LC-MS/MS METHOD DEVELOPMENT FOR QUANTITATIVE ANALYSIS OF CYANOGENIC GLYCOSIDES IN ELDERBERRY AND LIPID PEROXIDATION PRODUCTS IN MICE

A Dissertation

presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

MICHAEL KWAME APPENTENG

Dr. C. Michael Greenlief, Dissertation Supervisor

May 2021

The undersigned, appointed by the dean of the Graduate School at the University of Missouri-Columbia, have examined the dissertation entitled.

LC-MS/MS Method Development for Quantitative Analysis of Cyanogenic Glycosides in Elderberry and Lipid Peroxidation Products in Mice

presented by Michael Kwame Appenteng,

a candidate for the degree of Doctor of Philosophy in Chemistry,

and hereby certify that, in their opinion, it is worthy of acceptance.

Professor C. Michael Greenlief

Professor Silvia S. Jurisson

Professor John D. Brockman

Professor Chung-Ho Lin

DEDICATION

This dissertation is dedicated to my dear wife, Naa Adjorkor Sowah and delightful son, Frank Cudjoe Appenteng, for their love, unwavering support, and inspiration. Also, to my selfless parents, Frank Cudjoe and Comfort Asaku, who in little, never compromised on quality education.

ACKNOWLEDGMENTS

I have received a great deal of support and assistance throughout my research, dissertation writing and entire graduate school experience. I would first like to express my deepest appreciation to my advisor, Dr. C. Michael Greenlief for his unparalleled mentorship, patient and invaluable scientific insights and contributions to my work. Being part of the Greenlief research group has exposed me to great research projects, which have shaped me into an effective and efficient researcher. This has been a life changing experience and I am deeply indebted to him for his unwavering support and profound belief in me and my abilities. I would also like to thank the rest of my dissertation committee members (Silvia S. Jurisson, Chung-Ho Lin and John D. Brockman) who contributed immensely to the success of my research through their nurturing, constructive criticism and wise counsel. I am also very grateful to Renee D. JiJi who contribute immensely to my work as a committee member before leaving the University.

I would also like to extend my gratitude to all members of the collaborative research team (Research Council Grant) especially Dr. Grace Sun, Dr. David Q. Beversdorf, Dr. Kevin L. Fritsche, Dr. Jiankun Cui, Li Runting and Kille Braina, for their rich insight and enthusiasm towards research. Special thanks to Dr. Andrew L. Thomas at the Division of Plant Sciences, Southwest Research Center, University of Missouri, Mt. Vernon, for providing different varieties of American elderberry samples at different growth stages for analysis. I would also like to specially acknowledge Dr. Brain Mooney and Dr. Pei Liu at the Gehrke Proteomics Center. I learned much as a graduate research assistant under their supervision. Their patience, clarity of direction and decisiveness in research work was second to none. Under their watch, I was nurtured to excel not only in team research but also as an independent thinker. I also want to acknowledge present and past members of the Greenlief research group especially Mitch Johnson and Bo Yang for

their support and for enabling an intellectually stimulating environment during my research. Mitch Johnson was very instrumental in walking me through the basics of method development using the Xevo TQS-MS instrument. I cannot forget the assistance and input from undergrads like Ritter Krueger and Richard Bell who worked with me to optimize the picrate method. I am also very grateful to the Chemistry Department and Gehrke Proteomics Center for providing the necessary facilities, instruments, and equipment for my research. Notable mentions include Jerry Brightwell and Holly Oswald who provided all the needed help and support.

To my parents Mr. Frank Cudjoe and Mrs. Comfort Asaku and all my siblings, I am extremely grateful for your continuous prayers, support, and love. To my spiritual and God parents, Reverend Mr. and Mrs. Acheampong and Dr. Mr. and Mrs. Appenteng respectively, I say, a big thank you for your prayers and wise counsel all these years. I cannot begin to express my immense appreciation to my lovely wife, Naa Adjorkor Sowah and son, Frank Cudjoe Appenteng. You always give me a reason to smile each morning and keep my focus amidst any challenges. I am extremely grateful to you. Finally, to Jehovah God, I say thank you so much for your grace, direction, good health, sound mind and the gift of life without which none of this success would be possible.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

FIGURES

SUPPLEMENTARY FIGURES

LIST OF TABLES

TABLES

SUPPLEMENTARY TABLES

LC-MS/MS METHOD DEVELOPMENT FOR QUANTITATIVE ANALYSIS OF CYANOGENIC GLYCOSIDES IN ELDERBERRY AND LIPID PEROXIDATION PRODUCTS IN MICE

Michael Kwame Appenteng

Dr. C. Michael Greenlief, Dissertation Supervisor

ABSTRACT

Cyanogenic glycosides (CNGs) are naturally occurring plant molecules (nitrogenous plant secondary metabolites) which consist of an aglycone and a sugar moiety. Hydrogen cyanide (HCN) is released from these compounds following enzymatic hydrolysis causing potential toxicity issues. The presence of CNGs in American elderberry (AE) fruit, *Sambucus nigra* (subsp. *canadensis*), is uncertain. A sensitive, reproducible and robust LC-MS/MS method was developed and optimized for accurate identification and quantification of the intact glycoside. A complimentary picrate paper test method was modified to determine the total cyanogenic potential (TCP). TCP analysis was performed using a camera-phone and UV-Vis spectrophotometry. A method validation was conducted, and the developed methods were successfully applied to the assessment of TCP and quantification of intact CNGs in different tissues of AE samples. Results showed no quantifiable trace of CNGs in commercial AE juice. Levels of CNGs found in various fruit tissues of AE cultivars studied ranged from between 0.12-6.38 μg/g. In pressed juice samples, the concentration range measured was 0.29-2.36 μg/mL and in seeds the amounts was 0.12-2.38 μg/g. TCP was highest in the stems and green berries. CNG levels in all tissues were generally low and at a level that poses no threat to consumers of fresh and processed AE products.

The abundance of docosahexaenoic acid (DHA) in phospholipids in the brain and retina has generated interest to search for its role in mediating neurological functions. Besides the source of many oxylipins with pro-resolving properties, DHA also undergoes peroxidation, producing 4-hydroxyhexenal (4-HHE), although its function remains elusive. Despite wide dietary consumption, whether supplementation of DHA may alter the peroxidation products and their relationship to phospholipid species in brain and other body organs have not been explored sufficiently. In this study, adult mice were administered a control or DHA-enriched diet for three weeks, and phospholipid species and peroxidation products were examined in brain, heart and plasma. Results demonstrated that this dietary regimen increased (n-3) and decreased (n-6) species to different extent in all major phospholipid classes (PC, dPE, PE-pl, PI and PS) examined. Besides changes in phospholipid species, DHA-enriched diet also showed substantial increases in 4-HHE in brain, heart and plasma. Among different brain regions, the hippocampus responded to the DHA-enriched diet showing significant increase in 4-HHE. Considering the pro- and anti-inflammatory pathways mediated by the (n-6) and (n-3) polyunsaturated fatty acids, unveiling the ability for DHA-enriched diet to alter phospholipid species and lipid peroxidation products in the brain and in different body organs may be an important step forward towards understanding the mechanism(s) for this (n-3) fatty acid on health and diseases.

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by restricted social communication and repetitive behaviors. Prenatal stress is critical in neurodevelopment and increases risk for ASD, particularly in those with greater genetic susceptibility to stress. Docosahexaenoic acid (DHA) is one of the most abundant ω-3

fatty acids in mammalian brain, and dietary ω-3 fatty acid affects the development and maintenance of brain structure. We investigated whether prenatal supplementation of DHA alleviates autistic-like behaviors in a gene/stress mouse model and how it alters lipid peroxidation activity in the brain. Pregnant heterozygous serotonin transporter knockout (SERT-KO) and wild-type (WT) dams were placed in either non-stressed control conditions or chronic variable stress conditions and fed either a control diet or a DHA-rich (1% by wt) diet. Offspring of each group were assessed for anxiety and autism-associated behavior at post-natal day 60, including an open field test, elevated-plus maze test, repetitive behavior, and the 3-chamber social approach test. Our LC-MS-based method was used to follow changes in peroxidation product concentrations in mouse plasma, heart, and cerebral cortex.

CHAPTER 1: Introduction

American Elderberry

Elderberries (*Sambucus spp*) are large deciduous shrubs native to the northern hemisphere and naturalized throughout the temperate and subtropical regions.[1] Fruit of cultivated elderberry range from bright red to blue and dark purple depending on species (Figure 1.1). Nearly every part of the elderberry plant has some culinary use. The fruit and flowers are used in a variety of foods, jellies, syrups, pie, tea, wines, food colorants and other products like cosmetics and dietary supplements.[2,3] The wood is used by craftsmen to make toys and pipes. New elderberry bush varieties have also been bred for their attractive and ornamental characteristics.[1,4] Elderberry is known for its high nutritional and medicinal health benefits.[4,5] It is rich in carbohydrates, fatty acids, organic acids, minerals, vitamins (A, B6 and C), essential oils and is high in fiber.[5] Researchers have linked elderberry products to anti-inflammatory, anti-oxidant, anti-carcinogenic, anti-viral, anti-influenza, and antibacterial activities.[1,6,7,8,9,10,11,12]

The taxonomy of elderberry is still evolving due to its widespread and complex morphology. Donoghue[13], concluded that the genus Sambucus belongs to the family Adoxaceae[14,15] instead of Caprifoliaceae[16,17]. Bolli[18] earlier proposed a revision of the genus *Sambucus* in which the phylogenetic tree was simplified by submerging many species to the rank of subspecies. By emphasizing morphological similarities within the group, he concluded that only nine species are reputed to be part of this genus. Among this group are the two most common and economically important members of the genus; American elderberry (AE) [*Sambucus nigra* sbsp. *canadensis* (L.) R. Bolli] and European

elderberry (EE) [*Sambucus nigra* sbsp. *nigra* (L.) R. Bolli]. Native to eastern and midwestern North America, AE has rapidly developed as a specialty crop. Whereas little scientific research has been conducted on AE, as compared to its close relative, the EE, both species are excellent sources of flavonoids, polyphenols and anthocyanins, which give the fruit a diverse range of antioxidant properties.[1,19,20,21,22,23,24]

In-depth studies have been made to examine the botany and horticultural performance[2,3, 25, 26] and fruit characteristics[27] of elderberry. Elderberry grows on a wide range of soil types with a broad spectrum of nitrogen, phosphorous, and potassium content simplified as 0.l0 kg of l0 N-10 P_2O_5-10 K_2O for every year of the plant's age. Although it can tolerate occasional flooding, spring floods are usually detrimental. While seed dispersal is mainly done by birds and mammals, the seedlings hardly support any competition from faster growing weeds. Plants can reach up to 9 m in height. The main vector for pollen distribution is wind rather than insects. Fruit ripening happens over a 2-month period and a single cluster can contain as many as 2000 berries.

Figure 1. 1 Elderberry plant

Dietary Supplements

As defined by Congress in the Dietary Supplement Health and Education Act (DSHEA), which became law in 1994, a dietary supplement is a product (other than tobacco) that is intended to supplement the diet; contains one or more dietary ingredients (including vitamins; minerals; herbs or other botanicals; amino acids; and other substances) or their constituents; is intended to be taken by mouth as a pill, capsule, tablet, or liquid; and is labeled on the front panel as being a dietary supplement.[28,29] A new report reveals the dietary supplement global market reached $132.8 billion in 2016 and is on track to hit $220.3 billion by 2022. In the US alone, sales reached $42.6 billion in 2018.[30]

Botanical / Herbal Dietary Supplements

Botanical dietary supplements, sometimes called herbals or herbal dietary supplements, are products made from plants, plant parts, or plant extracts.[31] Like other dietary supplements, botanicals are not required by federal law to be tested for safety and effectiveness before they are marketed, so the amount of scientific evidence available for various botanical ingredients varies widely. Scientists over the years have used several approaches to evaluate botanical dietary supplements for their potential health benefits and risks. Investigation into the history of use, laboratory studies using cell or tissue cultures, experiment with animals, human studies (e.g., individual case reports, observational studies, and clinical trials) provide the most direct evidence of a botanical/herbal supplement's effects on health and patterns of use.[29]

Herbal Supplements sales in US

Herbal dietary supplements continued to experience strong sales growth in the United States in 2019, with total sales increasing by an estimated 8.6% from 2018, according to the Nutrition Business Journal (NBJ).[32] Consumers spent a total of $9.602 billion on herbal supplements across all market channels in 2019. More than three-quarters of a billion dollars of 2018 sales. Figure 1.2 shows the total US retail sales of herbal supplements from 2000 to 2019. SPINS, a market research firm based in Chicago, Illinois provided sales data for the 40 top selling herbal supplements in both mainstream and natural retail channels. Elderberry ranked 3rd in sales for both mainstream ($107.574 million) and natural retail channels ($31.952 million) in 2019.[32] Figure 1.3 shows mainstream elderberry sales performance from 2011 to 2019. Sambucol, based on EE, is popularly known as black elderberry (as shown in Figure 1.4) and is a typical elderberry supplement used to treat symptoms of the common cold and flu.[33]

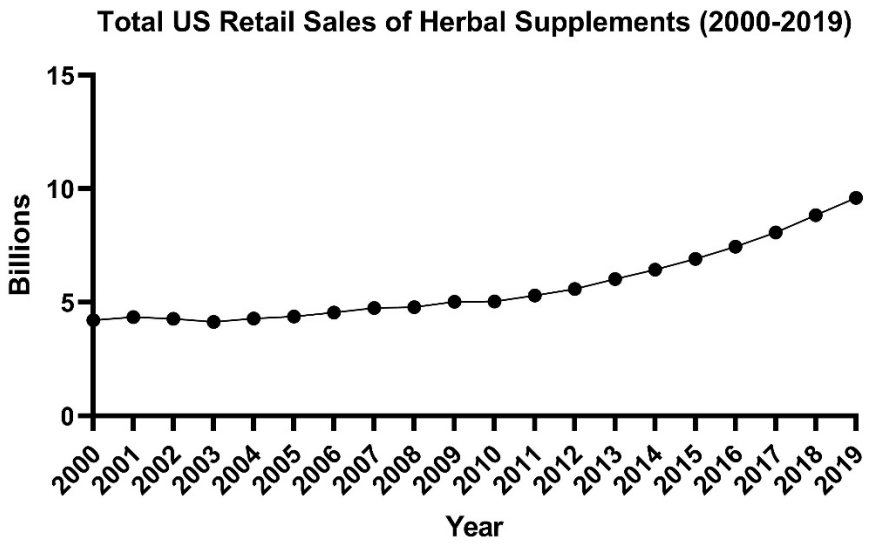

Figure 1.2 Total US Retail Sales of Herbal Supplements (2000-2019)
NB: Data for this graph was retrieved from 2019 herbal supplement sales report in the United States [32].

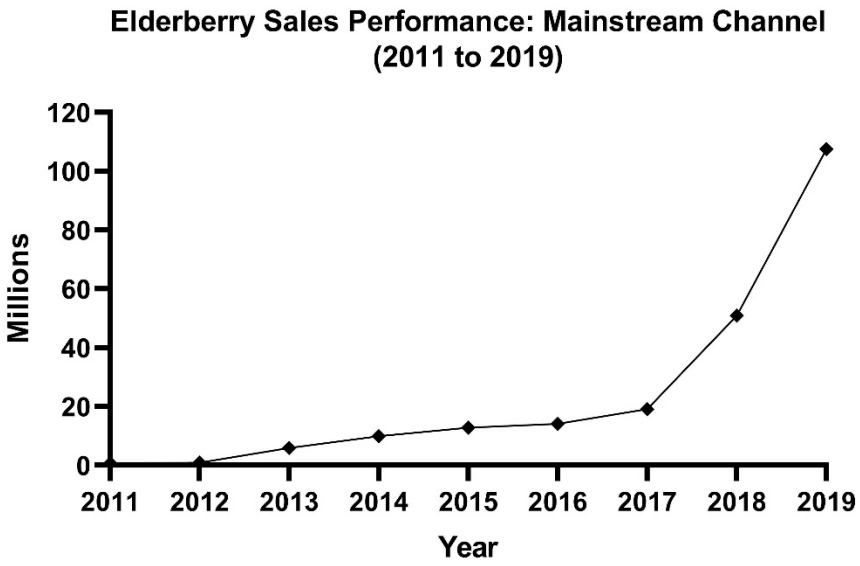

Figure 1.3. Elderberry Sales Performance: Mainstream Channel (2011 to 2019)
NB: Data for this graph was retrieved from 2011 to 2019 herbal supplement sales reports in the United States [32].

Figure 1.4. Sambucol (black elderberry); elderberry dietary supplement

Plant Metabolites

Metabolites are compounds synthesized by plants for both essential functions, such as growth and development, and specific functions, such as pollinator attraction or defense against herbivory.[34,35,] A plant cell produces two types of metabolites: primary metabolites involved directly in growth and metabolism (carbohydrates, lipids and proteins), and secondary metabolites considered as end products of primary metabolism and not involved in metabolic activity.[35] Whereas primary metabolites are universal in the plant kingdom, secondary metabolites are variously distributed in the plant kingdom, and their functions are specific to the plants in which they are found.[34,36]

Plant Secondary Metabolites or Bioactive Compounds

Plant secondary metabolites or bioactive compounds are biosynthetically derived from primary metabolites. They are synthesized in specialized cells at particular developmental stages and accumulated in smaller quantities making extraction and purification difficult.[35] In the plant kingdom, they are limited and may be restricted to a particular taxonomic group genus, species, or family. They are often colored, fragrant or flavorful compounds, and they typically mediate the interaction of plants with other organisms. Such interactions include those of plant-pollinator, plant-pathogen, and plant-herbivore.[34,] Secondary plant metabolites plays an important role in alleviating several aliments in traditional medicine and folk uses. In modern medicine, they provide lead compounds for the production of medications for treating various diseases.[36] Based on their biosynthetic origins, plant secondary metabolites can be divided into three major groups: terpenoids; flavonoids and allied phenolic and polyphenolic compounds; and

nitrogen-containing alkaloids and sulfur-containing compounds.[35, 37, 38] Table 1 shows the classes and number of secondary metabolites reported from higher plants.[35]

Table 1.1 Classes and number of secondary metabolites reported from higher plants [36]

Type of secondary metabolite	Approximate numbers
Nitrogen-containing Secondary metabolites	
Alkaloids	21,000
Non-protein amino acids (NPAAS)	700
Amines	100
Cyanogenic glycoside*	60*
Glucosinolates	100
Alkamides	150
Lectins, peptides, polypeptide	2000
Secondary metabolites without nitrogen	
Monoterpenes including iridoids	
Sesquiterpenes	5000
Diterpenes	2500
Triterpenes, steroids, saponins	5000
Tetraterpenes	500
Tannins, Flavonoids*	5000*
Phenylpropanoids, lignin, coumarins, lignans	2000
Polyacetylenes, fatty acid, waxes	1500
Anthraquinones and other polyketides	750
Carbohydrates, organic acids	200

Phenolic compounds

The phenolic compounds are a vast class of secondary metabolites performing several roles for the defense and survival of plants.[39] Characterized by having at least one aromatic ring with one or more hydroxyl groups attached, there are in excess of 8000 phenolic structures reported and widely dispersed throughout the plant kingdom.[35] Depending on their structure they can be classified into simple phenols, phenolic acids, hydroxycinnamic acid derivatives and flavonoids.[35,40] The main subclasses of flavonoids are the flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins as shown in Figure 1.5.[35] From a human physiological standpoint, phenolic compounds are

vital in defense responses, such as anti-aging, anti-inflammatory, antioxidant and anti-proliferative activities.[41,42,43,] Phenolic compounds have received considerable attention for being potentially protective factors against diabetes, cancers, cardiovascular and neurodegenerative diseases, in part because of their potent antioxidative properties through the management of oxidative stress.[41,44,45,46,47,48]

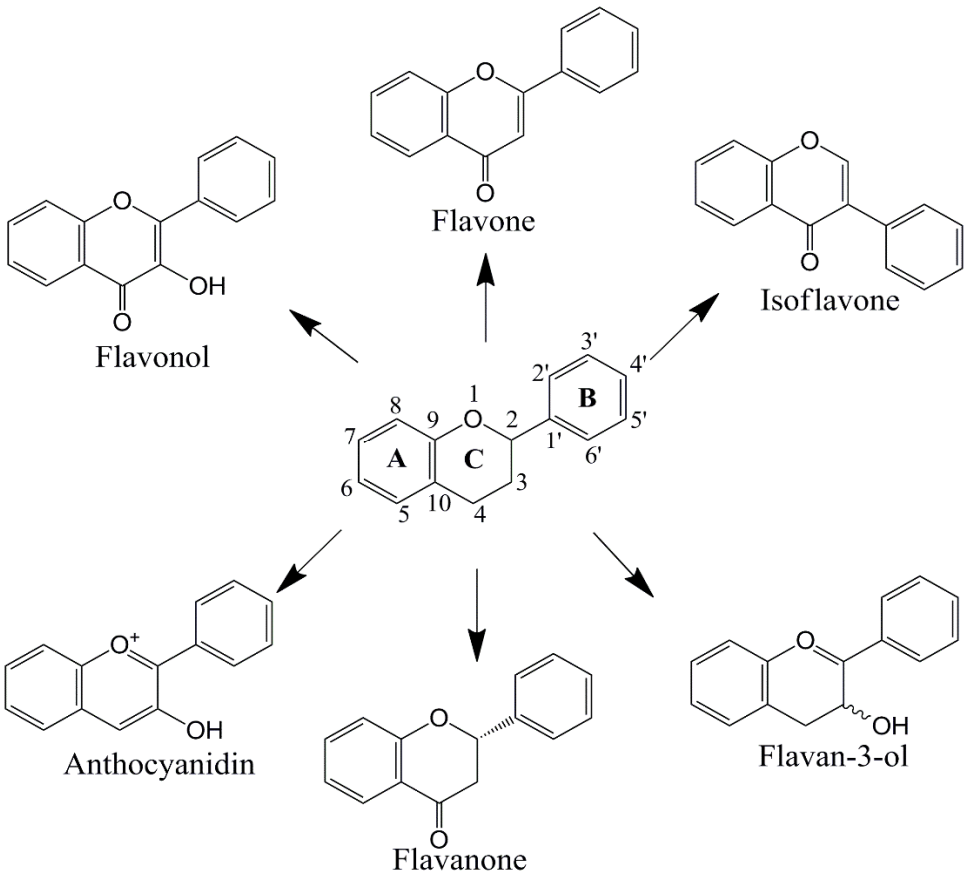

Figure 1.5. Generic structure of major flavonoids

Cyanogenic glycosides

Cyanogenic glycosides (CNGs) are naturally occurring plant toxins (nitrogenous secondary plant metabolites), which consist of aglycone and a sugar moiety [Figure 1.6].[49,50]

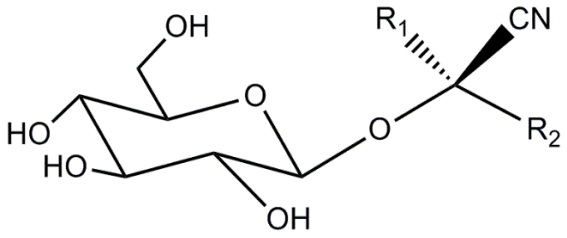

Figure 1.6. Generic structure for a cyanogenic glycoside, where R_1 is often methyl or a proton and R_2 is a variable organic group.

There are about 60 CNGs widely distributed in the plant kingdom and occurring in over 2600 plant species, representing more than 130 families.[51,52,53,54] Examples of different structural classes of cyanogenic compounds are found in Figure 1.7.[55] CNGs are stored in vacuoles within plant cells, separating them from plant hydrolyzing endogenous enzymes (β-1,6-glycosidases, and hydroxynitrile lyases).[49,56] Although intact CNGs are non-toxic, action by endogenous plant enzymes can release hydrogen cyanide (HCN) causing potential toxicity issues (as shown in Figure 1.8).[49,50,51,57,58] Plants already contain these endogenous enzymes so when their tissues are disrupted, for example by crushing, the CNGs come into contact with endogenous enzymes resulting in the release of HCN.[56,59] In plants, consequently, CNGs serve as important chemical defense compounds against herbivores and pathogens.[51,53,53.] Clinical trials have shown mixed results about the potency of amygdalin (a CNG) in cancer treatment and as cough suppressant in various preparations.[60,61] Excessive ingestion of CNGs can however be fatal. In humans, consumption of cyanogenic plants can cause sub-acute cyanide poisoning (depending on

dose) with symptoms including anxiety, headache, vomiting, nausea, abdominal cramps diarrhea, dizziness, weakness, and mental confusion. Acute cyanide toxicity in humans (0.5-3.5 mg kg^{-1} body weight)[50,51] can result in decreased consciousness, hypotension, paralysis, coma and even death.[49,50,51,57,58,62,63] Acute cyanide poisoning has been reported from the ingestion of apricot (intoxication may occur from eating 5 - 25 apricot seed) [64], bitter almonds (6.2 mg per each bitter almond) [65] and cassava(15-400 mg/kg fresh weight of hydrogen cyanide in cassava roots).[66]

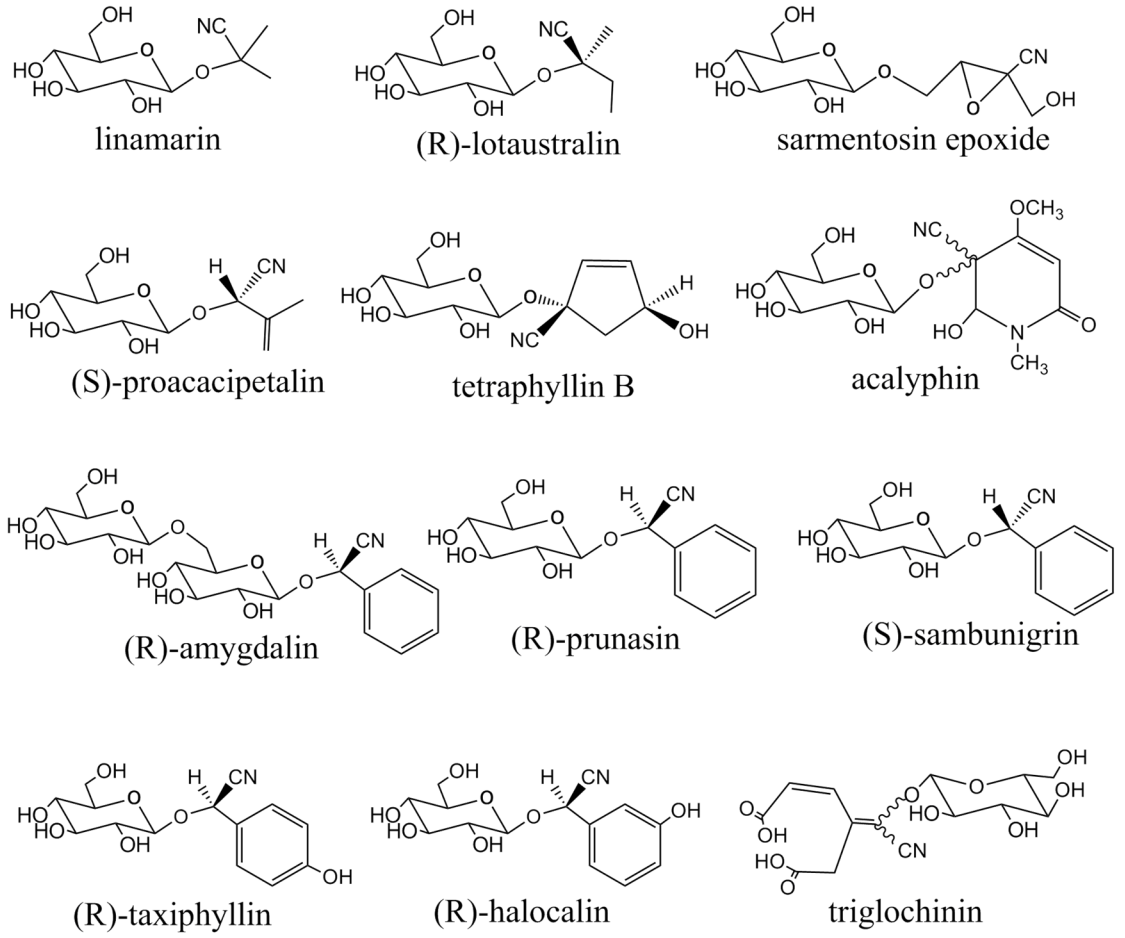

Figure 1.7. Examples of different types of cyanogenic glycosides

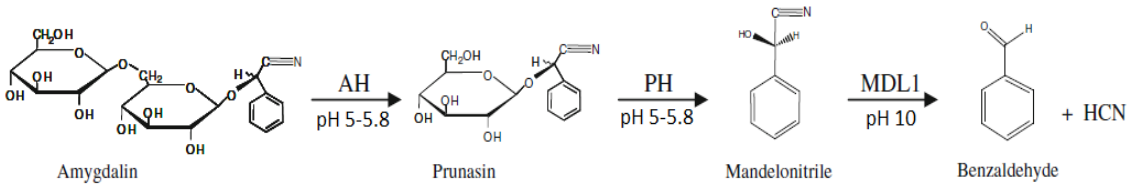

Figure 1.8. Enzymatic breakdown of amygdalin (a CNG) by endogenous enzymes: amygdalin hydrolase (AH), prunasin hydrolase (PH), mandelonitrile lyase 1(MDL1)[57]

Fatty Acids

Fatty acids (FAs) are carboxylic acids with long aliphatic chains, which may be straight or branched, saturated or unsaturated.[67,68] They are important component of lipids (fat-soluble components of living cells) in plants, animals, and microorganisms. FAs are present in cells as the acyl moieties of phospholipids which make up the structural matrix of cell and subcellular membranes.[69] They are classified into 3 groups according to the presence and number of double bonds in their carbon chain. Saturated fatty acids (SFAs) contain no double bonds, monounsaturated fatty acids (MUFAs) contain one, and polyunsaturated fatty acids (PUFAs) contain more than one double bond.

Fatty acids are either called by their systematic names according to the IUPAC (International Union of Pure and Applied Chemistry) nomenclature or by their trivial/common names.[70] The systematic naming can either be delta or omega nomenclature.[71] The common names and omega nomenclature are used more than the delta nomenclature when describing specific fatty acids.[71] The shorthand form of the omega nomenclature which has evolved over decades where fatty acids are named by the abbreviation FA, their number of carbon atoms, their number of double bonds after a colon and the positioning of the first double bond from the omega end written in

parenthesis (n).[71] An example is docosahexaenoic acid which is represented by DHA 22:16 (n=3) as shown in table 1.2.

Table 1. 2. Some common fatty acids with shorthand omega representation

Common Name	Omega Name	Number of carbons	Number of double bonds	n
Docosahexaenoic Acid	22:6 (n-3)	22	6	3
Arachidonic Acid	20:4 (n-6)	20	4	6
Eicosapentanoic Acid	20:5 (n-3)	20	5	3
Alpha-linolenic Acid	18:3 (n-3)	18	3	3
Linoleic Acid	18:2 (n-6)	18	2	6
Oleic Acid	18:1 (n-9)	18	1	9

Essential Fatty Acids

Although humans can synthesize saturated and monounsaturated fatty acids, n-6 and n-3 families of PUFAs cannot be formed *de novo* by mammalian cells.[72,69] The parent fatty acids of these families, α-linolenic acid (18:3n-3, ALA) and linoleic acid (18:2n-6, LA) are essential fatty acids and must be present in the diet.[72,69,73] Once obtained from the diet, ALA and LA can be further desaturated and elongated enzymatically to eicosapentaenoic acid (C20:5n-3), then to docosahexaenoic acid (C22:6n-3, DHA), whereas LA is converted to arachidonic acid (C20:4n-6, AA).[72,69] Figure 1.9 shows the pathway for conversion of ALA to DHA and LA to ARA.[72,73,74]

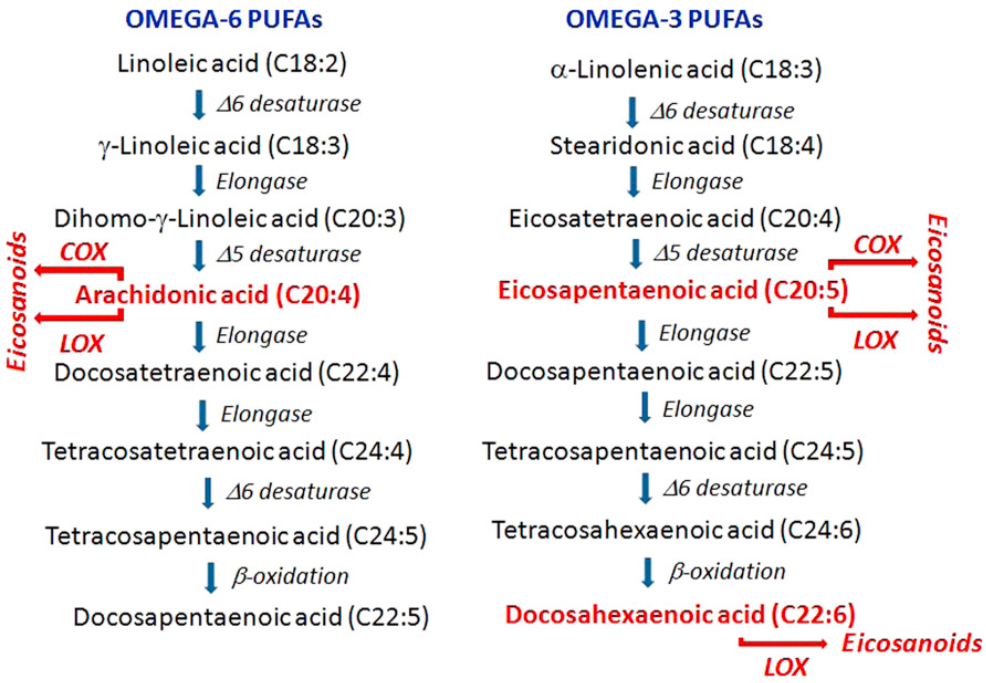

Figure 1.9. Metabolic conversion of the essential fatty acids, linoleic acid (n-6) and alpha-linolenic acid (n-3) to long-chain polyunsaturated fatty acids. *COX, cyclooxygenase; LOX, lipoxygenase.*

Docosahexaenoic acid and Arachidonic Acid

Docosahexaenoic acid (C22:6n-3, DHA) and arachidonic acid (C20:4n-6, ARA) are important structural components of the central nervous system.[73] The main source of n-3 and n-6 are through placental transfer and breastfeeding where they accumulate mostly in the brain and retina during fetal and infant development.[69,75,76] The high concentrations of DHA in the retina and of DHA and ARA in brain gray matter suggests that these fatty acids have important roles in retinal and neural function.[72,73,77] These essential FAs are commonly supplemented in diet due to insufficient endogenous synthesis. A balanced intake of both series of omega is particularly important, because n-3 and n-6 compete for incorporation into cell membranes[78] and have opposing physiological functions; whereas n-6 promotes systemic proinflammatory states, n-3 promotes anti-inflammatory

states.[75,79] Although there is no evidence that the ability to form ARA from linoleic acid is limiting, supplementation with DHA reduces tissue ARA, possibly creating a conditional need for ARA in infants with a dietary intake of DHA.[69]Animal studies have shown that depletion of DHA from the retina and brain results in reduced visual function, impaired memory, behavioral abnormalities and learning deficits.[69,73,80] Epidemiological studies also suggest that consumption of (n-3) PUFAs and fish oil may have significant effects on cardiovascular diseases (CVD).[81,82] Figure 1.10 shows the structure of DHA and ARA.

