen et al., 2016; Tessier et al., 2016; Alshehry et al., 2016; Dean & Lodh, 2018). Incorporating dietary sources of ether bonds is therefore essential for general human health. To my knowledge, this is the first demonstration of high ether linked polar lipids in eels found in the scientific literature.

The location of the habitat has a substantial effect on the molecular species of TG neutral lipids as well with Flat Bay eels contained more TG containing PUFA [22:5, 22:6, 20:4, 22:4] and MUFA [C18:1] molecular species (Figure 2.7B). The bioactivity associated with PUFAs from Flat Bay includes the function of Docosapentaenoic acid (DPA, C22:5 ω-6 or ω-3), which is very similar in structure to EPA, derivative in the regulation of immune phagocyte response (Gutiérrez et al., 2019). Docosahexaenoic acid (DHA, C22:6) is essential for normal brain, ocular, and skin function (Hashimoto and Hossain, 2018; Burdge and Calder, 2005; Sokoa-Wysoczaska et al., 2018). Arachidonic acid (AA, 20:4) is better recognised for its inflammatory response as it is converted to prostaglandins and leukotrienes; yet, it is essential for membrane fluidity and flexibility in all body cells due to its four cis double bonds (Fitzgerald, 2001). DHA and AA make up 20% of brain lipid (Weiser, Butt, & Hasan Mohajeri, 2016; Tallim & El Ridi, 2018); hence, it is plentiful in

breast milk as TG and phospholipids (Wijendran et al., 2002).

PUFA molecular species with 18 carbons [C18:2, C18:3] were more prevalent in eels from Robinson and Gander Bays (Figure 7B). The two most elementary necessary fatty acids are linoleic acid (LA, C18:2n-6) and -linolenic acid (ALA, C18:3n-3). ALA regulates the rhythm and pumping of the heart, as well as the influence or onset of cardiovascular disorders. LA is the principal omega-6 fatty acid that is converted to gamma-linoleic acid (GLA), which is effective in preventing various diseases (Sokoła-Wysoczańska et al., 2018). This study of the neutral lipids by HPLC-HESI-MS/MS is in accordance with the total fatty acid molecular species composition detected by GC-FID. According to Figure 2.7C, DG: MUFA & PUFA molecular species [18:1, 20:4, 22:5, 22:6] were more in eels from Flat Bay, while PUFA molecular species [18:2, 18:3] were greater in samples from Gander. Each place can serve as a source of health- promoting bioactive lipids and fatty acid species. Eels from Flat Bay are rich in polyunsaturated fatty acids, particularly C18:2n6 and EPA, while eels from Robinson are rich in omega-3 fatty acids (DHA and LN). MUFA- enriched anadromous fish products could be manufactured from either Gander or Robinson samples. Similar findings can be made about polar and neutral lipids. Flat Bay eels appear to be superior sources of PC and SM, whereas Robinson samples are superior producers of PE.

In addition, MUFA-enriched PE molecular species are more abundant in Flat Bay samples, whereas ARA- and DHA-enriched PE is more abundant in Robinson samples. Similarly, samples from gander and Robinson would be a suitable for eel-based products enriched with TG molecular species containing C18 PUFA and MUFA. Flat Bay would be a superior

supply of freshwater eels with TG molecular species enriched with DHA, ARA, EPA, and DPA in addition to oleic acid. The findings from this paper suggest undersized eels could be a good source of dietary or functional lipids which geography of the habitat location providing superior sources of different classes of fatty acids, polar and neutral lipids that could influence nutritional and functional qualities of the product.

2.6 Conclusion

Extensive investigation of freshwater American eels revealed that the smaller population shares a similar lipid profile as the bigger population. Nonetheless, the percent total fatty acid content was significantly different based on the eel's size and growth location. The overall PUFA content of the lipids of the eels was significantly different. Flat bay eels are rich in long-chain fatty acids with a high number of double bonds and -C=C carbons [C20:5, C22:3, C22:6]. Gander Bay's total MUFA content was high [C18:1]. The overall SFA content could not distinguish samples from various regions. Flat bay eels have larger concentrations of C16. The growth environment has a substantial effect on the total polar lipid classes of the eels. Total PC was greater in Robinson Bay-collected eels. Total PE was greater in Flat Bay-collected eels and a large number PE as well as a few PC had ether linkage, which is unique to this study. The examination of the molecular species in the various polar lipid classes revealed that Flat Bay samples included more MUFA molecular species and the concentration of PUFA molecular species was greater in samples from Robinson and Gander Bays. The growing environment greatly impacted the molecular species of the neutral lipids. Flat Bay eels had a greater concentration of PUFA molecular species [C20:5, C22:4, and C22:6] and MUFA [C18:1]. The PUFA molecular species

[C18:2, C18:3] are higher in eels from Robinson and Gander Bays. This investigation of neutral lipids by HPLC-HESI-MS/MS is consistent with the total fatty acid composition found by GC-FID. This study demonstrates that undersized freshwater American eels are great candidates for the production of secondary products; however, the nutritional benefits vary depending on growth area. Utilizing a combination of primary products from each site would increase the nutritional content of the secondary product through diversification.

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Chapter 3

3. Sensory Perception and Development of Kabayaki as a Functional Food using undersized Newfoundland Freshwater Eels (*Anguilla rostrata*)

3.1 Abstract

Eel is a superior source of high-quality functional lipids that has potential for development as high-quality functional food products. Undersized eels are underutilized for the production of eel products. This study aimed to develop high-quality Kabayaki products utilizing undersized Newfoundland American eel. In this study, we hypothesize that eel marinated and then cooked at reduced temperature (250°C) and oxygen exposure should have less formation of toxic lipid oxidation, harmful Maillard reaction products, and potentially carcinogenic HCAs typically generated during grilling. Furthermore, the nutritional quality of Kabayaki should improve with the incorporation of polyphenol-rich local Newfoundland cranberries. The results demonstrate that the cooking conditions did not significantly alter the eel fatty acid profile. Incorporating sous-vide cooking technique before grilling reduced the production of toxic HCAs, MRCs and VOCs in Kabayaki. Sous-vide before grilling maintained the nutritional value, increased the quality and safety of Kabayaki, and appealed more to consumers. Infusing fresh, unsweetened cranberry juice in Kabayaki marinade introduced more volatile compounds, including beneficial terpene D-limonene. Consumers preferred specific attributes of each product but were accepting of both 'Regular' and berry infused Kabayaki. Kabayaki, berry infused Kabayaki, and their corresponding marinades can serve as a ready-to-eat, frozen functional food product on the global market.

Key words: **Kabayaki, sous-vide, grilling, Maillard reaction, Lipid Oxidation (LO), Heterocyclic Amines (HCAs), Fatty acid methyl esters (FAMEs), Newfoundland cranberries**

3.2 Introduction

Kabayaki describes the Japanese method of grilling butterflied fillets of meat or fish marinated in a sweet soy-based sauce. Freshwater eel "*unagi*" is a popular protein of choice because the Japanese believe that eel can improve stamina and heal sickness/ fatigue caused by seasonal heat (Bestor 2004). Research shows that eel is rich in nutrients that can improve bodily function upon consumption. Eel contains vitamin E, A, D, K, essential amino acids, and fatty acids including essential unsaturated EPA (C22:6 n-3) and DHA (C20:5 n-3) (Harlioğlu & Yilmaz, 2011; Islam et al., 2020). These nutrients play vital roles in skin and gastrointestinal health, dental development, skeletal growth, DNA formation and replication (Balami et al., 2019; Kaźmierczak-Barańska et al., 2020). Kabayaki method of grilling is an excellent means of preparing freshwater eel as it has gained consumer acceptance across the world in the form of sushi. However, preserving the nutrients in eels can be challenging because many are susceptible to degradation and formation of toxic compounds upon exposure to oxygen and high heat. Unlike many other fish species, eel blood is poisonous; therefore, it cannot be eaten raw and must undergo some form of thermal processing (Yoshida et al., 2008). As a result, it is necessary to optimize preparation and cooking methods to preserve eel nutritional quality, reduce the generation

of toxins to ensure safety, as well as appeal to the sensory perception of consumers during the preparation of eel as a traditional or Kabayaki functional food product (Momenzadeh et al., 2017).

Thermal processing of raw freshwater eel eliminates pathogens and toxins while developing sought-after colour, taste, and aroma (Broncano et al., 2009). In the case of

Kabayaki, grilling over an open flame at high heat while continuously basting the eel with marinade generates a dark, crispy exterior while retaining a moist, chewy, succulent interior. Research shows that grilling changes the physicochemical properties of fish; however, the grilling technique and conditions control the extent of change. An increase in processing temperature and time and poor storage conditions encourages damage to essential fatty acids and overall nutritional quality which generates toxic compounds with profound health implications (Saldanha & Bragagnolo, 2010). A study conducted by Hangesti Emi Widyasari et al., (2014) characterized the fatty acid profile of fresh Indonesian eel compared to when prepared by roasting. It revealed a 0.45% and 3.87% reduction in EPA (C22:6 n-3) and DHA (C20:5 n-3) content, respectively, after roasting and steaming, followed by a second roasting. The effect of grilling (Kabayaki preparation) on American eel is unknown. However, the literature recognizes the link between extreme grilling conditions and the damage of heat-sensitive nutritional compounds, as well as the development of potential carcinogens heterocyclic amines (HCAs) and undesirable organoleptic attributes such as off-colour, off-flavour, etc. (Matsuda et al., 2013).

Maillard reaction (MR) and lipid oxidation (LO) are the main processes responsible for the changes in food nutritional value, safety, and sensory attributes during grilling. These processes occur almost simultaneously, and each pathway produces an array of volatile products that interact to promote or discourage the other reaction (Zamora and Hidalgo, 2005). Grilling conditions influence the extent to which MR and LO affect nutritional quality, safety, and sensory attributes of grill foods. Therefore, it is important to understand these pathways and interactions and to devise strategies to optimize the grilling technique or conditions to improve the safety, nutritional and sensory quality of the final product.

Maillard reaction is responsible for the characteristic colour, flavour, and texture that food develops during grilling (Nooshkam et al., 2019; Martin et al., 2000; Wang et al., 2011; & Peng et al., 2011). MR describes a cascade of reactions that occur whenever the carbonyl moiety of reducing sugars and the free amino groups on the surface of the food combine to produce volatile flavour and aroma compounds, and the brown colour (melanoidins pigment) observed in grilled foods. MR influences substantial functional and structural alterations that produce both pleasing and undesirable organoleptic qualities during cooking. LO products can also interact with intermediate MR products to create new compounds with distinct attributes (Zamora and Hidalgo, 2005). The extent of change depends on the composition of reactants and conditions, i.e., temperature and cooking time (Lund & Ray, 2017). High temperatures and prolonged cooking times drive the Maillard reaction pathway to convert desirable MR products (MRP) to prooxidants, carcinogens, and mutagens. This change is obvious during the Strecker degradation stage of MR where aldehydes, a significant contributor to flavour, accumulate and result in off-flavours (Jayasena et al., 2013). These compounds can harm human health, as well as decrease the nutritional value, sensory appeal, and shelf life of the product. The characteristic grill marks on Kabayaki are a good indicator of MR; however, the extent of MR and its effect on the nutritional quality, safety, and sensory perception of Kabayaki require identification and quantification of volatile MRPs.

Conversely, lipid oxidation occurs whenever high heat inactivates antioxidant enzymes releasing pro-oxidant nonheme iron, which denatures muscle fibres and ruptures membranes to expose phospholipids to molecular oxygen (Li & Liu, 2012; Morais De Lima Junior et

al., 2013; Amaral et al., 2018). Reactive oxygen species (ROS) act as free radicals and react with unsaturated fatty acids to form primary products (hydroperoxides) that can decompose to secondary products (hydrocarbons) (Tatiana & Bragagnolo, 2010). MR products can also affect LO as they can function as either pro-oxidants or antioxidants (Zamora and Hidalgo, 2005). At this stage, volatile organic compounds (VOCs), including alkanes, alkenes, acids, ketones, esters, and aldehydes (key contributors) begin to produce undesirable flavours and odours (Grebenteuch et al., 2021). Advanced lipid oxidation can cause further decline in sensory qualities (discolouration, texture modifications, rancidity, and off-flavour) which decreases consumer acceptance of the product (Purriños et al., 2011). An increase in processing temperature and time and poor storage conditions can degrade essential fatty acids contributing to the generation of toxic compounds with profound health implications (Saldanha & Bragagnolo, 2010). Freshwater American eels may be especially vulnerable to lipid oxidation because it is rich in highly unsaturated lipids, particularly omega-3 fatty acid (EPA) (Hamilton et al., 2022). However, identification and quantification of lipid oxidation products is important to determine the extent to which these products negatively impact the nutritional quality, safety, and sensory perception of Kabayaki.

Maillard reaction generates HCAs when muscle tissue becomes exposed to high temperatures (>100 degrees Celsius) (Gibis, 2016, Skog et al., 1998). HCAs are the reactionary products of sugars, amino acids, and creatinine. The generation of HCAs is heavily influenced by the presence of its precursors in combination with physical factors such as the nature of the muscle tissue, grilling temperature, time, and technique (Hur et al., 2019). High heat is the main contributor to the formation of HCAs during grilling, followed by the grilling time. HCAs then generate ROS which promotes oxidative stress

inside the body and heightens the risk of chronic diseases, such as pancreatic and colorectal cancers (Carvalho et al., 2015; Anderson et al., 2005; Sinha et al., 2005). Nevertheless, consumption of well-done muscle tissue is the leading source of HCA intake for humans, superseding that of tobacco (Matsumoto et al., 1981).

Cooked foods mainly contain thermic HCAs some of which can be categorized as *probable human carcinogen and include:* 2-amino-3-methyl-imidazo [4,5-f]quinoline (IQ), 2-amino-3-methylimidazo [4,5-f]quinoxaline (IQx) and *reasonably anticipated to be a human carcinogen:* 2-amino-3,4-dimethylimidazo [4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo [4,5-f]quinoxaline and (MeIQx), DiMeIQx (2-amino-3,4,8-trimethyl-imidazo [4,5-f]quinoxaline), and 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) (International Agency for Research on Cancer, 1993). As a result, it is important to identify, quantify, and reduce HCA presence in grilled foods such as Kabayaki to ensure consumer safety.

Kabayaki has great potential as a functional food; however, it requires preliminary evaluation of the nutritional quality and safety and subsequent reduction of toxic compounds, maintenance of nutritional quality, and assurance of sensory appeal. Research shows that regulating cooking time/temperature and incorporating sources of antioxidants can inhibit the formation of HCAs and toxic MRPs (Nooshkam et al., 2019; Cropotova et al., 2019; Platt et al., 2010). Furthermore, reducing oxygen exposure can also combat lipid oxidation (Cropotova et al., 2019).

Traditional cooking methods for fish usually require high heat in aerobic conditions. The

sous-vide cooking method is an alternative that cooks food to a precise temperature throughout and eliminates free oxygen molecules by vacuum sealing the food product. This cooking technique involves packing food in a heat-stable, food-grade vacuum sealing bag and suspending it in a water bath at a controlled, low temperature (50°C–65°C) for a prolonged period (4–6 hours) (Kim et al., 2019). While cooking, the warm water circulates the package to cook the food evenly without it being in contact with direct heat. Sous-vide food can be consumed after reaching the recommended internal temperature or kept in the package for longer shelf life (6–42 days) which restrict the growth of microbial organisms. Sous-vide meat/fish regained popularity and wide consumer acceptability in the early 2000s because the final product tends to be more tender, flavorful, and succulent. The sous-vide technique also effectively retains heat-sensitive vitamins, antioxidants, and unsaturated fatty acids due to the low cooking temperature (250°F) (Ortuño et al., 2021; Vaudagna et al., 2002) thus increasing the nutritional quality of the finished product. Admittedly, sous-vide food can appear dull after cooking, so searing on the grill for a short time can helpgenerate colour. Incorporating the sous-vide technique in the preparation of Kabayaki may assist in preserving nutritional quality as well as improving the safety and sensory quality. Sous-vide vacuum packaging is ideal for marinated food as it retains all the seasonings and juices during cooking. Marination is an easy method for infusing flavour, antioxidants, and other functional compounds into food before cooking. Marinades often have a liquid base seasoned with herbs and spices to add flavour, aroma, colour, and functional properties. In the case of Kabayaki, soy sauce, mirin, sake, and sugar impart an umami flavour. Soy sauce alone promotes digestion, antioxidant, antimicrobial activity, antihypertensive, and anticarcinogenic properties.

Antioxidant supplements can effectively disrupt the formation of free radicals via quenching and scavenging HCAs during MR (Vitaglione & Fogliano, 2004). These compounds donate extra electrons to neutralize free radicals; however, not all antioxidants have the same capacity or bioactivity. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butyl hydroquinone (TBHQ) are common synthetic antioxidants used in food production. These antioxidants are cheap, effective means of reducing oxidation compared to natural antioxidants; however, they require safety assessments because of potential side effects (Fasseas et al., 2007). Conversely, natural antioxidants, such as vitamin C (ascorbic acid) and vitamin E (α-tocopherol), are more expensive, but have a higher antioxidant capacity, consumer acceptance, and lower safety risks because antioxidants from plants or natural sources tend to be safer. Phenolic compounds have one of the most considerable antioxidant capacities among phytochemicals. These antioxidants arrest the formation of and scavenge ROS and prooxidants **(**Kumar et al., 2015). Nooshkam et al. (2019) reported that certain natural antioxidants could target toxic MRPs without disturbing the formation of desirable MRPs and color pigments. Natural antioxidants are ideal for functional food development because they contribute additional flavor and color, and some even soften the texture of the muscle tissue. Incorporating a good source of antioxidants in Kabayaki could potentially improve the nutritional or sensory quality of the product and offer protective effects against the formation of harmful oxidative compounds during grilling.

American cranberries (*Vaccinium macrocarpon*) have great potential as a functional ingredient because of their rich polyphenol, anthocyanins, proanthocyanidins (type A), vitamins, and phenol content (Côté et al., 2010; Odjo et al., 2022). Cranberry phenolics

have anticarcinogenic, anti-inflammatory, antioxidant, antiviral, antibacterial, anti-adhesion, antimutagenic and antiangiogenic properties (Caldas et al., 2018). Moore et al. (2019) determined that polyphenols and volatile extracts from cranberry inhibit NO formation before and after inflammation. Specifically, volatile monoterpenes, such as α-terpineol, play a significant role in reducing oxidation, thus cranberries are recognized in the scientific literature to be an effective source of natural antioxidants (Caldas et al., 2018). However, fresh cranberries have a sour/ tart taste that can become overwhelming; as a result, consumers typically repurposed them into juice, jams, etc. Cranberry juice is rich in terpenes, aliphatic alcohols, aliphatic aldehydes, and acids (Croteau & Fagerson, 1968). Incorporating cranberry juice into Kabayaki sauce formulation could improve the nutritional quality, safety, and sensory perception of the product.

To the best of our knowledge, this is the first study to incorporate sous-vide coupled with grilling to prepare Kabayaki. In this study, we hypothesize that eel marinated and then cooked at reduced heat and oxygen exposure should have reduced formation of toxic lipid oxidation and Maillard Reaction Products as well as potentially carcinogenic HCAs typically generated during grilling. Furthermore, the nutritional quality of Kabayaki should improve with the incorporation of polyphenol-rich local Newfoundland cranberries. The aim is to develop a high-quality Kabayaki product utilizing undersized Newfoundland freshwater American eel as a functional food. Thus, the objective of this study was to use sous vide along with grilling as a preparation technique to maintain the nutritional and sensory quality, as well as improve the safety of Kabayaki. Secondly, to develop Kabayaki as a functional food product by incorporating polyphenol rich Newfoundland cranberries.

3.3 Methods and Materials Preparation of Sample

Eel fillets were received from local fishermen and industry partners from Robinsons Bay, Flat Bay, and Gander Bay, in the province of Newfoundland and Labrador. The eels were separated into three pairs: (i) cleaned, (ii) skinned, and (iii) butterflied fillets and replicated four times. One of each pair was reserved for baseline analysis, and the other was used for marination. The samples were weighed in grams to determine the amount of marinade required for the experiment. Using the total weight of the fillets for marination, four times the volume of the marinade was initially prepared using mirin, soy sauce, sake, and granulated sugar in a 1:2:1:5-part ratio, respectively. This formulation was modified based on the traditional Japanese Kabayaki sauce. A new formulation was made using a 2:2:1:1 ratio and white was replaced with brown sugar. Each experiment was denoted with the letter ‘K’ + the number of the experiment - ‘M’ + marination time. For example, K1-M3 represents the Kabayaki sample from experiment one- marinated for 3 hours.

'Berry Infused' or 'BI' Kabayaki samples were made using two parts of the regular sauce to one part of Newfoundland cranberry juice. After 45 minutes of constant stirring and cooking, the berry-infused sauce was reduced to half the starting volume. Once the sauce had cooled, the eels were marinated in a 2:1 ratio of sauce to eel. Half of the samples were marinated for 3 hours, vacuum packed, then sous-vide for 15 minutes at 158 ºF before grilling at minimum heat for 3-minutes (flipped twice).

Extraction of the volatile components by solid-phase microextraction coupled to gas chromatography/ mass spectrometry (SPME/GC-MS)

A 1-gram ground sample from each replicate was placed in a 10 mL headspace vial. After equilibrating the sample at 50 ° C for 5 minutes, a fibre coated with divinylbenzene/ carboxyne / polydimethylsiloxane (DVB / CAR / PDMS) of the following dimensions: length 1 cm, film thickness 50/30 μm (Supelco, SigmaAldrich, St. Louis, MO, USA) was inserted into the headspace of the sample vial and held there for 60 minutes after which the sample was desorbed in a TSQ 8000 triple quadrupole mass spectrometer [GC/MS) (ThermoScientific, Brampton, Ontario, Canada) coupled to a Trace 1300 gas chromatograph for analysis. The extracted volatile compounds were purified using a non-polar stationary phase ZB5MS column (30 m x 0.25 mm i.d., 0.25 μm film thickness, Phenomenex, CA, USA) at a flow rate of 1 ml/min with Helium as the carrier.

After the extraction period, the fibres were desorbed at the injection port for 10 minutes. Operating conditions of the device with a purge time of 5 minutes: splitless mode was used for infusion with a flash time of 5 minutes. The oven temperature was initially set to 50 ° C (held for 5 minutes) and then increased to 290 ° C (held for 2 minutes) at 4 ° C / min. The ion source temperatures and the quadrupole mass spectrometer were set to 230 ° C and 150 ° C, respectively. The injector and detector temperatures were maintained at 250 ° C and 290 ° C. The mass spectrum was recorded with ionization energy of 70 eV, and the data acquisition was performed in scan mode. After desorption of each sample, the fibre was washed in a conditioning station at 250 ° C for 10 minutes. NIST / EPA / NIH (version 2.2, ThermoScientific) was used to identify volatiles present in the samples (Goicoechea and Guillen, 2014; Vidal et al., 2016; Vidal et al., 2020).

Extraction and Analysis of Heterocyclic Amines (HCAs)

Samples were prepared for accelerated solvent extraction (Dionex ASE 350, Thermo Scientific, MO, USA) as follows: One gram of ground Kabayaki sample was spiked with 50 uL of 250 ppb TriMeIQx (2 –amino-3, 4,7, 8- tetramethylimidazo [4,5-f] quinoxaline) as an Internal Standard solution and then mixed thoroughly with 2.5 mL of 0.5 M NaOH in MeOH/Water (70:30 v/v) for one hour until completely homogenous. The ASE (10 mL stainless steel) cell was preloaded with 1 g of neutral aluminum oxide (Al_2O_3). The mixture was then combined with diatomaceous earth (1:2 w/w) then loaded into the cell. The corresponding collecting batch of each cell was preloaded with 2.5 grams of sodium sulphate (Na_2SO_4) to absorb any remaining moisture. Dichloromethane/acetonitrile (CH_2Cl_2:CN 1:1, v/v) was used to extract the sample. The cells were loaded onto the rack

of the ASE 350 (Dionex ASE 350, Thermo Scientific, MO, USA) and programmed to run according to the following conditions: Static time: 10 minutes, Temperature: 80 C, Heat: 5 minutes, Static cycle: 1, Rinse volume: 20%, Purge time for 60 s, Pressure: 1500 psi. After extraction 10mL of the solution (extract) was collected from each sample and used for quantitative HCA analyses (Ouyang, Li, Tang, Jin, & Li, 2015). The extracts were dried using the rotating evaporator, resuspended in methanol, filtered using Mini-UniPrepTM G2 syringeless filter (0.2 m, Whatman, Buckinghamshire, UK), then analyzed with ultrahigh performance liquid chromatography coupled with high resolution tandem mass spectrometry (UHPLC-HRMS/MS).

Heterocyclic amines (HCAs) were analyzed following methods in Manful et al. (2019), except using Kabayaki samples. Briefly, prepared stock HCA standard solution (amine 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-Amino-3,4-dimethylimidazo[4,5-f]quinolone (MeIQ), 2-amino-3,4,7,8-tetramethylimidazo[4,5-f]quinoxaline (TriMeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 1-Methyl-9-H-pyrido[3,4-*b*]indole (Harman) and 19H-pyrido[4,3-b]indole (Nor-Harman)) was serially diluted using acetonitrile:water (10:90 v/v) and internal standard 0.05 g/mL TriMeIQx to form six calibration standard solutions (0-100 g/L).

The analytes were ran on an automated Dionex UltiMate 3000 UHPLC system under Chromeleon software on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, MO, USA) in positive ESI mode. Orbitrap was optimized to run under the follow conditions: sheath gas: 8, auxiliary gas: 2, ion spray voltage: 4.50 kV, capillary temperature: 320 °C;

S-lens RF: 100 V; capillary voltage: 30 V, mass range: 100–1000 m/z; full scan mode at a resolution of 60,000 m/z; top-3 data dependent MS/MS at a resolution of 30,000 m/z and collision energy of 35 (arbitrary unit); injection time 15 min; isolation window: 1.5 m/z; automatic gain control target: 2 e5 with dynamic exclusion setting of 30 s. Separation was conducted on the Luna C18 column (100 2.0 mm I.D., particle size: 3 m, pore diameter: 100; Phenomenex, California, USA) using the following solvent system: solvent A: pure acetonitrile; solvent B: H2O; and solvent C: 30 mM ammonium formate (pH 3.2) and in accordance with the following gradient: 0–1 min 10% B, 1–3 min 10–20% B, 3–6 min 20–30% B, 6–9 min 30–40% B, 9–12 min 40% B, 12–13 min 40–50% B, and re-equilibrated at 90% A for 2 min. Ten liters of the sample was put into the apparatus, and chromatographic separation was performed at 20 °C with a flow rate of 0.2 mL/min.

Fatty Acid Extraction, Methylation, and Analysis

Lipids were extracted from the eel samples using the modified Bligh and Dyer method (Bligh and Dyer, 1959) as follows: To 100 mg of eel sample, 1.5 mL of methanol and 1.5 mL of chloroform were added and the sample homogenized for 2 minutes. A total of 3.8 mL of distilled water was added to the mixture after which it was then centrifuged. The resulting mixture was separated into two parts, an aqueous top layer and an organic bottom layer.

The organic layer was carefully removed and transferred into a pre-weighed vial to which 100 μL of 2,2-Dimethoxypropane (DMP,a water scavenger) was added. The mixture was dried under nitrogen until there was no sign of water. The vial was then reweighed to acquire the total lipid content recovered. Next, 1mL of 1 mg/mL CHCl3:MeOH (1:1v/v)

was added to redissolve the extract from which 100 µL from the organic phase to a new 2mL vial. Next, 100 µL of internal standard (C18 ALK 0.1 mg/mL) was added to the organic phase and dried under nitrogen. Aliquot (400 µL) of freshly made methanolic- HCl 1.5N (Sigma-Aldrich, ON, Canada), was added, vortexed, and then heated at 80 °C for 1 hr in the drying oven. Next, 0.8mL of distilled water was added to the extract until it became cloudy, to which 0.5 mL of n-hexane was added and vortexed until clear separation was achieved. The upper layer was added to a new GC vial. This step was repeated in 0.5mL increments until 1.5mL of n-hexane was used for lipid recovery. DMP was then added to the mixture, then dried under nitrogen gas. The residue was then resuspended in 100 µL of hexane and ran on GC MS/FID. Fatty acid methyl esters (FAMEs) in the samples were determined using authentic standards (Supelco PUFA No. 3 mix, Supelco 37 component mix, Supelco FAME mix C8–C24; Sigma Aldrich, ON,Canada) and by comparison with the NIST database.

FAMEs analysis was performed using the Trace 1300 gas chromatography coupled to a Flame Ionization Detector (Thermo Fisher Scientific, Waltham, MA, USA). One microliter of FAMEs samples was injected by the Tri-plus auto-sampler in splitless mode. Analytes were carried through the column using He gas at 1 ml/min in the mobile phase and then effectively separated using DB-23 column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies, Santa Clara, CA, USA) in the stationary phase. Oven temperature was set at 50 °C and maintained for 1 minute, after which it was raised to 20 °C/min, 175 °C and maintained for 1 minute, and finally 230 °C at 4 °C/min and maintained for 5 minutes. Values are reported as nmole percent and were calculated using standard curves to determine the concentration of each fatty acid (Vidal et al., 2018).

Sensory Analysis

One and eight participants from Grenfell University and the wider Corner Brook community in Newfoundland and Labrador participated in an affective (consumer) test for a 15-minute survey. The survey was aimed at determining consumer perception of the sensory attributes and overall liking of the Kabayaki product. A follow up survey of 80 participants was also conducted to investigate consumer perception of the Berry Infused and Regular Kabayaki samples, and sauce recipes and packaging configurations. The participants agreed to a consent form and were made aware of potential allergens before starting. The Grenfell Campus Research Ethics Board at Memorial University of Newfoundland (MUN) approved the procedures for using human subjects in sensory panel evaluations. The products were evaluated using a 7-point hedonic scale (1: Extremely dislike to 7: Like very much). The samples were prepared as described above and then frozen. The samples were thawed at 30% power for 2 minutes in a temperature, time and power control microwave. Samples were reheated at the time of serving at 50% power for 30 seconds. The eels and sauce samples were assigned three-digit codes according to the respective treatment, and each was served in a quadrant of a white paper plate. The survey was conducted in the Functional Foods Sensory Lab at Grenfell Campus, equipped with individual booths where each participant completed the questionnaire. The survey was created, executed, and analyzed using SIMS 2000 sensory survey software (Sensory Computer Systems, NJ, USA).

Statistical Analysis

Four replicates were made for each treatment and the experiments repeated twice. A supervised multivariate analysis approach using XLSTAT (Addinsoft, New York, USA)

was applied to the eel fatty acids, volatiles, antioxidants, complex lipid classes and molecular species. The lipid classes and molecular species were identified using the Lipid Search version 4.1 (Mitsui Knowledge Industry, Tokyo, Japan) and manually confirmed with XCalibur 4.0 software (ThermoScientific, MO, USA). Principal component analysis (PCA) was used to ordinate the samples based on similarity and Analysis of variance (ANOVA) applied to determine the significant differences between treatments ordinated in different quadrants of the PCA biplot. Fisher's least significant difference (LSD) was used to separate the means whenever significant effects were observed at $\alpha = 0.05$. Figures were created using the XLSTATs premium version (Addinsoft, Long Island, NY, USA) and Sigma plot 13.0 (Systat Software Inc., San Jose, CA).

3.4 Results

Analysis of fatty acid content in Regular and BI Kabayaki samples

Kabayaki fatty acid content analysis

The fatty acid profile of Kabayaki is essential in determining the effect of grilling on eel nutritional quality. Eel fatty acid content (SFA, MUFA, PUFA, omega 3 and 6) was not affected by grilling at high heat after 1.5 and 3 hours of marination (see Figure 3.1).