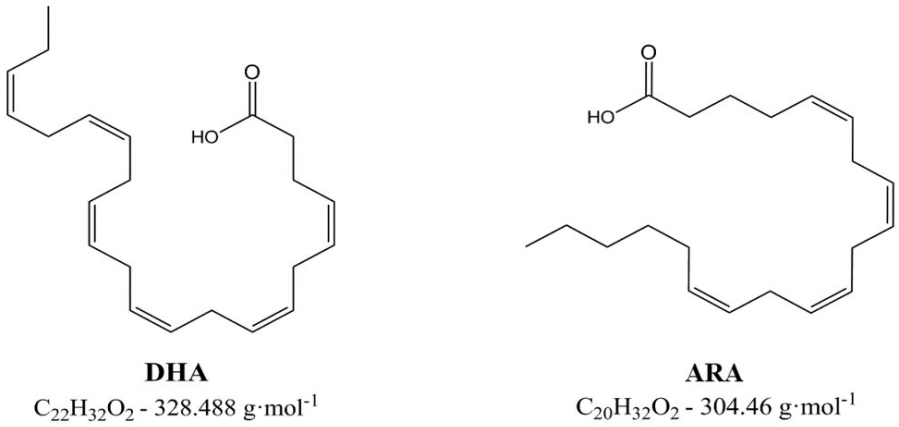

Figure 1.10. Molecular structures of docosahexaenoic acid (DHA) and arachidonic acid (ARA)

Phospholipids

Phospholipids are a key component of all cell membranes.[83] They are amphipathic in nature and can form lipid bilayers because of its hydrophilic phosphate group head.[84] Brain lipids, including phospholipids, sphingolipids, and cholesterol, are known to play critical roles in the structure and functions of cell membranes. Phospholipids in mammalian brain, including phosphatidylcholine (PC), phosphatidylethanolamine (PE),

phosphatidylethanolamine plasmalogen (PEpl), phosphatidylserine (PS) and phosphoinositides (PI, PIP and PIP2) have PUFAs esterified in the sn-2 position.[77] DHA is the major PUFA in the outer segments of the retina rods and cones, where it can constitute as much as 50% of the FAs in PE and PS, and as much as 80% of all the PUFAs.[85,73] The phospholipids of brain gray matter contain high proportions of DHA in PE and PS and high amounts of ARA in phosphatidylinositol (PI). ARA is also present in membrane phospholipids, particularly in PI throughout the body. Figure 1.11 shows phosphatidylcholine, a major component of lecithin.

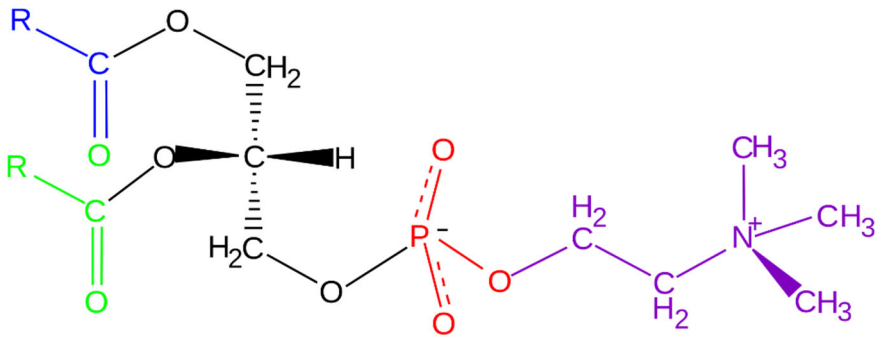

Figure 1.11. Phosphatidylcholine (PC), a major component of lecithin.

Lipids peroxidation products

Lipid peroxidation is a general term, which refers to different mechanisms classified as enzymatic, non-enzymatic non-radical and non-enzymatic free-radical mediated peroxidation.[86] Free-radical non-enzymatic peroxidation of PUFAs is the dominant pathway in oxidative stress induced by radiation, heat, free radicals, xenobiotics, metal ions or reactive oxygen or nitrogen species (ROS or RNS). Once initiated, results in an oxidative deterioration of polyunsaturated lipids.[87] The PUFAs in membrane phospholipids are metabolically active and undergo turnover through a deacylation-

reacylation mechanism mediated by enzymes.[77] DHA, as well as arachidonic acid (20:4 n-6, ARA), are two major PUFAs that undergo enzymatic reactions to produce active lipid mediators as well as interaction with other oxygen radical species. While arachidonic acid (AA) is released from membrane phospholipids by cytosolic phospholipase A2 (cPLA2), DHA is linked to action of the Ca^{2+}-independent iPLA2. Lipid peroxidation may target these PUFAs in the cell membranes, as well as in the free form, releasing 4-hydroxyhexenal (4-HHE) from DHA and 4-hydroxynonanal (4-HNE) from ARA as shown in Figure 1.12.[77,88] These alkenal products are readily detected in biological tissues and plasma. Overwhelming data suggest that reactive lipid mediators generated from this process are biomarkers for oxidative stress and important players for mediating a number of signaling pathways.[87,86] Moreover, they have been implicated in the pathogenesis of various diseases including, adult respiratory distress syndrome diabetes, cancer, and atherosclerosis.[87] Emerging evidence however, shows that sublethal concentrations of these compounds exhibit adaptive responses and can actually protect neurons against oxidative stress and act as important players for mediating a number of signaling pathways. (i.e. induce the production of heme oxygenase-1 (HO1), a potent antioxidant enzyme downstream of Nrf2/ARE activation).[77,89]

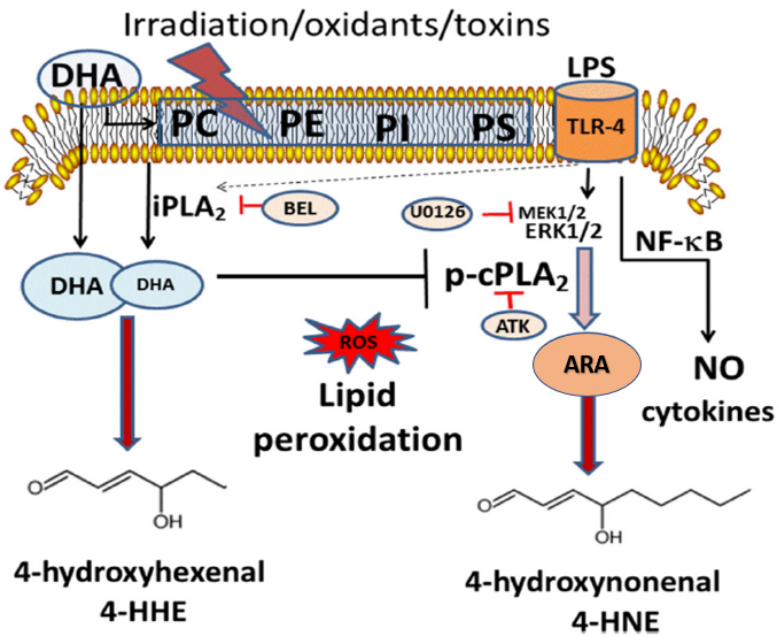

Figure 1.12. A scheme depicting metabolic pathways for production of 4-HHE and 4-HNE through different enzymatic (cPLA2 and iPLA2) and non-enzymatic (lipid peroxidation) mechanisms. *Diagram from Yang et al., 2018 [86].*

Chromatography

Chromatography Overview

Chromatography is an important analytical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.[90] Separation of components is based on the differential affinities of the analytes for a stationary phase relative to a mobile phase. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus affect the separation.[91] Chromatography was first employed in Russia by the Italian-born scientist Mikhail Tsvet in 1900, primarily for the separation of plant pigments. Since then, the technology has advanced rapidly and applied in many different ways, resulting in the different varieties of chromatography.

Gas Chromatography (GC)

The invention of Gas Chromatography (GC) is generally attributed to A.T. James and A.J.P. Martin in their 1952 paper.[92] GC is used to analyze volatile substances in the gas phase. In gas chromatography, the components of a sample are dissolved in a solvent and vaporized in order to separate the analytes by distributing the sample between two phases: a stationary phase and a mobile phase. The mobile phase is a chemically inert gas that serves to carry the molecules of the analyte through the heated column.

High-Performance Liquid Chromatography (HPLC)

HPLC relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow through the column. The acronym HPLC was coined by Prof. Csaba Horváth for his 1970 Pittcon paper. In the beginning, pumps only had a pressure capability of 500 psi (35 bar). This was called high pressure liquid chromatography, or HPLC. The early 1970s saw a tremendous leap in technology. These new HPLC instruments could develop up to 6,000 psi [400 bar] of pressure, and incorporated improved injectors, detectors, and columns. With continued advances in performance, the acronym HPLC remained the same, but the name was changed to high performance liquid chromatography.[93]

Ultra-high Performance Liquid Chromatography (UHPLC)

In 2004, further advances in instrumentation and column technology were made to achieve very significant increases in resolution, speed, efficiency and sensitivity in liquid

chromatography. Columns with smaller particles (1.7 μm) and instrumentation with specialized capabilities designed to deliver mobile phase at 15,000 psi (1,000 bar) were needed to achieve a new level of performance. This new system was historically created to perform ultra-high performance liquid chromatography, trademarked UPLC technology by Waters. This allows for a more rapid method development at a lower cost. Basic research is being conducted today by scientists working with columns containing even smaller 1-μm-diameter particles and instrumentation capable of performing at 100,000 psi (6,800 bar).[94,95]

Mass Spectrometry

Mass Spectrometry overview

Mass spectrometry is an analytical technique that involves the study in the gas phase of ionized molecules with the aim of one or more of the following: qualitative and quantitative analysis of components in a mixture, molecular weight determination, structural characterization and gas phase reactivity studies. The birth of mass spectrometry in 1912 began with the cathode ray experiments performed by Joseph John (J. J.) Thomson in Cambridge.[96] Before considering mass spectrometry (MS), one should consider the types of analyses and results you expected from them: Do you want to analyze large molecules, such as proteins and peptides, or acquire small, aqueous-molecule data? Do you want to look for target compounds at a determined level of detail, or characterize unknown samples? Are your current separations robust, or must you develop methods from complex matrices? Do you require unit mass accuracy-say, 400 MW-or accuracy to 5 ppm, as in 400.0125 MW? Must you process hundreds of samples a day? Thousands? Tens of thousands? Mass spectrometry uses an instrument called a mass

spectrometer. The main components of a mass spectrometer are: Inlet system, Ion source, Mass analyzer, Detector and Data Acquisition as shown in Figure 1.13.

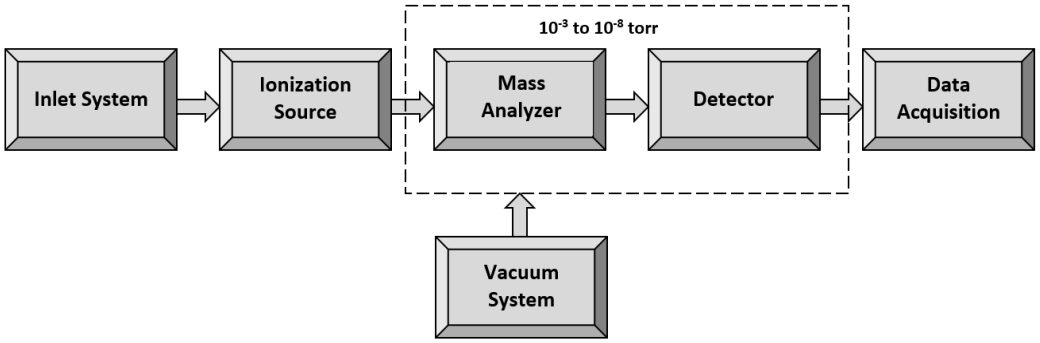

Figure 1.13. Schematic of mass spectrometry instrument layout

Inlet System

Samples can be introduced to the mass spectrometer directly via solids probe, or in the case of mixtures, by the intermediary of chromatography device (e.g. gas chromatography, liquid chromatography, capillary electrophoresis etc.) as earlier explained.

Ionization Sources

Electron Ionization (EI)

Electron ionization (EI) was first described in 1918 by Canadian-American Physicist Arthur J. Dempster in the article of "A new method of positive ray analysis".[97] EI, often referred to as "hard" ionization method, is suitable for non-thermolabile compounds. The volatility of the sample is required. Sample molecules in the vapor state are bombarded by fast moving electrons, conventionally 70 eV energy. The energy of the electrons interacting with the molecule of interest is generally much greater than that contained in

its bonds, so ionization occurs. The excess energy breaks bonds in a well-characterized way. The result is predictable, identifiable fragments from which we can deduce the molecule's identity. Abstraction of only an electron from the outer shell yields a radical cation in the positive mode ($M^{\cdot+}$) and a rich spectrum of fragments which is fairly independent of the source design as shown in equation 1.

$$M + e^- (70eV) \text{ -----> } M^{+\cdot} + 2e^- \quad (1.1)$$

Electrospray Ionization (ESI)

Among the most used spray ionization techniques is electrospray ionization (ESI). The ESI technique was first reported by Masamichi Yamashita and John Fenn in 1984.[98] It provides the softest ionization for the highly polar compounds that have low volatility or are thermally unstable . ESI creates ions at atmospheric pressure rather than in a vacuum. It uses an electrical potential between the ESI probe exit (e.g., capillary) and the counter electrode, which is located few millimeters from the probe. The process results in the generation of highly charged droplets directly from the infused solution as shown in Figure 1.14. Multiply and/or singly charged analyte molecules desorbed from the sprayed droplets are sampled through the rest of the mass spectrometer. ESI has been distinguished for its ability to produce multiply charged molecular ions from a large variety of molecules such as protein and DNA fragments; it allows for the sensitive detection of singly charged low molecular weight polar species such as drugs and drug metabolites. The formation of positive or negative ions (depending on the sign of the applied electric field) occurs in high yield. In the positive ion mode, protonated and/or alkali adduct analyte molecules are

generally observed in the mass spectra. In the negative, ion mode operation peaks corresponding to deprotonated analyte molecules are observed. ESI is described as a very "soft" ionization technique where the surrounding bath gas has a moderating effect on the internal and translational energies of desorbed ions.

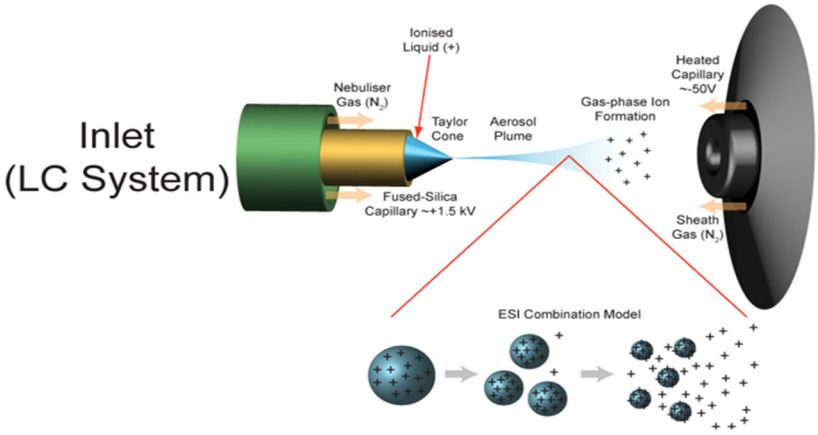

Figure 1.14. Electrospray Ionization (ESI)

Atmospheric pressure chemical ionization (APCI)

Horning was the first to introduced APCI in 1973 to analyze volatile compounds using various introduction techniques like HPLC.[99] APCI is also a soft ionization technique but not as soft as ESI. It operates at higher temperatures (350-550 °C). APCI is a techniques which creates ions at atmospheric pressure. A sample solution flows through a heated tube where it is volatilized in a mist and sprayed into a corona discharge with the aid of nitrogen nebulization. Sample molecules are ionized by ion molecule reactions from the ambient corona discharge ions. Ions are produced in the discharge and extracted into the mass spectrometer. APCI is best suited to relatively polar, thermally stable, and

semi-volatile samples. An APCI mass spectrum usually contains the quasi-molecular ion, $[M\text{-}H]^-$ or $[M + H]^{+\cdot}$

Mass Analyzers

A mass analyzer is the component of the mass spectrometer that takes ionized masses and separates them based on charge to mass ratios and outputs them to the detector where they are detected and later converted to a digital output. There are a variety of mass analyzers commercially available today that are suitable for different applications. Common types include the quadrupole, ion trap (three-dimensional quadrupole, linear or cylindrical ion trap), Orbitrap, time-of-flight (ToF), ion cyclotron resonance and magnetic /electrostatic double sector mass analyzers. Lately, there is a growing trend toward combining different analyzer designs in order to increase the versatility and allow multiple experiments to be performed simultaneously using a single instrument. One of the most recent examples of such a novel hybrid instrument is the linear ion trap-Fourier transform ion cyclotron resonance mass spectrometer (LIT-FT-ICR).[100]

Quadrupole Mass Analyzers

A quadrupole mass analyzer or mass filter consists of four hyperbolic- or circular shaped rods that are accurately positioned parallel to each other (Figure 1.15). Opposite rods are charged by either a positive or a negative direct-current (DC) potential, at which an alternating-current (AC) potential in the radiofrequency region is superimposed. At a given DC/RF combination, only the ions of a particular m/z show a stable trajectory and are transmitted to the detector, while ions with unstable trajectories do not pass the mass filter, because the amplitude of their oscillation becomes infinite. Thus, the quadrupole

acts as a variable band pass filter.[101] By changing DC and RF in time, usually at a fixed ratio, ions with different m/z values can be transmitted to the detector one after another.

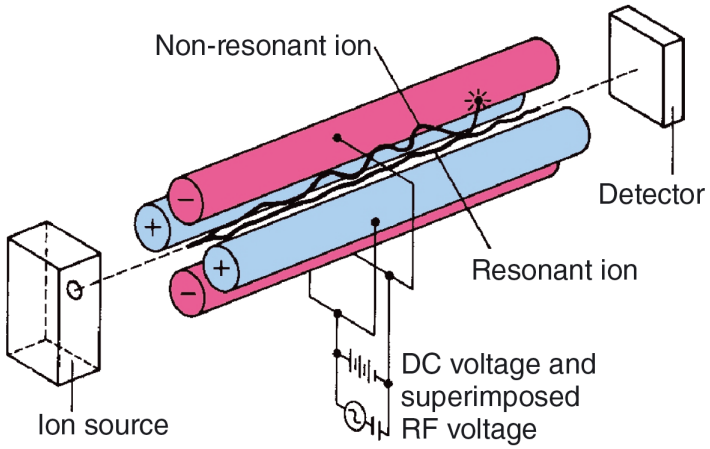

Figure 1.15. A Quadrupole mass analyzer or mass filter (*diagram from Wilfried M.A. Niessen and David Falck, 2015)*

Detectors

The final element of the mass spectrometer is the detector. The detector generates a signal from incident ions by either generating secondary electrons, which are further amplified or by inducing a current generated by a moving charge. The detector records either the charge induced or the current produced when an ion passes by or hits a surface. The most common types of ion detector used in modern instruments are Faraday cup detector (or cylinder electrode), electron multiplier and photomultiplier (or scintillation counter).

Photomultiplier

Photomultipliers (PM) have emerged as the most common detectors in modern mass spectrometers. In a photomultiplier, the ions initially strike a dynode, which results in

electron emission. These electrons then strike a phosphorous screen, which in turn releases a burst of photons. The photons then pass into the multiplier where amplification occurs in a cascade fashion, much like with the electron multiplier. The main advantage of using photons is that the multiplier can be kept sealed in a vacuum preventing contamination and greatly extending the lifetime of the detector.

Tandem Mass Spectrometry

Tandem Mass Spectrometry, usually referred to as MS/MS, involves the use of 2 or more mass analyzers. It is often used to analyze individual components in a mixture. This technique adds specificity to a given analysis. Tandem mass spectrometry can be referred to MS/MS, MS/MS/MS, etc.

Triple Quadrupole (QqQ)

A triple quadrupole mass spectrometer (TQMS), is a tandem mass spectrometer consisting of two quadrupole mass analyzers in series, with a (non-mass-resolving) radio frequency (RF)–only a quadrupole between them to act as a cell for collision-induced dissociation as shown in Figure 1.16. This configuration is often abbreviated QqQ. The arrangement of three quadrupoles was first developed by J.D. Morrison of La Trobe University, Australia for the purpose of studying the photodissociation of gas-phase ions.[102] The triple quadrupole is the most frequently used mass spectrometer for MS/MS, because of the cost and ease of use among other factors.

Essentially the triple quadrupole mass spectrometer operates under the same principle as the single quadrupole mass analyzer. Each of the two mass filters (Q1 and Q3) contains four parallel, cylindrical metal rods. Both Q1 and Q3 are controlled by direct current (dc) and radio-frequency (rf) potentials, while the collision cell, q, is only

subjected to RF potential. The RF potential associated with the collision cell (q) allows all ions that were selected for to pass through it. The arrangement of the TQMS allows for four different scan types to be performed: a precursor ion scan, neutral loss scan, product ion scan, and selected reaction monitoring or multiple reaction monitoring (MRM) modes.

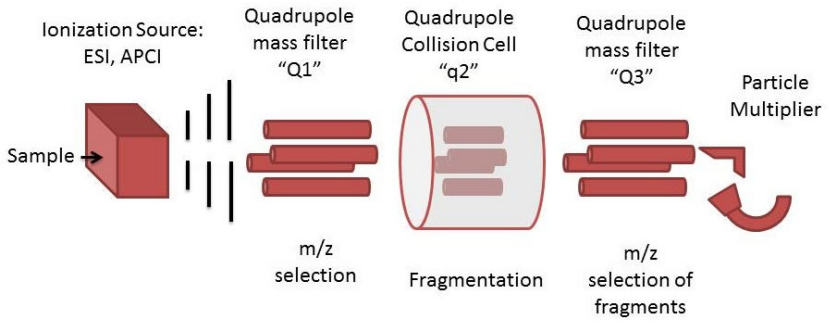

Figure 1.16. Schematic of a triple quadrupole mass spectrometer (*By MiaJ601 (talk) 18:17, 19 March 2015 (UTC) -CC BY-SA 3.0*

Multiple Reaction Monitoring (MRM)

Selected or multiple reaction monitoring (SRM or MRM) are methods used in tandem mass spectrometry in which an ion of a particular mass is selected in the first stage of a tandem mass spectrometer and an ion product of a fragmentation reaction of the precursor ion is selected in the second mass spectrometer stage for detection.[103] MRM mode is highly selective. Because both MS1 and MS2 are static, greater dwell time on the ions of interest is possible, so the sensitivity achieved in better. This mode is the most commonly used acquisition mode for quantitative analysis.

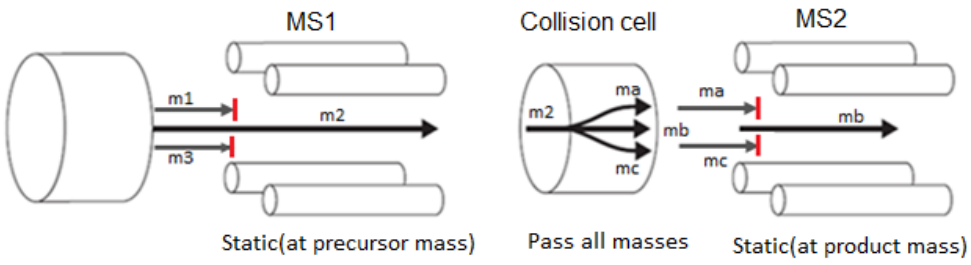

Figure 1.17. Data acquisition mode: Selected or Multiple Reaction Monitoring (SRM or MRM)

Liquid Chromatography Mass Spectrometry (LC-MS)

Although the coupling of chromatography with MS dates back from the 1950s, development of LC-MS systems took longer and was directly related to the development of proper interfaces. V.L. Tal'roze and collaborators started the development of LC-MS in the early 1970s, when they first used capillaries to connect LC columns and MS ion sources. While direct infusion is suitable for use with samples that are pure or that are simple mixtures composed of only a small number of constituents, LC-MS is a very powerful tool for mass analysis of a complex mixture by separating each analyte into an individual component before mass spectrometric analysis. It provides superior specificity and sensitivity and serves as powerful tool for toxicology applications in the clinical laboratory. LC-MS is the main method for detecting drugs and their major metabolites in vivo, and can provide high enough sensitivity, specificity, and molecular structural information for the qualitative assay of drugs and their metabolites.

References

1. Lee J, Finn CE. Anthocyanins and other polyphenolics in American elderberry (Sambucus canadensis) and European elderberry (S. nigra) cultivars. *J Sci Food Agric*. 2007;87(14):2665-2675. doi:10.1002/jsfa.3029
2. Charlebois, Denis, Patrick L. Byers, Chad E. Finn and ALT. *Elderberry: Botany, Horticulture, Potential*.
3. Thomas AL, Byers PL, Avery JD, Kaps M, Gu S. Horticultural performance of eight American elderberry genotypes at three Missouri locations. *Acta Hortic*. 2015;1061:237-244. doi:10.17660/ActaHortic.2015.1061.26
4. Native American Ethnobotany - Workman Publishing. Accessed June 4, 2020. https://www.workman.com/products/native-american-ethnobotany
5. Młynarczyk K, Walkowiak-Tomczak D, Łysiak GP. Bioactive properties of Sambucus nigra L. As a functional ingredient for food and pharmaceutical industry. *J Funct Foods*. 2018;40(November 2017):377-390.
6. Barak V, Birkenfeld S, Halperin T, Kalickman I. The effect of herbal remedies on the production of human inflammatory and anti-inflammatory cytokines. *Isr Med Assoc J*. 2002;4(11 SUPPL.):919-922.
7. Wu H, Johnson MC, Lu CH, Fritsche KL, Thomas AL, Cai Z, Greenlief CM. Determination of anthocyanins and total polyphenols in a variety of elderberry juices by UPLC-MS/MS and Other Methods. *Acta Hortic*. 2015;1061:43-52. doi:10.17660/ActaHortic.2015.1061.3
8. Roschek B, Fink RC, McMichael MD, Li D, Alberte RS. Elderberry flavonoids bind to and prevent H1N1 infection in vitro. *Phytochemistry*. 2009;70(10):1255-

1261. doi:10.1016/j.phytochem.2009.06.003

9. Uncini Manganelli RE, Zaccaro L, Tomei PE. Antiviral activity in vitro of Urtica dioica L., Parietaria diffusa M. et K. and Sambucus nigra L. *J Ethnopharmacol*. 2005;98(3):323-327. doi:10.1016/j.jep.2005.01.021

10. Mohammadsadeghi S, Malekpour A, Zahedi S, Eskandari F. The antimicrobial activity of elderberry (Sambucus nigra L.) extract against gram positive bacteria, gram negative bacteria and yeast. *Res J Appl Sci*. 2013;8(4):240-243. doi:10.3923/rjasci.2013.240.243

11. Antolak H, Czyzowska A, Kregiel D. Antibacterial and antiadhesive activities of extracts from edible plants against soft drink spoilage by Asaia spp. *J Food Prot*. 2017;80(1):25-34. doi:10.4315/0362-028X.JFP-16-134

12. Abdramanov A, Massanyi P, Sarsembayeva N, Usenbayev A, Alimov J, Tvrdá E. The in vitro effect of elderberry (Sambucus nigra) extract on the activity and oxidative profile of bovine spermatozoa. *J Microbiol Biotechnol Food Sci*. 2017;6(6):1319-1322. doi:10.15414/jmbfs.2017.6.6.1319-1322

13. Donoghue MJ, Bell CD, Winkworth RC. The evolution of reproductive characters in Dipsacales. In: *International Journal of Plant Sciences*. Vol 164. The University of Chicago Press ; 2003. doi:10.1086/376874

14. Testoni LD, De Souza AB, De Krueger CMA, Quintão NLM, Couto AG, Bresolin TMB. Quantification of sambucus nigra (Adoxaceae) markers related to tincture stability. *Nat Prod Commun*. 2019;14(1):59-62. doi:10.1177/1934578X1901400116

15. Mikulic-Petkovsek M, Ivancic A, Todorovic B, Veberic R, Stampar F. Fruit

Phenolic Composition of Different Elderberry Species and Hybrids. *J Food Sci*. 2015;80(10):C2180-C2190. doi:10.1111/1750-3841.13008

16. Deineka VI, Sorokopudov VN, Deineka LA, Shaposhnik EI, Kol'tsov S V. Anthocyans from fruit of some plants of the Caprifoliaceae family. *Chem Nat Compd*. 2005;41(2):162-164. doi:10.1007/s10600-005-0102-2
17. Guangwan H, Chunlin L, Murrell G, Keming L. Lectotypification of Sambucus chinensis (Caprifoliaceae) and a New Variety from Hunan, China. *Novon A J Bot Nomencl*. 2008;18(1):61-66. doi:10.3417/2006055
18. Bolli R. *Revision of the Genus Sambucus*. J. Cramer; 1994.
19. Thole JM, Kraft TFB, Sueiro LA, Kang Y-H, Gills JJ, Cuendet M, Pezzuto JM, Seigler DS, Lila MA. A Comparative Evaluation of the Anticancer Properties of European and American Elderberry Fruits. *J Med Food*. 2006;9(4):498-504. doi:10.1089/jmf.2006.9.498
20. Johnson MC, Dela Libera Tres M, Thomas AL, Rottinghaus GE, Greenlief CM. Discriminant Analyses of the Polyphenol Content of American Elderberry Juice from Multiple Environments Provide Genotype Fingerprint. *J Agric Food Chem*. 2017;65(20):4044-4050. doi:10.1021/acs.jafc.6b05675
21. Johnson MC, Thomas AL, Greenlief CM. Impact of Frozen Storage on the Anthocyanin and Polyphenol Contents of American Elderberry Fruit Juice. *J Agric Food Chem*. 2015;63(23):5653-5659. doi:10.1021/acs.jafc.5b01702
22. Ozgen M, Scheerens J, Reese R, Miller R. Total phenolic, anthocyanin contents and antioxidant capacity of selected elderberry (Sambucus canadensis L.) accessions. *Pharmacogn Mag*. 2010;6(23):198-203. doi:10.4103/0973-1296.66936

23. Mudge E, Applequist WL, Finley J, Lister P, Townesmith AK, Walker KM, Brown PN. Variation of select flavonols and chlorogenic acid content of elderberry collected throughout the Eastern United States. *J Food Compos Anal*. 2016;47:52-59. doi:10.1016/j.jfca.2015.12.003

24. Simonyi A, Chen Z, Jiang J, Zong Y, Chuang DY, Gu Z, Lu CH, Fritsche KL, Greenlief CM, Rottinghaus GE, Thomas AL, Lubahn DB, Sun GY. Inhibition of microglial activation by elderberry extracts and its phenolic components. *Life Sci*. 2015;128:30-38. doi:10.1016/j.lfs.2015.01.037

25. Thomas AL, Byers PL, Ellersieck MR. Productivity and characteristics of American elderberry in response to various pruning methods. *HortScience*. 2009;44(3):671-677. doi:10.21273/hortsci.44.3.671

26. Atkinson MD, Atkinson E. *Sambucus nigra* L. *J Ecol*. 2002;90(5):895-923. doi:10.1046/j.1365-2745.2002.00698.x

27. Thomas AL, Perkins-Veazie P, Byers PL, Finn CE, Lee J. A comparison of fruit characteristics among diverse elderberry genotypes grown in Missouri and Oregon. *J Berry Res*. 2013;3(3):159-168. doi:10.3233/JBR-130054

28. FDA 101: Dietary Supplements | FDA. Accessed June 11, 2020. https://www.fda.gov/consumers/consumer-updates/fda-101-dietary-supplements

29. Background Information: Botanical Dietary Supplements - Health Professional Fact Sheet. Accessed June 13, 2020. https://ods.od.nih.gov/factsheets/BotanicalBackground-HealthProfessional/

30. Natural Medicines - Dietary Supplement Sales Soar: Find Out Which Market Segments Lead the Surge. Accessed June 13, 2020.

https://naturalmedicines.therapeuticresearch.com/news/news-items/2019/september/dietary-supplement-sales-soar-find-out-which-market-segments-lead-the-surge.aspx

31. Botanical Dietary Supplements. Accessed June 11, 2020. https://ntp.niehs.nih.gov/whatwestudy/topics/botanical/index.html

32. US Herbal Supplement Sales Increase by 8.6% in 2019, Record-Breaking Sales Predicted for 2020. Accessed September 10, 2020. https://www.globenewswire.com/news-release/2020/08/31/2086400/0/en/US-Herbal-Supplement-Sales-Increase-by-8-6-in-2019-Record-Breaking-Sales-Predicted-for-2020.html

33. Zakay-Rones Z, Thom E, Wollan T, Wadstein J. Randomized study of the efficacy and safety of oral elderberry extract in the treatment of influenza A and B virus infections. *J Int Med Res*. 2004;32(2):132-140. doi:10.1177/147323000403200205

34. Seigler DS, Seigler DS. Introduction. In: *Plant Secondary Metabolism*. Springer US; 1998:1-15. doi:10.1007/978-1-4615-4913-0_1

35. Irchhaiya R, Kumar A, Yadav A, Gupta N, Kumar S, Gupta N, Kumar S, Yadav V, Prakash A, Gurjar H. Metabolites in Plants and Its Classification. *World J Pharm Pharm Sci*. 2015;4(1):287-305.

36. A. Hussein R, A. El-Anssary A. Plants Secondary Metabolites: The Key Drivers of the Pharmacological Actions of Medicinal Plants. In: *Herbal Medicine*. IntechOpen; 2019. doi:10.5772/intechopen.76139

37. Mahmoud SS, Croteau RB. Strategies for transgenic manipulation of monoterpene biosynthesis in plants. *Trends Plant Sci*. 2002;7(8):366-373. doi:10.1016/S1360-

1385(02)02303-8

38. Guerriero G, Berni R, Muñoz-Sanchez JA, Apone F, Abdel-Salam EM, Qahtan AA, Alatar AA, Cantini C, Cai G, Hausman JF, Siddiqui KS, Hernández-Sotomayor SMT, Faisal M. Production of plant secondary metabolites: Examples, tips and suggestions for biotechnologists. *Genes (Basel)*. 2018;9(6):309. doi:10.3390/genes9060309

39. Tsimogiannis D, Oreopoulou V. Classification of Phenolic Compounds in Plants. In: *Polyphenols in Plants*. Elsevier; 2019:263-284. doi:10.1016/b978-0-12-813768-0.00026-8

40. Cartea ME, Francisco M, Soengas P, Velasco P. Phenolic Compounds in Brassica Vegetables. *Molecules*. 2010;16(1):251-280. doi:10.3390/molecules16010251

41. Lin D, Xiao M, Zhao J, Li Z, Xing B, Li X, Kong M, Li L, Zhang Q, Liu Y, Chen H, Qin W, Wu H, Chen S. An overview of plant phenolic compounds and their importance in human nutrition and management of type 2 diabetes. *Molecules*. 2016;21(10). doi:10.3390/molecules21101374

42. Martins N, Barros L, Ferreira ICFR. In vivo antioxidant activity of phenolic compounds: Facts and gaps. *Trends Food Sci Technol*. 2016;48:1-12. doi:10.1016/j.tifs.2015.11.008

43. Limmongkon A, Janhom P, Amthong A, Kawpanuk M, Nopprang P, Poohadsuan J, Somboon T, Saijeen S, Surangkul D, Srikummool M, Boonsong T. Antioxidant activity, total phenolic, and resveratrol content in five cultivars of peanut sprouts. *Asian Pac J Trop Biomed*. 2017;7(4):332-338. doi:10.1016/j.apjtb.2017.01.002

44. van't Veer P, Jansen MC, Klerk M, Kok FJ. Fruits and vegetables in the

prevention of cancer and cardiovascular disease. *Public Health Nutr*. 2000;3(1):103-107. doi:10.1017/s1368980000000136

45. Aune D, Giovannucci E, Boffetta P, Fadnes LT, Keum NN, Norat T, Greenwood DC, Riboli E, Vatten LJ, Tonstad S. Fruit and vegetable intake and the risk of cardiovascular disease, total cancer and all-cause mortality-A systematic review and dose-response meta-analysis of prospective studies. *Int J Epidemiol*. 2017;46(3):1029-1056. doi:10.1093/ije/dyw319

46. Ramassamy C. Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: A review of their intracellular targets. *Eur J Pharmacol*. 2006;545(1):51-64. doi:10.1016/j.ejphar.2006.06.025

47. Vauzour D, Vafeiadou K, Rodriguez-Mateos A, Rendeiro C, Spencer JPE. The neuroprotective potential of flavonoids: A multiplicity of effects. *Genes Nutr*. 2008;3(3-4):115-126. doi:10.1007/s12263-008-0091-4

48. Cai Y, Luo Q, Sun M, Corke H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci*. 2004;74(17):2157-2184. doi:10.1016/j.lfs.2003.09.047

49. Bolarinwa IF, Orfila C, Morgan MRA. Determination of amygdalin in apple seeds, fresh apples and processed apple juices. *Food Chem*. 2015;170:437-442. doi:10.1016/j.foodchem.2014.08.083

50. Mazza G, Cottrell T. Carotenoids and cyanogenic glucosides in saskatoon berries (Amelanchier alnifolia Nutt.). *J Food Compos Anal*. 2008;21(3):249-254. doi:10.1016/j.jfca.2007.11.003

51. Bolarinwa IF, Orfila C, Morgan MRA. Amygdalin content of seeds, kernels and

food products commercially- available in the UK. *Food Chem*. 2014;152:133-139. doi:10.1016/j.foodchem.2013.11.002

52. Vetter J. Plant cyanogenic glycosides. *Toxicon*. 2000;38(1):11-36. doi:10.1016/S0041-0101(99)00128-2

53. Ganjewala D, Kumar S, Devi S A, Ambika K. Advances in cyanogenic glycosides biosynthesis and analyses in plants: A review. *Acta Biol Szeged*. 2010;54(1):1-14.