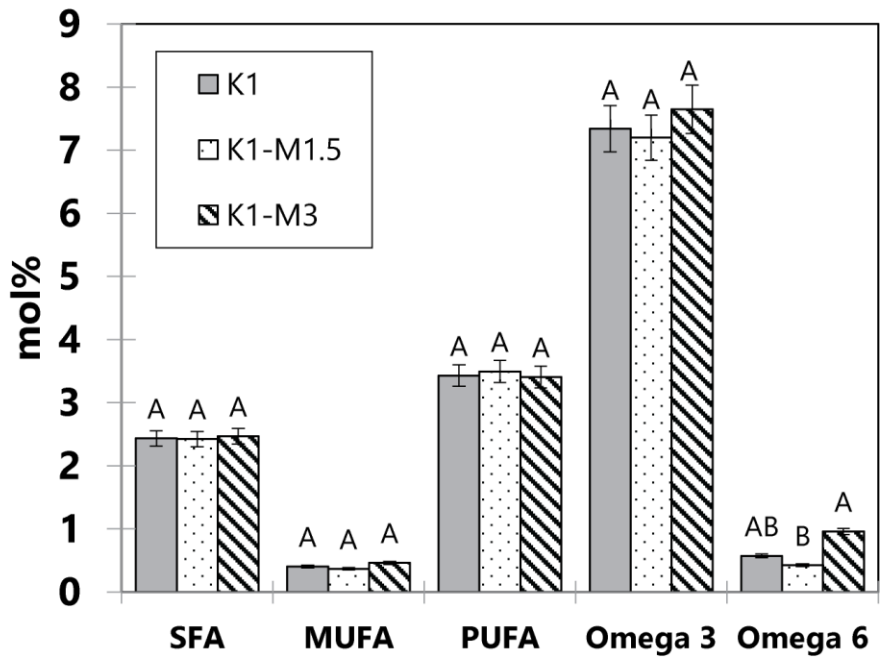

Figure 3.1: Total fatty acid content of raw eel samples (K1) and samples grilled at high heat after 1.5 hours (K1-M1.5) and 3 hours (K1-M3) of marination. Samples were randomly selected from Flat Bay, Gander Bay, and Robinson Bay located on the island of Newfoundland in the province Newfoundland and Labrador, Canada. Values in bar chart represent meant ± standard errors of the scores. Means accompanied by different superscripts are significantly different at LSD $\propto = 0.05$, n=40.

Analysis of fatty acid content in BI samples

Even though cranberries do not contain many fatty acids, their effect on maintaining the fatty acid content of grilled eel is unknown. Figure 3.2. showed that there were a few changes in fatty acids among samples. The most abundant MUFAs were C18:1n9cis, C16:1, and C18:1n9cis in BI samples, followed by C24:1n9. The other identified MUFAs were in negligible amounts. BI and regular Kabayaki had significantly higher than K1-M3 in C16:1, C18:1n9cis, and C24:1n9. BI samples had a significantly higher C18-1n9trans fatty acids than regular and K1-M3 samples. The most abundant PUFA species included C22:5n3, C20:5n3C20:3n3 (in K1-M3), followed by C22:5n3. BI samples were significantly higher in C22:6n3 and C20:5n3 than in regular (non-berry infused) Kabayaki samples. K1-M3 samples were significantly higher in C20:3n3 and C22:6n3. Notably, Regular samples had significantly more C20:4n6, while the K1-M3 samples had more C18:2n6cis. The most abundant SFAs were C16, C11, and C18, followed by C14. Compared to BI and Regular samples, K1-M3 had significantly higher levels of C14. Analysis of the total FAs showed that the MUFA content was significantly greater in Regular Kabayaki and BI samples. BI and K1-M3 had higher omega-3 content compared to Regular Kabayaki samples. Omega-6 content was significantly greater in Regular Kabayaki samples compared to K1-M3 samples. SFAs content was not significantly different across samples.

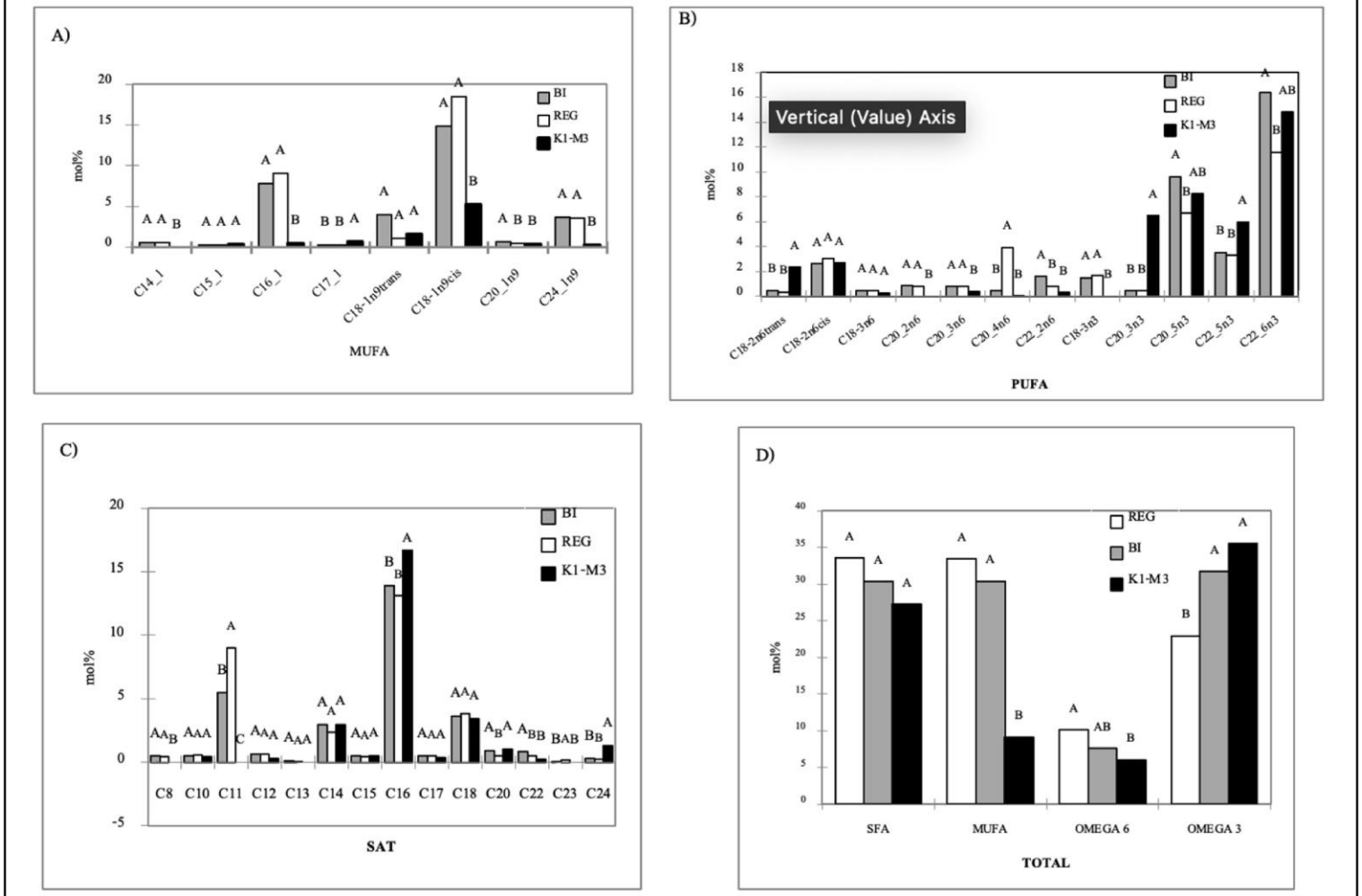

Figure 3.2 Fatty acid content of Kabayaki experiment K1-M3: samples grilled at maximum temperature (450) after 3 hours of marination. Regular K7-M1.5: Kabayaki samples sous-vide (158), grilled at minimum temperature (250), and marinated for 1.5 hours. Berry Infused (BI): regular samples infused with Newfoundland cranberry juice. A) Monounsaturated (MUFA) B) Polyunsaturated (PUFA) Saturated (SFA) fatty acid profile and) summary of total fatty acids for each level of saturation and Omega 3 & 6 content. Samples were randomly selected from Flat Bay, Gander Bay, and Robinson Bay located on the island of Newfoundland in the province Newfoundland and Labrador, Canada. Values in bar chart represent means ± standard errors of the scores. Means accompanied by different superscripts are significantly different at LSD = 0.05, n=22.

Analysis of Heterocyclic Amine (HCA) content in Kabayaki

Heterocyclic Amines (HCAs) are a grilling by-product of concern due to their carcinogenic properties. To identify and quantify the HCAs generated from Kabayaki, extraction from samples after varying marination times and grilling temperatures were analyzed using Liquid Chromatography-Mass Spectrometry (LC-MS). IQ, MelQ, MelQx, and NorHarman were present in all samples; but was in low in abundance (<100 µg/L). The samples marinated in the original sauce formulation for 3 hours and grilled at a temperature of 450°F for 3 minutes on each side (K1-M3) had the highest quantity of HCA (see Figure 3.3A). In addition, these samples had significant charring on their surface. There were significantly lower abundances of total HCAs in samples cooked using the same parameters but marinated for 1.5 hours (Figure 3.3B). HCAs were also in low abundances in subsequent experiments where samples were marinated for 1.5 and 3 hours in the original sauce formulation and grilled at a temperature of 350°F for 1.5 minutes on each side (K2- M1.5 and M3, respectively). However, samples sous-vided (158°F) and grilled at a temperature of 350°F for 1.5 minutes on each side (K3-M1.5 and M3) had the most significant reduction in total HCAs for both marination times (Figure 3.3B).

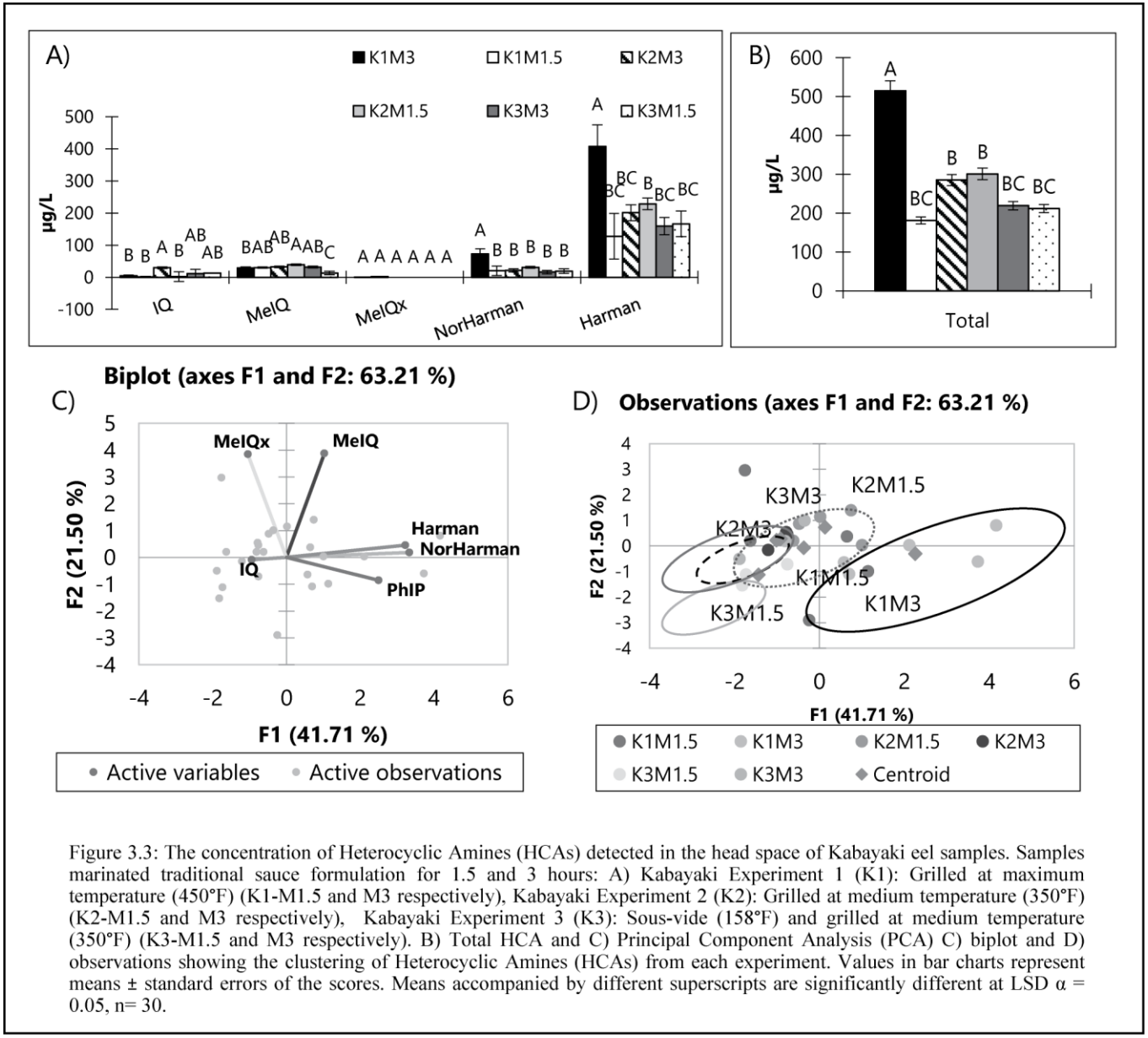

Figure 3.3: The concentration of Heterocyclic Amines (HCAs) detected in the head space of Kabayaki eel samples. Samples marinated traditional sauce formulation for 1.5 and 3 hours: A) Kabayaki Experiment 1 (K1): Grilled at maximum temperature (450°F) (K1-M1.5 and M3 respectively), Kabayaki Experiment 2 (K2): Grilled at medium temperature (350°F) (K2-M1.5 and M3 respectively), Kabayaki Experiment 3 (K3): Sous-vide (158°F) and grilled at medium temperature (350°F) (K3-M1.5 and M3 respectively). B) Total HCA and C) Principal Component Analysis (PCA) C) biplot and D) observations showing the clustering of Heterocyclic Amines (HCAs) from each experiment. Values in bar charts represent means ± standard errors of the scores. Means accompanied by different superscripts are significantly different at LSD α = 0.05, n= 30.

Principal component analysis (PCA) was conducted to discern how the treatments clustered together (Figure 3.3C-D) based on HCA formation. The output of the PCA observation and biplots accounted for 63.21% of the total variation (F1 41.71% and F2 21.50%) in the HCA data set. Data Points representing K1-M3 samples clustered in quadrant 2 with Phlp, while the samples in treatment K2M1.5 clustered in quadrant 1 with MeIQ; while MeIQx clustered with treatments K2M3 and K3M3 in quadrant 4 (see Figure 3.3C). The corresponding biplot indicates that there may exist a potential relationship between Harman, NorHarman, and Phlp and samples marinated in the original sauce for three hours and grilled at a temperature of 450°F for three minutes on each side (K1-M3).

Subsequent experiments (K4-K7) modified the conditions even further to improve safety. The results of the change in the formulation of the traditional marinade recipe of 1:2:1:5 to 2:2:1:1 (mirin, soy sauce, salted sake, and granulated sugar) showed a 200x reduction in the intensity of the HCA peaks, indicating a decrease in abundance of HCAs generated after grilling and application of sous-vide prior to grilling (K4 and K5 respectively) (see Figure 3.4C and D). Subsequent experiments incorporated the new sauce formulation and reduced the temperature to the lowest heat for each cooking method. Figure 3.4A through G shows a consistent reduction in the intensity of HCA peaks as the temperature decreases and incorporates the sous-vide cooking technique. At minimum grilling temperature (250°F), the chromatograph showed no distinct peaks indicating the generation of HCAs were restricted in experiments utilizing the grilling and sous-vide before grilling at minimum temperature (K6 and K7, respectively) (Figure 3.4F and G).

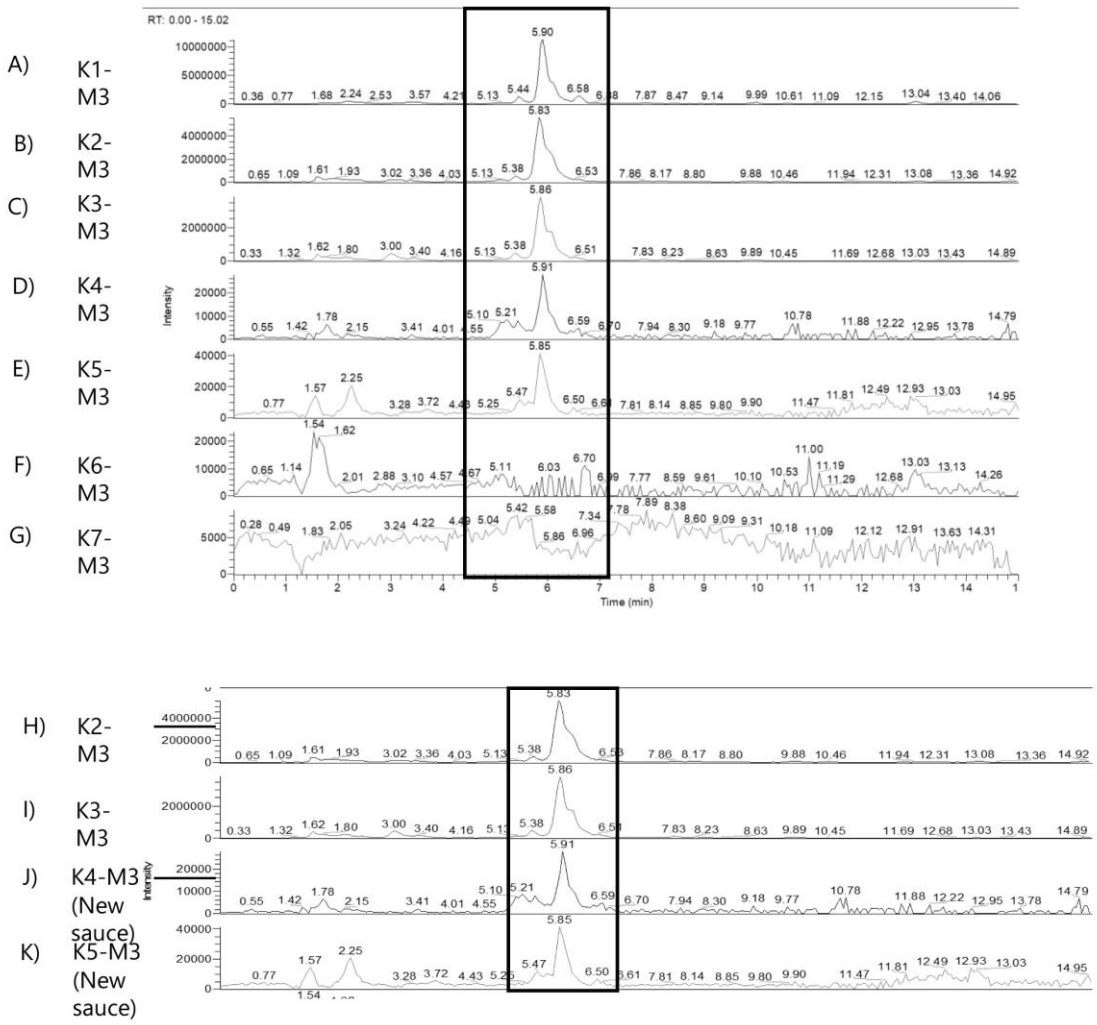

Figure 3.4: Chromatograms of the headspace compounds extracted from samples marinated traditional sauce formulation for 3 hours: A) Kabayaki Experiment 1 (K1): Grilled at maximum temperature (450°F) (K1-M3), B) Kabayaki Experiment 2 (K2): Grilled at medium temperature (350°F) (K2-M3), C) Kabayaki Experiment 3 (K3): Sous-vide (158°F) and grilled at medium temperature (350°F) (K3-M3). Samples marinated new sauce formulation for 3 hours: Kabayaki Experiment 4 (K4): D) Grilled at medium temperature (350°F) (K4-M3). E) Kabayaki Experiment 5 (K5): Sous-vide (158°F) and grilled at medium temperature (350°F) (K5-M3), F). Kabayaki Experiment 6 (K6): Grilled at minimum temperature (250°F) and marinated for 3 hours (K6-M3), G) Kabayaki Experiment 7 (K7): Sous-vide (158°F) and grilled at minimum temperature (250°F) (K7-M3). H-K) Chromatogram showing the reduction in peak intensity with the change in sauce formulation.

Analysis of Volatile Compounds in Regular Kabayaki samples

The analysis of volatile compounds provides insight into the changes happening in food during processing. In this experiment, the GC-MS detected varying abundances of volatiles including volatile oxidative compounds, Maillard reaction compounds, and terpenes depending on sample preparation and processing. The chromatograph in Figure 3.5 showed the effect of cooking temperature on the number and abundance of volatile compounds. Kabayaki samples marinated in the original sauce formulation and then grilled at maximum temperature (450°F) for 3 minutes on each side (K1-M3) showed the greatest abundance of volatile oxidation compounds. The number of peaks in grilled samples (K2-M3 and K3-M3) appeared to decrease with the decrease in temperature and cooking technique. Sous-vide samples (K3-M3 and K7-M3) had even fewer volatile compounds compared to samples grilled at medium and low temperatures (250°F) (Figure 3.5). The lowest abundance of volatile oxidation products was observed in samples marinated in the new sauce formulation and sous-vide (158°F) and then grilled at minimum temperature (250°F) for three minutes on each side (K7-M3).

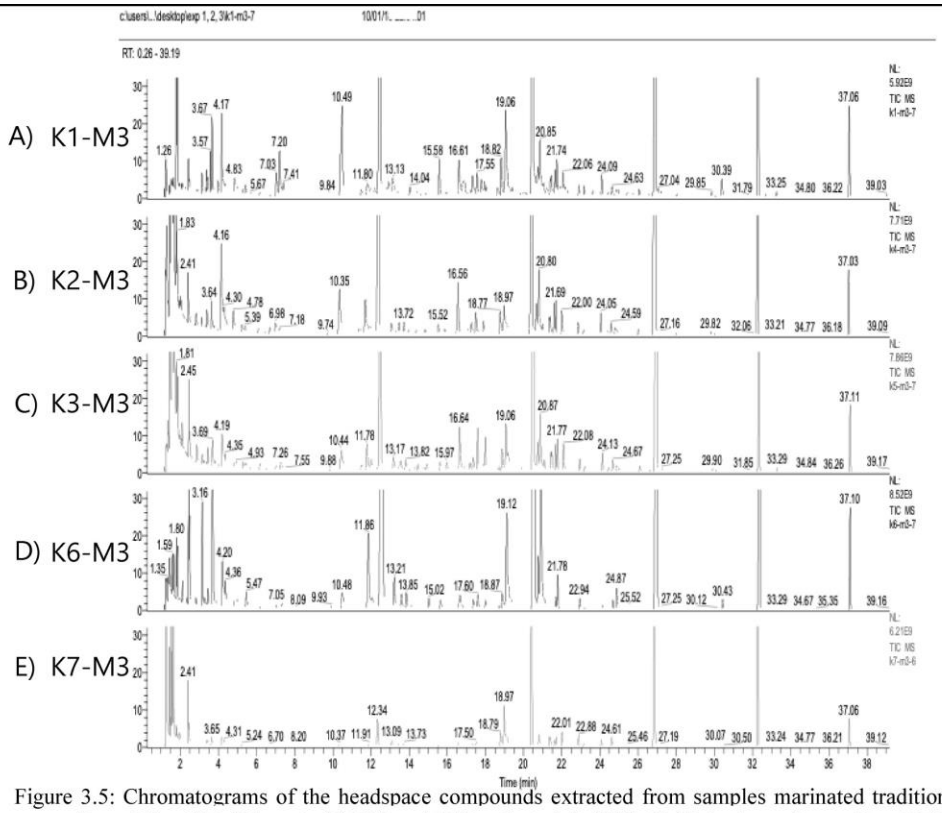

Figure 3.5: Chromatograms of the headspace compounds extracted from samples marinated traditional sauce formulation for 3 hours: A) Kabayaki Experiment 1 (K1): Grilled at maximum temperature (450°F) (K1-M3), B) Kabayaki Experiment 2 (K2): Grilled at medium temperature (350°F) (K2-M3), C) Kabayaki Experiment 3 (K3): Sous-vide (158°F) and grilled at medium temperature (350°F) (K3-M3). Samples marinated new sauce formulation for 3 hours: D) Kabayaki Experiment 6 (K6): Grilled at minimum temperature (250°F) and marinated for 3 hours (K6-M3), E) Kabayaki Experiment 7 (K7): Sous-vide (158°F) and grilled at minimum temperature (250°F) (K7-M3).

Evaluation of volatile content in berry-infused samples

An analysis of the volatile compounds in regular and BI samples determined if cranberries enhanced the product's quality and safety. Figure 3.6 compared chromatographs of VOCs generated from regular and berry infused Kabayaki samples. There was an observable difference in abundance of VOC in each experiment. Regular Kabayaki samples had a greater abundance of VOCs at retention time (RT) 1.22 min, but lower levels at 3.06 min and 12.33 min than BI samples. Conversely, BI samples had a lower abundance of VOCs at RT 1.58, and greater abundances at RTs 3.57, 11.53 and 12.26 min respectively.

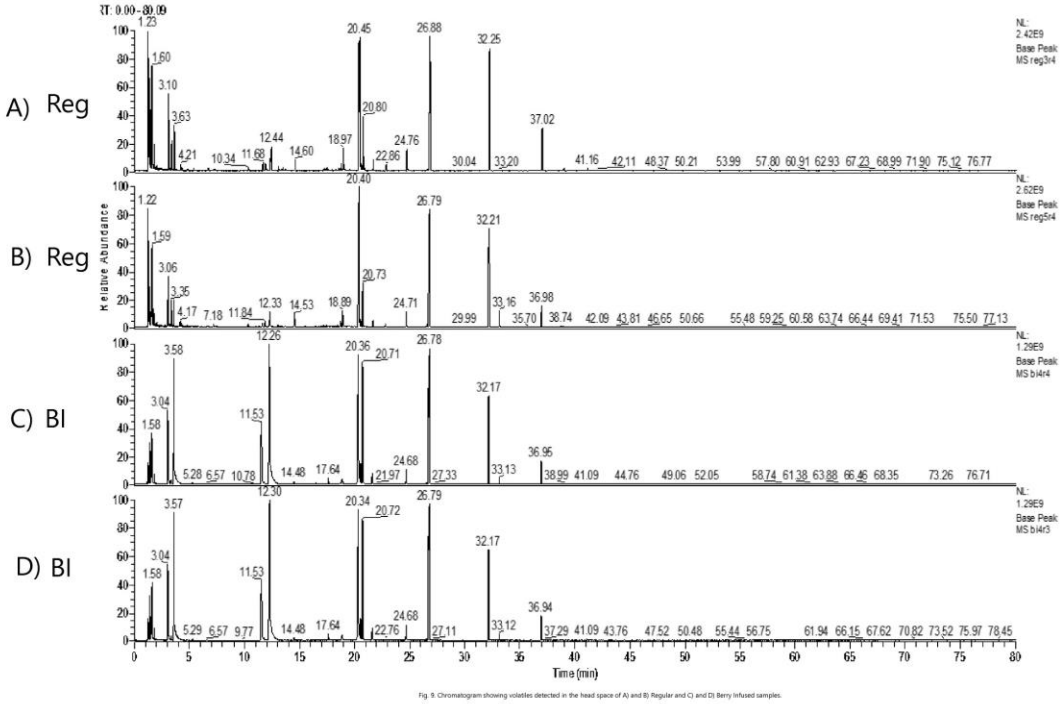

Figure 3.6: Chromatograms of the headspace compounds extracted from A-B) Regular 'Reg' Kabayaki: marinated in new sauce formulation, sous-vide (158°F) and grilled at low temperature (250°F). C-D) Berry-infused 'BI' Kabayaki': marinated in new sauce formulation infused with cranberry juice, sous-vide (158°F), and grilled at low temperature (250°F).

Volatile Oxidation Compounds in Kabayaki

An analysis of each peak identified and quantified for the volatile compounds produced during cooking is presented in Figure 3.7. Results from the GC-MS showed that all the Kabayaki samples contained VOCs; however, the type and abundance varied according to cooking conditions and technique (Figure 3.7). Samples marinated for three hours at high temperature had a higher abundance of oxidation products. Figure 3.7A-B showed that in experiments K1-K3, benzeneacetaldehyde was the most abundant. It was significantly greater in samples marinated in the original sauce formulation and grilled at maximum temperature (450°F) for 3 minutes on each side (K1-M3). Lower abundances were observed in samples prepared at 1.5 hours marination time than those marinated for 3-hour and cooked using the same parameters. This decrease was present in benzeneacetaldehyde (Figure 3.7A). Figures 3.7A- B) showed aldehydes reducing in abundance in experiments where there was a decrease in cooking temperature and marination time. However, the most significant reduction was in samples marinated for 1.5 hours, sous-vided (158°F), and finished off on the grill at minimum grilling temperatures (250°F).

Maillard Reaction Compounds

Figures 3.7C- D) showed volatile compounds coming from Maillard reactions. The abundance of furans generated were relatively low except for furfural and furancarboxaldeyde-5-methyl. These two remained in notable amounts but reduced significantly in samples marinated in the new sauce formulation, sous-vided (158°F), and then grilled at a minimum temperature (250°F) for 3 minutes on each side (K7-M3).

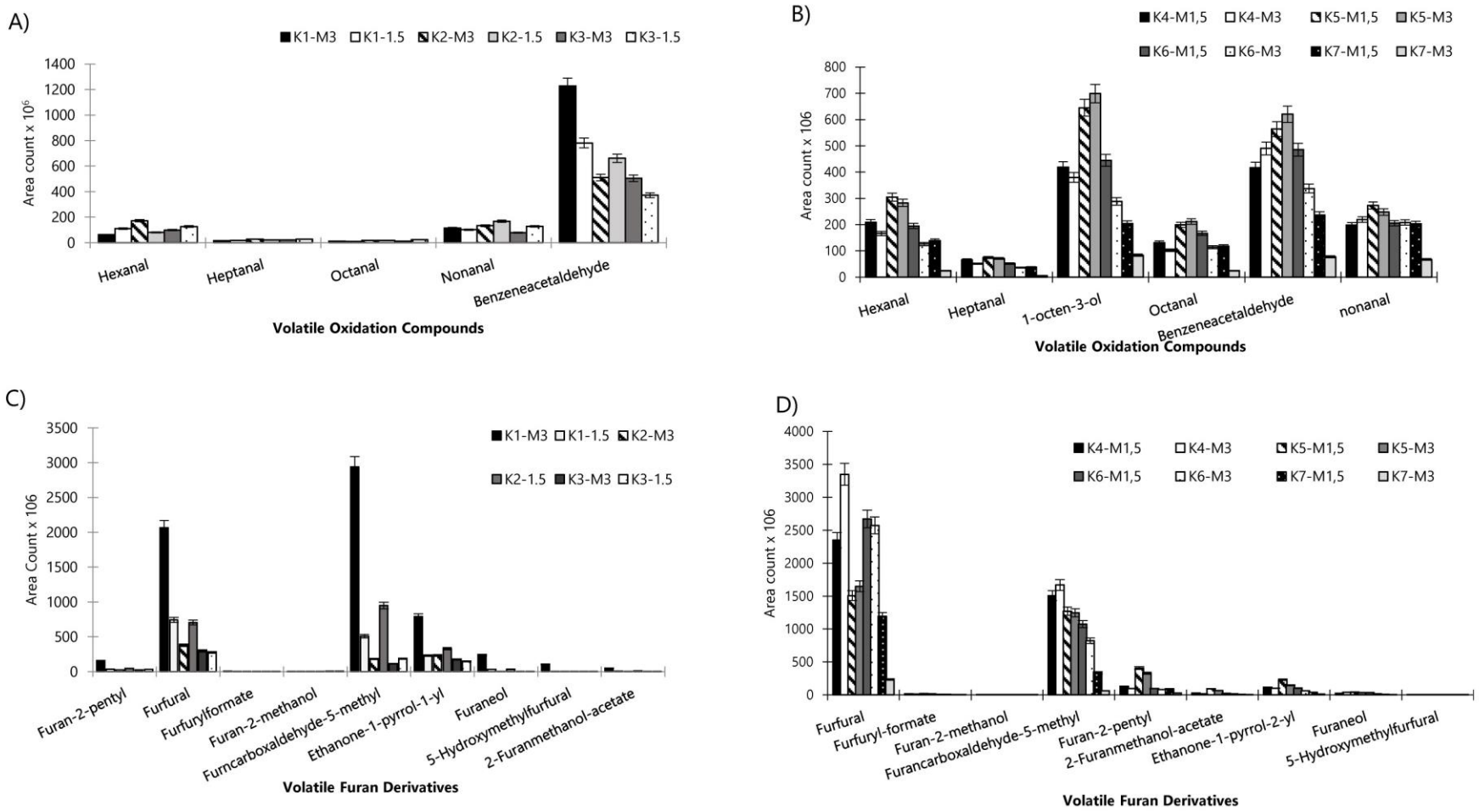

Figure 3.7: A) and B) Volatile oxidation and C and D) Maillard Reaction Compounds (MRPs) detected in the head space of samples marinated in traditional sauce formulation for 3 hours: Kabayaki Experiment 1 (K1): Grilled at maximum temperature (450°F) (K1-M3), Kabayaki Experiment 2 (K2): Grilled at medium temperature (350°F) (K2-M3), Kabayaki Experiment 3 (K3): Sous-vide (158°F) and grilled at medium temperature (350°F) (K3-M3). Samples marinated new sauce formulation for 3 hours: Kabayaki Experiment 4 (K4): Grilled at medium temperature (350°F) (K4-M3). Kabayaki Experiment 5 (K5): Sous-vide (158°F) and grilled at medium temperature (350°F) (K5-M3), Kabayaki Experiment 6 (K6): Grilled at minimum temperature (250°F) and marinated for 3 hours (K6-M3), Kabayaki Experiment 7 (K7): Sous-vide (158°F) and grilled at minimum temperature (250°F) (K7-M3) Values in bar chart represent means ± standard errors of the scores. Means accompanied by different superscripts are significantly different at LSD $\propto = 0.05$, n=30