54. Buhrmester RA, Ebinger JE, Seigler DS. Sambunigrin and cyanogenic variability in populations of Sambucus canadensis L. (Caprifoliaceae). *Biochem Syst Ecol*. 2000;28(7):689-695. doi:10.1016/S0305-1978(99)00105-2

55. Plant Toxin Analysis: Modern Methods Of Plant Analysis: Linskens H.F.: 9788184891102: Amazon.com: Books. Accessed July 21, 2020. https://www.amazon.com/Plant-Toxin-Analysis-Modern-Methods/dp/8184891105

56. Abraham K, Buhrke T, Lampen A. Bioavailability of cyanide after consumption of a single meal of foods containing high levels of cyanogenic glycosides: a crossover study in humans. *Arch Toxicol*. 2016;90(3):559-574. doi:10.1007/s00204-015-1479-8

57. Lee J, Zhang G, Wood E, Rogel Castillo C, Mitchell AE. Quantification of amygdalin in nonbitter, semibitter, and bitter almonds (prunus dulcis) by UHPLC-(ESI)QqQ MS/MS. *J Agric Food Chem*. 2013;61(32):7754-7759. doi:10.1021/jf402295u

58. Senica M, Stampar F, Veberic R, Mikulic-Petkovsek M. Transition of phenolics and cyanogenic glycosides from apricot and cherry fruit kernels into liqueur. *Food Chem*. 2016;203:483-490. doi:10.1016/j.foodchem.2016.02.110

59. Sánchez-Pérez R, Howad W, Garcia-Mas J, Arús P, Martínez-Gómez P, Dicenta F. Molecular markers for kernel bitterness in almond. *Tree Genet Genomes*. 2010;6(2):237-245. doi:10.1007/s11295-009-0244-7

60. Sarker SD, Nahar L. *Chemistry for Pharmacy Students*. Wiley; 2007. doi:10.1002/9781118687529

61. PDQ Integrative, Alternative, and Complementary Therapies Editorial Board. *Laetrile/Amygdalin (PDQ®): Health Professional Version*.; 2002. Accessed July 21, 2020. http://www.ncbi.nlm.nih.gov/pubmed/26389425

62. Geller RJ, Barthold C, Saiers JA, Hall AH. Pediatric Cyanide Poisoning: Causes, Manifestations, Management, and Unmet Needs. *Pediatrics*. 2006;118(5):2146-2158. doi:10.1542/peds.2006-1251

63. Burns AE, Bradbury JH, Cavagnaro TR, Gleadow RM. Total cyanide content of cassava food products in Australia. *J Food Compos Anal*. 2012;25(1):79-82. doi:10.1016/j.jfca.2011.06.005

64. Cyanide Poisoning in a Children Caused by Apricot Seeds | Abstract. Accessed July 21, 2020. https://www.hilarispublisher.com/abstract/cyanide-poisoning-in-a-children-caused-by-apricot-seeds-25161.html

65. Sanchez-Verlaan P, Geeraerts T, Buys S, Riu-Poulenc B, Cabot C, Fourcade O, Mégarbane B, Genestal M. An unusual cause of severe lactic acidosis: Cyanide poisoning after bitter almond ingestion. *Intensive Care Med*. 2011;37(1):168-169. doi:10.1007/s00134-010-2029-8

66. Akintonwa A, Tunwashe OL. Fatal Cyanide Poisoning from Cassava-Based Meal. *Hum Exp Toxicol*. 1992;11(1):47-49. doi:10.1177/096032719201100107

67. Wang HMD, Li XC, Lee DJ, Chang JS. Potential biomedical applications of marine algae. *Bioresour Technol*. 2017;244:1407-1415. doi:10.1016/j.biortech.2017.05.198

68. Venkata Mohan S, Rohit M V., Chiranjeevi P, Chandra R, Navaneeth B. Heterotrophic microalgae cultivation to synergize biodiesel production with waste remediation: Progress and perspectives. *Bioresour Technol*. 2015;184:169-178. doi:10.1016/j.biortech.2014.10.056

69. Innis SM. Essential fatty acid transfer and fetal development. *Placenta*. 2005;26(SUPPL.):S70-S75. doi:10.1016/j.placenta.2005.01.005

70. Köfeler HC. Nomenclature of Fatty Acids. In: *Encyclopedia of Lipidomics*. Springer Netherlands; 2016:1-3. doi:10.1007/978-94-007-7864-1_15-1

71. 2.33 Fatty Acid Naming & Food Sources | Nutrition Flexbook. Accessed April 14, 2021. https://courses.lumenlearning.com/suny-nutrition/chapter/2-33-fatty-acid-naming-food-sources/

72. Koletzko B, Lien E, Agostoni C, et al. The roles of long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: Review of current knowledge and consensus recommendations. *J Perinat Med*. 2008;36(1):5-14. doi:10.1515/JPM.2008.001

73. Innis SM. Perinatal biochemistry and physiology of long-chain polyunsaturated fatty acids. In: *Journal of Pediatrics*. Vol 143. Mosby Inc.; 2003:1-8. doi:10.1067/s0022-3476(03)00396-2

74. Metabolism and Pathophysiology of Bariatric Surgery | ScienceDirect. Accessed August 6, 2020.

https://www.sciencedirect.com/book/9780128040119/metabolism-and-pathophysiology-of-bariatric-surgery

75. López-Vicente M, Ribas Fitó N, Vilor-Tejedor N, et al. Prenatal Omega-6:Omega-3 Ratio and Attention Deficit and Hyperactivity Disorder Symptoms. *J Pediatr*. 2019;209:204-211.e4. doi:10.1016/j.jpeds.2019.02.022

76. Bernardi JR, Escobar RDS, Ferreira CF, Silveira PP. Fetal and neonatal levels of omega-3: Effects on neurodevelopment, nutrition, and growth. *Sci World J*. 2012;2012. doi:10.1100/2012/202473

77. Sun GY, Simonyi A, Fritsche KL, Chuang DY, Hannink M, Gu Z, Greenlief CM, Yao JK, Lee JC, Beversdorf DQ. Docosahexaenoic acid (DHA): An essential nutrient and a nutraceutical for brain health and diseases. *Prostaglandins Leukot Essent Fat Acids*. 2018;136:3-13. doi:10.1016/j.plefa.2017.03.006

78. Burdge G. α-Linolenic acid metabolism in men and women: Nutritional and biological implications. *Curr Opin Clin Nutr Metab Care*. 2004;7(2):137-144. doi:10.1097/00075197-200403000-00006

79. Simopoulos AP. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother*. 2002;56(8):365-379. doi:10.1016/S0753-3322(02)00253-6

80. Hashimoto M, Hossain S, Al Mamun A, Matsuzaki K, Arai H. Docosahexaenoic acid: one molecule diverse functions. *Crit Rev Biotechnol*. 2017;37(5):579-597. doi:10.1080/07388551.2016.1207153

81. Marckmann P, Grønbæk M. Fish consumption and coronary heart disease mortality. A systematic review of prospective cohort studies. *Eur J Clin Nutr*.

1999;53(8):585-590. doi:10.1038/sj.ejcn.1600832

82. Marik PE, Varon J. Omega-3 dietary supplements and the risk of cardiovascular events: A systematic review. *Clin Cardiol*. 2009;32(7):365-372. doi:10.1002/clc.20604

83. Vance JE. Phosphatidylserine and phosphatidylethanolamine in mammalian cells: Two metabolically related aminophospholipids. *J Lipid Res*. 2008;49(7):1377-1387. doi:10.1194/jlr.R700020-JLR200

84. Goda T, Goto Y, Ishihara K. Cell-penetrating macromolecules: Direct penetration of amphipathic phospholipid polymers across plasma membrane of living cells. *Biomaterials*. 2010;31(8):2380-2387. doi:10.1016/j.biomaterials.2009.11.095

85. Giusto NM, Pasquaré SJ, Salvador GA, Castagnet PI, Roque ME, Ilincheta De Boschero MG. Lipid metabolism in vertebrate retinal rod outer segments. *Prog Lipid Res*. 2000;39(4):315-391. doi:10.1016/S0163-7827(00)00009-6

86. Csala M, Kardon T, Legeza B, Lizák B, Mandl J, Margittai É, Puskás F, Száraz P, Szelényi P, Bánhegyi G. On the role of 4-hydroxynonenal in health and disease. *Biochim Biophys Acta - Mol Basis Dis*. 2015;1852(5):826-838. doi:10.1016/j.bbadis.2015.01.015

87. Grotto D, Santa Maria L, Valentini J, Paniz C, Schmitt G, Garcia SC, Pomblum VJ, Rocha JBT, Farina M. Importance of the lipid peroxidation biomarkers and methodological aspects for malondialdehyde quantification. *Quim Nova*. 2009;32(1):169-174. doi:10.1590/S0100-40422009000100032

88. Yang B, Li R, Michael Greenlief C, Fritsche KL, Gu Z, Cui J, Lee JC, Beversdorf DQ, Sun GY. Unveiling anti-oxidative and anti-inflammatory effects of

docosahexaenoic acid and its lipid peroxidation product on lipopolysaccharide-stimulated BV-2 microglial cells. *J Neuroinflammation*. 2018;15(1):1-16. doi:10.1186/s12974-018-1232-3

89. Yang B, Li R, Woo T, Browning JD, Song H, Gu Z, Cui J, Lee JC, Fritsche KL, Beversdorf DQ, Sun GY, Greenlief CM. Maternal dietary docosahexaenoic acid alters lipid peroxidation products and (N-3)/(n-6) fatty acid balance in offspring mice. *Metabolites*. 2019;9(3). doi:10.3390/metabo9030040

90. Coskun O. Separation Tecniques: CHROMATOGRAPHY. *North Clin Istanbul*. 2016;3(2):156. doi:10.14744/nci.2016.32757

91. Free Download Organic Chemistry with Biological Applications 2e By John McMurry | Chemistry.Com.Pk. Accessed August 29, 2020. https://chemistry.com.pk/books/organic-chemistry-with-biological-applications-2e/

92. JAMES AT, MARTIN AJ. Gas-liquid partition chromatography; the separation and micro-estimation of volatile fatty acids from formic acid to dodecanoic acid. *Biochem J*. 1952;50(5):679-690. doi:10.1042/bj0500679

93. Babu S, Chem JSN. *Journal of Synthetic and Natural Chemistry ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR CERTINIB BY RP-HPLC METHOD*. Vol 2.; 2017. Accessed August 29, 2020. http://www.worldinventiapublishers.com/

94. Swartz ME. UPLC ™ : An Introduction and Review. *J Liq Chromatogr Relat Technol*. 2005;28(7-8):1253-1263. doi:10.1081/JLC-200053046

95. UPLC - Ultra Performance Liquid Chromatography Beginner's Guide : Waters. Accessed August 29, 2020. https://www.waters.com/waters/en_US/UPLC---Ultra-

Performance-Liquid-Chromatography-Beginner%27s-Guide/nav.htm?cid=134803622&locale=en_US

96. Downard KM. 1912: a titanic year for mass spectrometry. *J Mass Spectrom*. 2012;47(8):1034-1039. doi:10.1002/jms.3071

97. Dempster AJ. A new method of positive ray analysis. *Phys Rev*. 1918;11(4):316-325. doi:10.1103/PhysRev.11.316

98. Yamashita M, Fenn JB. Electrospray ion source. Another variation on the free-jet theme. *J Phys Chem*. 1984;88(20):4451-4459. doi:10.1021/j150664a002

99. Horning EC, Horning MG, Carroll DI, Dzidic I, Stillwell RN. New Picogram Detection System Based on a Mass Spectrometer with an External Ionization Source at Atmospheric Pressure. *Anal Chem*. 1973;45(6):936-943. doi:10.1021/ac60328a035

100. Weisbrod CR, Hoopmann MR, Senko MW, Bruce JE. Performance evaluation of a dual linear ion trap-Fourier transform ion cyclotron resonance mass spectrometer for proteomics research. *J Proteomics*. 2013;88:109-119. doi:10.1016/j.jprot.2013.04.009

101. Miller PE, Denton MB. The quadrupole mass filter: Basic operating concepts. *J Chem Educ*. 1986;63(7):617-622. doi:10.1021/ed063p617

102. Morrison JD. Personal reminiscences of forty years of mass spectrometry in Australia. *Org Mass Spectrom*. 1991;26(4):183-194. doi:10.1002/oms.1210260404

103. De Hoffmann E. Tandem mass spectrometry: A primer. *J Mass Spectrom*. 1996;31(2):129-137. doi:10.1002/(SICI)1096-9888(199602)31:2<129::AID-JMS305>3.0.CO;2-T

CHAPTER 2: Determining and mitigating the potential occurrence of cyanide in elderberries

2.1 Introduction

2.1.1 Background on cyanide in elderberry

The elderberry industry appears poised for major expansion and have increased significantly in sales over the years. However, one major concern to growers and consumers of raw and processed elderberry products is the possible occurrence of cyanide (as cyanogenic glycosides), a class of toxic compounds, in raw or processed elderberries. While some consumers remain in a dilemma as to the safety of elderberry, others believe that raw ripe elderberries contain cyanide, but that heat treatment or fermentation destroys or removes the cyanide. Conventional wisdom and google searches make this situation murkier with numerous unsubstantiated claims to that effect.

A review of the medical literature revealed no reports of elderberry juice poisoning within the past 37-years. However, there was a CDC report (Centers for Disease Control and Prevention) on a poisoning incident on August 26, 1983 involving several religious people in California.[1] This was attributed to earlier consumption of juice prepared from raw wild elderberries along with leaves and stems of the plant. Cyanide is often implicated in that incident, but this was not definitively proven. There remains an uncertainty as to the presence of this cyanide containing compound called cyanogenic glycosides (CNGs) s in American elderberry (AE). The fact remains that no exhaustive work has been done on *subsp. canadensis* (AE) to conclusively ascertain the presence, forms, and levels of CNGs in ripe and unripe berries. And if present, in which tissues of the plant (juice, skin, pulp, seed, stem, and pedicel) or ripening stage do they occur more

and how safely can we remove or mitigate its effects in AE foods and products? Nevertheless, hearsay and uncertainty regarding the occurrence of cyanide in elderberry continues to foster hesitancy among both producers and consumers, which is most likely significantly hindering the full development of this potentially lucrative agricultural specialty crop in Missouri.

Traditional and modern food-processing techniques such as chopping, grinding, soaking, fermentation, drying, roasting, boiling, and steaming have been used to reduce the potential toxicity of dietary cyanogenic plants.[2,3,4] The effectiveness of these techniques however, depends on the processing method, the plant tissues and the intended processed forms.[2] Soaking for instance could be effective only with discarded soaking water due to solubilization of CNGs without enzymatic degradation.[2,3,5] Boiling can inhibit the activity of endogenous β-glucosidase due to high temperatures to halt HCN release.[3] However, this is only partially effective in reducing HCN toxicity because the CNGs are relatively heat stable[3] and HCN is water soluble.[6] Therefore, if the CNGs does not hydrolyze due to enzyme inactivation, toxicity may still result from the catabolism of these cyanogens in the gastrointestinal tract either by microfloral β-glucosidase or by β-glucosidase contributed by other components of the diet.[7,8] This implies any inefficient processing may expose consumers to some doses of the glycosides and HCN.

Quantification of CNGs can be made either indirectly (by determining HCN released after hydrolysis) or directly (by determining the intact glycoside).[9, 10] In this study, a very sensitive, reproducible and robust LC-MS/MS method was developed and optimized for accurate identification and quantification of intact CNGs. Ultra-high-performance liquid chromatography triple-quadruple mass spectrometry (UHPLC-QqQ MS/MS) was used

for this purpose. A complimentary picrate paper method was modified to assess the total cyanogenic potential (TCP) by determining the total cyanide concentration following action of endogenous enzymes on CNGs. Analysis was performed using a camera-phone and UV-Vis spectroscopy. This study will provide definitive and much needed answers to lingering questions regarding the absolute safety of AE. This will enhance confident in production, processing and marketing of safe healthy AE products which can compete more aggressively with other high antioxidant herbal dietary supplement products within and beyond the US.

2.1.2 Previous cyanide studies

Numerous studies have confirmed significant cyanide levels and specific types of cyanogenic glycosides in several food plants, seeds, and commercially available food products.[11,12,13,14,15] A study by Islamiyat et al.[9] showed significantly high levels of amygdalin in the seeds of 15 apple varieties (1000- 4000 µg/g) and much lower levels in fresh (10-40 µg/g) and processed (1-7 µg/g) apple juices. Amygdalin has also been detected in the seeds of several other fruits related to Rosaceae species (apricot, cherry, plum, pear, peach) and non-Rosaceae species (cucumber, melon, Courgette and Marrow), kernels and commercially-available food.[10,16,17] Although these commercially available foods have undergone some kind of processing, minimal levels of cyanogenic compounds were still detected in most of them. Lee et al.[18] quantified increasing mean concentration of amygdalin from nonbitter (63.13 ± 57.54 µg/g), semibitter (992.24 ± 513.04 µg/g) to bitter almonds (40060.34 ± 7855.26 µg/g) and suggested the possibility of phenotypic classification achieved on the basis of amygdalin levels. Several studies have been done to determine the total cyanide levels in cassava[14,19,20] and also confirm linamarin as the

predominant cyanogenic glycoside in cassava[21,22,23] and lima beans.[24,25]The presence of the cyanogenic glucoside dhurrin was also detected in isolated vacuoles from sorghum.[26]

Research on cyanogenic glycoside content of berries is very sparse. A study by Mazza and Cottrell[27] detected prunasin and amygdalin in the seeds of several cultivars of Saskatoon berries at several stages of ripeness. Interestingly, the prunasin and amygdalin content was high in the seeds and unripe berries in two of the cultivars but was not detected in the unripe berries of the other three cultivars. The cyanogenic glycoside content was generally higher in the ripe berries. Whereas few reports exist on cyanogenic glycosides in European elderberry [*Sambucus nigra* (subsp. *nigra*)], no extensive work has been made on the subsp. *Canadensis* (American elderberry). Earlier work by Senica et al.[2] on the subsp. *nigra* reported average levels of sambunigrin in fresh control juice (18.8 ± 4.3 µg/g) to processed products; juice (10.6 ± 0.7µg/g), liqueur (0.8 ± 0.21µg/g), tea (3.8 ± 1.7 µg/g) and spread (0.8 ± 0.19µg/g). This result shows that higher temperatures decreased the levels of harmful cyanogenic glycosides 44% in processed elderberry juice, 80% in tea and as much as 96% in elderberry liqueur and spread. In another study with sub.sp *nigra*, Senica et al.[28] investigated the change of CNG in elder leaves, flowers, and berries induced by different altitudes and locations. Findings from this study showed that elderberries contained the lowest levels of harmful CNG (0.11 µg/g at the foothills and 0.59 µg/ g at the hilltop) and highest levels in the leaves (153.31 µg/g at the foothills and 209.61 µg/ g at the hilltop). A recent work by Senica et al.[29] on the leaves of three different Sambucus species (*Sambucus nigra* - black elderberry, *Sambucus ebulus* – dwarf elder and *Sambucus racemosa* - red elderberry)

showed highest values of sumbunigrin accumulated in the leaves of black elderberry (1030 μg/g) and the lowest in the leaves of red elderberry (1 μg/g).

The only study previously made on *Sambucus canadensis* (American elderberry) is by Buhrmester et. al.[30] They examined the presence or absence of cyanogenic glycosides for individuals from nine populations of *Sambucus canadensis* L. (elderberry) in east-central Illinois. The study tested for cyanogenic glycosides in the leaves. Of the nine elderberry populations examined, only one population had a test positive for HCN production each of the three times tested. In another population the production of HCN was highly variable. The cyanogenic glycoside was determined to be (S)-sambunigrin by gas chromatographic separation of the TMS-derivative.

2.2 Experimental

2.2.1 Plant material

Elderberry fruit samples were harvested from experimental field plots that were previously described in detail.[31,32] Briefly, a replicated evaluation of eight American elderberry genotypes was established in Missouri (USA) in 2008. Fruits from six genotypes (Bob Gordon, Ocoee, Ozark, Ozone, Wyldewood, and York) were harvested from one of the study sites (Mt. Vernon, MO) at peak ripeness in August 2016, and promptly frozen. Frozen, de-stemmed, whole berries (> 400 g) from the five genotypes were provided to the laboratory, along with frozen unripe and almost-ripe berries (green and red-colored, respectively). Additionally, hundreds of individual berries from each genotype were thawed and painstakingly separated into skins (epicarp), pulp (mesocarp), seeds, juice, and small green stems (pedicels) that connect the berry to the infructescence. After dissection, samples were re-frozen. For detailed CNG analysis, tissue and juice samples from the

genotypes Ozone and Ozark were analyzed separately. Further, tissue and juice from five genotypes (Bob Gordon, Ocoee, Ozark, Wyldewood, and York) were combined into pooled samples for a broader analysis. Sufficient material was dissected to produce samples exceeding 10 mg. Commercially processed elderberry juice was purchased from River Hills Harvest, Hartsburg, MO.

2.2.2 Chemicals and reagents

Water, acetonitrile (ACN), methanol, ethanol and formic acid (FA) were purchased from Fisher Scientific (Fair Lawn, NJ, USA, HPLC grade). β-Glucosidase enzymes (250 mg lyophilized powder ≥ 6 U/mg), amygdalin (1 g, ≥ 99%), dhurrin (1 mg ≥ 98%), prunasin (1 mg ≥ 95%) and linamarin standards (1 mg ≥ 95%), together with picric acid (100 g moisten with water ≥ 98%) and potassium cyanide (≥98%) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Whatman no.1 filter paper, sodium carbonate, sodium hydroxide and pH 8 phosphate buffer (500 mL) were also purchased from Fisher Scientific (Fair Lawn, NJ, USA). Plastic backing and hobby glue (adhesive neutral pH) were purchased from the Mizzou Store (Columbia, MO, USA).

2.2.3 Picrate paper method for cyanide analysis

The picrate paper method is based on the reaction of enzymes that catalyze the release of HCN gas, which reacts with the picric acid on the paper test strip producing 2,6-dinitro-5-hydroxy-4-hydroxylamino-1,3 dicyclobenzne with an indication color change [33] as shown in Figure 2.1. The paper strip can then be analyzed using a camera method or UV-Vis spectrometry.

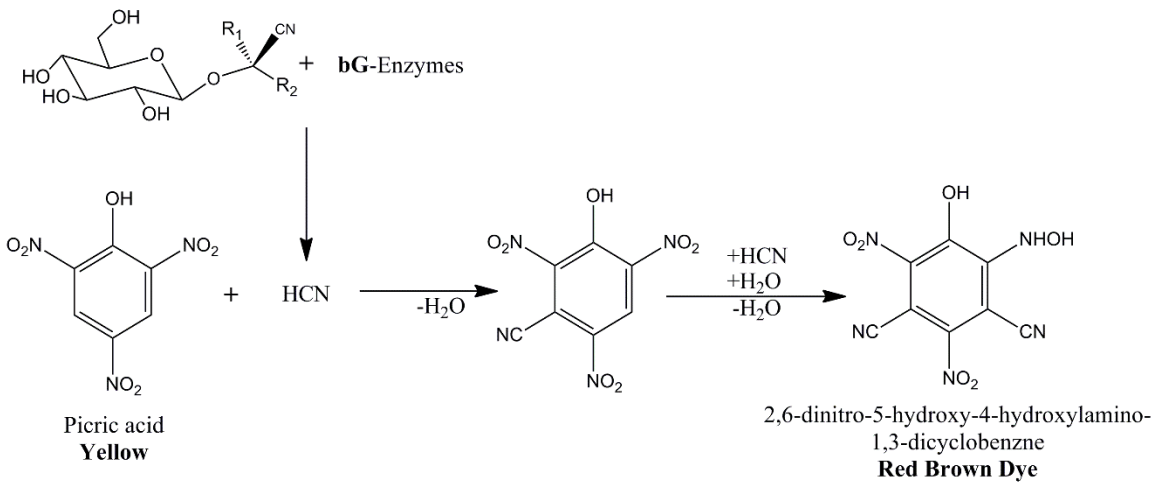

Figure 2.1 Indicator reaction of picric acid with cyanide to form 2,6-dinitro-5-hydroxy-4-hydroxylamino-1,3-dicyclobenzne (*bG; β-1,6-glycosidases*)

2.2.3.1 Preparation of picrate paper

A previously published picrate method described by Meredith et al. [20] for determination of total cyanogens in cassava roots was adapted and modified for use. The picrate paper was prepared beforehand by dipping a sheet of Whatman 3MM filter paper in a picrate solution (1.4% w/v moist picric acid diluted in 2.5% w/v Na_2CO_3 solution), allowing the paper to air dry and cutting it into 3 cm x 1 cm strips. These were glued using a drop of PVA hobby glue to 5 cm x 1.2 cm clear plastic strips to keep the paper clear of the liquid. The 2.5% w/v sodium carbonate diluent was prepared by diluting 2.5 g sodium carbonate to 100 mL of water. The 1.4% w/v (saturated) solution of picric acid was prepared by diluting 0.75 g of moist picric acid to 50 mL of Na_2CO_3 diluent (extra 0.05 g of picric acid adjusts for mass of wet powder.) and stored at room temperature.

2.2.3.2 Standard calibration

Different cyanide amounts (1, 3, 5, 10, 20, 50, and 100 µg) were prepared from 100 µg/mL of CN^- equivalent amygdalin solution following success from initial trials using

KCN/NaOH stock. 100 µg/mL of CN^- equivalent solution from amygdalin (457.17 g/mol) was prepared by dissolving 0.017 g amygdalin in 10 mL of deionized water and stored at 4°C in the refrigerator. One of the most complicated portions of this analysis is the enzymatic hydrolysis of amygdalin. Enzymes are macromolecular biological catalysts whose amount for a specific enzymatic activity is measured in Units (U). One U is defined as the amount of enzyme needed to catalyze the conversion of 1 micromole of substrate per minute.[34] Enzymatic degradation of amygdalin was achieved using the β-glucosidase enzyme.

Approximately 3 U/mL was calculated (mass dependent on U/mg of stock powder) as a concentration of β-glucosidase for the preparation of standards. 10 µL of this solution will completely catalyzed the conversion of 100 µg of amygdalin solution in 2 hours, leaving enough time for the released HCN to completely interact with picric acid. An excess volume of 50 µL of 3 U/mL β-glucosidase enzyme solution in pH 8 phosphate buffer was added to each cyanide equivalent amount of amygdalin in 20 mL scintillation vials. To each vial, a picrate test strip was added, capped and incubated overnight (16-24 hours) in an oven between 30-40 °C as shown below in Figure 2.2.

Figure 2.2 Set up for amygdalin (0-100 µg CN^- equivalent) + 50 µL of 3 U/mL β-glucosidase enzymes in pH 8 phosphate buffer and the picrate paper test strip.

2.2.3.3 Control test

Two control experiments were run, first to test the effectiveness of the picrate paper method using samples from known literature and second to test the extent of self-hydrolysis due to endogenous enzymes in the plant tissue. Seeds and juice from two apple varieties, Granny Smith (GS) and Gala (G), were used for this test. The seeds were pretreated by either grinding raw or following lyophilization. The juice was prepared in two ways, juice from the core and juice from the flesh. In effect both apple varieties were tested for total cyanogenic potential (TCP) by measuring total cyanide levels in the seeds (S), core juice (Jc) and Juice from flesh (J). 100 mg of juice/seeds were treated with the same protocol as in the standards to test for total cyanide levels.

Because cyanogenic plants already contain endogenous enzymes, which are released when plant tissues are disrupted[35,36] (for example by crushing), there is a need to assess the extent of plant self-hydrolysis of CNGs. To achieve this, a control test using ground seeds from the two-apple variety and the stems and green berries for AE composite samples were made. Two different picrate paper set-ups were made, first a control (without an external β-glucosidase solution) and the other, a typical picrate set-up (with an external β-glucosidase solution). Set-ups were made in three replicates using 100 mg of sample. The same set-up protocol as used for the standards were used.

2.2.3.4 Sample preparation and test

Processed elderberry juice was tested first for TCP before all tissues of American elderberry variety (Ozone, Ozark and composite samples). While all varieties were tested for juice, seeds, skin and stem, composite samples were additionally tested for pulp, green, ripe and red berry. Juice from hand pressed berries were tested raw while other tissues were

lyophilized and ground into a powder before testing. 100 mg of lyophilized or raw tissues of pure (Ozone, Ozark) and composite AE samples were weighed into clean 20 mL scintillation vials. 50 µL of β-glucosidase solution (3U/mL) in pH 8 phosphate buffer were added alongside the picrate paper test strip. Vials were immediately closed with a screw stopper. Each set-up was made in three replicates. These were left overnight (16-24 hours) in an oven to warm at temperatures between 30-40 °C.

2.2.3.5 Sample analysis

A simple and quick method of analyzing the reacted picric acid is by qualitative inspection. This appears to be an effective estimate for quantities of CN^- equivalents ranging between 1 and 100 µg. However, as the amount of CN^- eq. increases, the ability to differentiate between the colors decreases. A color chart shown in Figure 3 can be used to relate the color to total CN^- evolved.

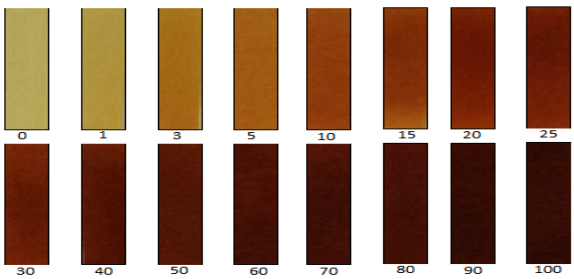

Figure 2.3. A picrate-paper cyanide color chart for qualitative analysis of CNGs for the concentration range of 0-100 µg CN^- eq.

A semi-quantitative approach using a camera-phone as a detector was used. Here, the image (Figure 2.3) was converted from color to greyscale so that the contribution of all colors to the depth of the image can be measured. This was done using "image J" software. The method generated mean intensity values corresponding to each CN^- eq. greyscale filter strip, which was used to generate a calibration curve. Quantification was alternatively done

using a UV-Vis spectrometer (Aligent 8454 photodiode array). The reacted picrate paper test strip was extracted in 3.5 mL of water in cuvettes and the resulting solution analyzed at a wavelength (λ_{max}) of 510 nm after standing for 30 min.

2.2.4 UHPLC-MS/MS method for cyanogenic glycosides analysis

2.2.4.1 Sample preparation and extraction

Berries were transferred into mini Ziploc bags and gently pressed. Juice was transferred into new vial and seeds separated from skin. Sample tissues (excluding juice) were transferred into Eppendorf tubes, flash frozen for about 5 mins in liquid nitrogen and freeze-dried for 24 hrs using a Labconco FreeZone -105°C 4.5 Liter Benchtop Lyophilizer. The lyophilized sample tissues were ground into powder and stored in a desiccator cabinet prior to extraction. In order to optimize the extraction process, equal volumes of elderberry juice (River Hills Harvest, Springfield, MO) were spiked in three replicates with varying amounts of cyanogenic standards (CNS) from a stock mixture (amygdalin, dhurrin, prunasin and linamarin) of 10 µg/mL to attain intended spike concentrations of 1000, 100, and 10 ng/mL (use for validation). The solutions were extracted with 1 mL of different ethanol/methanol and water proportions from 60:40 to 80:20 (v/v). Extraction was done via sonication (15-30 mins) at 30 °C or overnight shaking (16-24 hr) or 2 mins vortexing at room temperature on a vortex Genie 2 Shaker Mixer at 600 rpm. Extracts were centrifuged for 15 mins, dried under nitrogen gas and reconstituted in 1mL of mobile phase (0.1% FA in ACN) for SPE clean up. Sample extraction was done on both raw and lyophilized samples.

2.2.4.2 Solid phase extraction

An SPE method[18] previously used for the determination of amygdalin in almonds was adapted and optimized for our use. A Supelco Visiprep™ SPE vacuum manifold was used for this purpose. 10 inches Hg (35 kPa) pressure and a flowrate of about 1-2 drops/s was maintained throughout the process. To obtain the best washing and elution solvent strength for all four CNS mixture, a ten bottle SPE optimization was made with varying proportions of methanol/water (0, 10, 20, 30 to 100% v/v). SPE cartridges (Sep-Pak Vac 3cc, 500 mg certified C18 cartridge, Waters, USA) were conditioned with 2 mL of methanol and equilibrated with 2 mL of water. 1 mL of the sample was loaded onto the column. An additional 1 mL of 0.1% FA in water was used to remove remaining residues in the extraction tube. The column was flushed with 2 mL of 0.1% FA in water. CNGs were finally, eluted with 2 mL of methanol and water proportions. The extracts were dried under nitrogen gas, reconstituted into 0.1% FA in water and filtered through a 0.45 μm filter prior to UHPLC-MS/MS analysis.

2.2.4.3 Standard calibration

Standard curves were initially prepared as follows: top concentration was 10 μg/mL for a standards mixture for amygdalin, dhurrin, prunasin and linamarin in water spiked with 0.1% FA. The standard mixture was prepared from 1 mg/mL stock (10 μL each diluted to 1 mL) provided for each standard. Subsequent standards were made by serial dilution to produce calibration curves from which the upper limit of quantification for each standard was determined. Once the upper limits of quantification were determined for each standard, the top concentration was reduced from 10 μg/mL to 1000 ng/mL to enable a more accurate quantification of lower analytes concentration for each standard.

Standards were made by 1:2 serial dilution to obtain the following concentration range (1000, 500, 250, 125, 62.5, 32.5, 15.63, 7.81, 3.91, 1.95, 0.97, 0 ng/mL). Calibration curves were made using the analyte peak areas across the entire range of concentrations used (0.97 to 1000 ng/mL). Each concentration was made in three replicates (n).

2.2.4.4 UHPLC-MS/MS method development

2.2.4.4.1 Mass spectrometry conditions

A Waters Xevo TQ-S triple quadrupole mass spectrometer with an electrospray ionization source operated in the positive-ionization mode was used. Multiple reaction monitoring (MRM) scans were conducted by selecting parent and daughter ion pairs of a specific cyanogenic glycoside, which were optimized using the IntelliStartTM software. A desolvation temperature of 350°C and a source temperature of 150°C were used. A capillary voltage of 3.07 kV, cone voltage 21, and nebulizer gas 500 $L \cdot h^{-1}$ N_2 were used. Argon was used as the collision gas. The collision energies were optimized and ranged from 17 to 30 eV for individual analytes. Column and sample temperature were 40° and 10°C respectively. Instrument control and data acquisition were performed by using MassLynx software (version 4.1, Waters, Milford, MA, USA). Peak integration and data processing were made using the TargetLynx software (version 4.1, Waters, Milford, MA, USA). Analyte identity was determined based on integrated areas of retention times.

2.2.4.4.2 UPLC gradient

A Waters Acquity UHPLC system equipped with a quaternary solvent manager was used in conjunction with a C18 column (Acquity BEH, 1.7 µm, 100 mm × 2.1 mm^2, Waters, Milford, MA, USA) for separation of cyanogenic glycosides. A previously published gradient for the quantification of amygdalin in almonds was reduced from 20

min to 10 min.[18] Mobile phases used were 0.1% FA in water (A) and 0.1% FA in acetonitrile (B). The gradient used was 95% A, 0−1 min; 95−80% A, 1−3 min; 80−40% B, 3−7 min; 40% B, 7−8 min; 95% B 8.1−10 min re-equilibration. The flow rate was 0.2 mL/min, and the injection volume was 10 µL in full loop mode. The column was heated to 40°C and the sample chamber was cooled to 10°C.

2.2.5 Method validation for UHPLC-MS/MS

An analytical method for validation was performed, including analysis of lower limit of detection, lower limit of quantification, linearity, range with upper limit of quantification, precision, recovery, and matrix effect.

2.2.5.1 Lower limit of detection and quantification

The lower limit of quantitation (LLOQ) was taken as the lowest concentration of analyte in a sample that could be determined with acceptable precision and accuracy and the lower limit of detection (LLOD), was taken as the lowest absolute concentration of analyte in a sample that could be detected, but not necessarily quantified.[37] The LLOD and LLOQ were determined experimentally by performing serial dilutions on cyanogenic standards stock until the signal-to-noise ratio (S/N) of candidate concentration chromatograms were approximately 3 and 10, respectively. Subsequent repeated injections (n= 7) at this predicted LLOD and LLOQ concentration were performed at targeted coefficient of variation (CV% $\leq$ 20%) for confirmation.[38]

The LLOD and LLOQ values were also theoretically determined using the blank and low concentration sample approach for comparison.[39] This calculation explicitly takes both the blank and the low concentration samples into account using equations 1 and 2 below for LLOD and LLOQ, respectively. From equation 2.1, µB represents the

estimated mean of the blank samples, σB is the standard deviation of the blank samples and σS is the standard deviation of the low concentration samples. The equation assumes that analyte concentration is estimated using the mean of n replicates, and t(1-β,ν) represent the critical value of student's t distribution with ν degrees of freedom, which equals to the number of sample replicates.[40]

$$\mathrm{LLOD} = \mu B + \frac{t(1-\beta)(\sigma B + \sigma S)}{\sqrt{n}} \quad (2.1)$$

$$\mathrm{LLOQ} = 3 \times \mathrm{LLOD} \quad (2.2)$$

2.2.5.2 Linearity, range and upper limits of quantification

The linearity (R^2-value) of an analytical procedure is the ability (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. The range is the interval between the upper and lower concentration limits of analyte at a suitable level of precision, accuracy, and linearity.[41] The upper limit was determined as the highest concentration of the linear curve beyond which the linearity breaks. Cyanogenic standards were prepared at a top concentration of 10 µg/mL with serial dilutions to 1 ng/mL, to analyze the linearity, range, and upper limits of the analytical method.

2.2.5.3 Precision and reproducibility

The precision of an analytical procedure is defined as the closeness of agreement between a series of analytical measurements obtained from multiple sampling of the same homogeneous sample under the same developed conditions.[41] Intraday precision and

reproducibility were determined by analyzing replicate QC samples (n = 6). The criteria for acceptability of the data included a precision of within ±15% (RSD) (±20% at the LLOQ).[42]

2.2.5.4 Recovery and matrix effects

Recovery (RE) is the yield of a preconcentration or extraction stage of an analytical process for an analyte divided by amount of analyte in the original sample.[43] Matrix effects (ME) are the combined effects of all components of the sample other than the analyte of interest. RE and ME were evaluated by an approach based on responses from pre-extraction spike matrix (a), post-extraction spike matrix (b) and a neat spike standard (c). RE and ME were calculated using equations 2.3 and 2.4.[44] A positive ME implies ion enhancement and a negative ME signifies ion suppression.[45]

$$\mathbf{RE} = \frac{a}{b} * 100 \tag{2.3}$$

$$\mathbf{ME} = \left[\left(\frac{b}{c}\right) - 1\right] * 100 \tag{2.4}$$

2.2.6 Statistical analysis

For the determination of cyanide by UV-Vis and cyanogenic glycosides by LC-MS/MS, samples were prepared in three biological and three analytical replicates for each sample. Statistical analyses were performed in Excel (Microsoft Office 2016). Results are expressed as the mean ± standard deviation (SD, n=3). Also, the coefficient of variation for six (6) repeated injections (CV% ≤ 20%) was used to confirm candidate concentrations for LLOQ and LLOD.

2.3 Results and Discussion

2.3.1 Picrate paper analysis of cyanide

2.3.1.1 Standard calibration

The observed picrate color change for amygdalin standards improved significantly and became consistent with cyanide amounts only when the adapted method[20] was modified by using minimal water (<0.5 mL water). This was to enhance HCN reaction with picric acid since HCN is highly water-soluble.[6] A standard calibration curve showing the amount of CN^- eq. with its corresponding absorbance is shown in Figure 2.4, using amygdalin as the cyanide source. Also shown in Appendix 2 are Figure A2.1 which shows the corresponding standard curve using a camera-phone as a detector and Figure A2.2 which shows the expanded UV-Vis data from 0 to 10 µg/g. Table 2.1 summarizes the LLOD, LLOQ, ULOQ and the Pearson coefficients (R^2-values) for camera phone and UV-Vis analysis. UV-Vis showed better linearity compared to camera phone method in repeated analysis.

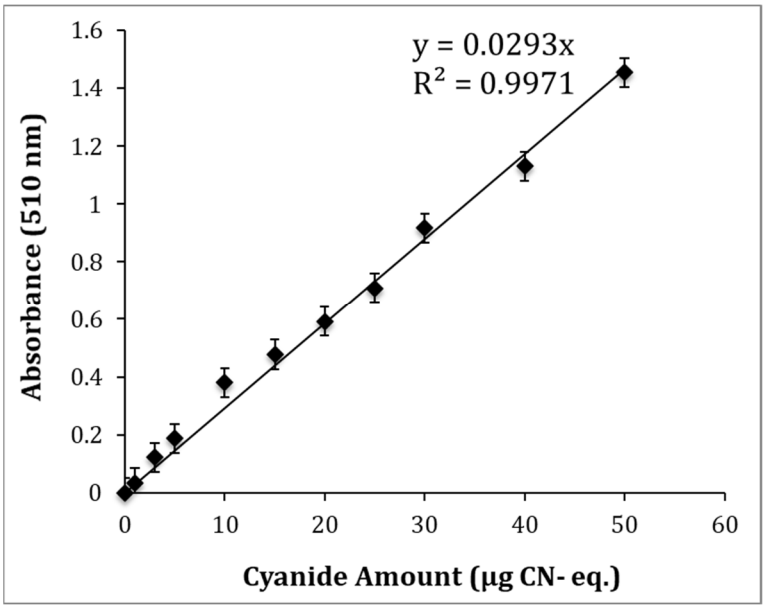

Figure 2.4. Calibration curve for the amount of CN^- eq. produced by reaction of picric acid with HCN using amygdalin as a CNG standard measured by UV-Vis spectrophotometry at λ_{max} = 510 nm (standard deviation for three replicate samples, n=3)

Table 2.1. LLOD, LLOQ, ULOQ, Pearson coefficients (R^2-values) for calibration curves from camera-phone and UV-Vis analysis

Methods/µg CN^-eq.	ULOQ	LLOQ	LLOD	R^2
UV-Vis (A)	50	0.14	0.047	0.9971
Camera phone (B)	50	1.59	0.530	0.9889

The LLOQ and LLOD for both methods were determined using equations 2.5 and 2.6 below. Where "Sy" (Equation 2.5) represents the standard deviation of the blank for three replicates analysis and for Equation 2.6 represents the standard deviation of the ordinate intercept. "m" in both cases represents the slope from the regression of the calibration curve.

$$\mathbf{LLOQ} = \frac{10 \text{ x Sy}}{\text{m}} \tag{2.5}$$

$$\mathbf{LLOD} = \frac{3 \text{ x Sy}}{\text{m}} \tag{2.6}$$

2.3.1.2 Control test

Color change on the picrate paper test strip for the control apple seeds occurred swiftly at room temperatures even before sample set-ups were transferred into the oven for overnight heating (30-40°C). A deep red color change was observed on inspection (as shown in Appendix 2 as Figure A2.3) for both raw and lyophilized seeds, juice (core) and juice (flesh) for Granny Smith (GS) and Gala (G) apple varieties. Analysis via UV-Vis at a wavelength of 510 nm showed high average cyanide concentrations (TCP) ranging from (497.50 ± 19.60 to 603.20 ± 28.41 µg CN^- eq.)/g of apple seeds as shown in Figure 2.5. TCP levels in analyzed seeds were higher in Granny Smith as compared to Gala apple varieties. These results were comparable to available literature.[9,46] The mean levels

of cyanide determined in the juice were very low, slightly higher in core compared to the flesh.

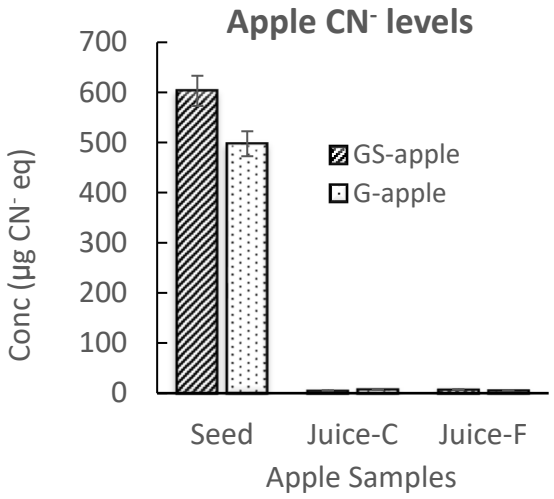

Figure 2.5**.** Picrate Paper results for apple seeds and juice. The error bars represent the standard deviation of at least three replicate samples (n=3).

Results from the endogenous enzymes test made using composite AE stems and green berry revealed higher cyanide levels in samples with added β-glucosidase than those without added β-glucosidase (as shown in Figure 2.6 and in Appendix 2 as Figure A2.4). Approximately 43.4% and 24.7% more cyanide were determined in composite AE stems and whole green berries respectively, with added β-glucosidase. These findings indicated that, AE contains appreciable amounts of endogenous β-glucosidase enzymes to initiate self-hydrolysis (56.6 and 75.3% for stems and green berries, respectively) of CNG's. This may not be sufficient for complete hydrolysis of all CNG's when the tissues are disrupted. This also implied that not all available CNG's in elderberry may necessarily translate into HCN. Deductions based on these observations are supported in a similar analysis by Miller et al.[47] using *B. collina foliage* and *Mischocarpus spp* assay. Apple seeds however showed no appreciable change in cyanide concentration with or

without addition of β-glucosidase enzymes (as shown in Figure 2.7 and Appendix 2 as Figure A2.5). It can be suggested that the seeds of the apple varieties used contain sufficient endogenous beta glucosidase for complete hydrolysis of all CNG's when the tissues are disrupted. The picrate paper test method is quick and simple and can serve as an effective field test for elderberry producers.

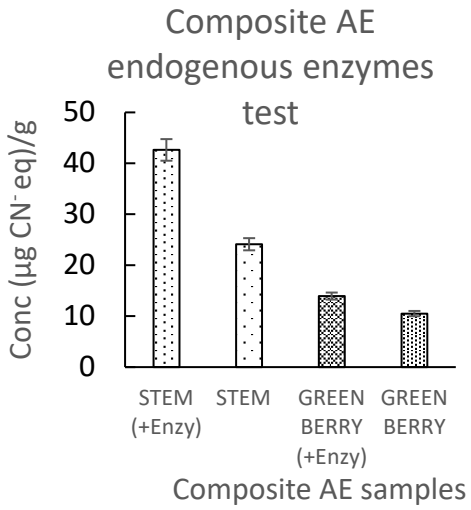

Figure 2.6. Picrate paper results for endogenous enzymes test using composite elderberry samples. The error bars represent the standard deviation of at least three replicate samples (n=3).

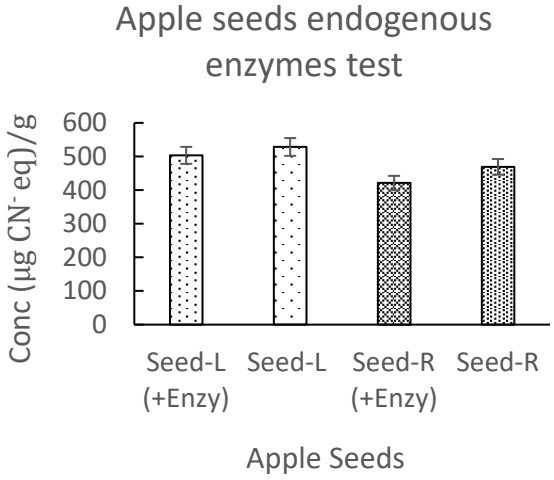

Figure 2.7**.** Picrate paper results for endogenous enzymes test using apples seeds. The error bars represent the standard deviation of at least three replicate samples (n=3).

2.3.1.3 Cyanide analysis in elderberry

Qualitative inspection of the picrate paper strips showed no visible color change for commercial elderberry juice sample. UV-Vis analysis of the picrate paper test stripes showed no detectable trace of cyanide (Concentration < LLOD: 0.047 µg CN^- eq.) as shown in Appendix 2 as Figure A2.6. All sample tissues (juice, skin, stem, seeds) of Ozone and Ozark AE also showed no visible color change on qualitative assessment. Generally, results obtained for lyophilized samples were comparable to raw samples. Quantitative determination by UV-Vis at 510 nm (λ_{max}) however revealed very low traces of cyanide detection with average concentrations ranging from (2.60 ± 0.25 - 9.20 ±0.30 µg CN^- eq.) / g of sample. TCP levels obtained were comparable for both genotypes and ranges between (2.60 ± 0.25 - 9.20 ± 0.30) and (3.05 ± 0.20 - 8.20 ± 0.21) µg CN^- eq. / g for all tissues of Ozone and Ozark samples, respectively. TCP concentration increased in the order juice < seeds < skin < stem for both genotypes as shown in Figure 2.8.

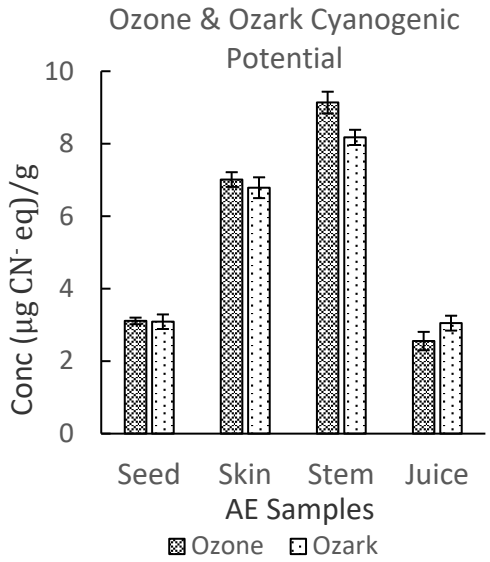

Figure 2.8**.** Total cyanogenic potential for different types of tissue of Ozone and Ozark AE genotypes using UV-Vis spectrophotometry. The error bars represent the standard deviation of at least three replicate samples (n=3).

Qualitative inspection of picrate paper test strip for composite AE tissues (seeds, skin, pulp, stem, juice and whole green, red and ripe berries) showed a faintly visible color change for composite green berries and stem tissues (as shown in Appendix 2 as Figure A2.7). UV-Vis analysis showed average concentrations of sample tissues ranging between (8.03 ± 0.41-37.43 ±1.87 µg CN^- eq.) / g of sample (as shown in Figure 2.9). TCP levels in analyzed tissues increased in the order: whole ripe berries < whole red berries < juice < seeds < skin < pulp < whole green berries < stem, with highest average concentrations in the stems (37.43 ± 1.87 µg CN^- eq. / g) and whole green berries (25.60 ± 1.28 µg CN^- eq. / g). Koss-Mikolajczyk et al.[49] in a recent study observed a weak and unstable signal for a peak corresponding to sambunigrin, which happened to decrease with the stage of ripeness in elderberry fruit. Analysis by Zahmanov et al.[48] also reported metabolic differences in mature and immature fruits, and plant leaves of *Sambucus ebulus*. Moreover, immature fruits (green berries) and leaves were shown to have similar metabolomes, which apparently undergoes significant changes during the fruit ripening stage. These observations may have accounted for the slightly higher levels recorded in green berries. Although concentration levels in the stems and green berries are not significant enough to pose any threat, a cumulative dose especially in larger quantities may be problematic. It is therefore advisable to carefully exclude green elder berries and stems during juice preparation.

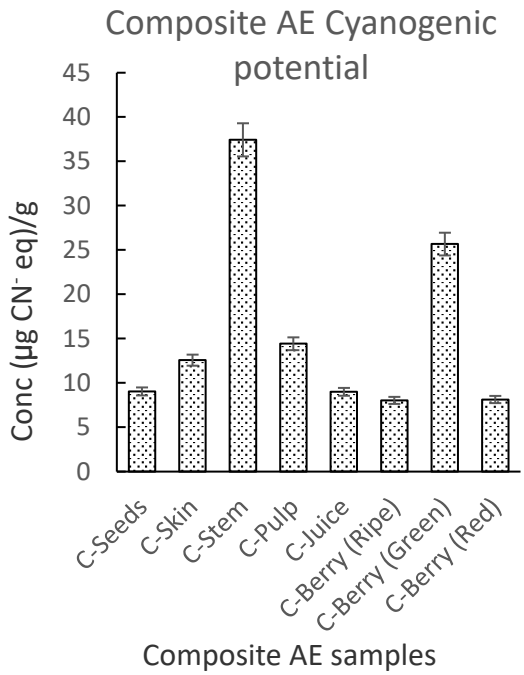

Figure 2.9**.** Total cyanogenic potential for different types of AE tissue of pooled samples made up of five different genotypes using UV-Vis spectrophotometry. The error bars represent the standard deviation of at least three replicate samples (n=3).

2.3.2 UHPLC-MS/MS analysis cyanogenic glycosides

2.3.2.1 Optimized extraction method

Selecting the most appropriate extraction solvent was key to development of the extraction methodology. Recoveries from aqueous ethanol or methanol combinations were evaluated. Higher recoveries were obtained with 75% methanol extraction with overnight shaking (16-24 hrs) at room temperature and 30 mins sonication at 30ºC as compared to other extraction methods and conditions. Table 2.2 below compares the mean recoveries and standard deviations for 30 mins sonication at 30°C to overnight shaking (16-24 hrs) at room temperature with intended spike concentration of 1,000 ng/mL and 100 ng/mL CNS mixture. Figure 2.10 also compares recoveries for 2 mins vortexing at room temperature to 30 mins sonication at 30°C.

Table 2.2. Mean recoveries and standard deviations for intended spike concentration of 100 and 1000 ng/mL

CNG standards/ mean recovery%	Conc. (ng/mL)	MRM		SIR							
		Amygdalin		Amygdalin		Dhurrin		Prunasin		Linamarin	
		Mean RE%	SD%	Mean RE%	SD%	Mean RE%	SD %	Mean RE%	SD %	Mean RE%	SD %
Sonication	1000	85.40	1.01	86.14	1.61	95.34	2.1	91.35	0.95	95.26	1.98
(30 min at 30°C)	100	91.43	0.92	92.14	3.11	88.54	2.04	79.14	5.1	92.1	0.96
Overnight shaking	1000	93.69	0.31	91.40	0.98	98.19	0.73	106.98	0.86	112.21	0.62
(16-24 hrs)	100	81.70	3.61	101.54	2.98	109.99	2.75	87.94	1.85	98.11	2.13

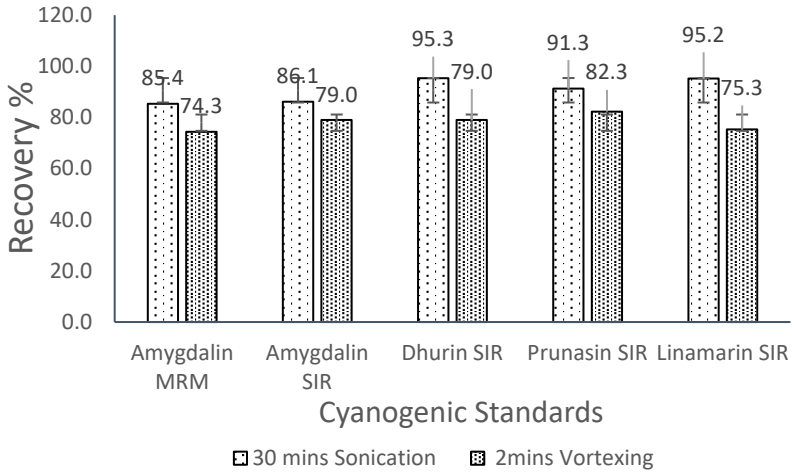

Figure 2.10. Recoveries for 75% MeOH (sonication vs vortexing) The error bars represent the standard deviation of at least three replicate samples (n=3).

2.3.2.2 Optimized SPE method

To assess and evaluate the elution solvent strength in the SPE method, an elution profile showing methanol and water proportions from 0:100 to 100:0 (v/v) versus peak area was made for all four standards. Figure 2.11 shows the elution profile for amygdalin for 2 µg/mL spike (sonication) and 1 µg/mL spike (overnight shaking). Evaluation of these profile diagrams revealed 30-40% methanol as the best solvent strength for elution

of all four CNS. Confirmation using water methanol proportions from 0:40 to 40:0 (v/v) was made to conclusively determine the elution solvent as 35% methanol.

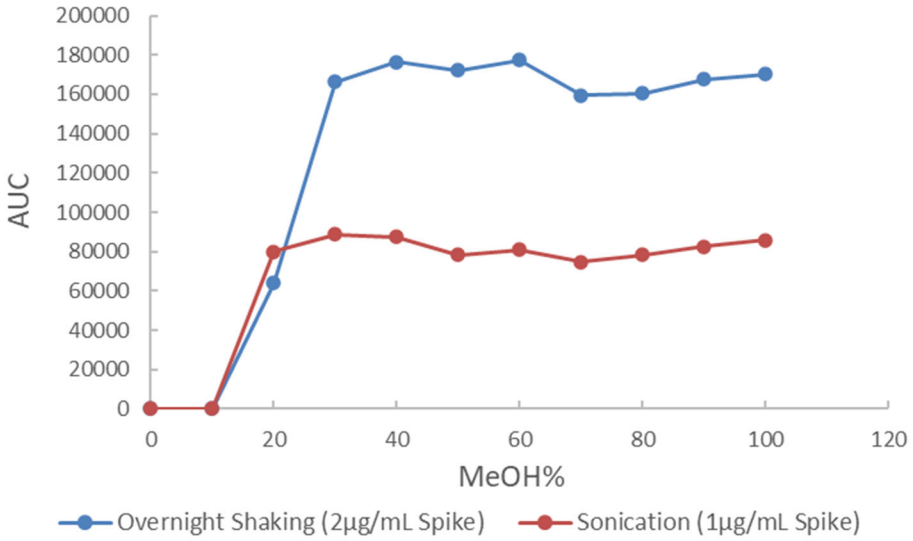

Figure 2.11. Elution profile for amygdalin as a function of methanol content in the extraction solvent. (AUC is the area under the curve).

2.3.2.3 Intellistart® optimization

Stock solutions (1 µM) for four cyanogenic standards (CNS); amygdalin (≥ 99%), dhurrin (≥ 95%), linamarin (≥ 98%), and prunasin (≥ 90%) were prepared for each standard in HPLC grade water (0.1% formic acid). An attempt was made to find multiple reaction monitoring (MRM) transitions for the four CNS's using both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources for both positive and negative modes. Direct infusion of stock standards into the Xevo TQ-S mass spectrometer at flow rates of 5 µL/min was made and IntelliStart software was used to generate the optimal cone voltages and collision energies that produce daughter ions with the highest intensity. Intellistart was only successful for amygdalin in ESI positive mode

with a parent-daughter ion transition of m/z = 458.2 → 296.1 and m/z = 458.2 → 158.1 as part of a multiple reaction monitoring protocol for quantification (as shown in Figure 2.12).

IntelliStart found the following compounds:

Compound	Formula/Mass		Parent m/z	Cone Voltage	Daughters	Collision Energy	Ion Mode
Amygdalin	457.1	1	458.20	46	296.12	17	ES+
		2	458.20	46	158.05	29	ES+

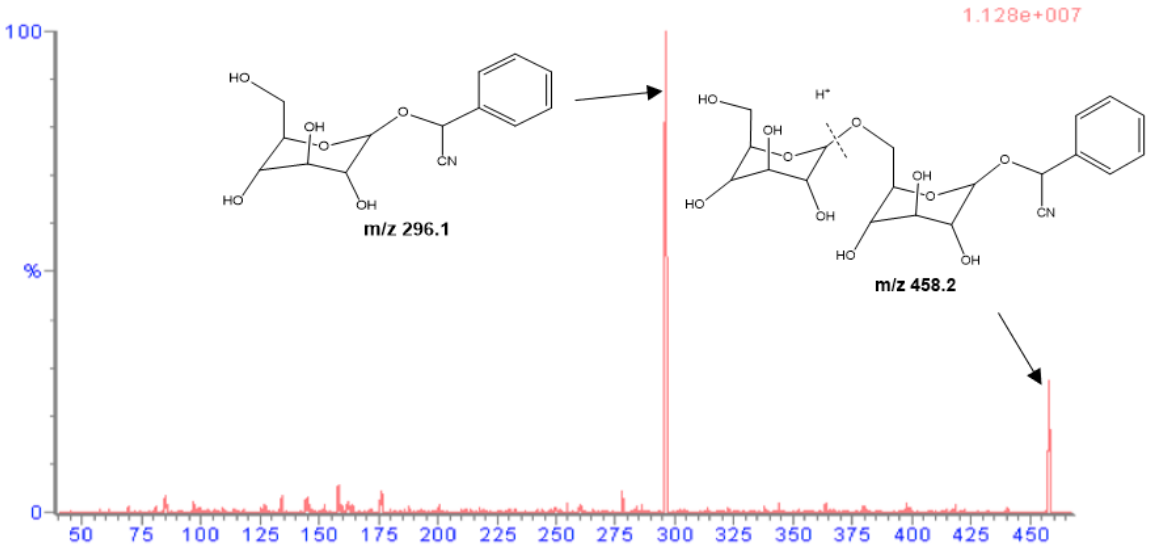

Figure 2.12**.** Product ion mass spectrum for amygdalin (MRM mode).

Unfortunately, Intellistart was unable to identify parent-daughter ion transitions for dhurrin, prunasin and linamarin compounds using the $[M + H]^+$ or $[M – H]^-$ parent ions. After further analysis, it was discovered that these three compounds exist primarily as sodium adducts, resulting in an increase of m/z = 23 and formed dimers (as shown in Table 2.3 and Figure 2.13 and 2.14). IntelliStart optimization was attempted using $[M + Na]^+$ as the parent ion for dhurrin, linamarin and prunasin, however, these adducts are very stable and the energy applied in the collision cell is not sufficient to cause ample

fragmentation. Daughter ions were found, but their intensities were very low (roughly 2% relative to the parent), which is unsuitable for proper MRM quantitation. Atmospheric pressure chemical ionization (APCI) is an alternative to (ESI) that was used to create the ions in this experiment. APCI also failed to produce parent ions that are more suitable for fragmentation.

Table 2.3 Observed mass to charge (m/z) peaks observed for direct infusion of cyanogenic standards.

Compound	Monoisotopic mass (amu)	Observed Peaks (m/z)
Amygdalin	457.2	458.2
Dhurrin	311.1	333.9, 360.0
Linamarin	247.1	269.9, 516.9, 248.1
Prunasin	295.1	318.1

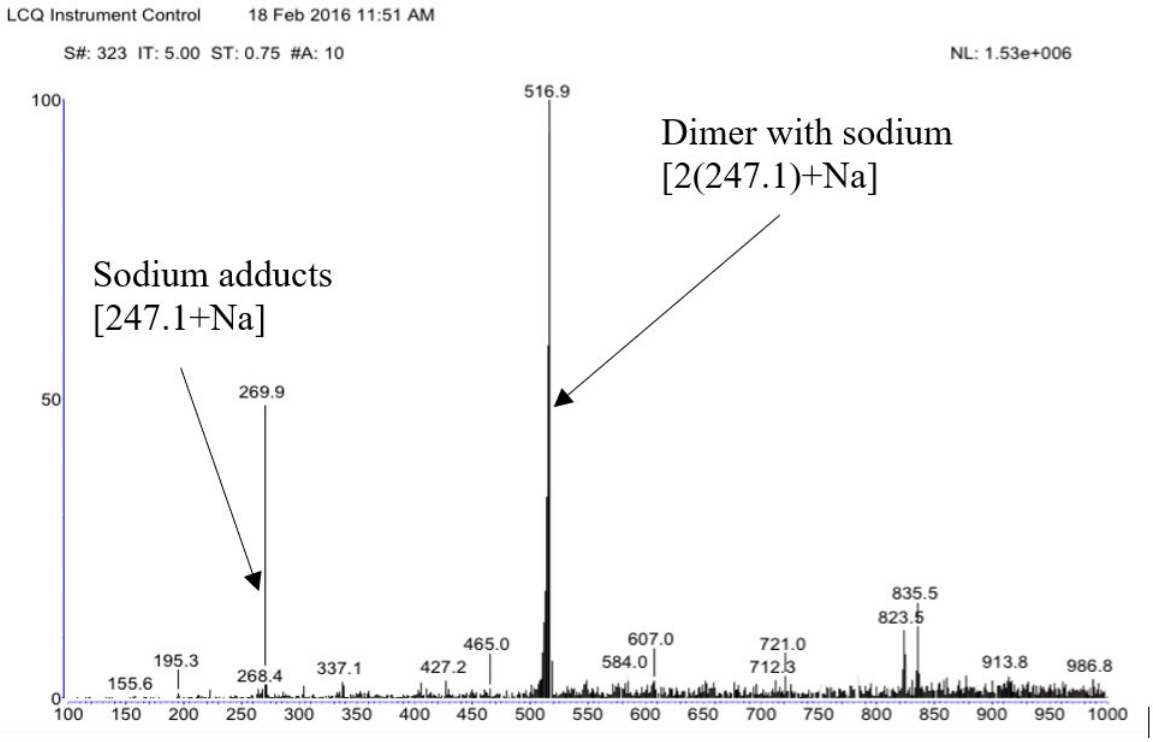

Figure 2.13**.** Parent ion mass spectrum for linamarin as dimers and sodium adduct.

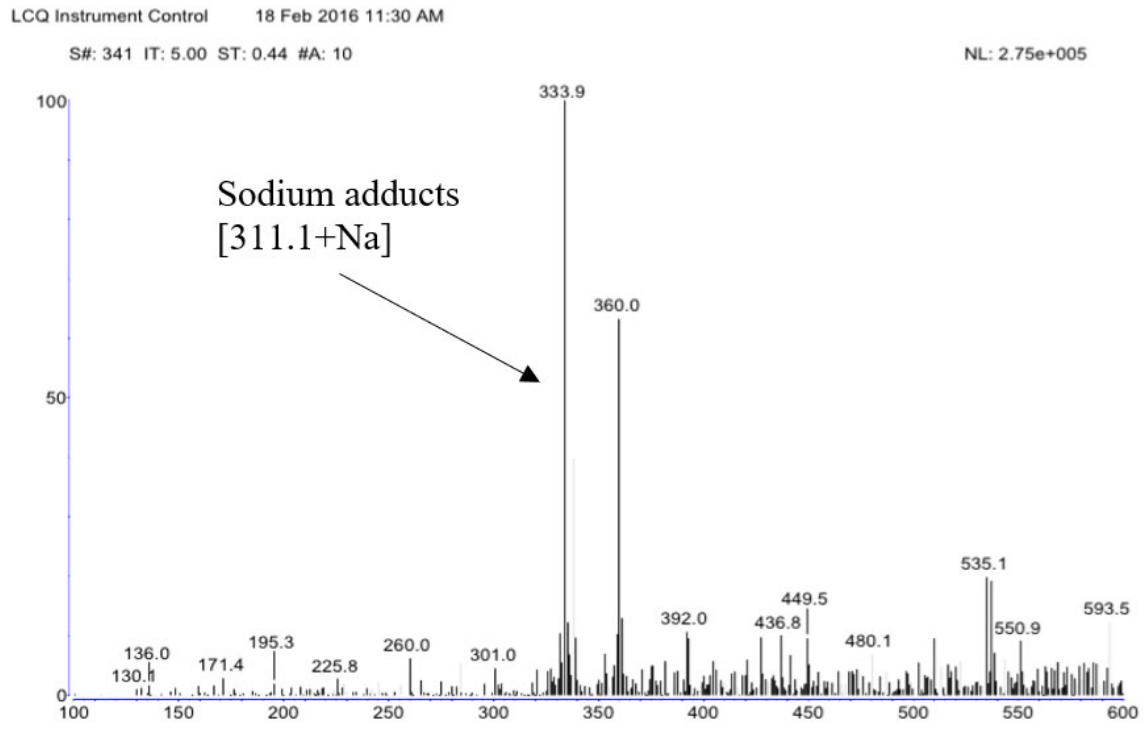

Figure 2.14**.** Parent ion mass spectrum for dhurrin as sodium adduct.

This problem can be overcome by investigating alternate mass spectrometry scans. Quantification can also be performed using selected ion recording (SIR), which only allows particles of a specific mass-to-charge ratio through the third quadrupole mass analyzer. SIR mode, like MRM is also very sensitive but it is not as selective as MRM. This is because only one ion is being monitored, instead of a parent and daughter ion pair. However, given the fact the cyanogenic glycosides do not form typical $[M+H]^+$ or $[M-H]^-$ parent ions, we feel this is the best option for quantitative analysis moving forward. Thus, Intellistart was used to optimize cone voltages for $[M+Na]^+$ ions for all four standards.

2.3.2.4 UPLC gradient performance

The developed UHPLC and MS method displayed excellent separation of the four standards and exhibited retention time repeatability and good peak shape. A chromatogram for the separation with retention times (RT) and scanning modes are shown in Figure 2.15. Although the run time was 10 mins, it took less than 6 mins to separate and elute all 4 CNGs.

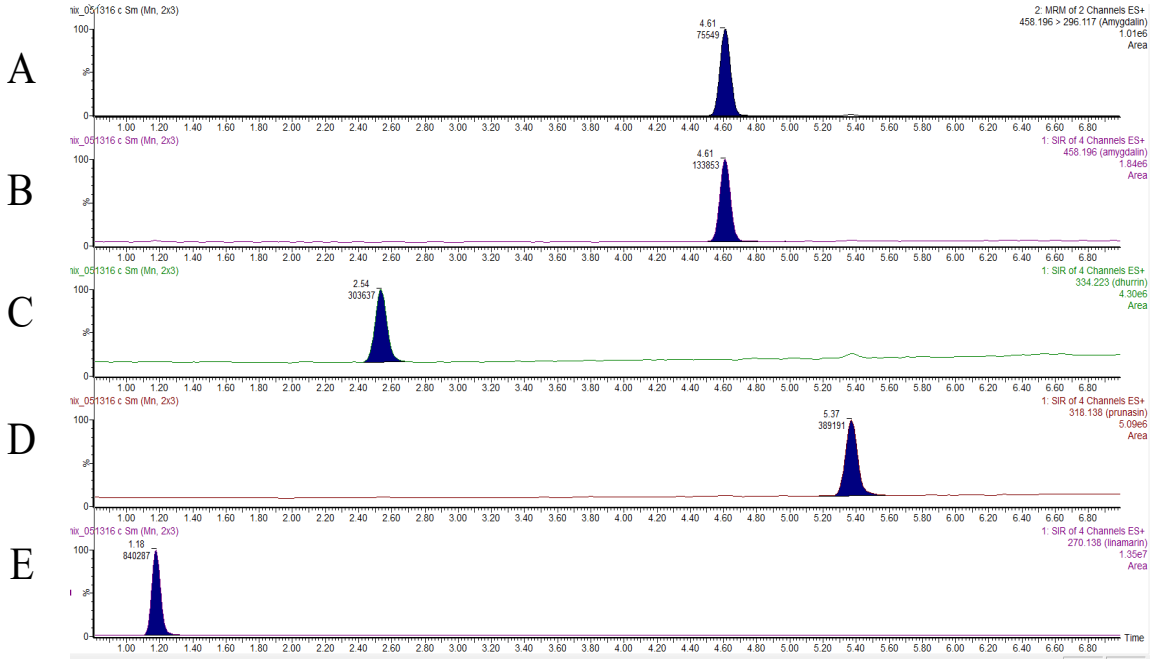

Figure 2.15**.** Chromatograms for A) amygdalin (MRM), B) amygdalin, C) dhurrin, D) prunasin and E) linamarin (SIR) RT: 4.61; 4.61; 2.54; 5.37 and 1.18 min respectively.

2.3.2.5 Method validation

The performance of the analytical method was verified by performing a method validation. This included tests for lower limit of detection (LLOD), lower limit of

quantification (LLOQ), linearity, linear range with the upper limit of quantification (ULOQ), precision, recovery, and matrix effect. Values for these parameters are listed in Table 2.2 and 2.4. The dynamic working range was identified for each standard and excellent linearity was also observed for all four standards. The calibration curve showing the linear range and linearity for amygdalin standard for both MRM and SIR method is shown in Figure 2.16. Results from the determination of LLOD and LLOQ showed comparable results for both the experimental dilution approach (using the S/N, candidate concentration and targeted coefficient of variation) and the theoretical approach based on the blank and low concentration. The former appeared more consistent as detailed in Table 2.4. Evaluation of recoveries (RE) and matrix effect (ME) based on responses from pre-extraction spike matrix (a), post-extraction spike matrix (b) and a neat spike standard (c) using equations 2 and 3. Mean recoveries were very high and range from 79-110% for all cyanogenic standards and for both sonication and overnight-shaking extraction methods (as shown in Table 2.2). ME estimation was found to range between 10.20-18.34% for all cyanogenic standards in raw juice. This was a negative estimation and as such indicated some degree of ion suppression. Although further dilution of sample matrix from 10 to 1000-fold reduced this value significantly, it also decreased the sensitivity of sample detection. Hence a 10-fold dilution was maintained. Reproducibility/precision calculated using data for all standard replicates yielding reasonable counts were well within the threshold of 15% CV for each matrix within batches. The coefficient of variation for all standards was within $3\% < CV < 6\%$.