Volatile Oxidation Compounds in berry infused Kabayaki

Figure 10 identified and quantified the volatile oxidation products and MRCs present in regular, and berry-infused samples cooked at high temperatures (K1-M3). Of the VOCs in Figure 3.8A), benzeneactaldehyde was significantly reduced in regular and BI samples. Significantly higher hexanal, heptanal, octanal, and nonanal levels were present in regular Kabayaki samples; however, these occurred at low abundances. These levels were lower in K1-M3 and BI samples. The MRCs in Figure 3.8B) were significantly reduced in regular and BI Kabayaki samples. Furfural was, however, significantly higher in BI compared to regular Kabayaki samples.

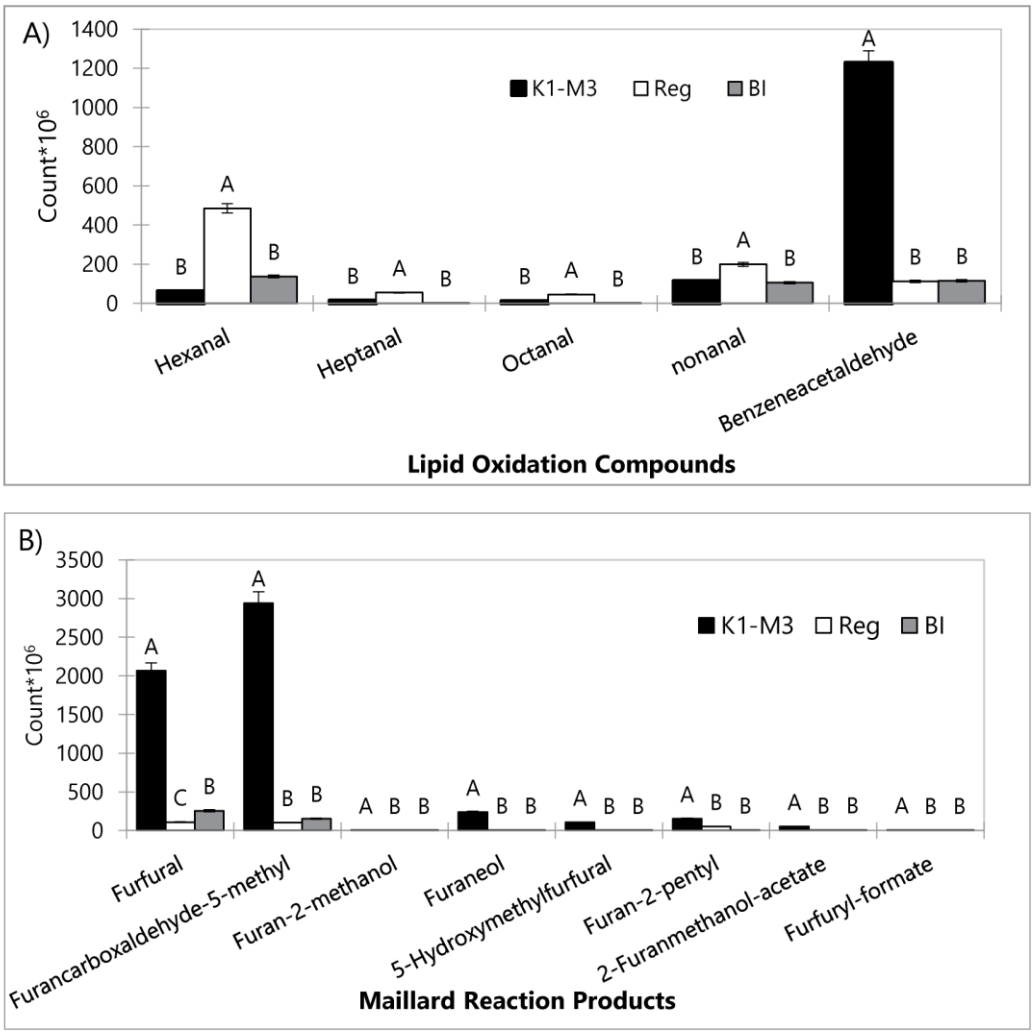

Figure 3.8: A) Lipid oxidation and B) Maillard Reaction Products (MRPs) detected in the head space of samples marinated in traditional sauce formulation for 3 hours: Kabayaki Experiment 1 (K1): Grilled at maximum temperature (450°F) (K1-M3), Regular 'Reg' Kabayaki: marinated in new sauce formulation, sous-vide (158°F) and grilled at minimum temperature (250°F), Berry-infused 'BI' Kabayaki': marinated in new sauce formulation infused with cranberry juice, sous-vide (158°F), and grilled at minimum temperature (250°F). Values in bar chart represent meant ± standard errors of the scores. Means accompanied by different superscripts are significantly different at LSD $\propto = 0.05$, n=30

PCA of Volatile Oxidation Compounds in Kabayaki

A follow-up PCA highlighted underlying correlations that may exist between sample cooking conditions and the volatiles generated (Figure 3.9). This observation accounted for 61.80 % of the total variance (F1- 51.18% and F2- 10.61= 61.80 %) in the data. The y-axis separated samples cooked at low (250°F) and medium (350°F) temperatures; as a result, samples cooked at low temperature clustered in quadrants 1 and 2 (Q1 & Q2), while samples cooked at medium temperature clustered in quadrants 3 and 4 (Q3 & Q4). Conversely, the x-axis separated grilled samples and samples sous-vide before grilling. As a result, most grilled samples clustered in Q2 and Q3, while sous-vide samples clustered in Q1 and Q4. Minimum temperature and samples sous-vided prior to grilling were distinctly grouped as clusters in the negative quadrant (Q1). Based on the corresponding biplot, little to no VOCs were likely associated with K7-M1.5 and K7-M3 samples.

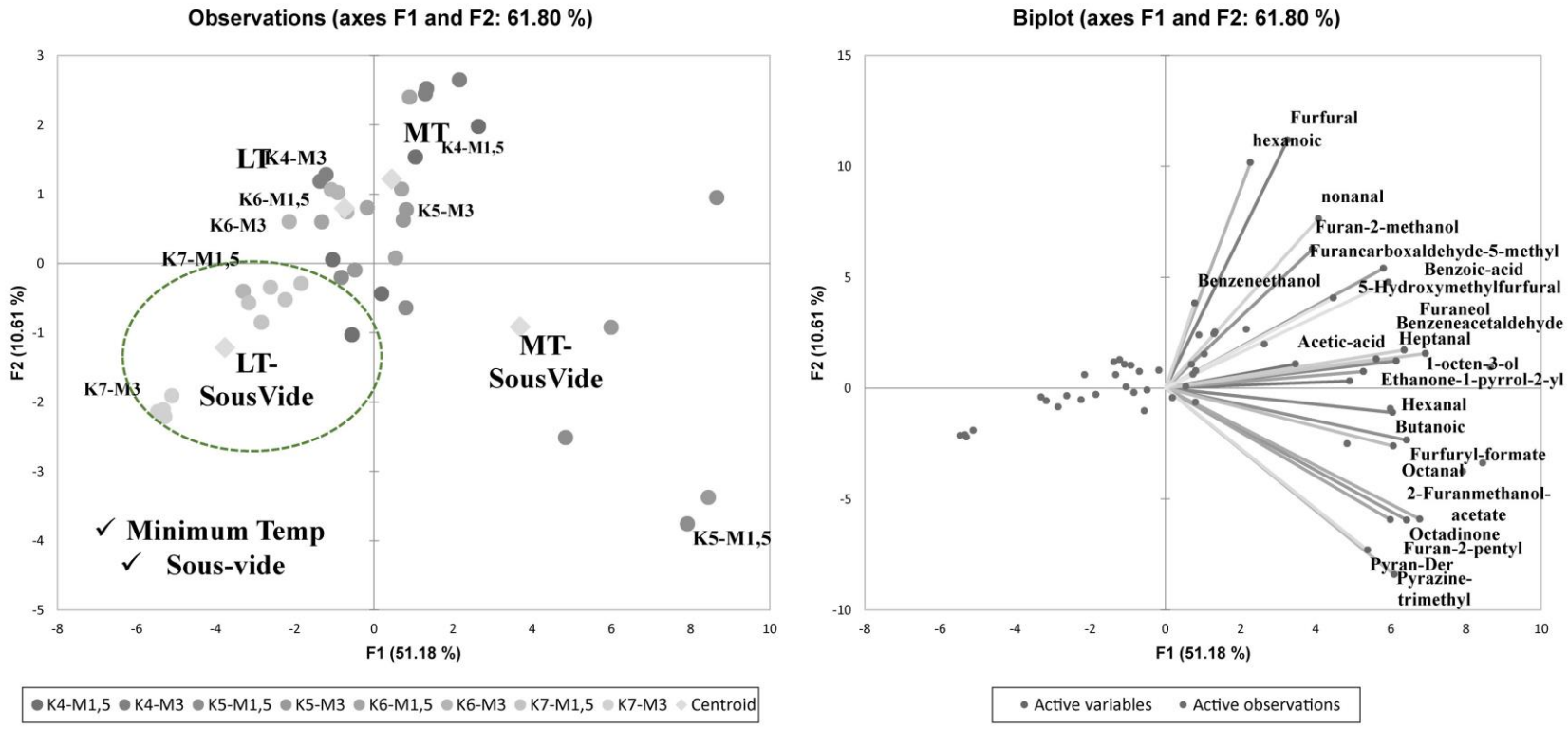

Figure 3.9: Principal Component analysis showing the cluster of Kabayaki samples marinated new sauce formulation for 1.5 and 3 hours: Kabayaki Experiment 4 (K4): Grilled at medium temperature (350°F) (K4-M1.5 and M3). Kabayaki Experiment 5 (K5): Sous-vide (158°F) and grilled at medium temperature (350°F) (K5-M1.5 and M3), Kabayaki Experiment 6 (K6): Grilled at minimum temperature (250°F) and marinated for 3 hours (K6-M1.5 and M3), Kabayaki Experiment 7 (K7): Sous-vide (158°F) and grilled at minimum temperature (250°F) (K7-M1.5 and M3).

PCA of Volatile Oxidation Compounds in berry infused Kabayaki

The PCA in Figure 3.10A-B demonstrated the potential relationship between the volatiles present in the sample and the treatment. The observation plot showed the clear separation of the samples based on oxidation products and nitrogenous compounds present in the Kabayaki samples with and without berry infusion, and this clustering accounted for 51.92% of the total variation present in the data (Figure 3.10A).

Samples associated with certain terpenes may have more functional properties because of their bioactivity (see Table 2.1). In Figure 3.10C-D, there was a clear separation in the clustering of BI and the regular Kabayaki samples. The BI samples were spread across Q1 and Q2, while Reg samples clustered in Q3 and Q4 (Figure 3.10C) and showed the terpenes identified following GC-MS analysis. The resulting PCA accounted for 94.42% of the total variation in both the observation and biplots (F1 75.58% and F2 18.85%). Regular samples almost exclusively cluster in Q1, while BI in Q3. Terpenes were mainly in Q3 and Q4 of the corresponding biplot near BI samples.

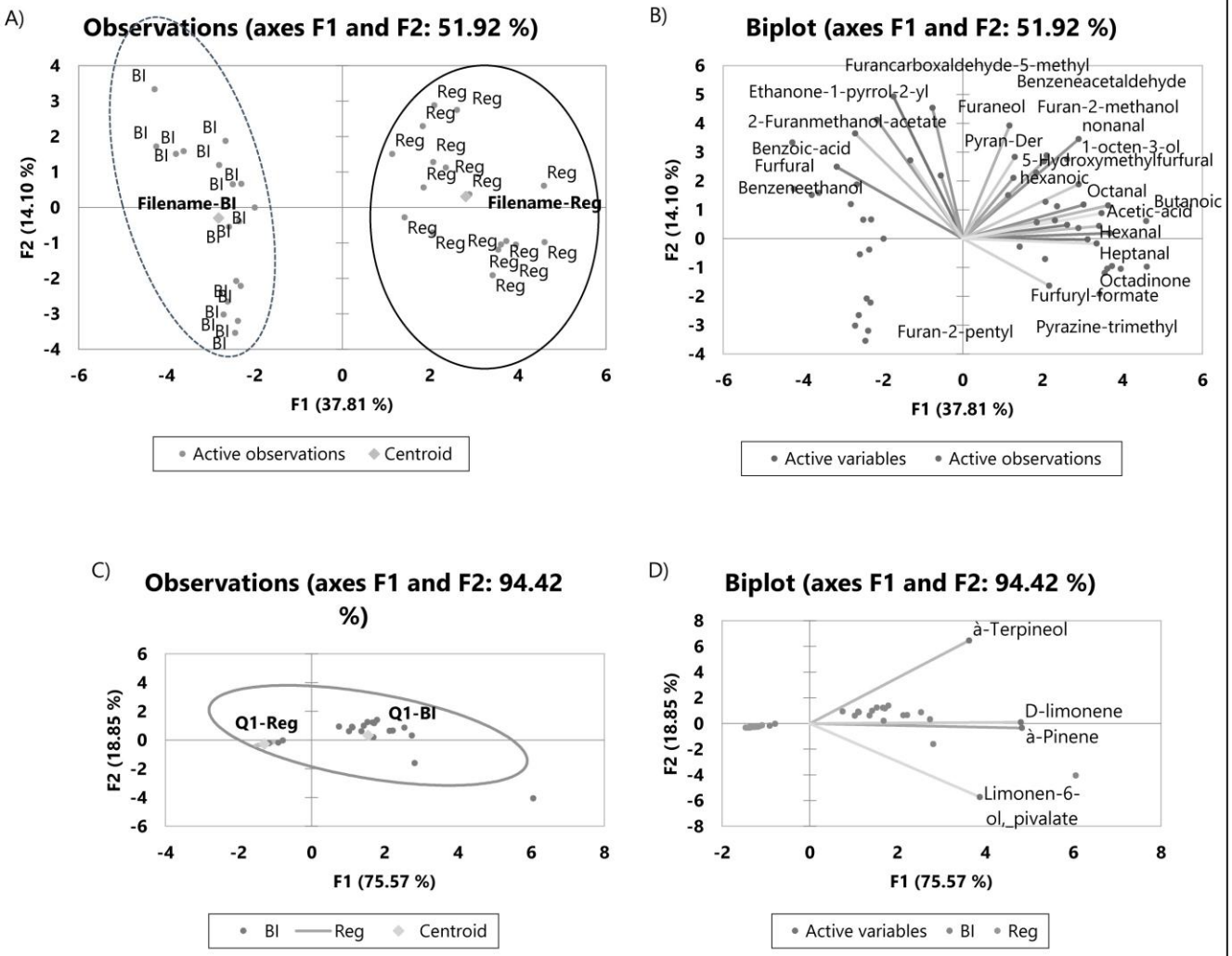

Figure 3.10: Principal component analysis (PCA) showing the clustering of the a) volatile oxidation products and b) nitrogenous compounds detected in the headspace of Regular 'Reg' Kabayaki: marinated in new sauce formulation, sous-vide (158°F) and grilled at minimum temperature (250°F) and Berry-infused 'BI' Kabayaki': marinated in new sauce formulation infused with cranberry juice, sous-vide (158°F), and grilled at minimum temperature (250°F). B) and D) are the respective biplots. Centroids of the BI and Reg samples are indicated with a diamond.