Table 2.4. Summary of Pearson coefficient, detection and quantification limits (ng/mL) for CNGs

Parameters ng/mL	MRM Amygdalin	SIR			
		Amygdalin	Dhurrin	Prunasin	Linamarin
LLOD	0.3	3	3	3	1
LLOQ	1	10	10	5	5
ULOQ	8000	8000	6000	6000	2000
R^2	0.9998	0.9998	0.9983	0.9984	0.9910
Reproducibility	CV=3.09%	CV=3.50%	CV=3.09%	CV=5.80%	CV=3.54%

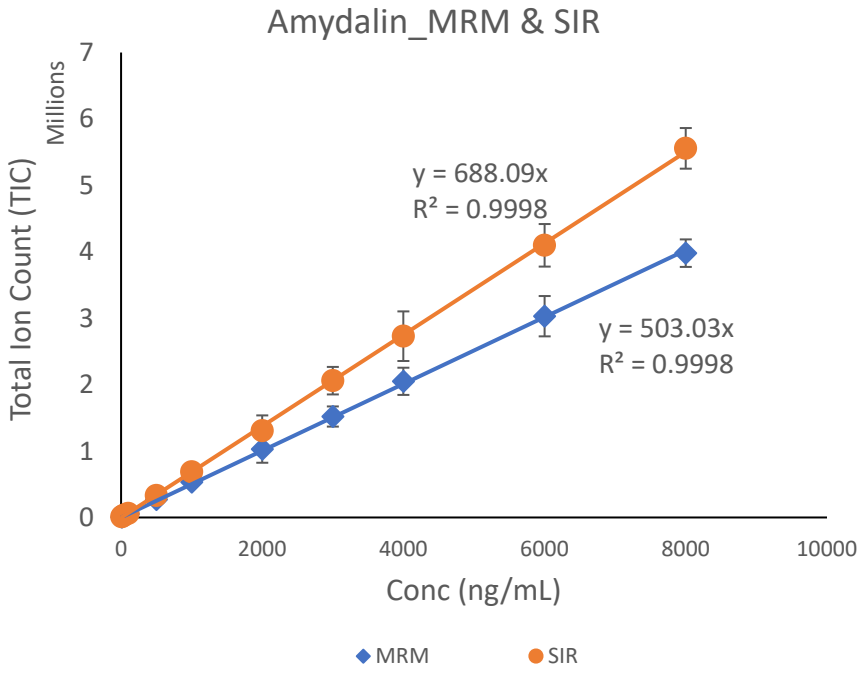

Figure 2.16**.** Calibration curve for amygdalin for MRM and SIR quantification method. The error bars represent the standard deviation of at least three replicate samples (n).

2.3.2.6 Cyanogenic glycosides analysis in elderberry

The developed UHPLC-MS/MS method was used to determine the levels of intact CNGs in different AE samples. Analysis of commercial elderberry juice showed no detectable trace of CNGs. However, extracts of Ozark and Ozone elderberry tissues

(seeds, juice, skin and stem) for both lyophilized and raw pretreated samples showed low traces of CNGs (amygdalin, dhurrin, linamarin and prunasin). Levels of CNGs detected in lyophilized samples were comparable to raw samples. Concentration levels (μg/g) in tissues were generally higher in Ozone (0.12-6.38) compared to Ozark (0.13-3.48). Higher concentration (μg/g) levels were recorded in the stems (0.48-5.42; 0.57-3.07) and skin (0.12-6.38; 0.90-3.47) tissues as compared to levels in the seeds (0.12-2.38; 0.13-0.68) and juice (0.29-1.57; 0.31-2.36), respectively, for Ozone and Ozark sample tissues. A detailed summary of concentration levels for each detected CNGs in AE tissues is shown in Table 2.5. Figure 2.17 and 2.18 shows the concentrations of these cyanogens in tissues for Ozone and Ozark respectively. Concentration levels (μg/g) of CNGs in analyzed tissues increased in the order: linamarin (0.12-0.75; 0.13-0.90) < dhurrin (0.12-0.94; 0.22-1.91) < prunasin (0.58-2.84; 0.36-3.07) < amygdalin (1.57-6.38; 0.36-3.48), respectively, for Ozone and Ozark samples tissues. In contrast to this trend, prunasin levels were highest in the juice and stems of Ozark AE.

Table 2.5. Mean recoveries and standard deviations for spike concentrations of 100 and 1000 ng/mL

Elderberry samples		**Concentration ± standard deviation (μg/g)**			
		Amygdalin	**Dhurrin**	**Prunasin**	**Linamarin**
Seeds	Ozone	2.38 ± 0.09	0.27 ± 0.05	0.58 ± 0.04	0.12 ± 0.06
	Ozark	0.68 ± 0.12	0.22 ± 0.03	0.36 ± 0.05	0.13 ± 0.05
Juice	Ozone	1.57 ± 0.08	0.70 ± 0.12	1.45 ± 0.06	0.29 ± 0.03
	Ozark	0.36 ± 0.03	0.63 ± 0.04	2.36 ± 0.08	0.31 ± 0.01
Skin	Ozone	6.38 ± 0.40	0.12 ± 0.08	2.39 ± 0.04	0.75 ± 0.06
	Ozark	3.48 ± 0.14	1.46 ± 0.20	2.53 ± 0.08	0.90 ± 0.11
Stem	Ozone	5.42 ± 0.12	0.94 ± 0.06	2.84 ± 0.02	0.48 ± 0.04
	Ozark	2.15 ± 0.17	1.91 ± 0.03	3.07 ± 0.06	0.57 ± 0.06

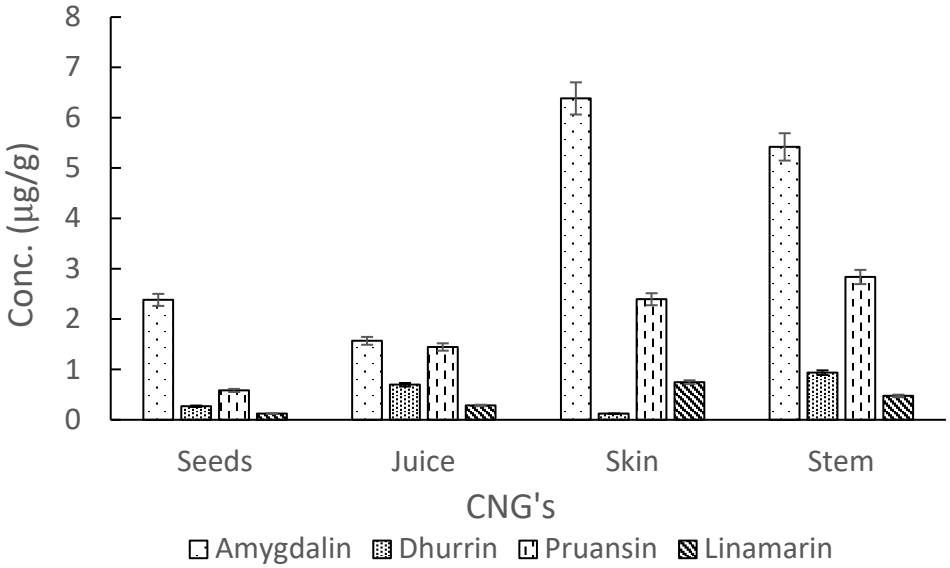

Figure 2.17. Concentrations of CNGs (µg/g) in tissues (seeds, juice, skin and stem) of Ozone elderberry samples as measured by UHPLC-MS/MS. The error bars represent the standard deviation of at least three replicate samples (n=3).

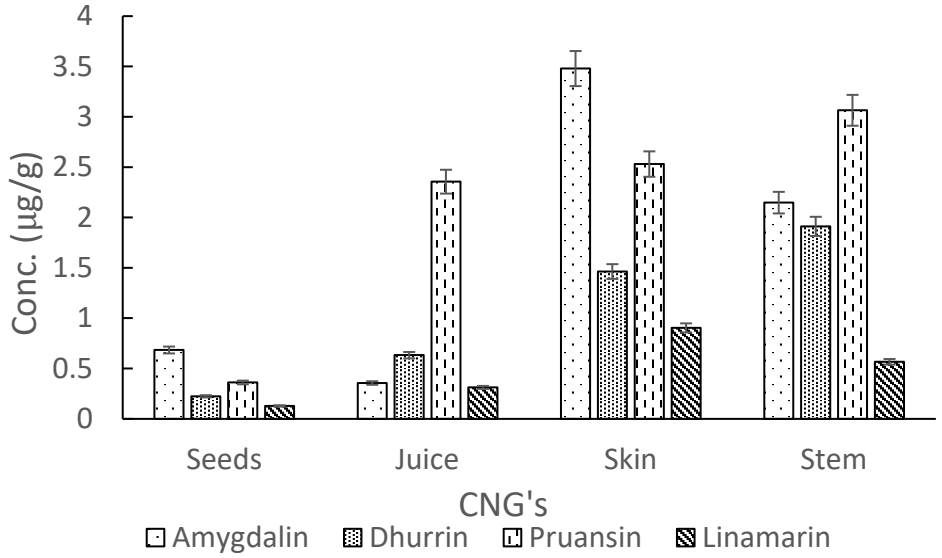

Figure 2.18. Concentrations of CNGs (µg/g) in tissues (seeds, juice, skin and stem) of Ozark elderberry samples as measured by UHPLC-MS/MS. The error bars represent the standard deviation of at least three replicate samples (n=3).

The trend of results from this study is comparable to results obtained from European elderberry (EE) studies. Senica et al.[2] studies on EE (subsp. *nigra*) reported average levels of sambunigrin (μg/g) in fresh berries (18.8 ± 4.3), processed juice (10.6 ± 0.7), tea (3.8 ± 1.7), spread (0.8 ± 0.19) and liqueur (0.8 ± 0.21). In another study, Senica et al.[16] reported the highest amounts of sambunigrin in elder leaves (27.68–209.61 μg/g FW), lower amounts in flowers (1.23–18.88 μg/g FW) and lowest amounts in berries (0.08–0.77 μg/g FW). They added that the content of sambunigrin in elderberry changes depending on the growing altitudes (high on hill tops and low at the foothill). Another study by Koss-Mikolajczyk et al.[49] on EE (subsp. *nigra*) observed the highest signal for a peak detected as sambunigrin in the elder leaves although this peak became undetectable after one day of storage of extracts in the fridge. It was also reported that the level of cyanogens in cassava leaves are 10 times more than in the roots.[3] Deductions from this trend of results suggest that the leaves of most cyanogenic plants accumulate large amount of CNGs to which elder leaves are no exception. The trend of results also corroborates the fact that elderberry juice, being it AE or EE showed very low levels of CNGs.

2.3.3 Cyanide toxicity

In humans, consumption of cyanogenic plants can cause sub-acute or acute cyanide poisoning depending on the dose. Depending on the body weight (BW), acute cyanide toxicity can occur in humans at doses between 0.5 and 3.5 mg/ kg BW.[50] This means the extent of cyanide toxicity can vary from person to person depending on weight and the ingested amount. For cyanide in whole blood, the toxicity threshold for cyanide alone ranges from 0.5 to 1.0 mg/L, and the lethal threshold ranges from 2.5 to 3.0 mg/L.[51,52]

Acute cyanide poisoning has been reported from the ingestion of apricots (intoxication may occur from eating 5 - 25 apricot seed)[52,53,54], almonds (6.2 mg per each bitter almond) [55,56], cassava (15-400 mg/kg fresh weight of hydrogen cyanide in cassava roots).[57] A typical case of acute cyanide poisoning in bitter almonds was recorded by Mouaffak et al.[56] of a 67-year-old woman weighing 60 kg (132 lb) and diagnosed as having carcinoma of the large bowel after consuming a slurry of water with 12 bitter almonds the night of admission. The average cyanide content was 6.2 mg per each bitter almond. Considering the minimum lethal dose of cyanide of 0.5 mg/kg BW and the weight of the woman (60 kg), her calculated lethal dose will be 30 mg of cyanide. She however, ingested at least 70-75 mg of cyanide beyond the calculated lethal dose resulting in the cyanide poisoning. Fortunately, the patient rapidly and dramatically responded to treatment. Another case of cyanide poisoning with apricots was reported by Sahin et al.[52] for a 28 month old girl presented to the emergency department with sudden onset of unconsciousness and seizures following consumption of approximately ten apricot seeds. The result of her whole blood cyanide level was more than 3 mg/L at the 20th hour of presentation. She unfortunately died on the 22nd day of hospitalization following supportive care in the intensive care unit.

High levels of cyanogenic glycosides (1000- 4000 µg/g) are reported in apple seeds (for 15 apple varieties)[9,19] and further confirmed in this study, with high cyanide levels from a control picrate test (497.50 -603.20 µg/g). However, the possibility of cyanide toxicity may occur for an average adult male of weight 82 kg, only after consuming about 14 or more apples along with chewing all seeds. This estimation was made considering the threshold value of 0.50 mg/kg BW for cyanide toxicity, average

toxicity per seed (550 μg/g or 0.55mg/g), the average weight of an apple seed (0.75 g), the average number of seeds per apple (7 or 8) and assuming maximum enzyme activity.[58] Although high levels of cyanide or CNGs are present in apple seeds, an estimated high number of apples are needed to cause any kind of toxicity at threshold lethal levels. Comparing the levels of cyanide or CNGs in apple seeds to much lower levels in fresh (10-40 μg/g) and processed (1-7 μg/g) apple juices, which are about 10x higher than levels recorded in elderberry juice (0.29-2.36 μg/g), and shows American elderberry is safe and poses no threat to consumers.

2.3.4 Processing techniques for elderberry juice

Different processing techniques like chopping, grinding, soaking, fermentation, drying, roasting, boiling, and steaming have been used to remove or reduce the potential toxicity of cyanogens in plants.[2,3,4] The effectiveness of these processes are however dependent on the specific processing method[2], the plant tissues and the intended processed forms. Boiling of juice for instance may have a different effect from boiling or soaking cassava chips where boiling or soaking water can easily be discarded.[2,3,5] This may be due to enzyme inactivation and solubilization of CNGs in discarded water.[3] A study done by Montagnac et al.. further indicated that the effectiveness of these techniques depends on the processing steps, the sequence utilized, and often time dependent. They proposed that to increase the efficiency of cyanogen removal, efficient processing techniques should be combined with others.[3] For example, soaking, fermenting and roasting removes about 98% of cyanogens.[39] Recent study by Senica et al.[2] also showed that thermal processing, time and type of extraction solution greatly affected phenolics and cyanogenic glycosides in different elderberry products. This study applied higher temperatures to decrease the levels

of harmful cyanogenic glycosides by 44% in elderberry juice, 80% in tea and as much as 96% in elderberry liqueur and spread.[2] Heat treatment significantly diminished the levels of cyanogenic glycosides in elderberry liqueur and spread. It has been confirmed that pasteurization effectively decreases the levels of harmful compounds, such as cyanogenic glycosides.[2] Islamiyat et. al[9] observed that holding the apple juice at room temperature for 120 min either before or after pasteurizing decreased the amygdalin content by about 19% compared to the original juice. These methods are very effective and can be applied to remove or further reduce the levels of CNGs in elderberry tissues. It is however important to establish that the types and levels of CNGs observed in AE are very low and pose no threat to consumers in the use of raw or processed AE products.

2.4 Conclusions

The UHPLC-MS/MS and picrate paper methods developed were used to reliably determine the intact CNGs and assess the TCP in various AE fruit tissue. No quantifiable trace of cyanide or CNG was detected in commercial elderberry juice. Moreover, traces of CNGs (amygdalin, dhurrin, prunasin, and linamarin) detected in tissues of AE samples were generally low with lower levels in the juice and seeds as compared to stems and skin. TCP assessed in both pure and pooled AE sample tissues were generally low with higher concentrations recorded in pooled stems and unripe (green) berries. The picrate paper method can also be used to help detect the presence of CNGs. A camera-phone and UV-Vis spectrophotometer can both be used as a detector. The camera-phone can give results easily with limits of detection that are useful for CNG analysis. Although the TCP and CNGs levels in tissues of AE pose no threat to consumers, it is advisable to separate out the stems, green berries and leaves[2] from AE ripe berries during product preparation.

References

1. Poisoning from Elderberry Juice -- California. Accessed October 19, 2020. https://www.cdc.gov/mmwr/preview/mmwrhtml/00000311.htm
2. Senica M, Stampar F, Veberic R, Mikulic-Petkovsek M. Processed elderberry (Sambucus nigra L.) products: A beneficial or harmful food alternative? *LWT - Food Sci Technol*. 2016;72:182-188. doi:10.1016/j.lwt.2016.04.056
3. Montagnac JA, Davis CR, Tanumihardjo SA. Processing techniques to reduce toxicity and antinutrients of Cassava for use as a staple food. *Compr Rev Food Sci Food Saf*. 2009;8(1):17-27. doi:10.1111/j.1541-4337.2008.00064.x
4. Gleadow RM, Møldrup ME, O'Donnell NH, Stuart PN. Drying and processing protocols affect the quantification of cyanogenic glucosides in forage sorghum. *J Sci Food Agric*. 2012;92(11):2234-2238. doi:10.1002/jsfa.5752
5. Akande KE, Fabiyi EF. Effect of processing methods on some antinutritional factors in legume seeds for poultry feeding. *Int J Poult Sci*. 2010;9(10):996-1001. doi:10.3923/ijps.2010.996.1001
6. Plant Toxin Analysis: Modern Methods Of Plant Analysis: Linskens H.F.: 9788184891102: Amazon.com: Books. Accessed July 21, 2020. https://www.amazon.com/Plant-Toxin-Analysis-Modern-Methods/dp/8184891105
7. Jeon IJ, Ikins WG. *Analyzing Food for Nutrition Labeling and Hazardous Contaminants*. CRC Press; 2020. doi:10.1201/9781003067351
8. Handbook of Natural Toxins - Google Livres. Accessed October 20, 2020. https://books.google.bi/books?id=DtUO6fgC4w4C
9. Bolarinwa IF, Orfila C, Morgan MRA. Determination of amygdalin in apple seeds, fresh apples and processed apple juices. *Food Chem*. 2015;170:437-442.

doi:10.1016/j.foodchem.2014.08.083

10. Bolarinwa IF, Orfila C, Morgan MRA. Amygdalin content of seeds, kernels and food products commercially- available in the UK. *Food Chem*. 2014;152:133-139. doi:10.1016/j.foodchem.2013.11.002

11. Ganjewala D, Kumar S, Devi S A, Ambika K. Advances in cyanogenic glycosides biosynthesis and analyses in plants: A review. *Acta Biol Szeged*. 2010;54(1):1-14.

12. Vetter J. Plant cyanogenic glycosides. *Toxicon*. 2000;38(1):11-36. doi:10.1016/S0041-0101(99)00128-2

13. Francisco IA, Pinotti MHP. Cyanogenic glycosides in plants. *Brazilian Arch Biol Technol*. 2000;43(5):487-492. doi:10.1590/S1516-89132000000500007

14. Burns AE, Bradbury JH, Cavagnaro TR, Gleadow RM. Total cyanide content of cassava food products in Australia. *J Food Compos Anal*. 2012;25(1):79-82. doi:10.1016/j.jfca.2011.06.005

15. Nhassico D, Muquingue H, Cliff J, Cumbana A, Bradbury JH. Rising African cassava production, diseases due to high cyanide intake and control measures. *J Sci Food Agric*. 2008;88(12):2043-2049. doi:10.1002/jsfa.3337

16. Senica M, Stampar F, Veberic R, Mikulic-Petkovsek M. Transition of phenolics and cyanogenic glycosides from apricot and cherry fruit kernels into liqueur. *Food Chem*. 2016;203:483-490. doi:10.1016/j.foodchem.2016.02.110

17. Chaouali N, Gana I, Dorra A, Khelifi F, Nouioui A, Masri W, Belwaer I, Ghorbel H, Hedhili A. Potential Toxic Levels of Cyanide in Almonds (Prunus amygdalus), Apricot Kernels (Prunus armeniaca), and Almond Syrup. *ISRN Toxicol*. 2013;2013:610648. doi:10.1155/2013/610648

18. Lee J, Zhang G, Wood E, Rogel Castillo C, Mitchell AE. Quantification of amygdalin in nonbitter, semibitter, and bitter almonds (prunus dulcis) by UHPLC-(ESI)QqQ MS/MS. *J Agric Food Chem*. 2013;61(32):7754-7759. doi:10.1021/jf402295u

19. Rezaul Haque M, Howard Bradbury J. Total cyanide determination of plants and foods using the picrate and acid hydrolysis methods. *Food Chem*. 2002;77(1):107-114. doi:10.1016/S0308-8146(01)00313-2

20. Bradbury MG, Egan S V., Bradbury JH. Picrate paper kits for determination of total cyanogens in cassava roots and all forms of cyanogens in cassava products. *J Sci Food Agric*. 1999;79(4):593-601. doi:10.1002/(SICI)1097-0010(19990315)79:4<593::AID-JSFA222>3.0.CO;2-2

21. Mcmahon JM, White WLB, Sayre RT. Review article: Cyanogenesis in cassava (manihot esculenta crantz). *J Exp Bot*. 1995;46(7):731-741. doi:10.1093/jxb/46.7.731

22. Yeoh HH, Truong V Den. Quantitative analysis of linamarin in cassava using a cassava β-glucosidase electrode. *Food Chem*. 1993;47(3):295-298. doi:10.1016/0308-8146(93)90164-B

23. Nambisan B, Sundaresan S. Distribution of linamarin and its metabolising enzymes in cassava tissues. *J Sci Food Agric*. 1994;66(4):503-507. doi:10.1002/jsfa.2740660413

24. Clegg DO, Conn EE, Janzen DH. Developmental fate of the cyanogenic glucoside linamarin in Costa Rican wild lima bean seeds [8]. *Nature*. 1979;278(5702):343-344. doi:10.1038/278343a0

25. Frehner M, Conn EE. The Linamarin β-Glucosidase in Costa Rican Wild Lima Beans (Phaseolus lunatus L.) Is Apoplastic . *Plant Physiol*. 1987;84(4):1296-1300. doi:10.1104/pp.84.4.1296

26. Saunders JA, Conn EE. Presence of the Cyanogenic Glucoside Dhurrin in Isolated Vacuoles from Sorghum. *Plant Physiol*. 1978;61(2):154-157. doi:10.1104/pp.61.2.154

27. Mazza G, Cottrell T. Carotenoids and cyanogenic glucosides in saskatoon berries (Amelanchier alnifolia Nutt.). *J Food Compos Anal*. 2008;21(3):249-254. doi:10.1016/j.jfca.2007.11.003

28. Senica M, Stampar F, Veberic R, Mikulic-Petkovsek M. The higher the better? Differences in phenolics and cyanogenic glycosides in Sambucus nigra leaves, flowers and berries from different altitudes. *J Sci Food Agric*. 2017;97(8):2623-2632. doi:10.1002/jsfa.8085

29. Senica M, Stampar F, Mikulic-Petkovsek M. Harmful (cyanogenic glycoside) and beneficial (phenolic) compounds in different Sambucus species. *J Berry Res*. 2019;9(3):395-409. doi:10.3233/JBR-180369

30. Buhrmester RA, Ebinger JE, Seigler DS. Sambunigrin and cyanogenic variability in populations of Sambucus canadensis L. (Caprifoliaceae). *Biochem Syst Ecol*. 2000;28(7):689-695. doi:10.1016/S0305-1978(99)00105-2

31. Thomas AL, Byers PL, Avery JD, Kaps M, Gu S. Horticultural performance of eight American elderberry genotypes at three Missouri locations. *Acta Hortic*. 2015;1061:237-244. doi:10.17660/ActaHortic.2015.1061.26

32. Thomas AL, Byers PL, Gu S, Avery JD, Kaps M, Datta A, Fernando L, Grossi P,

Rottinghaus GE. Occurrence of polyphenols, organic acids, and sugars among diverse elderberry genotypes grown in three Missouri (USA) locations. *Acta Hortic*. 2015;1061:147-154. doi:10.17660/ActaHortic.2015.1061.14

33. Drochioiu G, Pui A, Dănac R, Băsu C, Murariu M. Improved spectrophotometric assay of cyanide with picric acid. *Rev Roum Chim*. 2003;48(8):601-606.

34. Bergmeyer H. *Methods of Enzymatic Analysis*. 2d English ed. /. Verlag Chemie ;;Academic Press; 1974.

35. Abraham K, Buhrke T, Lampen A. Bioavailability of cyanide after consumption of a single meal of foods containing high levels of cyanogenic glycosides: a crossover study in humans. *Arch Toxicol*. 2016;90(3):559-574. doi:10.1007/s00204-015-1479-8

36. Sánchez-Pérez R, Howad W, Garcia-Mas J, Arús P, Martínez-Gómez P, Dicenta F. Molecular markers for kernel bitterness in almond. *Tree Genet Genomes*. 2010;6(2):237-245. doi:10.1007/s11295-009-0244-7

37. Brum L, Leal MG, De Toni Uchoa F, Kaiser M, Guterres SS, Dalla Costa T. Determination of quinine and doxycycline in rat plasma by LC-MS-MS: Application to a pharmacokinetic study. *Chromatographia*. 2011;73(11-12):1081-1088. doi:10.1007/s10337-011-1949-y

38. González O, Blanco ME, Iriarte G, Bartolomé L, Maguregui MI, Alonso RM. Bioanalytical chromatographic method validation according to current regulations, with a special focus on the non-well defined parameters limit of quantification, robustness and matrix effect. *J Chromatogr A*. 2014;1353:10-27. doi:10.1016/j.chroma.2014.03.077

39. Mani DR, Abbatiello SE, Carr SA. Statistical characterization of multiple-reaction monitoring mass spectrometry (MRM-MS) assays for quantitative proteomics. *BMC Bioinformatics*. 2012;13 Suppl 16(16):1-18. doi:10.1186/1471-2105-13-S16-S9

40. 1.3.6.7.2. Critical Values of the Student's-t Distribution. Accessed October 29, 2020. https://www.itl.nist.gov/div898/handbook/eda/section3/eda3672.htm

41. Naz S, Vallejo M, García A, Barbas C. Method validation strategies involved in non-targeted metabolomics. *J Chromatogr A*. 2014;1353:99-105. doi:10.1016/j.chroma.2014.04.071

42. Wang H, Yang G, Zhou J, Pei J, Zhang Q, Song X, Sun Z. Development and validation of a UPLC-MS/MS method for quantitation of droxidopa in human plasma: Application to a pharmacokinetic study. *J Chromatogr B Anal Technol Biomed Life Sci*. 2016;1027:234-238. doi:10.1016/j.jchromb.2016.04.056

43. Burns DT, Danzer K, Townshend A. Use of the terms "recovery" and "apparent recovery" in analytical procedures (IUPAC Recommendations 2002). *Pure Appl Chem*. 2003;74(11):2201-2205. doi:10.1351/pac200274112201

44. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal Chem*. 2003;75(13):3019-3030. doi:10.1021/ac020361s

45. Łozowicka B, Rutkowska E, Jankowska M. Influence of QuEChERS modifications on recovery and matrix effect during the multi-residue pesticide analysis in soil by GC/MS/MS and GC/ECD/NPD. *Environ Sci Pollut Res*. 2017;24(8):7124-7138. doi:10.1007/s11356-016-8334-1

46. Drochioiu G, Arsene C, Murariu M, Oniscu C. Analysis of cyanogens with resorcinol and picrate. *Food Chem Toxicol*. 2008;46(11):3540-3545. doi:10.1016/j.fct.2008.09.005

47. Miller RE, Tuck KL. Reports on the distribution of aromatic cyanogenic glycosides in Australian tropical rainforest tree species of the Lauraceae and Sapindaceae. *Phytochemistry*. 2013;92:146-152. doi:10.1016/j.phytochem.2013.04.007

48. Zahmanov G, Alipieva K, Simova S, Georgiev MI. Metabolic differentiations of dwarf elder by NMR-based metabolomics. *Phytochem Lett*. 2015;11:404-409. doi:10.1016/j.phytol.2014.11.021

49. Koss-Mikołajczyk I, Lewandowska A, Pilipczuk T, Kusznierewicz B, Bartoszek A. Composition of bioactive secondary metabolites and mutagenicity of *Sambucus nigra* L. Fruit at different stages of ripeness. *Acta Aliment*. 2016;45(3):442-451. doi:10.1556/066.2016.45.3.16

50. 763. Cyanogenic glycosides (WHO Food Additives Series 30). Accessed October 25, 2020. http://www.inchem.org/documents/jecfa/jecmono/v30je18.htm

51. Borron SW. Recognition and Treatment of Acute Cyanide Poisoning. *J Emerg Nurs*. 2006;32(4 SUPPL.). doi:10.1016/j.jen.2006.05.011

52. Sahin S. Cyanide Poisoning in a Children Caused by Apricot Seeds. Published online 2011. doi:10.4172/2157-7420.1000106

53. Akyildiz BN, Kurtoğlu S, Kondolot M, Tunç A. Cyanide poisoning caused by ingestion of apricot seeds. *Ann Trop Paediatr*. 2010;30(1):39-43. doi:10.1179/146532810X12637745451951

54. Sayre JW, Kaymakcalan S. Cyanide Poisoning From Apricot Seeds Among Children In Central Turkey. *N Engl J Med*. 1964;270:1113-1115. doi:10.1056/NEJM196405212702109

55. Sanchez-Verlaan P, Geeraerts T, Buys S, Riu-Poulenc B, Cabot C, Fourcade O, Mégarbane B, Genestal M. An unusual cause of severe lactic acidosis: Cyanide poisoning after bitter almond ingestion. *Intensive Care Med*. 2011;37(1):168-169. doi:10.1007/s00134-010-2029-8

56. Mouaffak Y., Zegzouti F., Boutbaoucht M., Najib M., El Adib AG., Sbihi M., Younous S. Cyanide poisoning after bitter almond ingestion. *Ann Trop Med Public Heal*. 2013;6(6):679-680. doi:10.4103/1755-6783.140262

57. Akintonwa A, Tunwashe OL. Fatal Cyanide Poisoning from Cassava-Based Meal. *Hum Exp Toxicol*. 1992;11(1):47-49. doi:10.1177/096032719201100107

58. Are Apple Cores Poisonous? | Questions | Naked Scientists. Accessed October 26, 2020. https://www.thenakedscientists.com/articles/questions/are-apple-cores-poisonous

CHAPTER 3: Docosahexaenoic acid supplementation alters phospholipid species and lipid peroxidation products in adult mouse brain, heart, and plasma

3.1 Introduction

Docosahexaenoic acid (DHA, 22:6n-3) is an (n-3) polyunsaturated fatty acid (PUFA) abundant in the phospholipids in the brain and retina. DHA not only plays a role in the maintenance of cell membrane fluidity, it also is involved in the production of lipid mediators important in regulating intracellular signaling activities.[1,2,3,4] Its pleiotropic properties have generated interest in examining its role and mechanism(s) of action in health and diseases.[5,6,7] There is a suggestion that abnormalities in DHA during brain development may be an underlying factor for some psychiatric disorders including attention deficit, hyperactivity, and autism spectrum disorders.[8,9,10,11] Furthermore, studies with animal models have demonstrated the ability for (n-3) PUFAs to ameliorate age-related neurodegenerative diseases, and to facilitate brain recovery after ischemic stroke and traumatic brain injuries.[12,13,14] Aside from its effects on the central nervous system, DHA and other (n-3) PUFAs also offer beneficial effects on other body systems, including the cardiovascular system.[15,2,16] Subsequently, it is not surprising that DHA is widely used as a dietary supplement and a nutraceutical across different human populations.[17]

DHA and arachidonic acid (ARA, 20:4n-6) are major PUFAs linked to the *sn*-2 position of membrane phospholipids. These PUFAs are metabolically active and are mediated by phospholipases A2 (PLA2), energy dependent fatty acid CoA ligase, and acyltransferases through the deacylation-reacylation cycle, also known as the "Land's cycle".[1819] However, depending on the cell type and conditions, different types of PLA2

are responsible for regulating the metabolism of DHA and ARA.[20,4,21] For example, ARA in phospholipids is released through the action of cytosolic PLA2 (cPLA2), and this pathway is known to play a role in the production of prostaglandins, thromboxanes, and leukotrienes, and lipid mediators, which are largely pro-inflammatory.[20] In contrast, the release of DHA through the Ca^{+2}-independent PLA2 (iPLA2) is linked to production of resolvins, protectins and marisins, which are pro-resolving mediators.[22,23]

Besides interactions with cyclooxygenases and lipoxygenases, PUFAs are also susceptible to enzymatic and non-enzymatic reactions with oxygen free radicals, leading to the production of reactive aldehydes, namely, 4-hydroxyhexenal (4-HHE) from DHA and 4-hydroxynonenal (4-HNE) from ARA.[24,21] There is evidence that these lipid peroxidation products may offer important physiological functions, due mainly to their ability to form adducts with proteins, nucleic acids and phospholipids.[25,24,26,25] However, in light of the "Yin-Yang" mechanism for the metabolism of DHA and ARA, there is also evidence that these peroxidation products are also regulated differently.[21] For example, our study with microglial cells demonstrated the increase in 4-HNE but not 4-HHE upon stimulation of the cPLA2/ARA pathway by lipopolysaccharides.[27] Increases in 4-HNE are also observed in brain associated with neuroinflammatory diseases, including stroke and spinal cord injury.[28,29,30] On the other hand, increases in 4-HHE have been shown upon consumption of diets enriched in DHA or fish oil.[17,26,31] Since both peroxidation products are readily detected in tissues and body fluids, there is increasing interest to examine regulation of these products in health and diseases.

Our recent study demonstrated the effects of maternal DHA-enriched diet to alleviate stress-induced behavioral deficits in offspring mice.[32] Subsequently, a follow-up

study using the same dietary regimen showed that administration of the DHA-enriched diet to pregnant mothers resulted in altered fatty acid composition in all brain regions, but increases in 4-HHE levels mainly in the cerebral cortex and hippocampus in the offspring pups.[31] In addition to brain tissue, this study also demonstrated substantial changes in fatty acid composition and levels of lipid peroxidation products in the heart and plasma.[31] Considering that (n-3) and (n-6) PUFAs are linked to different classes of membrane phospholipids, an important goal in this study is to determine whether the DHA-supplemented diet may alter lipid peroxidation products and molecular species in different phospholipids in adult mice administered a control or DHA-enriched diet. In addition to examining the changes in the brain, this study also included determination of lipid peroxidation products and phospholipids species in the heart and plasma.

3.2 Experimental

3.2.1 Chemicals and reagents

4-Hydroxyhexenal (4-HHE, 1 mg in 100 μL of ethanol), 4-hydroxynonenal (4-HNE, 1 mg in 100 μL of ethanol), and 4-hydroxy hexenal-d3 (4-HHE-d3, 100 μg in 100 μL of methyl acetate) were purchased from Cayman Chemical Co. (Ann Arbor, MI). 1,3-Cyclohexanedione (CHD, 97%), ammonium acetate (HPLC grade), acetic acid (ACS grade) and formic acid (mass spectrometry grade), were purchased from Sigma-Aldrich (St. Louis, MO). C18 Sep-Pak cartridges (1 mL, 100 mg) were obtained from Waters Corporation (Milford, MA). Phospholipid removal cartridges (PhreeTM, 1 mL) were purchased from Phenomenex Inc. (Torrance, CA). All solvents (HPLC grade) used for sample preparation, UHPLC, and MS analysis were obtained from Thermo Fisher Scientific Inc. (Fair Lawn, NJ).

3.2.2 Animals and diets

Three-month-old male mice (C57Bl/6J) were purchased from Jackson Laboratories (Bar Harbor, ME), and were given a nutritionally complete, control (CTL) diet (modified AIN-93G #103619) from Dyets Inc. (Bethleham, PA). The CTL diet contained no preformed DHA (as shown in Appendix 3 as Table A3.1), but did contain sufficient amounts of α-linolenic acid (ALA,18:3n-3) to meet normal brain DHA requirements.[33,] At the beginning of the study, animals (n = 12) were randomly divided into two groups. The control group (n = 5 mice) remained on the CTL diet, whereas the experimental group (n = 7 mice) was fed a diet containing 1%, by weight, DHA (#103598) for three weeks. The reason for the three-week diet is because this period of time was used in a study in which adult mice fed a fish oil diet with a similar amount of DHA resulted in substantial changes in fatty acids and lipid peroxidation products in different body organs.[26] The composition of the control and DHA diets have been previously described,[34,35] as well as, the final fatty acid profiles (as shown in Appendix 3, Tables A3.1 and A3.2). The diets were stabilized against auto-oxidation with 0.02 g tertiary-butylhydroquinone/100 g fat. All experiments were approved by the University of Missouri Animal Care and Use Committee (#8945) and were performed in compliance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals.

3.2.3 Sample preparation

After the feeding regimen, animals were anesthetized with isoflurane and blood was obtained by heart puncture using heparin as an anticoagulant, followed by perfusion with physiological saline. The brain was dissected to obtain left and right cerebral cortices,

striatum, hippocampus, and cerebellum. In addition, the perfused heart was also obtained. The blood was centrifuged at 16,100 g for 5-8 min to obtain plasma and was transferred to Eppendorf tubes and frozen at -80 °C until use. Samples were placed in sterilized aluminum foil, immediately flash frozen in liquid nitrogen, and stored at -80 °C until use.

For the experiments using LC-MS/MS to determine 4-HHE and 4-HNE, the left cerebral cortex, hippocampus, striatum and cerebellum, along with the heart were weighed and homogenized in 8-fold volumes of HPLC water using a bullet blender (Next Advance, Inc., Averill Park, NY) as describe previously.[35] The heart tissue was first pulverized in liquid nitrogen prior to homogenization, and the homogenates were centrifuged at 16,100 *g* for 20 min at 4°C. The supernatant was collected and stored at 80°C prior to use. Total protein concentration in the supernatant was determined using the Bicinchoninic Acid Assay (Sigma-Fisher, St. Louis, MO).

3.2.4 LC-MS/MS analysis of 4-HHE and 4-HNE

The sample preparation stage involved three main steps: phospholipid removal, derivatization, and desalting as previously described.[36,35] Briefly, phospholipids were removed via solid phase extraction using a 1 mL Phree^{TM} cartridge. This step removes phospholipids and helps eliminate matrix effects to allow for lower limits of detection of the peroxidation products. The cartridge was loaded with 30 µL of homogenized sample and an equal volume of internal standard (4-HHE-d_3, 1000 ng/mL). Acetonitrile (500 µL) was spiked with 1% formic acid and was used to elute the analyte of interest. The clear eluate was dried under a stream of nitrogen and used for derivatization with the freshly prepared acidified CHD reagent. The mixture was incubated at 60°C for 1 h and then cooled on ice prior to desalting using a C18 SPE cartridge. The C18 cartridge was pre-

conditioned with methanol (0.7 mL) and equilibrated with water (0.7 mL). The cooled derivatized mixture was loaded onto the cartridge, washed twice with water (0.7 mL) and followed by 5% acetonitrile in water (0.7 mL). The derivatized analytes of interest (4-HHE, 4-HNE, and 4-HHE-d3 derivatives) were eluted with 100% acetonitrile (0.7 mL) and dried with a steady stream of nitrogen gas. The samples were reconstituted in 40% methanol in water with 0.1% formic acid (300 µL) and were ready for LC-MS/MS analysis. A Waters Acquity UHPLC system equipped with a quaternary solvent manager was used in conjunction with a C18 reversed phase column, Luna Omega100 Å (1.6 µm × 50 × 2.1 mm, Phenomenex). A previously developed and validated UHPLC-MS/MS method with a 7 min LC gradient including equilibration was used for the separation and detection of the 4-HHE and 4-HNE derivatives.[35] A Waters Xevo TQ-S triple quadrupole mass spectrometer with an electrospray ionization source operated in the positive-ion mode was used. Multiple reaction monitoring transitions m/z 326.3 -> 216.1Da, m/z 284.2 -> 216.1 Da and 287.2 -> 216.1 Da were chosen for simultaneous monitoring of 4-HNE, 4-HHE and 4- HHE-d_3 (internal standard) derivatives, respectively. Tissue samples (~10 mg) were pulverized and homogenized in 0.5 mL of ice-cold diluted PBS (0.1x) with a Potter-Evejar tissue grinder. Protein assays on individual homogenates were conducted. An aliquot of homogenate was transferred to a disposable glass test tube. A mixture of lipid internal standards for quantification of all reported lipid classes was added to the tube based on the tissue protein content.[37] Lipid extraction was performed by a modified Bligh and Dyer method as previously describe.[38]

3.2.5 Analysis of phospholipid species

3.2.5.1 Sample preparation

Four randomly selected frozen samples (half of the left cerebral cortex, half of the heart tissue, and plasma) from the control and DHA groups were sent to Prof. Xianlin Han's laboratory (University of Texas Health Science Center at San Antonio, TX) for analysis of phospholipid species. The reason for selecting only cerebral cortex was because a substantial amount of brain tissue is required for this type of lipid analysis, and in our previous study, similar changes in fatty acid composition (i.e., decrease in ARA and increase in DHA) were observed in all brain regions.[35] All of the lipid extracts were flushed with nitrogen, capped, and stored at -20ºC.

3.2.5.2 Mass spectrometric analysis of lipids

Mass spectrometric analysis of lipids was performed with an Altis triple-quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) or a Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an automated nanospray device (Triversa Nanomate, Advion Biosciences, Ithaca, NY) and operated with the Xcalibur software as previously describe.[39, 40] Identification and quantification of all reported lipid molecular species were performed using an in-house automated software program.[37,40] All lipid levels were normalized to sample protein content.

3.2.6 Statistical analysis

For the determination of 4-HHE and 4-HNE levels via LC-MS/MS, three biological replicates of each tissue sample and three analytical replicates of each biological replicate were completed for each sample. Statistical analyses were performed with GraphPad Prism (version 8.3; GraphPad Prism Software Inc., San Diego, CA). Results are

expressed as the mean ± standard error of mean (SEM). Depending on the experiments, samples were analyzed by non-parametric t-tests or one-way ANOVA to compare among control and DHA diet groups. Differences were considered significant at $p < 0.05$ for all analyses.

3.3 Results

3.3.1 Feeding mice with control and DHA-enriched diets

In this study, adult mice were given control and 1% DHA-enriched diets for three weeks. Fresh diets were provided every other day, and body weights for each mouse were obtained every 4 days. As shown in Figure 3.1, there was no significant change in body weights between the two groups during the three-week period with the control and DHA-enriched diets.

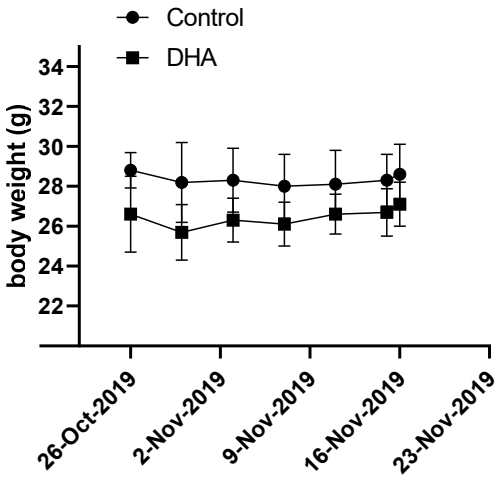

Figure 3.1. Body weights of mice in the control and DHA group over the time of the study

3.3.2 Determination of 4-HHE and 4-HNE

Levels of 4-HHE and 4-HNE in cerebral cortex, hippocampus, striatum and cerebellum in control and DHA supplement groups were determined. Among the

different brain regions, only the hippocampus showed a small but significant ($p < 0.01$) increase in 4-HHE (Figure 3.2). Although there was an apparent increase in 4-HHE in the cerebral cortex, this change was not significant ($p = 0.12$) due to the large variances. Except for the striatum, which showed a small but significant ($p < 0.05$) increase in 4-HNE levels in the DHA-enriched diet group, no apparent changes in 4-HNE levels were found in other brain regions (Figure 3.2).

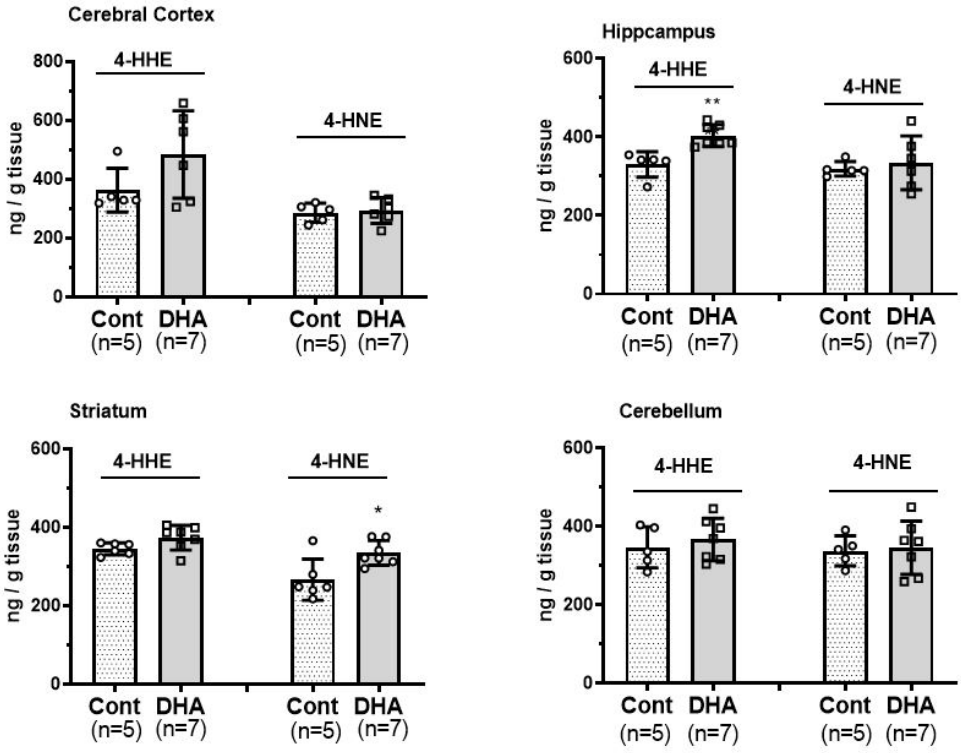

Figure 3.2. Levels of 4-HHE and 4-HNE in mouse brain regions after feeding with a control or DHA-enriched (1%) diet for three weeks. Brain tissues including cerebral cortex, hippocampus, striatum, and cerebellum, were dissected, and homogenized as described in the text. Levels of 4-HHE and 4-HNE were determined by LC-MS/MS protocol as described in text. Data are normalized to tissue weight. Results represent the mean ± SEM of control (n = 5) and DHA (n = 7) samples. Analysis using a two-tail unpaired t-test indicated significance between DHA group and controls. * $p < 0.05$; ** $p < 0.01$.

In heart tissue, the levels of 4-HNE were higher than levels of 4-HHE in the control group (Figure 3.3A). Nevertheless, supplement with the DHA-enriched diet resulted in an almost two-fold increase in 4-HHE ($p < 0.05$) as compared to the control group (Figure 3.3A). Again, DHA-enriched diet did not alter 4-HNE levels in the heart tissue (Figure 3.3A).

In the plasma samples, there were higher levels of 4-HHE as compared to 4-HNE in the control group (Figure 3.3B). Similarly, levels of 4-HHE in the DHA-enriched group were increased significantly ($p < 0.001$) as compared to the controls. In the plasma, the level of 4-HNE in the DHA-enriched group showed a small but significant increase ($p < 0.01$) (Figure 3.3B).

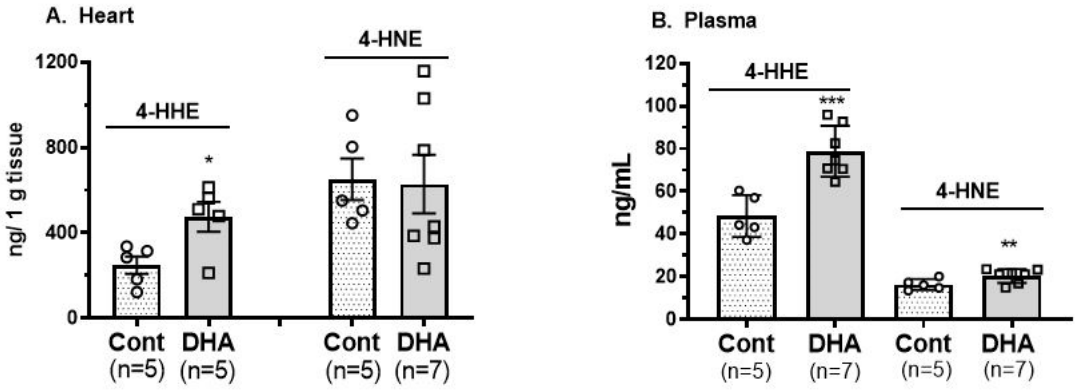

Figure 3.3. Levels of 4-HHE and 4-HNE in mouse (A) heart and (B) plasma after feeding with a control or DHA-enriched diet for three weeks. Procedures for processing the heart and plasma, and protocol for LC-MS/MS determination of 4-HHE and 4-HNE are described in text. Results represent the mean ± SEM of control (n = 5) and DHA (n = 7) samples. Analysis using two-tail unpaired t-test indicated significance between DHA group and controls. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3.3 Analysis of phospholipid species

The major phospholipid classes in the cerebral cortex are diacyl-PE, alkenylacyl-PE (PE-pl, also PE plamalogen), PC, PS and PI (Figure 3.4A). Analysis also detected small amounts of alkylacyl-PE and alkenylacyl-PC species (data not shown). The major phospholipids in the heart tissue are PC and diacyl-PE, and unlike those in the cerebral cortex, only low levels of PEpl, PI and PS were detected (Figure 3.4B). In the plasma, although PC was the major phospholipid, low levels of PI and PS were detected (Figure 3.4C). However, the levels of diacyl-PE in plasma were too low to allow proper analysis of its molecular species. Levels of individual phospholipid classes in the cerebral cortex and heart were not different when comparing the DHA supplement group with the control group (Figure 3.4). Nevertheless, levels of PC and PI in plasma were significantly lower ($p < 0.01$) in the DHA supplement group as compared with the control group (Figure 3.4C).

In the cerebral cortex, analysis of the diacyl-PE species containing (n-6) PUFAs showed primarily the 18:0/20:4 species, and those with (n-3) were primarily 18:0/22:6 (as shown in Appendix 3 as Table A3.3). For PE-pl, where linkage in the sn-1 position is in alkenyl (ake) form, the species with (n-6) were comprised mainly of 18:0 ake/20:4 and 18:0 ake/22:4, and the (n-3) species were mainly 16:0 ake/22:6. For PC, the (n-6) species were primarily 16:0/20:4 and 18:0/20:4, and the (n-3) species were mainly 16:0/22:6 and 18:0/22:6. For PI, there were high levels of (n-6) species, mainly 18:0/20:4, and low levels of the (n-3) species, mainly 16:0/22:6. For PS, there were low levels of (n-6) species, mainly 18:0/20:4, and high levels of (n-3) species, mainly 18:0/22:6.

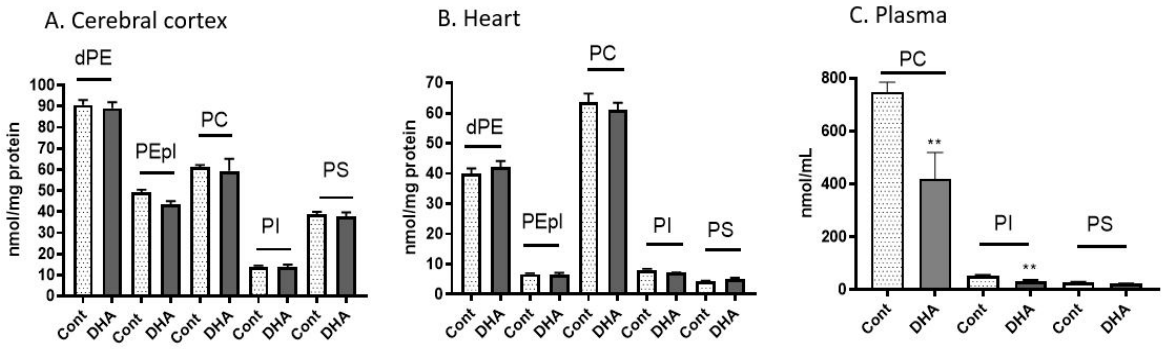

Figure 3.4. Levels of phospholipids (diacyl-PE, PEpl, PC, PI, and PS) in mouse (A) cerebral cortex, (B) heart, and (C) plasma after feeding a control and DHA-enriched diet for three weeks. Lipids were extracted and analyzed by the shotgun lipidomic platform as described in text. Data are expressed as nmol/mg protein and are mean ± SEM of control (n=4) and DHA (n=4) samples. Analysis using two-tail unpaired t-test indicated significance between DHA group and controls. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Abbreviations: dPE, diacyl phosphatidylethanolamine, PEpl, alkenylacyl phosphatidylethanolamine or ethanolamine plasmalogen, PC, phosphatidylcholine, PI, phosphatidylinositol, PS, phosphatidylserine

Among the diacyl-PE species in the cerebral cortex, there were more (n-3) species than (n-6) species, whereas in PE-pl, the (n-6) species were higher than (n-3) species (Figure 3.5A). PC had similar levels of (n-6) and (n-3) species, whereas PI contained high (n-6) species and PS contained high (n-3) species (Figure 3.5A). In all phospholipid species analyzed in the cerebral cortex, the DHA-enriched group showed a trend for increase in (n-3) species and a decrease in (n-6) species. In fact, significant ($p < 0.05$) decreases in the (n-6) species were observed in dPE, PE-pl and PS. When the ratios for (n-6)/(n-3) species in the control and DHA-enriched group were determined, all three major phospholipid classes (diacyl-PE, PE-pl, and PC) showed significant decreases ($p < 0.05$, $p < 0.01$) in (n-6) to (n-3) ratios in the DHA-enriched group as compared with the control group (Figure 3.5B).

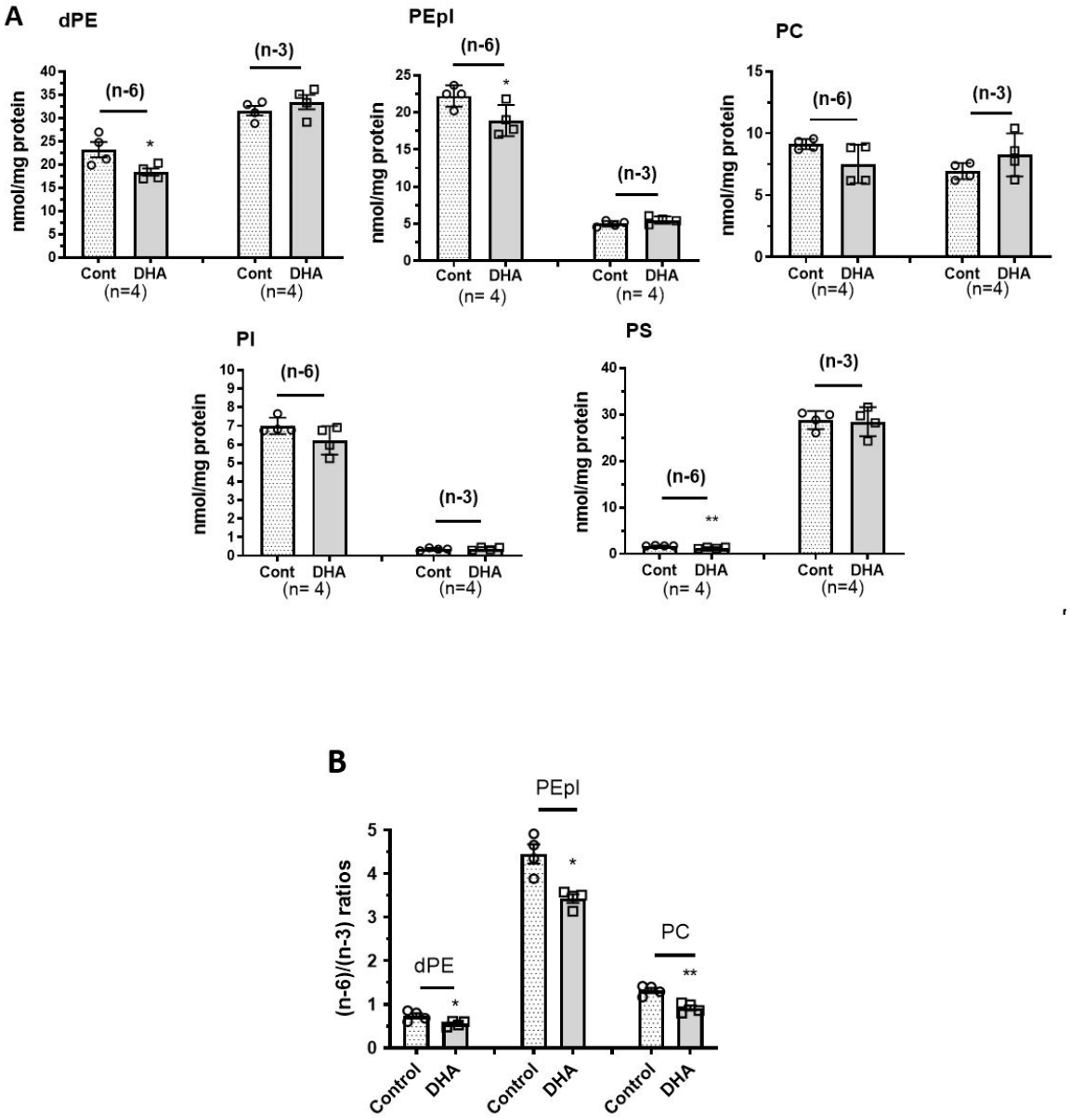

Figure 3.5. (A) Levels of (n-3) and (n-6) phospholipid species (dPE, PEpl, PC, PI and PS) in mouse cerebral cortex after feeding a control and DHA-enriched diet for three weeks. Lipids were extracted and analyzed by the shotgun lipidomics platform as described in text. In each phospholipid class, species with fatty acids containing (n-3) or (n-6) were grouped and expressed as nmol/mg protein. (B) Ratios of (n-6)/(n-3) phospholipid species of dPE, PEpl and PC from control and DHA group in the cortex using the data from (A). Data are mean ± SEM of control (n=4) and DHA (n=4) samples. Analysis using two-tail unpaired t-test indicated significance between DHA group and controls. * $p < 0.05$; ** $p < 0.01$.

Analysis of phospholipid species in the heart tissue showed significantly larger effects of DHA supplementation of all phospholipid classes. Among the phospholipid classes analyzed (diacyl-PE, PC, PI and PS), the DHA-enriched group showed significant decreases in (n-6) species ($p < 0.001$), and significant increases in the (n-3) species ($p < 0.05$) in all classes examined (Figure 3.6). These results clearly indicate that a 3-week DHA-enriched diet is sufficient to cause alterations of membrane phospholipids in the heart tissue.

In the plasma, PC with (n-6) PUFA is the predominant phospholipid species, and a DHA-enriched diet resulted in a large and significant decrease ($p < 0.001$) in this phospholipid species (Figure 3.7). For PI in plasma, a DHA-enriched diet resulted in a large decrease in (n-6) and a small increase in (n-3) species ($p < 0.001$, $p < 0.01$, respectively) (Figure 3.7). Despite low levels, PS in plasma also showed a decrease in (n-6) and only a small increase in (n-3) species in this phospholipid ($p < 0.01$, $p < 0.05$, respectively) (Figure 3.7).

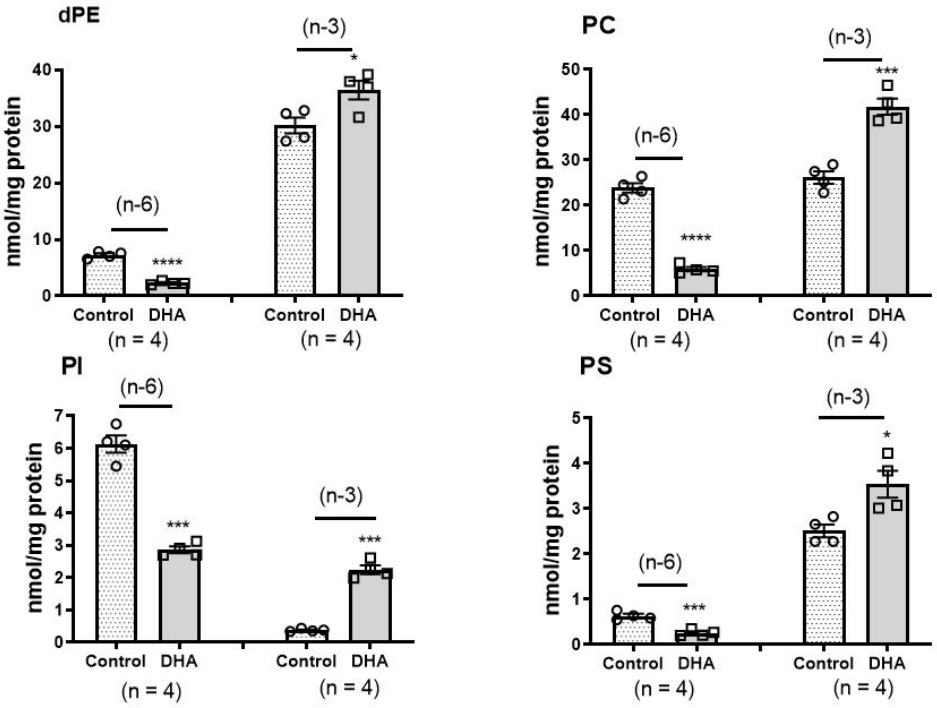

Figure 3.6. Levels of (n-3) and (n-6) phospholipid species (dPE, PC, PI and PS) in mouse heart after feeding a control and DHA-enriched diet for three weeks. In each phospholipid class, species with fatty acids containing (n-3) or (n-6) were grouped and expressed as nmol/mg protein. Data are mean ± SEM of control (n=4) and DHA (n=4) samples. Analysis using two tail unpaired t-test indicated significance between DHA group and controls. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

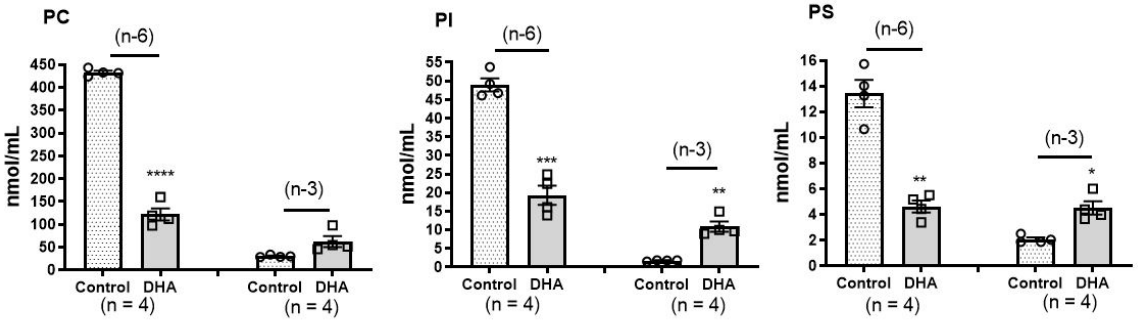

Figure 3.7. Levels of (n-3) and (n-6) phospholipid species (PC, PI, and PS) in mouse plasma after feeding a control and DHA-enriched diet for three weeks. In each phospholipid class, species with fatty acids containing (n-3) or (n-6) were grouped and expressed as nmol/mg protein. Data are mean ± SEM of control (n=4) and DHA (n=4) samples. Analysis using two tail unpaired t-test indicated significance between DHA group and controls. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.4 Discussion

In many studies, including our own, there is evidence for the ability of DHA-enriched a or diet with (n-3) fatty acids to alter the fatty acid composition in brain and other body organs.[26,35,41] In a study by Murphy's group, they further demonstrated increases in (n-3) and decreases in (n-6) fatty acids in different phospholipid classes in brain, heart and liver upon feeding rats with fish oil diet for 8 weeks.[41] Unlike the peripheral organs, linoleic acid (18:2n-6) is low in brain tissue, and thus ARA and DHA contributed to the major (n-6) and (n-3) PUFAs linked to the *sn*-2 position of phospholipids. Despite the fact that both PUFAs are abundant in the brain tissue, it is well recognized that they undergo different metabolic pathways and participate in different reactions: ARA is the precursor for multiple reactions leading to lipid mediators that are involved in pro-inflammatory functions, whereas DHA are known for production of oxylipins that are pro-resolving.[42] Recent studies also demonstrated involvement of both PUFAs in lipid peroxidation and production of 4-HNE from ARA and 4-HHE from DHA. Although the physiological role of these reactive aldehydes is not clearly understood, there is increasing evidence for their engagement in specific cellular metabolism.[24] In our previous study with the maternal mouse model, weaning pups nursed by mothers with the DHA-enriched diet showed an increase in 4-HHE mainly in the cerebral cortex and hippocampus, despite that changes in fatty acid composition were observed in all brain regions, including cerebellum.[35] In this study with adult mice, a three-week supplement with DHA-enriched diet again showed an increase in 4-HHE in the hippocampus. These results are in agreement with the contention that the hippocampus is responsive to a DHA-enriched diet by producing more 4-HHE.

Interestingly, despite that a supplement of DHA-enriched or fish oil diet resulted in a decrease in ARA in all brain regions, there was no obvious decrease in 4-HNE in these brain regions. The study by Nakagawa et al. reported changes in fatty acids and peroxidation products (4-HHE and 4-HNE) in plasma and in multiple organs after feeding adult mice with fish oil for three weeks.[26] In their study, besides increase in 4-HHE, a significant decrease in 4-HNE was observed in the plasma. The discrepancy between our results and those by Nakagawa et al. is not known. In a study by Calzada et al., healthy human subjects given a DHA supplement ranging from 200 to 1600 mg/day for two weeks also showed a dose-dependent increase in 4-HHE (but not 4-HNE) in plasma.[17] Therefore, these studies with animal models and humans provided consensus that DHA-enriched and fish oil diets are associated with an increase in 4-HHE. Taken together, these results also show that determination of 4-HHE levels in plasma can be a useful biomarker for assessing DHA consumption. Since there is a concern that the increase in 4-HHE due to DHA-enriched or fish oil diets may be due to contamination of this peroxidation product in the diet, an attempt was made to determine the 4-HHE and 4-HNE content in the control and DHA-enriched diets used in our experiments. Our results indicated no detectable 4-HHE in the diets although trace amounts of 4-HNE (control 7.62 ± 0.21versus DHA 5.73 ± 0.15 ng/g) were detected in both control and DHA-enriched diet. The rationale for detecting 4-HNE in both control and DHA-enriched diets is not clear but since we could not detect 4-HHE, these results clearly show that the increase in 4-HHE levels in brain and other organs upon feeding the DHA-enriched diet is a physiological phenomenon. Several studies with cell and animal models have demonstrated the hormetic effects of 4-HHE through upregulation of the Nrf2 pathway

and production of heme oxygenase-1, a potent antioxidant enzyme.[26,43,44,45] However, more studies are needed to examined possible physiological roles of these lipid peroxidation products in the heart and other body systems.[24]

In this study, we examined changes in phospholipid species in the brain, heart and plasma after feeding with DHA-enriched diet for three weeks. In agreement with the presence of the blood brain barrier, uptake of fatty acids to the brain and their turnover are slower as compared to peripheral tissues such as the heart.[46] Phospholipid classes in the cerebral cortex are comprised primarily of dPE, PE-pl, PC, PI and PS (Figure 3.4). Analysis of the molecular species of dPE, PE-pl, and PC in this brain region indicate small increases in (n-3) and decreases in (n-6) species for these phospholipids (Figure 3.5A). Although determination of (n-6)/(n-3) ratios showed significant decreases for dPE, PEpl and PC, it is obvious that a longer feeding time is required to obtain a significant outcome.[47,48] In this study, it is of interest that PE-pl showed a high level of (n-6) species, and the decrease in (n-3)/(n-6) ratios in PE-pl was greater for diacyl-PE and PC. In a study by Rosenberger et al., a two-hour i.v. injection of labeled hexadecanol (precursor for the alkyl group) showed a distribution of 97% in the gray matter and only 3% in myelin.[49] This study demonstrated at least two pools of ether phospholipids, a static pool in the myelin and a dynamic pool in the synaptic area. Their findings together with the present results highlight the active metabolic function of plasmalogens in brain as well as their ability to participate in membrane signaling activity.[50] Future investigation should examine whether dietary DHA may alter molecular species of this PE-pl in different brain regions and subcellular fractions.

Upon examining the molecular species of dPE and PC in heart, results showed significant decreases ($p < 0.001$) in (n-6) and concomitant increases ($p < 0.05$, 0.001, respectively) in (n-3) species in both phospholipids comparing the DHA-enriched diet group with controls (Figure 3.6). In a previous study by Murphy's group, analysis of fatty acids in phospholipid classes (PC, PE, PI and PS) in rat heart after an 8-week feeding with fish oil also indicated an increase in (n-3) and a decrease in (n-6) fatty acids in all phospholipid classes.[41] In another study, feeding rats with different levels of fish oil diet also showed a time- and dose-dependent decrease in (n-6) and increase in (n-3) phospholipids in the heart tissue.[51]As indicated in the review by Oppedisano et al., (n-3) fatty acids could offer vaso- and cardio-protective effects through modulation of membrane phospholipids and improve cardiac mitochondrial functions.[52] In our study, it seems that a three-week DHA-enriched diet is sufficient to change phospholipid species and peroxidation products in the heart tissue.

PC is the major phospholipid in plasma, and in the control group, plasma PC are comprised with high levels of (n-6) species and only low levels of (n-3) species (Figure 3.7). In this study, plasma obtained from a DHA-enriched diet showed a more than 70% decrease in the PC (n-6) species but only a small increase in the (n-3) species. As a result, the total level of plasma PC in the plasma from the DHA-enriched diet group declined by almost 50% as compared with the control group (Figure 3.4C).

In agreement with earlier studies,[47,53] PI in brain showed high proportions of 20:4n-6, and PS with high proportions of 22:6 n-3 (Figure 3.5A). It is also recognized that PI and PS have distinctive functions, i.e., PI is involved in inositol metabolism associated with second messengers and intracellular calcium mobilization,[54] whereas PS plays a role

in membrane trafficking and endocytosis.[55] In the study here, the three-week DHA-enriched diet did not greatly alter the (n-6) and (n-3) species in PI and PS in the cerebral cortex (Figure 3.5A), but this dietary regimen resulted in more than 50% decrease in (n-6) species and a concomitant increase in (n-3) species in both PI and PS in the heart (Figure 3.6). Considering the diverse functions of these two phospholipids, it is possible that a change in the fatty acid composition may link to an interaction to different enzymes and thus result in an altered ability to regulate myocardial functions.

Our results indicate that a DHA-enriched diet not only decrease phospholipid species containing 20:4(n-6), but also other (n-6) fatty acids, such as 20:4 (n-6) and 22:4 (n-6). These results suggest that changes in the (n-6) fatty acid pool are likely to occur at the initiation point of fatty acid elongation and desaturation. This is in agreement with the study showing that DHA could suppress metabolism of linoleic acid (18:2 n-6) and its ability to form elongation products, including 20:4(n-6) and 22:4(n-6).[56] Nevertheless, considering that phospholipid species with 20:4 (n-6) undergo active metabolism through the diacylation-reacylation cycle, and its release by cytosolic phospholipase A2 is linked to inflammatory pathways, the ability for DHA-enriched diet to decrease phospholipids with (n-6) species may be advantageous in suppressing cell membranes against inflammatory responses.

3.5 Conclusions

Albeit to different extents, feeding adult mice a DHA-enriched diet for three weeks resulted in changes in (n-6) and (n-3) phospholipid species and an alteration of lipid peroxidation products in the brain, heart and plasma. The increase in (n-3) phospholipids due to DHA-enriched diet is associated with an increase in 4-HHE but not 4-HNE. In

brain, the increase in 4-HHE appears to be mainly observed in the hippocampus, suggesting higher lipid peroxidation activity in this brain region. The decrease in (n-6) species in the heart tissue suggests the possibility that the DHA dietary regimen can mitigate ARA metabolism associated with inflammatory and oxidative activity.