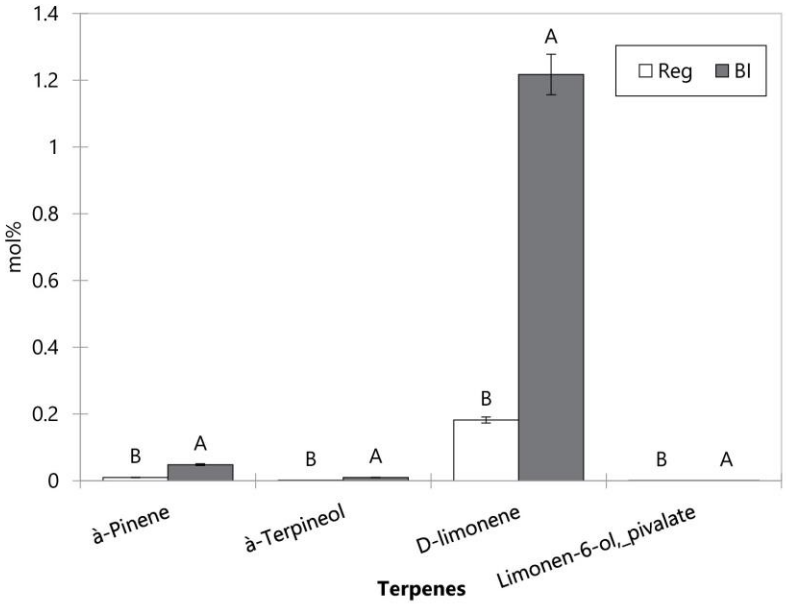

Figure 3.11: Terpenes detected in the headspace of berry infused Kabayaki samples. Values in bar chart represent meant ± standard errors of the scores. Means accompanied by different superscripts are significantly different at LSD ∝= 0.05, n=30.

Table 3.1: Describing odor and biological significance associated with the terpenes identified in the head space of the berry infused samples.

Terpene	Odour	Biological Significance
Alpha- Terpineol	Similar to lilac	antioxidant, anticancer, anticonvulsant, antiulcer, antihypertensive.
D-Limonene	Zesty-citrus	dissolve cholesterol-containing gallstones. Relief of heartburn and gastroesophageal reflux (GERD).
Alpha and beta-pinene	pine	antibiotic resistance modulation, anticoagulant, antitumor, antimicrobial, antimalarial, antioxidant, anti-inflammatory

Sensory Analysis of Kabayaki samples

The first sensory analysis provided insight into consumer perception of the different cooking and reheating techniques used to prepare the optimized Kabayaki samples (K6- M1.5 and K7-M1.5). The PCA representing 99.66 % of the total variance (F1- 77.43% and F2- 22.23) showed that sous-vide samples seem to be associated with the overall liking attributes clustered in the 3rd and 4th quadrants (Q3 & Q4) of the biplot (Figure 3.12A). Samples prepared by sous-vide prior to grilling followed by reheating in the microwave before serving scored the highest among samples for attribute scored on an 8-point hedonic scale.

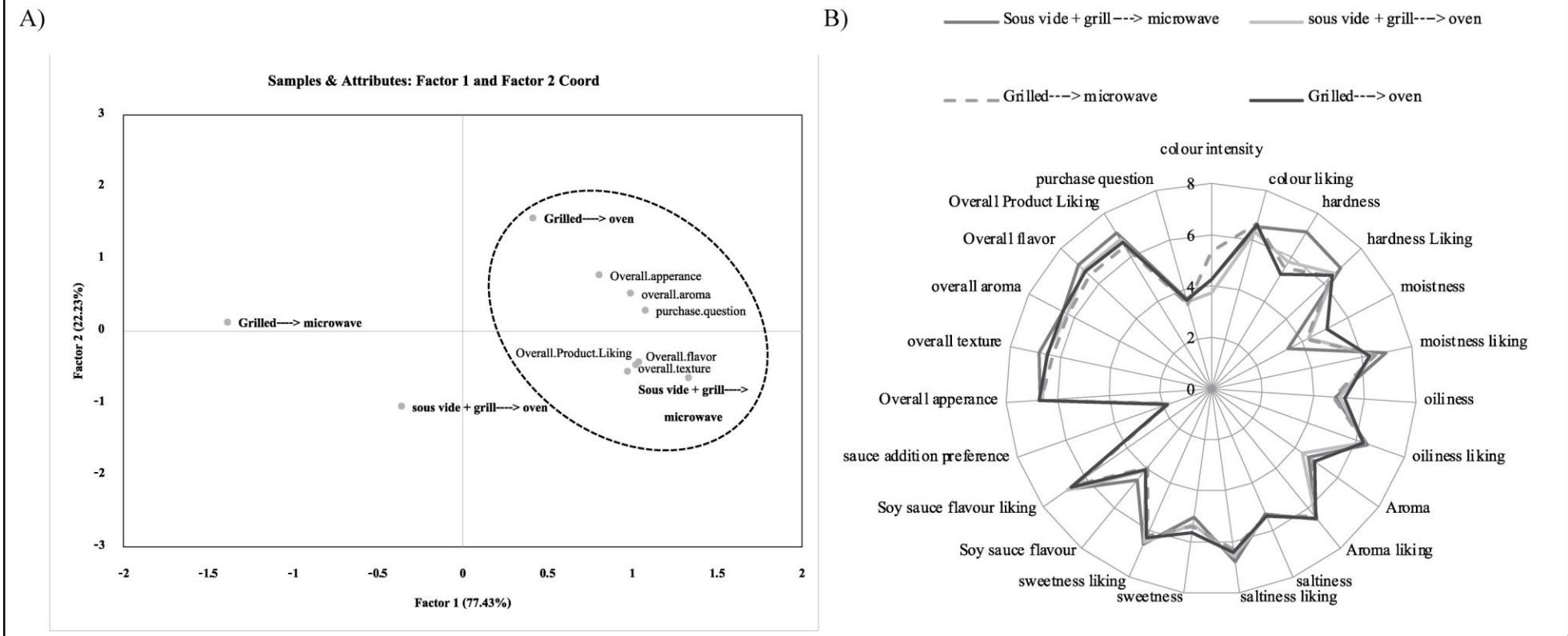

Figure 3.12 A) Principal Component Analysis (PCA) showing the potential association between attributes and preparation method and B) Spider plot (mean scores) showing consumer ranking on an 8-point hedonic scale their preferred Kabayaki preparation method: sous-vide prior to grilling followed by reheating in microwave, grilled then reheating in microwave, sous-vide prior to grilling followed by reheating in oven, grilled then reheat in over.

The second sensory analysis evaluated consumer perception of BI samples while comparing consumer preference for BI versus non berry infused (regular) Kabayaki samples. One-way ANOVA of participant scores (Figure 3.13 A) showed a significant difference between the participant scores for hardness (texture), oiliness, aroma liking, sweetness, sauce flavour, sauce flavour liking, and overall flavour. There were no significant differences between the overall product liking and purchase questions (consumer motivation to buy product) for BI and Regular Kabayaki. The spider plot showed the participant's rank of each attribute according to an 8-point hedonic scale (Figure 3.13 B). BI samples scored highest in terms of hardness, oiliness, sweetness, and soy sauce flavour among the significant attributes. Regular samples scored highest in aroma liking, soy sauce flavour liking, and overall taste.

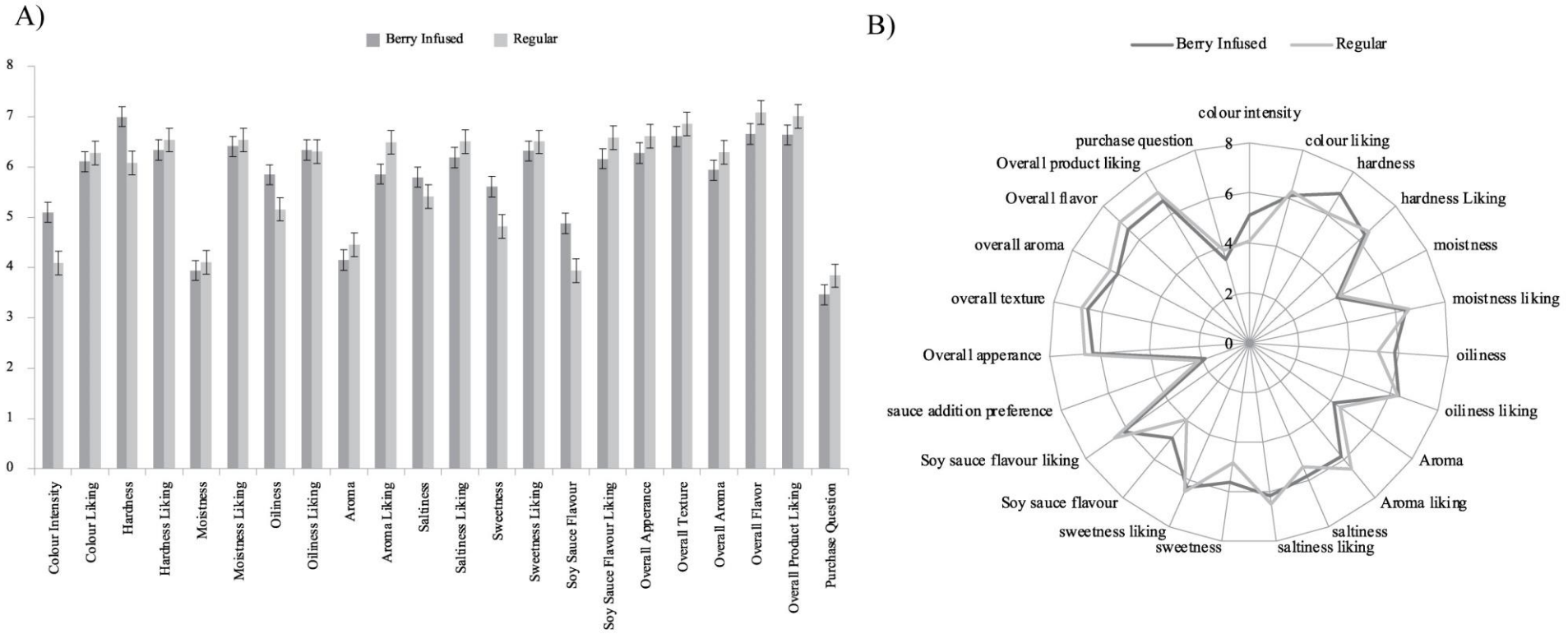

Figure 3.13: A) One-way ANOVA showing the significant difference between attributes Regular 'Reg' Kabayaki: marinated in new sauce formulation, sous-vide (158°F) and grilled at minimum temperature (250°F) and Berry-infused 'BI' Kabayaki': marinated in new sauce formulation infused with cranberry juice, sous-vide (158°F), and grilled at minimum temperature (250°F). B) Spider plot consumer ranking on an 8-point hedonic scale their perception and overall liking of each attribute. Values in bar chart represent meant ± standard errors of the scores. Means accompanied by different superscripts are significantly different at LSD $\propto = 0.05$, n=12

3.5 Discussion

Undersized American eels are rich in highly unsaturated fatty acid and are ideal for the development of high-value function Kabayaki products. PUFA are susceptible to deterioration after prolonged exposure to the high temperatures used to grill Kabayaki. This study developed four functional food Kabayaki product with an emphasis on the nutritional value, sensory quality, and food safety. The outcome of this study determined that grilling Kabayaki at high temperature for a short time does not cause significant change in fatty acid content. Incorporating sous vide cooking technique before grilling at low temperature (250°F) can effectively reduce the production of toxic HCAs, MRCs, lipid oxidation products in Kabayaki. This study also improved the functional properties of Kabayaki by introducing cranberry as source of bioactive monoterpenes. The high participant ratings of the Kabayaki products developed in this study indicate that this product has the potential to be successful in the global market.

This study was primarily concerned with optimising the Kabayaki cooking method while continuing to maintain the initial fatty acid content. The rich fatty acid content of American eels was not affected by the high grilling temperature traditionally used in making Kabayaki (Figure 3.1). In a similar study, Kusharto et al., (2014) reported a decrease from 28.29% to 2.39% of fat in Indonesian eels after it was used to prepare Kabayaki. Further investigation showed that the unsaturated fatty acid classes were mainly affected by the thermal processing. In this experiment, it is likely that the short grilling time of 3 minutes per side may have prevented/ limited the fatty acid depletion,

unlike the comparative study that roasted, steamed, re-roasted and then added then the

Kabayaki sauce (Kusharto et al., 2014).

The use of sous-vide cooking prior to grilling at low-temperature was effective in maintaining omega-3 content and preventing the loss of EPA and DHA content in both berry infused and regular Kabayaki (Figure 3.2B). Kusharto et al., (2014) reported an increase in fatty acid class content after preparation of Kabayaki using Indonesian eel and explained that percentage of body fat appeared to increase because the loss of moisture during grilling. Similarly, an 'increase' in mass was apparent in this study as well (see Figue 3.2B). There was no significant difference in total MUFA between regular and berry infused Kabayaki, indicating sous-vide (low temperature-long time) approach had the greatest influence on MUFA retention in Kabayaki (Figure 3.2B). Sous-vide also prevented the loss of total omega-6s which can also be attributed to the low temperature used during this process. These results demonstrated that sous vide followed by grilling at low temperature, as well as the infusion of cranberry juices into the Kabayaki sauce was very effective in maintaining the dietary lipids and nutritional quality of Kabayaki.

This investigation supports the hypothesis that eel marinated and then cooked at reduced temperature and oxygen exposure should have reduced formation of toxic lipid oxidation and MRCs as well as potentially carcinogenic HCAs typically generated during grilling. All the samples from experiments conducted at high (450°F) and medium (250°F) temperatures contained HCAs (K1-K3) (Figure 3.3A- B). These results are consistent with findings in the literature suggesting that temperature, duration, cooking technique are important contributors to the generation of HCA (Jahurul et al., 2010; Sinha & Snyderwine, 2001; Oz & Kaya, 2011a). After optimization, there was a significant reduction of HCAs in samples sous-vided before grilling at medium temperature for 1.5

minutes on each side (Figure 3.3B). The sous vide technique used indirect heating to cook the samples in an airtight package, which reduced the effects of the Maillard reaction and inhibited HCA formation. Vacuum sealing technology deters HCA formation by reducing lipid oxidation and free radical production (Adeyeye 2020; Zamora et al. 2013; Oz et al., 2010; Rönner et al., 2000). Oz and Zikirov (2015) reported as well that sous-vide meat generates low concentration of HCAs compared to the recommended daily intake. This study demonstrates that sous vide combined with grilling at low temperature (250°F) is an effective approach for preparing Kabayaki with undetectable levels of HCAs, and it could be a suitable cooking technique to improve the safety quality of grilled fish.