3.6 References

1. Belkouch M, Hachem M, Elgot A, Ugi S, Kondo H, Makino T, Kashiwagi A, Maegawa H. The pleiotropic effects of omega-3 docosahexaenoic acid on the hallmarks of Alzheimer's disease. *J Nutr Biochem*. 2016;38:1-11. doi:10.1016/j.jnutbio.2016.03.002
2. Jump DB, Depner CM, Tripathy S. Omega-3 fatty acid supplementation and cardiovascular disease. *J Lipid Res*. 2012;53(12):2525-2545. doi:10.1194/jlr.R027904
3. Maulucci G, Cohen O, Daniel B, Sansone A. Petropoulou I, Filou S, Spyridonidis A, Pani G,De Spirito M, Chatgilialoglu C, Ferreri, Kypreos C, Sasson S Fatty acid-related modulations of membrane fluidity in cells: detection and implications. *Free Radic Res*. 2016;50(sup1):S40-S50. doi:10.1080/10715762.2016.1231403
4. Sun GY, Simonyi A, Fritsche KL, Chuang D Y, Hannink M, Gu Z, C. Greenlief M C, Yao J K, Lee J C, Beversdorf D Q. Docosahexaenoic acid (DHA): An essential nutrient and a nutraceutical for brain health and diseases. *Prostaglandins Leukot Essent Fat Acids*. 2018;136:3-13. doi:10.1016/j.plefa.2017.03.006
5. Avallone R, Vitale G, Bertolotti M. Omega-3 Fatty Acids and Neurodegenerative Diseases: New Evidence in Clinical Trials. *Int J Mol Sci*. 2019;20(17):4256. doi:10.3390/ijms20174256
6. Bazan NG, Molina MF, Gordon WC. Docosahexaenoic Acid Signalolipidomics in Nutrition: Significance in Aging, Neuroinflammation, Macular Degeneration, Alzheimer's, and Other Neurodegenerative Diseases. *Annu Rev Nutr*. 2011;31(1):321-351. doi:10.1146/annurev.nutr.012809.104635

7. Lauritzen L, Brambilla P, Mazzocchi A, Harsløf LBS, Ciappolino V, Agostoni C. DHA effects in brain development and function. *Nutrients*. 2016;8(1). doi:10.3390/nu8010006

8. Agostoni C, Nobile M, Ciappolino V, et al. The Role of Omega-3 Fatty Acids in Developmental Psychopathology: A Systematic Review on Early Psychosis, Autism, and ADHD. *Int J Mol Sci*. 2017;18(12):2608. doi:10.3390/ijms18122608

9. Clayton EH, Hanstock TL, Garg ML, Hazell PL. Long chain omega-3 polyunsaturated fatty acids in the treatment of psychiatric illnesses in children and adolescents. *Acta Neuropsychiatr*. 2007;19(2):92-103. doi:10.1111/j.1601-5215.2007.00189.x

10. Hashimoto M, Maekawa M, Katakura M, Hamazaki K, Matsuoka Y. Possibility of Polyunsaturated Fatty Acids for the Prevention and Treatment of Neuropsychiatric Illnesses. *J Pharmacol Sci*. 2014;124(3):294-300. doi:10.1254/jphs.13R14CP

11. Müller CP, Reichel M, Mühle C, Rhein C, Gulbins E, Kornhuber J. Brain membrane lipids in major depression and anxiety disorders. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2015;1851(8):1052-1065. doi:10.1016/j.bbalip.2014.12.014

12. Bazan NG. Docosanoids and elovanoids from omega-3 fatty acids are pro-homeostatic modulators of inflammatory responses, cell damage and neuroprotection. *Mol Aspects Med*. 2018;64(September):18-33. doi:10.1016/j.mam.2018.09.003

13. Zhang M, Wang S, Mao L, Leak R K, Shi Y, Zhang W, Hu X, Sun B, Cao G, Gao Y, Xu Y, Chen J, Feng Zhang F.Omega-3 Fatty Acids Protect the Brain against Ischemic Injury by Activating Nrf2 and Upregulating Heme Oxygenase 1. *J*

Neurosci. 2014;34(5):1903-1915. doi:10.1523/JNEUROSCI.4043-13.2014

14. Zhu W, Ding Y, Kong W, Li T, Chen H. Docosahexaenoic Acid (DHA) Provides Neuroprotection in Traumatic Brain Injury Models via Activating Nrf2-ARE Signaling. *Inflammation*. 2018;41(4):1182-1193. doi:10.1007/s10753-018-0765-z
15. Duda MK, O'Shea KM, Stanley WC. ω-3 polyunsaturated fatty acid supplementation for the treatment of heart failure: Mechanisms and clinical potential. *Cardiovasc Res*. 2009;84(1):33-41. doi:10.1093/cvr/cvp169
16. Sakamoto A, Saotome M, Iguchi K, Maekawa Y. Marine-Derived Omega-3 Polyunsaturated Fatty Acids and Heart Failure: Current Understanding for Basic to Clinical Relevance. *Int J Mol Sci*. 2019;20(16):4025. doi:10.3390/ijms20164025
17. Calzada C, Colas R, Guillot N, Guichardant M, Laville M, Véricel E, Lagarde M. Subgram daily supplementation with docosahexaenoic acid protects low-density lipoproteins from oxidation in healthy men. *Atherosclerosis*. 2010;208(2):467-472. doi:10.1016/j.atherosclerosis.2009.07.049
18. Granger MW, Liu H, Fowler CF. Distinct disruptions in Land's cycle remodeling of glycerophosphocholines in murine cortex mark symptomatic onset and progression in two Alzheimer's disease mouse models. *J Neurochem*. 2019;149(4):499-517. doi:10.1111/jnc.14560
19. SUN GY, MacQUARRIE RA. Deacylation-Reacylation of Arachidonoyl Groups in Cerebral Phospholipids. *Ann N Y Acad Sci*. 1989;559(1 Arachidonie A):37-55. doi:10.1111/j.1749-6632.1989.tb22597.x
20. Sun GY, Chuang DY, Zong Y, Jiang J, Lee JCM, Gu Z, Simonyi A Role of Cytosolic Phospholipase A2in Oxidative and Inflammatory Signaling Pathways in

Different Cell Types in the Central Nervous System. *Mol Neurobiol*. 2014;50(1):6-14. doi:10.1007/s12035-014-8662-4

21. Yang B, Fritsche KL, Beversdorf DQ, Gu Z, Lee ZJC, Folk WR, Greenlief CM, Sun GY. Yin-yang mechanisms regulating lipid peroxidation of docosahexaenoic acid and arachidonic acid in the central nervous system. *Front Neurol*. 2019;10(JUN):1-14. doi:10.3389/fneur.2019.00642

22. Bazan NG. Docosanoids and elovanoids from omega-3 fatty acids are pro-homeostatic modulators of inflammatory responses, cell damage and neuroprotection. *Mol Aspects Med*. 2018;64:18-33. doi:10.1016/j.mam.2018.09.003

23. Serhan CN, Chiang N, Dalli J. The resolution code of acute inflammation: Novel pro-resolving lipid mediators in resolution. *Semin Immunol*. 2015;27(3):200-215. doi:10.1016/j.smim.2015.03.004

24. Long EK, Picklo MJ. Trans-4-hydroxy-2-hexenal, a product of n-3 fatty acid peroxidation: Make some room HNE... *Free Radic Biol Med*. 2010;49(1):1-8. doi:10.1016/j.freeradbiomed.2010.03.015

25. Cohen G, Riahi Y, Sunda V, Deplano S, Chatgilialoglu C, Ferreri C, Kaiser N, Sasson S. Signaling properties of 4-hydroxyalkenals formed by lipid peroxidation in diabetes. *Free Radic Biol Med*. 2013;65:978-987. doi:10.1016/j.freeradbiomed.2013.08.163

26. Nakagawa F, Morino K, Ugi S, et al. 4-Hydroxy hexenal derived from dietary n-3 polyunsaturated fatty acids induces anti-oxidative enzyme heme oxygenase-1 in multiple organs. *Biochem Biophys Res Commun*. 2014;443(3):991-996.

doi:10.1016/j.bbrc.2013.12.085

27. Yang B, Li R, Michael Greenlief C, Fritsche KL, Gu Z, Cui J, Lee JC, Beversdorf DQ & Sun GY. Unveiling anti-oxidative and anti-inflammatory effects of docosahexaenoic acid and its lipid peroxidation product on lipopolysaccharide-stimulated BV-2 microglial cells. *J Neuroinflammation*. 2018;15(1):202. doi:10.1186/s12974-018-1232-3

28. Lee WC, Wong HY, Chai YY, WeiShi C, Amino N, Kikuchi S, HaiHuang S Lipid peroxidation dysregulation in ischemic stroke: Plasma 4-HNE as a potential biomarker? *Biochem Biophys Res Commun*. 2012;425(4):842-847. doi:10.1016/j.bbrc.2012.08.002

29. Ungerer G, Cui J, Ndam T, et al. Harpagophytum procumbens Extract Ameliorates Allodynia and Modulates Oxidative and Antioxidant Stress Pathways in a Rat Model of Spinal Cord Injury. *NeuroMolecular Med*. Published online 2020. doi:10.1007/s12017-019-08585-z

30. Zhang M, Wang S, Mao L, et al. Omega-3 fatty acids protect the brain against ischemic injury by activating Nrf2 and upregulating heme oxygenase 1. *J Neurosci*. 2014;34(5):1903-1915. doi:10.1523/JNEUROSCI.4043-13.2014

31. Yang B, Li R, Woo T, et al. Maternal dietary docosahexaenoic acid alters lipid peroxidation products and (N-3)/(n-6) fatty acid balance in offspring mice. *Metabolites*. 2019;9(3). doi:10.3390/metabo9030040

32. Matsui F, Hecht P, Yoshimoto K, et al. DHA Mitigates Autistic Behaviors Accompanied by Dopaminergic Change in a Gene/Prenatal Stress Mouse Model. *Neuroscience*. 2018;371:407-419. doi:10.1016/j.neuroscience.2017.12.029

33. Domenichiello AF, Kitson AP, Bazinet RP. Is docosahexaenoic acid synthesis from α-linolenic acid sufficient to supply the adult brain? *Prog Lipid Res*. 2015;59:54-66. doi:10.1016/j.plipres.2015.04.002

34. Matsui F, Hecht P, Yoshimoto K, et al. DHA Mitigates Autistic Behaviors Accompanied by Dopaminergic Change in a Gene/Prenatal Stress Mouse Model. *Neuroscience*. 2018;371:407-419. doi:10.1016/j.neuroscience.2017.12.029

35. Yang B, Li R, Woo T, et al. Maternal dietary docosahexaenoic acid alters lipid peroxidation products and (N-3)/(n-6) fatty acid balance in offspring mice. *Metabolites*. 2019;9(3):1-14. doi:10.3390/metabo9030040

36. Sun G, Li R, Yang B, et al. Quercetin Potentiates Docosahexaenoic Acid to Suppress Lipopolysaccharide-induced Oxidative/Inflammatory Responses, Alter Lipid Peroxidation Products, and Enhance the Adaptive Stress Pathways in BV-2 Microglial Cells. *Int J Mol Sci*. 2019;20(4):932. doi:10.3390/ijms20040932

37. Wang M, Wang C, Han X. Selection of internal standards for accurate quantification of complex lipid species in biological extracts by electrospray ionization mass spectrometry—What, how and why? *Mass Spectrom Rev*. 2017;36(6):693-714. doi:10.1002/mas.21492

38. Wang M, Han X. Multidimensional mass spectrometry-based shotgun lipidomics. *Methods Mol Biol*. 2014;1198:203-220. doi:10.1007/978-1-4939-1258-2_13

39. Han X, Yang K, Gross RW. Microfluidics-based electrospray ionization enhances the intrasource separation of lipid classes and extends identification of individual molecular species through multi-dimensional mass spectrometry: Development of an automated high-throughput platform for shotgun lipidomics. *Rapid Commun*

Mass Spectrom. 2008;22(13):2115-2124. doi:10.1002/rcm.3595

40. Yang K, Cheng H, Gross RW, Han X. Automated lipid identification and quantification by multidimensional mass spectrometry-based shotgun lipidomics. *Anal Chem*. 2009;81(11):4356-4368. doi:10.1021/ac900241u

41. Barceló-Coblijn G, Collison LW, Jolly CA, Murphy EJ. Dietary α-linolenic acid increases brain but not heart and liver docosahexaenoic acid levels. *Lipids*. 2005;40(8):787-798. doi:10.1007/s11745-005-1440-y

42. Yang B, Fritsche KL, Beversdorf DQ, et al. Ying-yang mechanisms regulating lipid peroxidation of docosahexaenoic acid and arachidonic acid in the central nervous system. *Front Neurol*. 2019;10(JUN):642. doi:10.3389/fneur.2019.00642

43. Yang B, Li R, Greenlief CM, et al. Unveiling anti-oxidative and anti-inflammatory effects of docosahexaenoic acid and its lipid peroxidation product on lipopolysaccharide-stimulated BV-2 microglial cells. *J Neuroinflammation*. 2018;15(1):1-16. doi:10.1186/s12974-018-1232-3

44. Geng X, Yang B, Li R, et al. Effects of Docosahexaenoic Acid and Its Peroxidation Product on Amyloid-β Peptide-Stimulated Microglia. Published online 2035. doi:10.1007/s12035-019-01805-4

45. Ishikado A, Nishio Y, Morino K, et al. Low concentration of 4-hydroxy hexenal increases heme oxygenase-1 expression through activation of Nrf2 and antioxidative activity in vascular endothelial cells. *Biochem Biophys Res Commun*. 2010;402(1):99-104. doi:10.1016/j.bbrc.2010.09.124

46. Patrick CB, McHowat J, Rosenberger TA, Rapoport SI, Murphy EJ. Arachidonic acid incorporation and turnover is decreased in sympathetically denervated rat

heart. *Am J Physiol Circ Physiol*. 2005;288(6):H2611-H2619. doi:10.1152/ajpheart.00549.2004

47. Bascoul-Colombo C, Guschina IA, Maskrey BH, Good M, O'Donnell VB, Harwood JL. Dietary DHA supplementation causes selective changes in phospholipids from different brain regions in both wild type mice and the Tg2576 mouse model of Alzheimer's disease. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2016;1861(6):524-537. doi:10.1016/j.bbalip.2016.03.005

48. Lamaziere A, Richard D, Barbe U, et al. Differential distribution of DHA-phospholipids in rat brain after feeding: A lipidomic approach. *Prostaglandins Leukot Essent Fat Acids*. 2011;84(1-2):7-11. doi:10.1016/j.plefa.2010.11.001

49. Rosenberger TA, Oki J, Purdon AD, Rapoport SI, Murphy EJ. *Rapid Synthesis and Turnover of Brain Microsomal Ether Phospholipids in the Adult Rat Microsome*. Vol 43.; 2002. Accessed November 20, 2020. www.jlr.org

50. Su XQ, Wang J, Sinclair AJ. Plasmalogens and Alzheimer's disease: A review. *Lipids Health Dis*. 2019;18(1). doi:10.1186/s12944-019-1044-1

51. Owen AJ, Peter-Przyborowska BA, Hoy AJ, McLennan PL. Dietary fish oil dose- and time-response effects on cardiac phospholipid fatty acid composition. *Lipids*. 2004;39(10):955-961. doi:10.1007/s11745-004-1317-0

52. Oppedisano F, Macrì R, Gliozzi M, et al. The Anti-Inflammatory and Antioxidant Properties of n-3 PUFAs: Their Role in Cardiovascular Protection. *Biomedicines*. 2020;8(9):306. doi:10.3390/biomedicines8090306

53. Sun GY, Huang HM, Kelleher JA, Stubbs EB, Sun AY. Marker enzymes, phospholipids and acyl group composition of a somal plasma membrane fraction

isolated from rat cerebral cortex: a comparison with microsomes and synaptic plasma membranes. *Neurochem Int*. 1988;12(1):69-77. doi:10.1016/0197-0186(88)90150-7

54. Lin TA, Zhang JP, Sun GY. Metabolism of inositol 1,4,5-trisphosphate in mouse brain due to decapitation ischemic insult: effects of acute lithium administration and temporal relationship to diacylglycerols, free fatty acids and energy metabolites. *Brain Res*. 1993;606(2):200-206. doi:10.1016/0006-8993(93)90985-V

55. Varga K, Jiang Z-J, Gong L. Phosphatidylserine is critical for vesicle fission during clathrin-mediated endocytosis. *J Neurochem*. 2020;152(1):48-60. doi:10.1111/jnc.14886

56. Madison Sullivan E, Pennington ER, Sparagna GC, Torres MJ, Neufer PD, Harris M, Washington J, Anderson EJ, Zeczycki TN, Brown DA, Shaikh SR.. Docosahexaenoic acid lowers cardiac mitochondrial enzyme activity by replacing linoleic acid in the phospholipidome. *J Biol Chem*. 2018;293(2):466-483. doi:10.1074/jbc.M117.812834

CHAPTER 4: Effect of maternal dietary DHA in prenatal gene/stress mouse model on lipid peroxidation activity and autistic-like behaviors in offspring

4.1 Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by restricted social communication and repetitive interests and behaviors.[1,2,3,4,5] Autism is among the most enigmatic disorders of child development, with a prevalence of about 1 in 54 among children in the United States according to estimates from the CDC.[6] Globally, the WHO estimates that 1 in 160 children has ASD.[7] Annual costs of autism have increased to $126 billion in the U.S. and £34 billion in the U.K in 2012.[2,8] Numerous studies have associated prenatal maternal stress to a risk of neurodevelopmental disorders in the offspring[9,10,11,12,13,14,15] A study by Breen et al.[16] indicate that prenatal exposure to maternal psychological distress induces neuronal, immunological and behavioral abnormalities in affected offspring. Several studies have also suggested that prenatal stress is a possible risk factor in the development of autism spectrum disorders.[17,18,19,20,21] However, many children exposed to stress prenatally are born healthy and develop normally, suggesting that other factors must contribute to autism.[22] Genes that contribute to stress reactivity may, therefore, exacerbate prenatal stress-mediated behavioral changes in offspring. One candidate gene linked to increased stress reactivity is the serotonin transporter gene.[23,24,25,] In humans, the short allele polymorphism of the serotonin transporter gene is linked to ASD in some studies. [24,26] In animal models, the heterozygous serotonin transporter (SERT) knockout mouse with reduced serotonin uptake activity and transporter binding availability[27] is similar to that seen in the human short allele carriers. In an earlier study by Beversdorf and co-workers,

they looked at the combined effect of maternal serotonin transporter genotype and prenatal stress in modulating offspring social interaction in mice.[1] Data from this study indicated a possible combined effect of maternal serotonin transporter genotype and prenatal stress contributing to the production of autistic-like behavior in offspring. In another study, the Beversdorf group examined a maternal genetic variation in the promoter region of the serotonin transporter gene (5-HTTLPR) affecting stress tolerance and its interaction with the effect of environmental stressors on ASD risk.[28]

Docosahexaenoic acid (DHA, C22:6, n-3), is an omega-3 polyunsaturated fatty acid (PUFA) enriched in membrane phospholipids in the brain.[29] Recent studies demonstrated pleiotropic properties[30] of DHA with diverse health effects.[31,32,33] While DHA is important for adequate brain development and cognition,[34,35, 36] its deficiency is associated with impaired visual, attention and cognition deficits, psychiatric symptoms precipitation and increased vulnerability to neuronal atrophy.[37,38,39,40,41] Other studies have found that DHA may have an anti-stress function[42,43,44] and can be converted to oxylipins, which regulate cell redox homeostasis and contribute to anti-oxidant pathways.[45] A study by Tang et. al[46] examining the mental health of weaning female rats found that altered dopamine or norepinephrine transmission in the brain may be a key neuronal mechanism that contributes to the potential detrimental effects of maternal dietary n-3 PUFAs deficiency. While some studies[47,48] claimed ω-3 long chain polyunsaturated fatty acid supplementation improved social interaction and repetitive and restricted interests and behaviors, outcomes from other studies[49,50,51,52,53,54] are inconclusive or suggest ω-3 PUFA's supplementation has no effect on ASD. An earlier study by the Beversdorf group indicated that autism associated behaviors and changes in

the dopaminergic system in offspring mice can be mitigated through maternal DHA supplementation.[48] In a related study by Feng et al.,[55] they found that maternal feeding of DHA exerts preventive effects on prenatal stress-induced brain dysfunction and that modulation of mitochondrial metabolism may play a critical role in DHA protection. A study by Gao et al.[56] showed that maternal DHA supplementation protected rat offspring against learning and memory impairment following prenatal exposure to valproic acid. However, a study by Kunio et al.[57] also suggested that supplementation with larger arachidonic acid (ARA) doses added to DHA improved social interaction in individuals with ASD by up-regulating signal transduction. The apparent Yin-Yang mechanism[58] demonstrated that the metabolism of DHA and ARA, via enzymatic and non-enzymatic pathways, yields 4-hydoxyhexenal (4-HHE) and 4-hydroxynonenal (4-HNE), respectively.[59] Our recent study[60] showed that a maternal DHA-enriched diet alters the (n-3)/(n-6) fatty acid balance and 4-HHE levels in the brain as well as in the heart of offspring mice. A follow up study[61] with a DHA-enriched diet in adult mice showed substantial 4-HHE increases in brain, heart and plasma ($p < 0.05$), with no significant change in 4-HNE levels. However, the role of maternal dietary DHA in a gene/stress mouse model, and how it may affect lipid peroxidation activity and autistic-like behaviors in offspring, is unknown.

In this study, pregnant heterozygous serotonin transporter knockout-SERT-KO (HT) and wild-type (WT) mice were placed in either a non-stressed control condition or a chronic variable stress condition and fed either a control diet or a DHA-rich (1% by wt) diet. Male offspring from each group were assessed for anxiety and autism-associated behavior at post-natal day 60, including an open field test, elevated-plus maze test,

repetitive behavior, and the 3-chamber social approach test. Our LC-MS-based method was used to follow changes in peroxidation product concentrations in mouse plasma, heart, and cerebral cortex. Investigations were carried out to ascertain whether prenatal supplementation of DHA alleviates autistic-like behaviors in a gene/stress mouse model and whether it alters lipid peroxidation activity in the brain.

4.2 Experimental

4.2.1 Chemicals and reagents

4-Hydroxyhexenal (4-HHE, 1 mg in 100 μL of ethanol), 4-hydroxynonenal (4-HNE, 1 mg in 100 μL of ethanol), and 4-hydroxy hexenal-d3 (4-HHE-d3, 100 μg in 100 μL of methyl acetate) were purchased from Cayman Chemical Co. (Ann Arbor, MI). 1,3-Cyclohexanedione (CHD, 97%), ammonium acetate (HPLC grade), acetic acid (ACS grade) and formic acid (mass spectrometry grade), were purchased from Sigma-Aldrich (St. Louis, MO). C18 Sep-Pak cartridges (1 mL, 100 mg) were obtained from Waters Corporation (Milford, MA). Phospholipid removal cartridges (PhreeTM, 1 mL) were purchased from Phenomenex Inc. (Torrance, CA). All solvents (HPLC grade) used for sample preparation, UHPLC, and MS analysis were obtained from Thermo Fisher Scientific Inc. (Fair Lawn, NJ)

4.2.2 Animals, diets, stress, experimental groups

4.2.2.1 Animals

Male homozygous serotonin transporter (SERT) knock-out (KO) mice with a C57BL6/J background and male/female C57BL6/J mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). From these mice, heterozygous SERT KO female (HT) dams and wild-type (WT) dams were generated in our laboratory. Eight-

week-old experimental dams were paired with WT males and inspected for a vaginal plug the following morning. Identification of a plug was designated as gestational day 0. Additionally, Hsd:NSA(CF-1) male and female mice were purchased from Jackson Laboratories and mated to give birth to induce lactation and maternal behavior (foster dams). Within 24 hours of experimental offspring birth, pups from foster dams were removed and replaced with experimental offspring. All pups were weaned on postnatal day (PD) 21 and sacrificed around post-natal day 70 for subsequent brain lipid analysis. Animals were maintained in a temperature- and humidity-controlled room at 25 ± 2°C on a 12-h light/dark cycle with food and water available *ad libitum*. All animals were housed in clear carbonate cages provided with aspen shaving bedding. All procedures were in accord with protocols approved by the University of Missouri Institutional Animal Care and Use Committee.

4.2.2.2 Diet

Two weeks prior to mating, all experimental and foster dams were randomly assigned to one of two dietary groups, control diet (AIN-93G, #103619, Dyets Inc., Bethlehem, PA) and 1% DHA diet (#103598, Dyets Inc., Bethlehem, PA), which they remained on throughout gestation, lactation, and offspring postweaning.

4.2.2.3 Gene/environment interaction

To generate gene/environment interaction mouse models and the effects of dietary DHA supplement, experimental dams were randomly assigned to one of eight conditions represented in a 2 × 2 × 2 design of genotype × prenatal stress × diet: WT/non-stressed/AIN-93G (WNA), WT/non-stressed/DHA diet (WND), WT/stressed/AIN-93G diet (WSA), WT/stressed/DHA diet (WSD), HT/non-stressed/AIN-93G (HNA), HT/non-

stressed/DHA (HND), HT/stressed/AIN-93G (HAS), and HT/stressed/DHA (HSD). The offspring from each experimental dam was also described by the same group name.

4.2.2.4 Prenatal chronic variable stress

Chronic variable stress (CVS) was administered on gestational day 6 through birth for all mice in the prenatal stress groups. Stressors included: constant light exposure (36 hours), exposure to fox urine (1 hour), overnight exposure to novel objects (marbles) in the home cage, restraint (10 minutes), overnight exposure to novel noise (radio static), and multiple cage changes during the light cycle. Each of these stressors were presented in succession over 6 days, with one stressor presented per day, and this pattern was repeated 2.5 times. Stressors were chosen on the basis that they do not cause pain and have minimal influence on food intake and weight gain.

In summary, we have 8 groups, 8 mice per group (4 males & females) as shown in Table 4.1. The focus of this study is on the male mice, so we have 8 groups, 4 mice per group (males), left cerebral cortex, plasma, and heart. The numbers in parenthesis represents the dams, while the other value represents the number of offspring.

Table 4.1. Summary of experimental groups

	Stressed DHA HT (HSD)		**Stressed AIN HT (HAS)**		**Stressed DHA WT (WSD)**		**Stressed AIN WT (WSA)**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Behavior	14 (7)	14(8)	18 (7)	15(6)	6 (3)	5(3)	10 (5)	11(6)
Brain & Tissue	4 (3)	4(3)	4 (2)	4(2)	4 (3)	4(2)	4 (3)	4(3)
	Non-stressed DHA HT (HND)		**Non-stressed AIN HT (HNA)**		**Non-stressed DHA WT (WND)**		**Non-stressed AIN WT (WNA)**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Behavior	17 (7)	12(7)	8 (5)	8(5)	10 (4)	7(4)	6 (4)	6(4)
Brain & Tissue	4 (4)	4(4)	4 (3)	4(3)	4 (4)	4(3)	4 (4)	4(4)

4.2.3 Behavioral assays/studies

This part of the study was made by the Beversdorf group in the Departments of Radiology and Psychological Sciences.

4.2.3.1 Open field/repetitive behavior

Mice were placed in an open arena and allowed to explore for a total of 20 minutes. Grooming behavior was measured manually through open field video recording.

4.2.3.2 Elevated plus maze

The mice were placed in an elevated plus-maze (EPM) that has two open and two closed arms for a duration of 10 minutes.

4.2.3.3 Three (3)-chamber social approach task

A 9" × 21" box is evenly divided by Plexiglass to form 3 chambers. The partitions have removable openings that allow experimental mice to freely explore all chambers. Prior to the task, experimental mice are habituated to the central chamber for 10 minutes. In the task, one stranger mouse is randomly assigned to a non-central chamber, while an empty, identical small wire cage is placed in the remaining non-central chamber. The experimental mouse is allowed to freely explore the 3 chambers for 10 minutes as shown in Figure 4.1.[62]

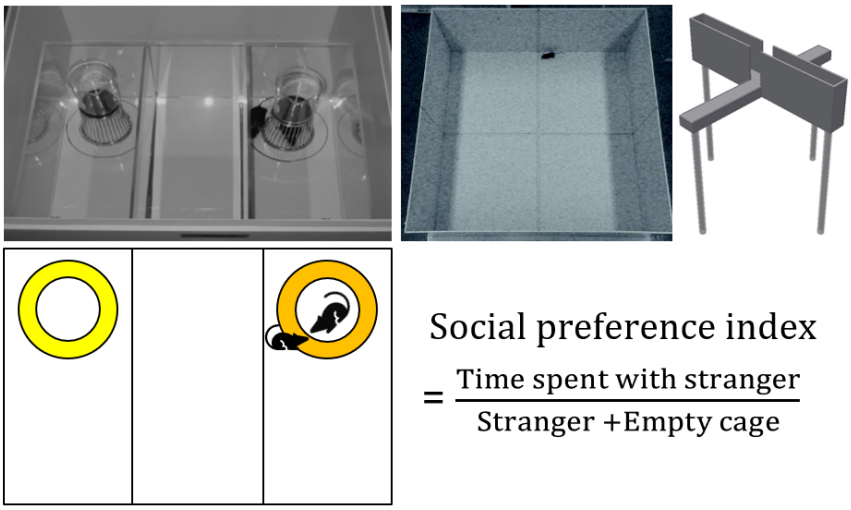

Figure 4.1. Social Preference index (time with stranger)/(time with stranger + time with empty cage) was calculated for the social interaction aspect [62].

4.2.4 LC-MS/MS analysis of 4-HHE and 4-HNE

Determination of 4-HHE and 4-HNE by LC-MS/MS analysis was performed as described by Yang et al.[60] Briefly, blood samples were collected with heparin treated syringes by cardiac puncture following CO_2 asphyxiation. Samples were immediately centrifuged at 1,500 rpm for 5-8 min and supernatant frozen at -80°C prior to analysis. The left cerebral cortex and heart were weighed and homogenized in double distilled water using a weight to water ratio of 1:8 (wt/vol) for 5 mins in a bullet blender (Next Advance, Inc., Averill Park, NY). The heart tissue was first pulverized in liquid nitrogen prior to homogenization, the homogenates were centrifuged at 17,000 rpm for 20 min at 4°C, and the supernatant was collected and stored at -80 °C prior to use. Total protein concentration in the supernatant was determined using the Bicinchoninic Acid Assay (Sigma-Fisher, St. Louis, MO).

The sample preparation stage involved three main steps: phospholipids removal, derivatization, and desalting as previously described.[59,60] Briefly, an aliquot of the sample

was added to an equal volume of internal standard (4-HHE-d3), and acetonitrile containing 1% formic acid was added to the mixture to elute the analyte of interest. Phospholipids were removed using solid-phase extraction (SPE) and was performed using a 1 mL Phree^{TM} cartridge. The clear eluate was dried under a study stream of nitrogen and derivatized by adding freshly prepared acidified 1,3-cyclohexanedione reagent. The mixture was incubated at 60°C for 1 h and then cooled on ice prior to desalting using a C18 SPE cartridge. The derivatization reaction is shown in Figure 4.2. The C18 cartridge was preconditioned with methanol (0.7 mL) and equilibrated with water (0.7 mL). The cooled derivatized mixture was loaded onto the cartridge, washed twice with water (0.7 mL) and followed by 5% acetonitrile in water (0.7 mL). The analytes of interest (4-HHE, 4-HNE, and 4-HHE-d3 derivatives) were eluted with 100% acetonitrile (0.7 mL) and dried with a steady stream of nitrogen gas. The samples were reconstituted in 40% methanol in water with 0.1% formic acid (300 µL) and were ready for LC-MS/MS analysis.

A Waters Acquity UHPLC system equipped with a quaternary solvent manager was used in conjunction with a C18 reversed phase column, Luna Omega 100Å (1.6 µm × 50 × 2.1 mm, Phenomenex). A previously developed and validated UHPLCMS/ MS method[60] with a 7 min LC gradient including equilibration was used for the separation and detection of the 4-HHE and 4-HNE derivatives. Water containing 0.1% formic acid (solution A) and methanol containing 0.1% formic acid (solution B) were used as mobile phases. The solvent gradient was 60% A 40% B at 0 min, 60% A 40% B at 0.25 min, 10% A 90% B at 3.55 min, 10% A 90% B at 3.80 min, 60% A 40% B at 3.90 min, and 60% A 40% B at 7.00 min. The flow rate was 0.3 mL/min. A Waters Xevo TQ-S triple

quadrupole mass spectrometer with an electrospray ionization source operated in the positive-ion mode was used. Multiple reaction monitoring transition m/z 326.3 -> 216.1 Da, m/z 284.2 -> 216.1 Da and 287.2 -> 216.1 Da were chosen for simultaneous monitoring of 4-HNE, 4- HHE and 4- HHE-d3 (internal standard) derivatives, respectively. Compound-dependent parameters in MS/MS analysis are shown in Table 4.2.

Table 4.2. Compound-dependent parameters in MS/MS analysis

Compound	Dwell time (ms)	Q1	Q3	Cone voltage (V)	Collision energy (eV)
4-HHE	25	284.2	216.1	22	25
4-HNE	25	326.3	216.1	20	23
4-HHE-d_3	25	287.2	216.1	14	23

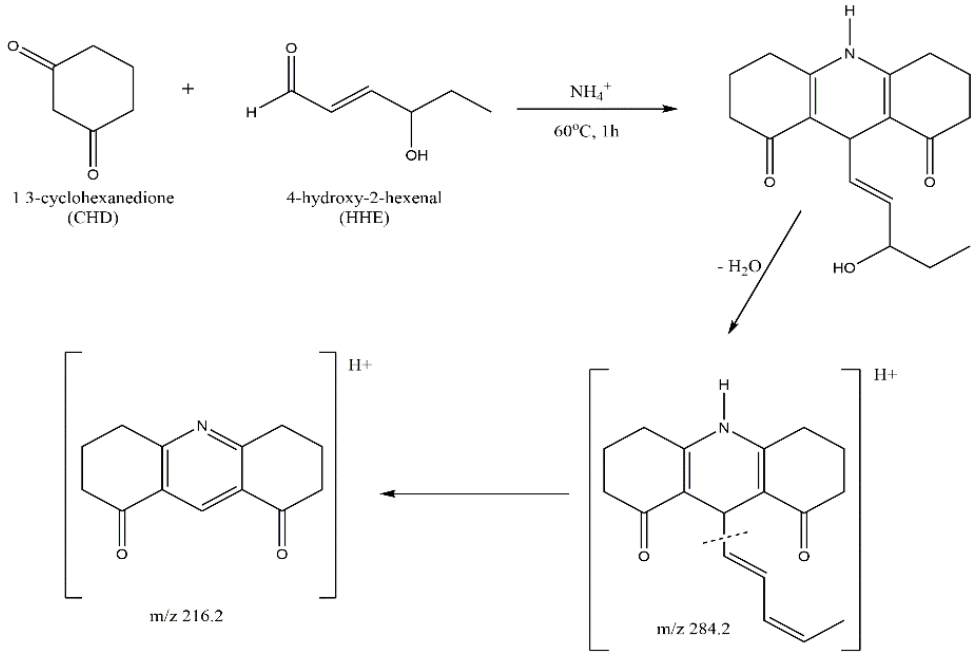

Figure 4.2. Reaction showing the derivatization of 4-HHE from freshly prepared acidified CHD reagent and the formation of product ions (m/z 216.2) from pseudo precursor ions (m/z 248.2).

4.2.5 Statistical analysis

For the determination of 4-HHE and 4-HNE levels via LC-MS/MS, samples were prepared in three biological replicates and three analytical replicates for each sample. Statistical analyses were performed with GraphPad Prism (version 8.3; GraphPad Prism Software Inc., San Diego, CA). Results are expressed as the mean ± standard error of mean (SEM). Depending on the experiments, samples were analyzed by non-parametric t-tests or one-way ANOVA to compare among control and DHA diet groups. Differences were considered significant at $p < 0.05$ for all analyses.

4.3 Results

4.3.1 4-HHE and 4-HNE analysis

Levels of 4-HHE and 4-HNE were determined in heart, plasma and left cerebral cortex for offspring of heterozygous serotonin transporter knockout-SERT-KO (HT) and wild-type (WT) dams placed in either a non-stressed control condition or a chronic variable stress condition and fed either a control diet or a DHA-rich (1% by wt) diet.

Results were obtained for the heart tissues. All values are reported as the mean ± SEM in units of ng/g. The values measured showed that there were no apparent changes in 4-HHE levels between the control and 1% DHA diet conditions for both stressed (2879.2 ± 2.5; 2876.5 ± 2.5) and non-stressed (2882.3 ± 2.5; 2880 ± 2.3) WT, respectively (Figure 4.3A). A similar trend was observed for the stressed (2878.4 ± 1.9; 2882.3 ± 4.6) and non-stressed (2880.9 ± 2.1; 2882.7 ± 2.7) HT groups (Figure 4.3A). The levels of 4-HHE determined for the control and 1% DHA diet groups were high and similar for WT and HT for both the stress and non-stress conditions (Figure 4.3A).

Nonetheless, the DHA-enriched diet resulted in about 1.6-fold increase in 4-HNE ($p < 0.05$) for non-stressed (43.1 ± 11.5; 70.1 ± 14.2) WT with a slight increase for the stressed (56.3 ± 9.9; 60.1 ± 16.9) WT group as compared to the control diet group (Figure 4.3A). The DHA-enriched diet did not alter the 4-HNE levels measured for both the DHA enriched and control diet groups in HT for both stress (54.1 ± 9.6; 54.1 ± 8.1) and non-stressed (72.9 ± 19; 75.2 ± 18.2) conditions (Figure 4.3A).

The ratios of 4-HHE/4-HNE showed an apparent increase in the dietary DHA supplement groups for stress and non-stress HT animals. There is significant decrease (about 2-fold) for the WT mice in non-stress conditions compared to the control group with a 1% DHA diet (Figure 4.3B). Moreover, supplementation with the DHA-enriched diet in stress WT mice remained unchanged compared to the control diet (Figure 4.3B).