Another means of ensuring safety involved adjusting the marinade formulation to reduce the production of HCAs. The presence of white granulated sugar and soy sauce in the marinade were ingredients of concern because both promote the formation of HCAs (Lan and Chen; 2002). Evidence of an increase in HCA content appear in samples marinated in the traditional formulation (1 mirin: 1soy sauce: 2 sake: 5 granulated sugar) for 3 hours and then cooked at high temperature (450° F) (K1-M3 and K1-M1.5 respectively) (see Figure 3.3A). Research shows that sugars react differently depending on their source; for example, brown sugar generates fewer HCAs than white, and honey even offers protective properties to reduce HCAs formed during grilling (Hasnol et al., 2014 & Shamsudin et al., 2020). The replacement of white sugar in the marinade with brown sugar in lesser amounts and shorten marination time resulted in a decrease in HCA content (see Figure 3.3B). In subsequent experiments using the new formulation, the HCA content decreased, and the length of marination time had no effect on the amount of HCA generated; thus, this study confirmed that large amounts of granulate sugar can increase HCA production during

grilling (see Figure 3.3 B and Figure 3.4 J-K). Therefore, reducing the sugar content in marinades in addition to incorporating sous-vide, prior to grilling at low temperature (250°F) was shown to be the most effective in reducing HCA concentrations to undetectable levels in Kabayaki (Figures 3.4 G-F).

Evaluation of volatile constituents in food is an important aspect of food production as it communicates knowledge about the relationship between food quality and sensory attributes (Starowicz, 2021). Cooking techniques have a strong influence on lipid oxidation, which can contribute to flavour, decrease in the shelf-life, and reflect loss of lipid content (Rasinska et al., 2019; Grebenteuch et al., 2021; Purriños et al., 2011; Saldanha & Bragagnolo, 2010). Dominguez-Hernandez et al., (2018) reported that the low temperature and long cooking time associated with sous vide reduced volatile compounds arising from lipid oxidation such as aldehydes. This is illustrated in Figure 3.9, with volatiles associated with higher grilling temperatures. On the other hand, high temperatures and long cooking times further increase volatile compounds from the Strecker degradations of amino acids and thermal degradation of thiamine (Roldán et al.,2015). Results of this study confirmed that volatile compounds reduced with temperature and the incorporation of sous-vide method. Kabayaki sous-vide prior to grilling at a low temperature (250°F) produced the fewest volatile peaks with the lowest intensities (see Figure 3.5).

Further identification and quantification of volatiles produced during the grilling of Kabayaki confirmed that a higher oxidation level exists in samples grilled at high temperature but were significantly lower in in abundance sous-vide samples (see Figure 3.7 A). For example, benzeneacetaldehyde decrease significantly as the experiment

progressed (see Figure 3.7A). The many aldehydes in the Kabayaki were the result of lipid peroxidation, which increases with temperature, unlikely ketones and alcohols, which decrease with temperature (see Figure 3.7A-B) (Roldán et al.,2015). In Figure 3.7 C-D, mainly furans were generated from Maillard reaction. Furans are derived from Maillard reaction at high heat and from the oxidation of unsaturated lipids at milder temperatures (Roldán et al.,2015; Pérez-Palacios, Petisca, Melo, & Ferreira, 2012; Elmore et al., 1999). The findings of this study agreed that lower temperature and long cooking time generated less MRPs (Maillard Reaction Products) (Figure 3.7C-D). Many of the aroma compounds (primarily aldehydes and ketones) generated in grilled foods can be attributed to the Maillard reaction in conjunction with lipid oxidation, whereas Strecker degradation of amino acids and thermal decomposition of thiamine generate flavour (Resconi, Escudero, & Campo, 2013). Maillard reaction and lipid oxidation appeared to be reduced in Kabayaki (K7-M3) experiment mostly likely because it was prepared using optimized method of sous-vide prior to grilling at low temperature (see Figure 3.7). This observation was made in berry infused and regular Kabayaki as well since they were made under the same optimized conditions (see Figure 3.8). The antioxidant properties of cranberries may have assisted in combating lipid oxidation in addition to low oxygen environment of the sous vide vacuum seal bag as well (Caldas et al., 2018). However, further tests will be required to evaluate the extent of the role of cranberry as antioxidant during the preparation of kabayaki. Roldán et al., 2015 suggested searing sous-vide meat at high temperature (130–150 °C) for a short time to intensify the flavor profile. However, it is unknown how sous-vide prior to grilling at high temperature will affect the nutrition and safety of the product.

Berry infused Kabayaki had improved the sensory, nutritional, and functional qualities

compared to regular Kabayaki. Cranberries are high in flavonoids (classes: anthocyanins, flavonols, flavan-3-ols, and proanthocyanidins), as well as phenolic acids, benzoates, hydroxycinnamic acids, terpenes, and organic acids. (Odjo et al., 2022; Bariexca et al., 2019). Cranberries also introduced new volatile peaks into the product (reference Figure 3.6). This is reflective of the abundance of volatile compounds associated with berry infused Kabayaki (see Figure 3.10), some of which has their own characteristic taste and aroma. MRPs such as furfural may have given Kabayaki cooked at high temperature (K-M3) a grainy, biscuity, or almond-like flavor, while Furancarboxyaldehyde-5-methyl introduced a burnt sugar, and caramel flavor (see Figure 3.8). Similarly, terpenes alpha-terpineol, D-limonene, and alpha and beta pinene identified in berry infused samples have notes of lilac, citrus, and pine (see Table 3.1).

Moore et al., (2019) identified volatile compounds esters, alcohols, monoterpenes, acids, sesquiterpenes, C13 isoprenoids, and others in cranberry extracts as the source of aroma. This study confirmed the presence of functional terpenes alpha-terpineol, D-limonene, and alpha and beta pinene in berry infused Kabayaki (see Figure 3.10C-D). Held et al. (2007) identified alpha-terpineol as the major volatile responsible for anti-inflammatory properties. Alpha terpineol possess anticancer, anticonvulsant, antiulcer, and antihypertensive properties (Khaleel et al., 2018; Sales et al., 2020). The abundance of D-limonene in berry infused samples is evidence of the high abundance of the compound in cranberry (see Figure 3.11). This terpene is common in citrus and has proven to be effective in dissolving cholesterol-containing gallstones, relieving heartburn and gastroesophageal reflux (GERD) (Sun, 2018). Alpha pinene, which was present in a lesser amount and is recognized for its antibiotic resistance modulation, anticoagulant,

antitumor, antimicrobial, antimalarial, antioxidant, and anti-inflammatory properties (Salehi et al., 2019) (Table 3.1). The presence of these functional compounds in berry infused Kabayaki increase the nutritional value of the product. Further tests will be required to determine the bioavailability of the functional compounds in cranberry after consumption.

Consumer assessment of organoleptic properties indicated that participants preferred Kabayaki samples that were sous-vide prior to grilling at low temperature in addition to being reheated in the microwave (see Figure 3.12). Cooking at a low temperature for an extended period of time improves flavour and aroma, tenderness and texture, colour and visual appeal. (Zavadlav et al., 2020). The vacuum sealed bags prevent water loss, facilitates eel to cook in its natural juices, and allow for deeper penetration of the marinade (Głuchowski et al., 2020; Baldwin, 2012). Participants liked and were willing the purchase both berry infused and regular kabayaki and preferred certain attributes of each product (see Figure 3.13). Infusion of fresh cranberry juice in Kabayaki marinade) introduced a large array of new compounds that can interact with the properties of the primary marinade. Cranberries are rich in anthocyanins which created a visibly reddish hue to the initially brown marinade and explained the perceived difference in color intensity (Bariexca et al., 2019) as well as volatile flavour or aroma compounds that can alter the sensory profile of Kabayaki. For example, the presence of terpenes alpha- terpineol, D-limonene, and alpha and beta pinene can contribute floral/lilac, citrus, and pine aroma respectively to the berry infused product (Table.3.1) (Zhu et al., 2016; Bourgou et al 2012; Vespermann et al., 2017). Cranberries also contain naturally occurring sugars, such as sucrose, glucose, and fructose which may contribute to the increase in sweetness (Pappas

& Schaich, 2009).

3.6 Conclusion

This study determined that sous-vide prior to grilling at a temperature of 250°F produced Kabayaki samples with the best safety, nutritional quality, and consumer acceptability. The fatty acid profile did not change dramatically in terms of nutritional quality. However, sous-vide had the greatest protective effect on protecting Fatty acids (MUFAs) that were affected by high grilling temperatures. Incorporating the sous vide cooking technique before grilling can effectively reduce the production of toxic MRCs and VOCs in Kabayaki. Sous-vide before grilling maintains the nutrition and increases the quality and safety of Kabayaki. Sous-vide before grilling treatment appealed more to consumers. Infusing cranberry juice in Kabayaki marinade does not make a significant contribution to the formation of lipid oxidation products nor MRCs. Heterocycle amines compounds (HCA) were present in all

the samples. However, the concentration of total HCAs was lower in sous-vided samples. Fresh cranberry juice in the marinade introduced more volatile compounds, including beneficial monoterpenes. Consumers preferred specific attributes of each product but were equally accepting of both regular and Berry Infused Kabayaki. BI Kabayaki increased the nutritional value of Kabayaki; however, additional tests are needed determine if the bioactive properties of cranberry induced functional compounds present in the BI infused Kabayaki conferred any health promotive benefits to consumers after consumption.

3.7 References

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Chapter 4

4.1. Summary of results and conclusion

The studies conducted in this thesis had the following objectives:

i. Investigate the influence of the colour, different habitat locations, and size on the nutritional value of Freshwater American eels sourced from Newfoundland.
ii. Determine the potential of undersize juvenile eels to be used in the production of high-value eel-based secondary food products
iii. Develop a high-quality Kabayaki eel product utilizing undersized Newfoundland freshwater American eel.
iv. Maintain nutritional quality and ensure safety and sensory appeal by adjusting cooking temperature/ time and using the sous-vide cooking technique.
v. Develop Kabayaki as a functional food product by incorporating polyphenol-rich Newfoundland Cranberries.

This thesis delves into the aforementioned objectives in two main chapters: Chapter two described the Assessment of the Nutritional Quality of Newfoundland Freshwater Eels (Anguilla rostrata) as Influenced by Size and Growth Habitats: Implications in the Production of High-Quality Value Eel-Based Food Products. Chapter 3 explored the Sensory Perception and Development of Kabayaki as a Functional Food using undersized Newfoundland American Eels (*Anguilla rostrata*).

The evaluation of American eel lipid content undertaken in this study provides insight into the nutritional value of small yellow and large silver eels as well as identifies the elements that contribute to their degree of enrichment. The ability of undersized freshwater American eels from Newfoundland to be utilised in the development of high-value eel-based

functional food products is highly dependent on the existence of necessary lipids species and fatty acid species with related bioactive qualities. In this study, large migratory American eels have a greater amount of fatty acids than the smaller sedentary yellow eels. The American eel samples in this study all had high MUFA levels. Despite having a slightly lower lipid content, undersized American eels are nutrient-dense, high-quality ingredients for the production of secondary functional food. Undersized American eels are abundant in highly unsaturated fatty acids and are therefore suited for the creation of high-value function Kabayaki products. This study determined that grilling Kabayaki at high temperatures for a brief period of time does not result in a significant change in fatty acid content and demonstrates the efficacy of sous-vide cooking technique combined with low temperature grilling to ensure safety by reducing toxic HCA and certain harmful Maillard reaction compounds and to ensure consumer acceptability. Incorporating polyphenol-rich American Cranberries into Kabayaki marinade resulted in the addition of many bioactive components, resulting in a Kabayaki product with additional functional constituents.

Eels are highly sought after because they contain high levels of important dietary lipids. Little, if any, research has been conducted on the lipid composition of American eels. This study confirmed that smaller eels have a more of a yellow hue than their larger counterparts, and that the colour difference between the sizes is substantial. These results are consistent with studies on the life stages of the American eel, which characterise immature eels as becoming darker and more yellow as they mature (Greene et al., 2009; COSEWIC, 2012; McCleave, 2001).

The presence of unsaturated fatty acids indicates nutritional and bioactive qualities that

have the potential to promote human health (Marchioli et al., 2002). Larger silver eels were connected with SFA, PUFA, omega-6s, and omega-3s, while smaller eels were associated with MUFA. This result is consistent with Van Ginneken et al., (2007)'s analysis of the fatty acid composition of European eels. Flat Bay eels contained the highest levels of polyunsaturated fatty acids, including DPA, EPA, DHA, and stearidonic acid (see Figure 2.5a). The presence of EPA and DHA may regulate inflammatory cytokines, vasodilation, and vasoconstriction. The availability and abundance of essential fatty acids in Flat Bay eels indicate that the area is conducive to the creation of eel-based goods rich in polyunsaturated fatty acids (PUFA). Flat Bay may have a healthy population of microalgae to supply its much greater omega-3s content (see Figure 2b D) and total food availability (Maltsev & Maltseva, 2021; Barkia et al. 2019; Sathasivam et al. 2019; Chalima et al. 2019, 2020). Gander had the greatest percentage of MUFA. Robinson eels were fortified with important -Linolenic acid (C18:3n3) and linoleic acid (C18:2n6cis), along with 11,14,17-Eicosatrienoic acid (C20:3n3cis) and C18:1n7. Each location's eel's fatty acid profile contains bioactive fatty acids. It is likely that the variation in fatty acids among individuals of the same species is influenced by their food.

Long-chain polyunsaturated fatty acids (LC-PUFA) concentrate in muscle tissue's polar lipids. Figure 2.6a demonstrates that Robinson eels are a source of PC (35%) and SM (36%). Flat Bay is the best source of PE (30%), whereas Gander Bay supplies the most LPC (16%). Additionally, freshwater eels are an important source of ether-linked polar lipids. Docosapentaenoic acid (DPA, C22:5) and arachidonic acid are responsible for the bioactivity linked with PUFAs from Flat Bay (AA, 20:4). DPA and AA contribute for 10-16% and 5-13%, respectively, of brain lipid. The neutral lipids identified by HPLC-HESI-

MS/MS correspond to the total fatty acid molecular species found by GC-FID. Each place can serve as a source of health-promoting bioactive lipids and fatty acid species.

Flat Bay eels appear to be superior suppliers of PUFA, specifically C18:2n6 and EPA. Robinson eels would be excellent suppliers of omega-3 fatty acids (DHA and LN). It is possible to manufacture MUFA-enriched anadromous eel products from Gander or Robinson samples.

The substantial fatty acid content of American eels was unaffected by the customarily high grilling temperature required to prepare Kabayaki (Figure 3.1). In a similar study, Kusharto et al. (2014) found that the fat content of Indonesian eels decreased from 28.29% to 2.39 % after preparation into Kabayaaki. Sous vide prior to low-temperature grilling proved successful at preserving omega-3 content and preventing the loss of EPA and DHA content in both berry-infused and traditional Kabayaki. This was observable in the fatty acid classes C16:1 (palmitoleic acid), C18:1n9cis (oleic acids), and C18:1n9trans (Elaidic acid). These results revealed that sous vide followed by low-temperature grilling, as well as the addition of cranberry juice to the Kabayaki sauce, were extremely successful at preserving the dietary lipids and nutritional value of Kabayaki.

Oz and Zikirov (2015) observed that sous-vide meat produces a low HCA concentration compared to the recommended daily allowance. The sous vide approach utilised indirect heating to cook the samples in an airtight packaging, which decreased the impacts of the Maillard reaction and prevented the development of HCA. Searing Kabayaki for a brief duration at a low temperature (250°F) did not contribute to the formation of HCA. Both

white granulated sugar and soy sauce contribute to the development of HCAs in marinades, which is cause for concern (Lan and Chen; 2002). The substitution of brown sugar for white sugar in smaller quantities and a reduction in marination time reduced the HCA concentration. Prior to grilling at a low temperature (250°F), sous vide Kabayaki produced the fewest volatile peaks with the lowest intensities (Roldán et al.,2015; Dominguez-Hernán et al.,2018). Many of the fragrance molecules (mainly aldehydes and ketones) produced in grilled foods are the result of the Maillard reaction and lipid oxidation. Roldán et al. (2015) proposed sous-vide cooking at a high temperature for a brief period of time to increase the flavour profile.

Berry-infused Kabayaki enhanced the flavour, nutritional value, and functionality. Flavonoids, phenolic acids, benzoates, hydroxycinnamic acids, terpenes, and organic acids are abundant in cranberries (Odjo et al., 2022; Bariexca et al., 2019). Cranberries added new volatile peaks to the product as well (reference Figure 3.7). This reflects the quantity of volatile chemicals found in Kabayaki flavoured with berries. This research revealed the existence of the functional terpenes alpha-terpineol, D-limonene, and alpha and beta pinene in Kabayaki infused with berries. Alpha terpineol is anticancer, anticonvulsant, antiulcer, and antihypertensive. To establish the bioavailability of the bioactive components in cranberry after eating, additional experiments will be required.

Consumer evaluation of organoleptic qualities revealed that participants preferred Kabayaki samples that were sous-vide before to low-temperature grilling over those that had been reheated in the microwave. Cooking at a low temperature for an extended time period enhances flavour and aroma, softness and texture, colour and appearance (Zavadlav

et al., 2020). Infusion of fresh cranberry juice in Kabayaki marinade) offered a vast number of additional compounds that may have interacted with the qualities of the primary marinade to account for some difference in the ranking of attributes by consumers; however, consumers did not favour one product over the other.

The experiment yields new information regarding the nutritional value of Newfoundland's freshwater American eels. The results of this study indicate that undersized eels could be a good source of dietary or functional lipids, with the geography of their habitat providing superior sources of different classes of fatty acids, polar (especially those with ether-linkages) and neutral lipids, which could influence the nutritional and functional qualities of potential products. To successfully prevent the generation of hazardous MRCs and lipid oxidation products in Kabayaki, sous-vide undersized eel before grilling at a low temperature. Cranberry juice added volatile components, including beneficial monoterpenes, to the marinade. Total HCA concentration was decreased in sous-vide samples. These findings enabled the manufacture of four functional food products: kabayaki, kabayaki sauce, berry infused kabayaki and berry infusion Kabayaki sauce. This project was completed in conjunction with North Atlantic Aquaponics, with whom my supervisor, Dr. Raymond Thomas, co-supervisor, Natalia Prieto-Vidal, and I created the Kabayaki products from conception until impending commercialization. Our role in process development is that of the academic and research sector. Our responsibilities in Stage 1 included collaborating with our industry partner to solve the problem of underutilized American eel, conducting research and identifying the knowledge gap regarding the nutritional qualities of the species, and conducting chemical analysis to determine the nutritional values and the possibility of undersized eels being used as a

secondary functional food product. In addition to applying for a license from the Canadian Food Inspection Agency (CFIA) (see Appendices) to import, export, produce, and handle food legally. Together with an industry partner, we formulated the four Kabayaki products in two phases (two eel products and their respective sauces). We did a market and sensory analysis at the Functional Foods Sensory Laboratory at Grenfell Campus, Memorial University. This project also funded the construction of a cooking facility in Black Duck Siding, Newfoundland, where the third round of testing was undertaken. In the near future, Black Duck Siding Facility will commence large-scale production, and the Kabayaki products produced for this project will be available to the global market.

4.2. Future Studies

The composition and molecular species of neutral and polar lipids linked with large and small American eels can be investigated further. In addition, taking measurements of significant environmental parameters at each location can provide additional insight into the external influences that influence lipid composition. Temperature readings, phytoplankton composition and density, eel diet, and ecosystem health measurements will shed more light on the site-specific variation in eel lipid content. It would have been interesting investigate the digestive contents of the eels from different locations to confirm that it was indeed dietary factors that caused the differences in the fatty acid contents and not something environmental. This study's product development section could be enhanced by addressing the processing impacts to the bioavailability of beneficial compounds, biological, allergy, and microbiological aspects of food safety. The functional features of the Kabayaki products developed in this investigation must also be validated using cell and/or animal studies.

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technique for preparing healthy and high-quality vegetable and seafood products. *Foods*, *9*(11). https://doi.org/10.3390/FOODS9111537

5.Appendices: Pictures demonstrating achievements during product development are as follows:

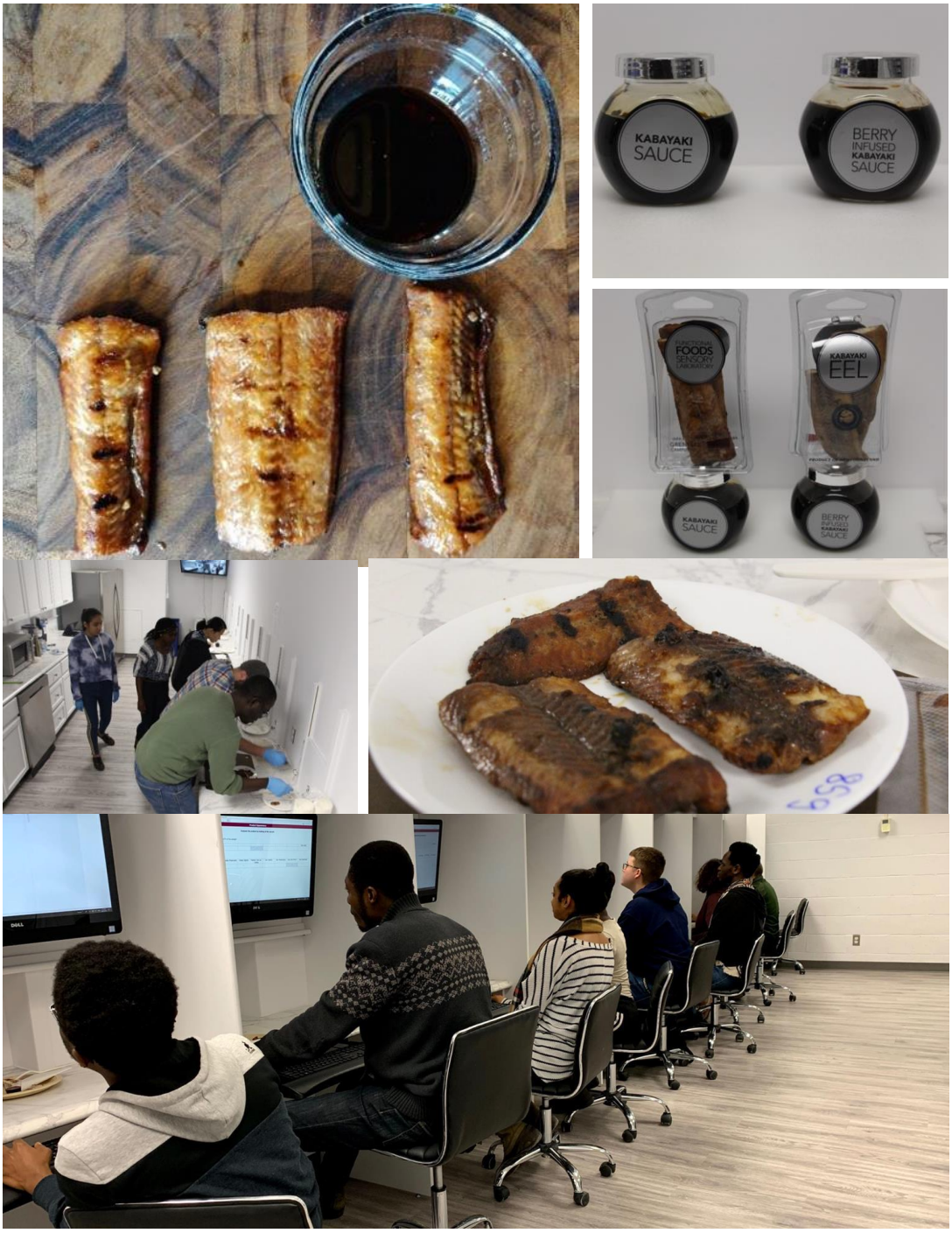
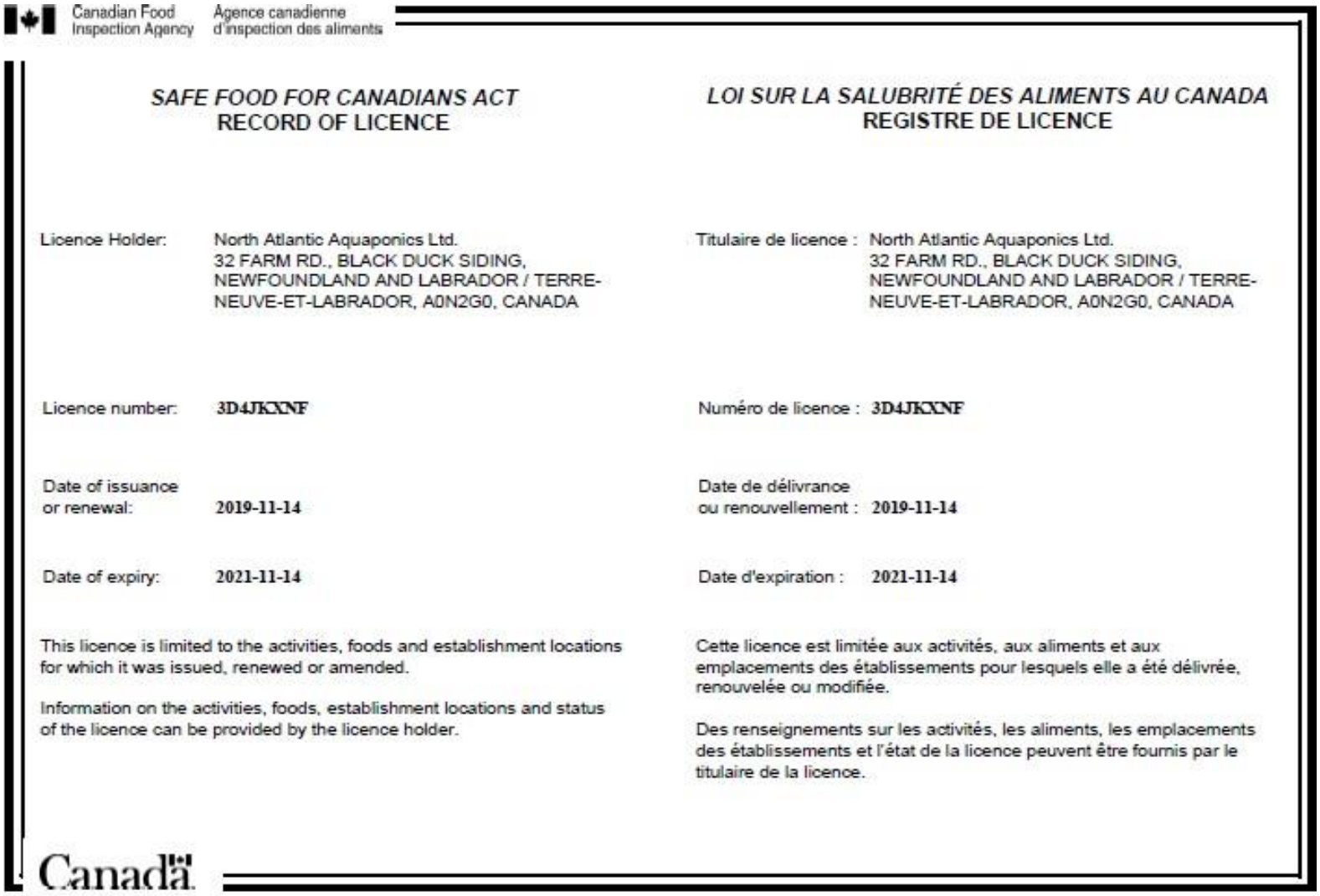


Figure: 5.1. Samples of Kabayaki marinades and packaged Kabayaki produced for the commercial market.



Canadian Food Inspection Agency | Agence canadienne d'inspection des aliments

SAFE FOOD FOR CANADIANS ACT
RECORD OF LICENCE

LOI SUR LA SALUBRITÉ DES ALIMENTS AU CANADA
REGISTRE DE LICENCE

Licence Holder: North Atlantic Aquaponics Ltd.
32 FARM RD., BLACK DUCK SIDING,
NEWFOUNDLAND AND LABRADOR / TERRE-NEUVE-ET-LABRADOR, A0N2G0, CANADA

Titulaire de licence : North Atlantic Aquaponics Ltd.
32 FARM RD., BLACK DUCK SIDING,
NEWFOUNDLAND AND LABRADOR / TERRE-NEUVE-ET-LABRADOR, A0N2G0, CANADA

Licence number: **3D4JKXNF**

Numéro de licence : **3D4JKXNF**

Date of issuance or renewal: **2019-11-14**

Date de délivrance ou renouvellement : **2019-11-14**

Date of expiry: **2021-11-14**

Date d'expiration : **2021-11-14**

This licence is limited to the activities, foods and establishment locations for which it was issued, renewed or amended.

Information on the activities, foods, establishment locations and status of the licence can be provided by the licence holder.

Cette licence est limitée aux activités, aux aliments et aux emplacements des établissements pour lesquels elle a été délivrée, renouvelée ou modifiée.

Des renseignements sur les activités, les aliments, les emplacements des établissements et l'état de la licence peuvent être fournis par le titulaire de la licence.

Canada

CFIA / ACIA 5852 (2018/12)

Figure: 5.2. Sample of CFIA certification that the research assisted in obtaining for the production of Kabayaki at the industry partner production site in Black Duck Siding, NL. The research assisted in the development of a CFIA certified facility for the production of Kabayaki working in collaboration with the industry partner (North Atlantic Aquaponics)

Kabayaki | Kabayaki Sauce | Berry Infused Kabayaki | Berry Infused Kabayaki Sauce

Figure: 5.3. Food labels showing the nutritional facts for the Kabayaki and Kabayaki sauces developed for commercialization

gazette MEMORIAL UNIVERSITY

Feb. 15, 2019

Understanding food

Functional Foods Sensory Laboratory officially opens at Grenfell Campus

Research

BY PAMELA GILL

"Currently there are no companies in Atlantic Canada making Kabayaki."

— Louis MacDonald

Industry partner Atlantic Aquaponics and its sister company, Atlantic Canada Eels Inc., have a keen interest in working with the lab. Their facilities, located in Stephenville, Robinsons and Black Duck Siding, use land-based aquaculture recirculating technology to produce American eels.

The aim is to produce a ready-to-eat eel product (Kabayaki) for markets in Japan. The new functional food product will incorporate the benefits of selected Newfoundland berries, enhancing the functionality of the Kabayaki products developed.

"The largest market for eels is the Japanese Kabayaki market," said Louis MacDonald, who co-owns the companies with Scott Madore, adding that discussions with existing Asian clients has already generated interest in N.L. Kabayaki. "Currently there are no companies in Atlantic Canada making Kabayaki, and there are no listed companies in Canada that purchase eels for processing."

"We believe several other industry partners will work with this program once the first set of outputs from this program are disseminated," said Dr. Thomas. "These collaborations will create the capacity and opportunity to develop value-added processed products from raw materials to enhance business growth and revenue generating potential of the companies."

Figure: 5.4. Coverage of the research study in Memorial university Gazette news site

Figure: 5.5. Coverage of the research study by SaltWire Network

MEMORIAL

sowing seeds of knowledge

Research labs advancing research capacity at Grenfell Campus and beyond

Dr. Raymond Thomas, associate professor and principal investigator of the functional foods research program, and post-doctoral fellow Dr. Natalia Prieto Vidal, manager of the Functional Foods Laboratory, supervise Ms. Hamilton's work.

Participants in sensory surveys at Grenfell's Functional Foods Laboratory provide valuable feedback to researchers.

https://www.mun.ca/presidentsreport/2019/features/sowing-seeds/

Figure: 5.6. Coverage of the research study in 2019 Presidents Report

CBC Sign In

Nfld. & Labrador

For the love of eel: N.L. company developing new product for international market

Jennifer Grudić · CBC News · Posted: Jan 20, 2019 11:09 AM NT | Last Updated: January 20, 2019

This week the public was asked to take part in a tasting experiment that got them to rate and rank four different kabayaki samples based on things like appearance, flavour and texture.

Graduate student Melissa Hamilton is overseeing the project. (Jennifer Grudic/CBC)