The levels of 4-HNE in plasma samples showed significant changes with no apparent changes in 4-HHE levels for control and 1% DHA diet respectively in (mean ±SEM) ng/mL. DHA-enriched diet resulted in significant increases in 4-HNE ($p<0.05$) for stressed (8.4±2.1; 12.1 ±13.5) and non-stressed (6.2±1.7; 8.7 ±1.7) HT mice with no significant changes in stressed (6.5±1.7; 7.4 ±1.7) and non-stressed (7.7±1.9; 7.4 ±1.4) WT groups compared to the control diet (Figure 4.4A).

Conversely, no changes in 4-HHE levels were observed for control and 1% DHA enriched diet groups, respectively, for both the stressed (359.0 ±0.2; 358.8 ±0.3) and non-stressed (360.3 ±0.5; 360.4±0.4) HT and stressed (359.3±0.5; 359.2±0.5) and non-stressed (360.7±0.4; 360.7 ±0.4) WT group (Figure 4.4A).

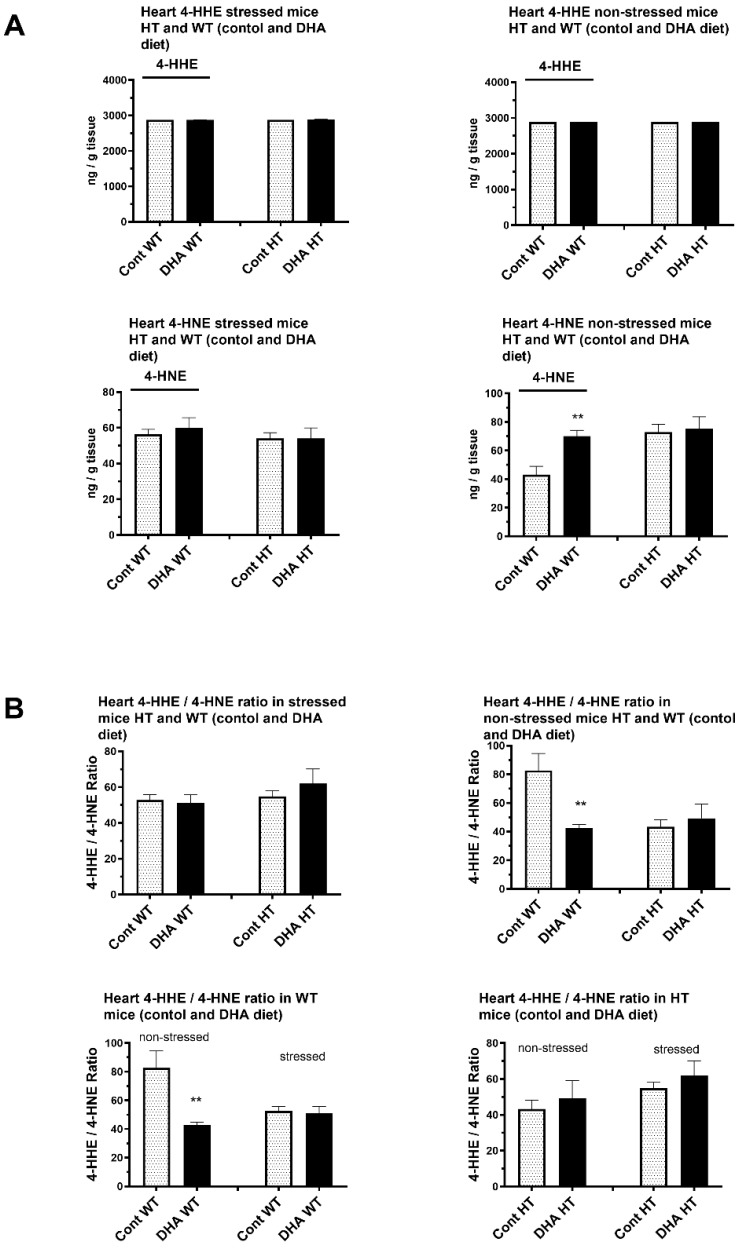

Figure 4.3. Levels of (A) 4-HHE, 4-HNE and (B) 4-HHE/4-HNE ratio of offspring ***heart*** from pregnant heterozygous serotonin transporter knockout-SERT-KO (HT) and wild-type (WT) dams placed in either non-stressed control condition or chronic variable stress condition and fed either control diet or DHA-rich (1% by wt.) diet. Procedures for processing the heart and protocol for LC-MS/MS determination of 4-HHE and 4-HNE are described in text. Data are normalized to tissue weight. Results represent the mean ± SEM of control (n = 4) and DHA (n = 4) samples. Analysis using two-tail unpaired t-test indicated significance between DHA group and controls. ** $p < 0.01$.

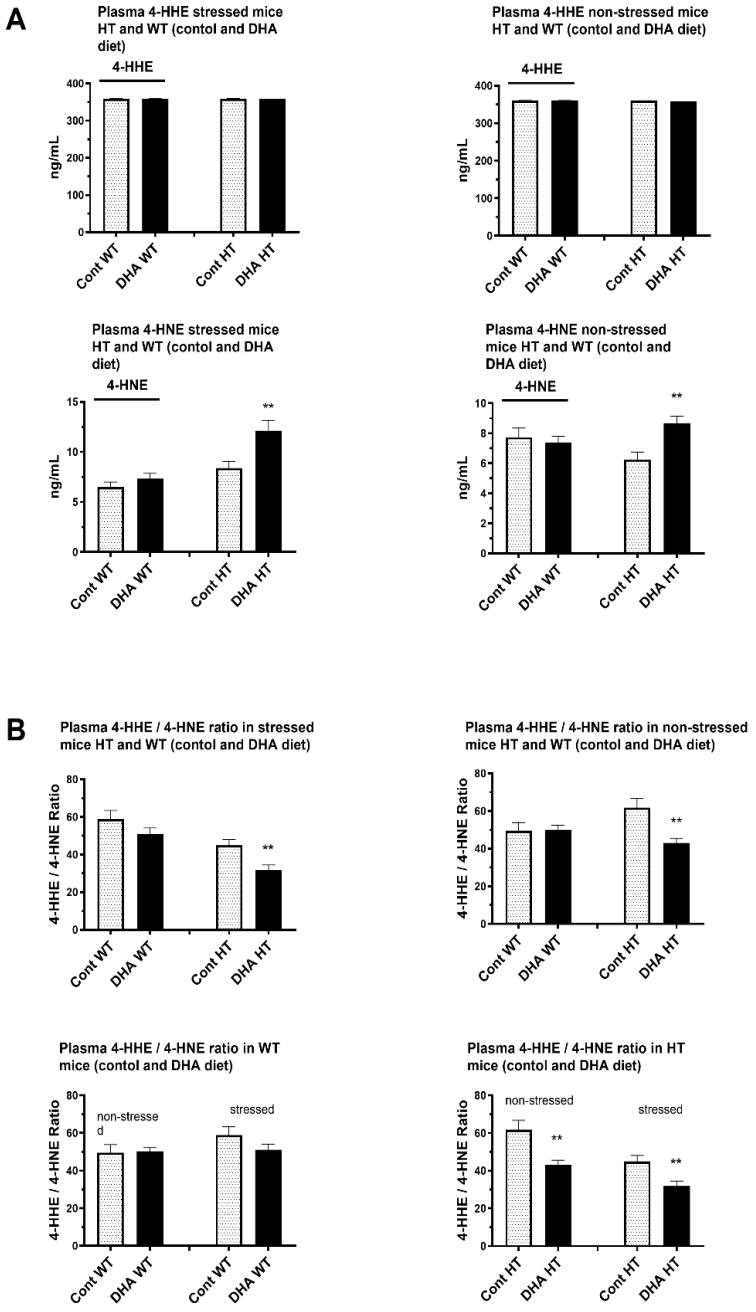

Figure 4.4. Levels of (A) 4-HHE, 4-HNE and (B) 4-HHE/4-HNE ratio of offspring ***plasma*** from pregnant heterozygous serotonin transporter knockout-SERT-KO (HT) and wild-type (WT) dams placed in either non-stressed control condition or chronic variable stress condition and fed either control diet or DHA-rich (1% by wt.) diet. Procedures for processing the heart and protocol for LC-MS/MS determination of 4-HHE and 4-HNE are described in text. Data are normalized to tissue weight. Results represent the mean ± SEM of control (n = 4) and DHA (n = 4) samples. Analysis using two-tail unpaired t-test indicated significance between DHA group and controls. ** $p < 0.01$.

The ratios of 4-HHE/4-HNE exhibited a significant decrease in the DHA supplement stress (42.8±0.1; 29.5 ±0.1) group and the non-stress (57.6±0.3; 41.6 ±0.2) HT group. There are no significant changes between the stress (55.2±0.3; 48.7 ±0.3) and non- stress conditions (46.7±0.2; 48.8 ±0.3) WT groups from control to 1% DHA diet (Figure 4.4B).

Among the different brain regions, the left cortex was analyzed for 4-HHE and 4-HNE. The concentrations are reported as the (mean ±SEM) in units of ng/g for all samples. A significant increase in 4-HHE levels for the control and 1% DHA groups was observed for the stressed (259.9 ±57.0; 321.0 ±74.9) HT group with no apparent change in non-stressed (288.0 ±97.6; 278.4±68.1) HT group. No apparent change was recorded for the stressed (362.1±43.9; 343.2±34.9) WT group. The 4-HHE levels for DHA enriched diet for the non-stressed (394.8±28.0; 230.1 ± 57.0) WT group showed a significant decrease compared to control diet group (Figure 4.5A).

Supplementation with a DHA-enriched diet resulted in a significant decrease in 4-HNE ($p<0.05$) for the non-stressed (76.5±22.2; 53.2 ±20.9) WT group and no apparent change in 4-HNE for the stressed (55.9±23.2; 54.3 ±24.4) WT group (Figure 4.5A). On the other hand, the levels of 4-HNE increased the DHA enriched diet in stressed (53.5±18.0; 65.1 ±16.5) HT mice and a slight decrease was observed for the non-stressed (65.6±7.0; 59.0 ±12.3) HT mice (Figure 4.5A).

No significant changes in the 4-HHE/4-HNE ratios were measured. A slight increase in the ratio for the dietary DHA supplement in the non-stress (4.3±1.3; 4.9 ±1.7) HT group was observed with a slight decrease in stress (5.1±0.9; 4.9 ±0.6) HT group (Figure 4.5B). A slight decrease was observed for the DHA-enriched diet in the stressed (7.0±2.1; 6.7 ±3.5) group and non-stressed (5.3±2.0; 4.9 ±2.1) WT group (Figure 4.5B).

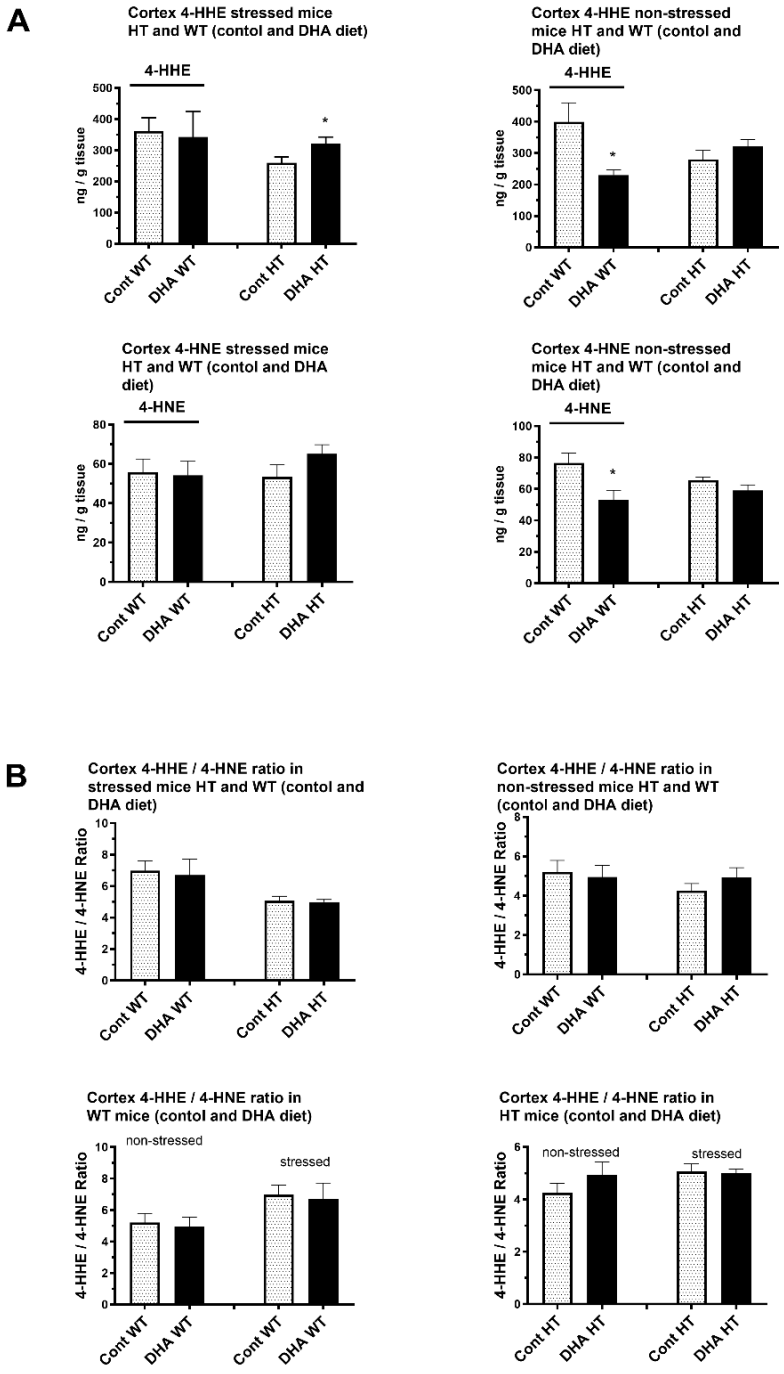

Figure 4.5. Levels of (A) 4-HHE, 4-HNE and (B) 4-HHE/4-HNE ratio of offspring ***left cortex*** from pregnant heterozygous serotonin transporter knockout-SERT-KO (HT) and wild-type (WT) dams placed in either non-stressed control condition or chronic variable stress condition and fed either control diet or DHA-rich (1% by wt.) diet. Procedures for processing the heart and protocol for LC-MS/MS determination of 4-HHE and 4-HNE are described in text. Data are normalized to tissue weight. Results represent the mean ± SEM of control (n = 4) and DHA (n = 4) samples. Analysis using two-tail unpaired t-test

indicated significance between DHA group and controls. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.3.2 Behavioral studies

In this part of the study, the group investigated whether prenatal supplementation of DHA alleviates autistic-like behaviors by using a serotonin transporter haplo-insufficient mouse model, which is similar with genetically stress-susceptible humans and giving them a prenatal stress for the final two thirds their pregnancy. Offspring from stressed HT dams showed a significantly decreased social preference index and increased grooming duration compared to WT non-stressed dams. Maternal DHA diet alleviated reduced social interaction and mitigated increased repetitive behaviors in stressed HT offspring as shown in Figure 4.6. No significant difference in EPM suggested increased grooming behavior was not associated with anxiety as shown in Figure 4.7.

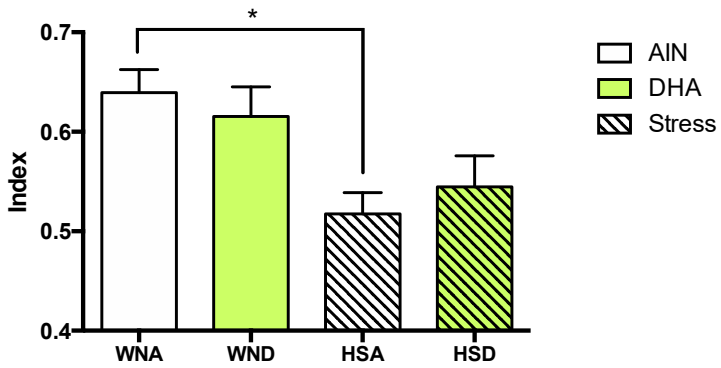

Figure 4.6. Three-chamber social approach task in male offspring. There was a significant decreased social preference index in HSA group compared to WNA. Values are means ± SEM. *p<0.05.

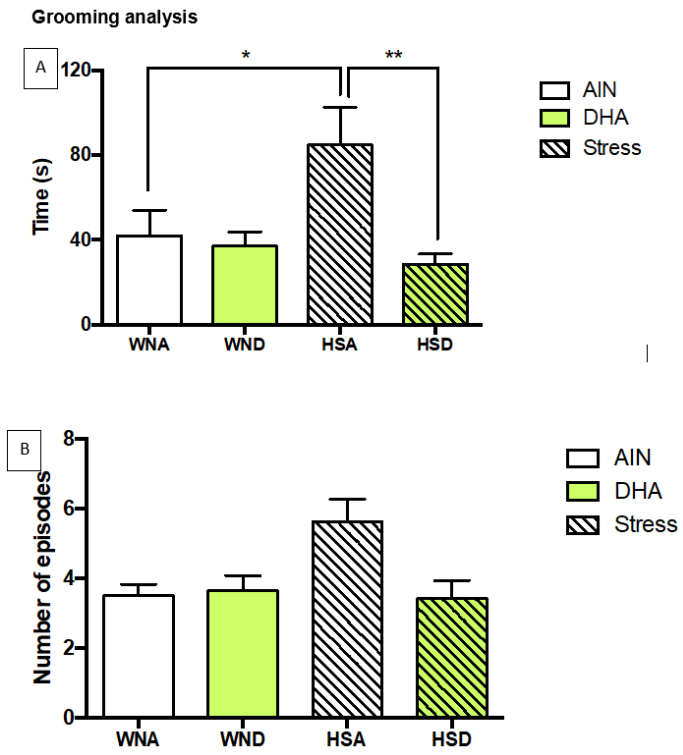

Figure 4.7. Total grooming time (A) and the number of episodes (B). HSA group showed significantly increased grooming time compared to WNA and HSD. Although there was no significant difference in number of total grooming episodes, HSA showed a trend toward a higher number. Values are means ± SEM. *p<0.05, **p<0.01.

4.4 Discussion

In the present study, pregnant heterozygous serotonin transporter knockout (SERT-KO) and wild-type (WT) dams were placed in either a non-stressed control condition or in a chronic variable stress condition and fed either a control diet or DHA-supplemented diet (1% by wt). A LC-MS/MS protocol[60] developed by the group was adapted and optimized for the determination of 4-HHE and 4-HNE levels in the left cerebral cortex of the brain, heart, and plasma of offspring. In agreement with previous studies by the group[60,61], endogenous levels of 4-HHE and 4-HNE are present in the left cerebral cortex as well as in the heart and plasma for all analyzed sample groups.

In contrast to the earlier study[60] where maternal DHA resulted in a large increases in the levels of 4-HHE (4.3 fold) in the heart tissues of offspring and about two-fold increase ($p < 0.05$) in the heart tissue of adult male mice[61], this current study showed no apparent changes in 4-HHE levels in heart tissue for a DHA-enriched diet and control diets for both the stressed and non-stressed conditions for HT and WT groups. The reason why the gene/non-stress condition also showed no variation in 4-HHE levels for DHA supplement diet is not known. A theoretical expectation was to at least observe some apparent increase in non-stressed DHA groups over stressed group but that was not the case for both WT and HT groups as no apparent differences were observed. In a recent review by De Crescenzo et al.[52], they looked at the impact of PUFAs on patient-important outcomes in children and adolescents with autism spectrum disorder. This study was based on randomized controlled trials (RCTs) comparing PUFAs versus placebo or a healthy diet for the treatment of ASD in children and adolescents. Results showed PUFAs were superior compared to placebo in reducing anxiety in individuals with ASD, but worsened quality of sleep compared to a healthy and were not better than placebo in reducing aggression, hyperactivity, adaptive functioning, irritability, restricted and repetitive interests and behaviors and communication. Findings from the De Crescenzo study[52] and other similar studies[49,50,51,53,54] were inconclusive or suggested that omega-3 PUFA's supplementation has no effect on ASD. In this study, although the 4-HNE showed no apparent variation for all heart tissues and group except for non-stress WT groups, these trends were consistent with earlier studies in heart tissues.[60,61] The reason why the non-stress WT groups showed significant increases in 4-HNE levels with no apparent change in control and dietary DHA supplement groups for stressed WT groups

is not known. The ratio of 4-HHE to 4-HNE did not show any clear trend for distinction between the stressed and non-stressed group for control and DHA supplement groups as HT showed similar increase for both stress and non-stress group. These results are not so conclusive to make any deduction as to the dynamics of 4-HHE/4-HNE ratios in heart tissues for gene/stress mice model. It should be noted that the length of time DHA is administered may make a difference. Our previous studies[60,61] in adult and offspring mice were short-term studies (3 weeks). The studies described in this Chapter examine a longer-term dose of DHA (2 months). The interplay between 4-HNE and 4-HHE levels has been observed in other long-term studies.[49,50,51,52,5354]

Results looking at the levels of 4-HHE in plasma showed no direct correlation with previous studies.[60,61] Maternal DHA supplementation saw about a two-fold increase in 4-HHE levels in offspring plasma[60] with similar levels recorded in the plasma of DHA dietary adult male mice.[61] Again, both of these studies looked at 4-HHE after three weeks of DHA supplementation. However, for both the stress and non-stress conditions with HT and WT groups, no apparent variations in 4-HHE were observed. These results showed that DHA supplementation had no effect on endogenous 4-HHE levels for both stress and non-stress conditions. Deducing from previous studies, an increase 4-HHE levels for non-stress group was expected compared to the stressed groups, but this was not the case. Levels of 4-HNE remained unchanged for non-stressed WT groups with a slight increase in stressed WT groups. This variations were consistent with observed variations in the plasma of DHA adult mice study.[61] The significant increase in 4-HNE ($p<0.05$) for stressed and non-stressed are not distinct enough to differentiate between stress and non-stress effect. The ratio of 4-HHE/4-HNE levels also showed a consistent significant

increase in dietary DHA supplementation groups compared to control groups for stress and non-stressed groups of HT group. An apparent increase was also observed in the stressed WT group for DHA supplement diet.

Although the cerebral cortex showed significant increase in 4-HHE levels for DHA enriched diet in stressed HT, results for the non-stressed conditions showed no apparent changes. This is interesting as an increase in 4-HHE levels for non-stressed instead of stressed HT conditions was expected. On the other hand, the HT stressed condition showed no changes in 4-HHE levels for a DHA supplement diet. Instead, a significant decrease in non-stressed conditions was observed. The reason for the apparent decrease in 4-HHE levels is not known. Levels for 4-HNE for the HT group however shows an interesting trend with an apparent decrease for non-stress conditions and increase in stressed conditions for dietary DHA supplementation. This was consistent with earlier studies for non-stressed groups.[60,61] The ratio of 4-HHE/4-HNE also showed an increase for DHA dietary supplement groups for non-stressed HT with no apparent changes for stressed groups. WT groups on the other hand showed a slight decrease in the ratio for DHA supplement groups for both stressed and non-stressed groups.

In our previous study, a prenatal diet in high ratio of omega-6 to omega-3 fatty acid decreased sociability of offspring.[63] Our previous work revealed that stress-reactive polymorphisms found on the serotonin transporter gene may interact with environmental stressors during the pregnancy to affect the risk for the development of ASD in the child.[64] Therefore, we hypothesize that a DHA-rich diet can mitigate the development of ASD-associated behaviors in offsprings which would be induced by prenatal stress exposure. Results from the behavioral study with dietary DHA appeared to mitigate the

repetitive behaviors in this model considering the fact that offspring from stressed HT dams showed a significantly decreased social preference index and increased grooming duration compared to WT non-stressed dams. Future studies are needed to determine whether maternal dietary DHA can reduce risk of autism in offspring of mothers exposed to stress during pregnancy in a clinical population, particularly for those who have genetic susceptibility to greater responses to stress.

Putting all these results together, although a clear link between determined levels of 4-HHE and 4-HNE levels from dietary DHA supplement and how it is impacted by a gene/stress mice model to influence conditions of ASD was not established due to lack of variability, a clear trend from the behavioral studies showed the tendency of dietary DHA to mitigate repetitive behaviors in this model. The lack of variability and clear trend of results from the lipid peroxidation activity may be due to the long supplementation period (70 days). Further work is however recommended to establish a clear trend and ascertain DHA impact on ASD. Analysis of phospholipids levels in future work is highly recommended to correlate these results and make conclusive deductions.

4.5 Conclusions

In summary, an adapted LC-MS method was successfully used to determine the concentrations of 4-HHE and 4-HNE in the plasma, heart, and left cortex for eight experimental mice groups represented in a 2 × 2 × 2 design of genotype × prenatal stress × diet. Although the findings from the study confirm endogenous levels of 4-HHE and 4-HNE in all analyzed tissues, variations in observed groups/conditions did not establish any conclusive trend. It is recommended that further studies with phospholipids levels

and multiple time point levels may provide supporting information to conclusively correlate levels of 4-HHE and 4-HNE to conditions of ASD. Results from the behavioral study however showed the tendency of dietary DHA able to mitigate repetitive behaviors in this model with offspring from stressed HT dams showing significantly decreased social preference index and increased grooming duration compared to WT non-stressed dams. Going forward, it will be interesting to examine whether maternal dietary DHA can reduce risk of autism in offspring of mothers exposed to stress during pregnancy in a clinical population, particularly for those who have genetic susceptibility to greater responses to stress.

4.6 References

1. Jones KL, Smith RM, Edwards KS, Givens B, Tilley MR, Beversdorf DQ. Combined effect of maternal serotonin transporter genotype and prenatal stress in modulating offspring social interaction in mice. *Int J Dev Neurosci*. 2010;28(6):529-536. doi:10.1016/j.ijdevneu.2010.05.002
2. Cohen S, Conduit R, Lockley SW, Rajaratnam SM, Cornish KM. The relationship between sleep and behavior in autism spectrum disorder (ASD): A review. *J Neurodev Disord*. 2014;6(1):1-10. doi:10.1186/1866-1955-6-44
3. Prater CD, Zylstra RG. *Autism: A Medical Primer*. Vol 66.; 2002. Accessed December 18, 2020. www.aafp.org/afpAMERICANFAMILYPHYSICIAN1667
4. DiCicco-Bloom E, Lord C, Zwaigenbaum L, Courchesne E, Dager SR, Schmitz C, Schultz RT, Crawley J, Young LJ. The developmental neurobiology of autism spectrum disorder. *J Neurosci*. 2006;26(26):6897-6906. doi:10.1523/JNEUROSCI.1712-06.2006
5. Autism Spectrum Disorder Fact Sheet | National Institute of Neurological Disorders and Stroke. Accessed December 18, 2020. https://www.ninds.nih.gov/Disorders/Patient-Caregiver-Education/Fact-Sheets/Autism-Spectrum-Disorder-Fact-Sheet
6. Maenner MJ, Shaw KA, Baio J, et al. Prevalence of autism spectrum disorder among children aged 8 Years-Autism and developmental disabilities monitoring network, 11 Sites, United States, 2016. *MMWR Surveill Summ*. 2020;69(4):1-12. doi:10.15585/MMWR.SS6904A1
7. Autism spectrum disorders. Accessed December 27, 2020.

https://www.who.int/news-room/fact-sheets/detail/autism-spectrum-disorders

8. New Research Finds Annual Cost of Autism Has More Than Tripled to $126 Billion in the U.S. and Reached £34 Billion in the U.K. | Autism Speaks. Accessed December 19, 2020. https://www.autismspeaks.org/press-release/new-research-finds-annual-cost-autism-has-more-tripled-126-billion-us-and-reached

9. Manzari N, Matvienko-Sikar K, Baldoni F, O'Keeffe GW, Khashan AS. Prenatal maternal stress and risk of neurodevelopmental disorders in the offspring: a systematic review and meta-analysis. *Soc Psychiatry Psychiatr Epidemiol*. 2019;54(11):1299-1309. doi:10.1007/s00127-019-01745-3

10. Kundakovic M, Jaric I. The Epigenetic Link between Prenatal Adverse Environments and Neurodevelopmental Disorders. *Genes (Basel)*. 2017;8(3):104. doi:10.3390/genes8030104

11. Say GN, Karabekiroğlu K, Babadağı Z, Yüce M. Maternal stress and perinatal features in autism and attention deficit/hyperactivity disorder. *Pediatr Int*. 2016;58(4):265-269. doi:10.1111/ped.12822

12. Ronald A, Pennell CE, Whitehouse AJO. Prenatal Maternal Stress Associated with ADHD and Autistic Traits in early Childhood. *Front Psychol*. 2011;1(JAN):223. doi:10.3389/fpsyg.2010.00223

13. Charil A, Laplante DP, Vaillancourt C, King S. Prenatal stress and brain development. *Brain Res Rev*. 2010;65(1):56-79. doi:10.1016/j.brainresrev.2010.06.002

14. Khashan AS, Abel KM, McNamee R, Pedersen MG, Webb RT, Baker PN, Kenny LC, Mortensen PB. Higher risk of offspring schizophrenia following antenatal

maternal exposure to severe adverse life events. *Arch Gen Psychiatry*. 2008;65(2):146-152. doi:10.1001/archgenpsychiatry.2007.20

15. Li J, Vestergaard M, Obel C, Christensen J, Precht DH, Lu M, Olsen J. A nationwide study on the risk of autism after prenatal stress exposure to maternal bereavement. *Pediatrics*. 2009;123(4):1102-1107. doi:10.1542/peds.2008-1734
16. Breen MS, Wingo AP, Koen N, Donald KA, Nicol M, Zar HJ, Ressler KJ, Buxbaum JD, Stein DJ. Gene expression in cord blood links genetic risk for neurodevelopmental disorders with maternal psychological distress and adverse childhood outcomes. *Brain Behav Immun*. 2018;73:320-330. doi:10.1016/j.bbi.2018.05.016
17. Beversdorf DQ, Manning SE, Hillier A, Anderson SL, Nordgren RE, Walters SE, Nagaraja HN, Cooley WC, Gaelic SE, Bauman ML. Timing of prenatal stressors and autism. *J Autism Dev Disord*. 2005;35(4):471-478. doi:10.1007/s10803-005-5037-8
18. Kinney DK, Munir KM, Crowley DJ, Miller AM. Prenatal stress and risk for autism. *Neurosci Biobehav Rev*. 2008;32(8):1519-1532. doi:10.1016/j.neubiorev.2008.06.004
19. Varcin KJ, Alvares GA, Uljarević M, Whitehouse AJO. Prenatal maternal stress events and phenotypic outcomes in Autism Spectrum Disorder. *Autism Res*. 2017;10(11):1866-1877. doi:10.1002/aur.1830
20. Mulder EJH, Robles De Medina PG, Huizink AC, Van Den Bergh BRH, Buitelaar JK, Visser GHA. Prenatal maternal stress: Effects on pregnancy and the (unborn) child. *Early Hum Dev*. 2002;70(1-2):3-14. doi:10.1016/S0378-3782(02)00075-0

21. Weinstock M. Does prenatal stress impair coping and regulation of hypothalamic-pituitary-adrenal axis? *Neurosci Biobehav Rev*. 1997;21(1):1-10. doi:10.1016/S0149-7634(96)00014-0

22. Kinney DK, Miller AM, Crowley DJ, Huang E, Gerber E. Autism prevalence following prenatal exposure to hurricanes and tropical storms in Louisiana. *J Autism Dev Disord*. 2008;38(3):481-488. doi:10.1007/s10803-007-0414-0

23. Abdulamir HA, Abdul-Rasheed OF, Abdulghani EA. Serotonin and serotonin transporter levels in autistic children. *Saudi Med J*. 2018;39(5):487-494. doi:10.15537/smj.2018.5.21751

24. Tordjman S, Gutknecht L, Carlier M, Spitz E, Antoine C, Slama F, Carsalade V, Cohen DJ, Ferrari P, Roubertoux PL, Anderson GM. Role of the serotonin transporter gene in the behavioral expression of autism. *Mol Psychiatry*. 2001;6(4):434-439. doi:10.1038/sj.mp.4000873

25. Huang CH, Santangelo SL. Autism and serotonin transporter gene polymorphisms: A systematic review and meta-analysis. *Am J Med Genet Part B Neuropsychiatr Genet*. 2008;147B(6):903-913. doi:10.1002/ajmg.b.30720

26. Brune CW, Kim SJ, Salt J, Leventhal BL, Lord C, Cook EH. 5-HTTLPR genotype-specific phenotype in children and adolescents with autism. *Am J Psychiatry*. 2006;163(12):2148-2156. doi:10.1176/ajp.2006.163.12.2148

27. Montañez S, Owens WA, Gould GG, Murphy DL, Daws LC. Exaggerated effect of fluvoxamine in heterozygote serotonin transporter knockout mice. *J Neurochem*. 2004;86(1):210-219. doi:10.1046/j.1471-4159.2003.01836.x

28. Hecht PM, Hudson M, Connors SL, Tilley MR, Liu X, Beversdorf DQ. Maternal

serotonin transporter genotype affects risk for ASD with exposure to prenatal stress. *Autism Res*. 2016;9(11):1151-1160. doi:10.1002/aur.1629

29. Sun GY, Simonyi A, Fritsche KL, Chuang DY, Hannink M, Gu Z, Greenlief CM, Yao JK, Lee JC, Beversdorf DQ. Docosahexaenoic acid (DHA): An essential nutrient and a nutraceutical for brain health and diseases. *Prostaglandins Leukot Essent Fat Acids*. 2018;136:3-13. doi:10.1016/j.plefa.2017.03.006

30. Belkouch M, Hachem M, Elgot A, Van A Lo, Picq M, Guichardant M, Lagarde M, Bernoud-Hubac N. The pleiotropic effects of omega-3 docosahexaenoic acid on the hallmarks of Alzheimer's disease. *J Nutr Biochem*. 2016;38:1-11. doi:10.1016/j.jnutbio.2016.03.002

31. Hashimoto M, Hossain S, Al Mamun A, Matsuzaki K, Arai H. Docosahexaenoic acid: one molecule diverse functions. *Crit Rev Biotechnol*. 2017;37(5):579-597. doi:10.1080/07388551.2016.1207153

32. Bazan NG, Molina MF, Gordon WC. Docosahexaenoic Acid Signalolipidomics in Nutrition: Significance in Aging, Neuroinflammation, Macular Degeneration, Alzheimer's, and Other Neurodegenerative Diseases. *Annu Rev Nutr*. 2011;31(1):321-351. doi:10.1146/annurev.nutr.012809.104635

33. Avallone R, Vitale G, Bertolotti M. Omega-3 Fatty Acids and Neurodegenerative Diseases: New Evidence in Clinical Trials. *Int J Mol Sci*. 2019;20(17):4256. doi:10.3390/ijms20174256

34. Lauritzen L, Brambilla P, Mazzocchi A, Harsløf L, Ciappolino V, Agostoni C. DHA Effects in Brain Development and Function. *Nutrients*. 2016;8(1):6. doi:10.3390/nu8010006

35. Kuratko C, Barrett E, Nelson E, Salem N. The Relationship of Docosahexaenoic Acid (DHA) with Learning and Behavior in Healthy Children: A Review. *Nutrients*. 2013;5(7):2777-2810. doi:10.3390/nu5072777

36. Bradbury J. Docosahexaenoic acid (DHA): An ancient nutrient for the modern human brain. *Nutrients*. 2011;3(5):529-554. doi:10.3390/nu3050529

37. DeMar JC, Ma K, Bell JM, Igarashi M, Greenstein D, Rapoport SI. One generation of n-3 polyunsaturated fatty acid deprivation increases depression and aggression test scores in rats. *J Lipid Res*. 2006;47(1):172-180. doi:10.1194/jlr.M500362-JLR200

38. McCann JC, Ames BN. Is docosahexaenoic acid, an n−3 long-chain polyunsaturated fatty acid, required for development of normal brain function? An overview of evidence from cognitive and behavioral tests in humans and animals. *Am J Clin Nutr*. 2005;82(2):281-295. doi:10.1093/ajcn.82.2.281

39. McNamara RK. DHA Deficiency and Prefrontal Cortex Neuropathology in Recurrent Affective Disorders. *J Nutr*. 2010;140(4):864-868. doi:10.3945/jn.109.113233

40. Moriguchi T, Loewke J, Garrison M, Catalan JN, Salem N. Reversal of docosahexaenoic acid deficiency in the rat brain, retina, liver, and serum. *J Lipid Res*. 2001;42(3):419-427.

41. Bhatia HS, Agrawal R, Sharma S, Huo YX, Ying Z, Gomez-Pinilla F. Omega-3 fatty acid deficiency during brain maturation reduces neuronal and behavioral plasticity in adulthood. *PLoS One*. 2011;6(12). doi:10.1371/journal.pone.0028451

42. Takeuchi T, Iwanaga M, Harada E. Possible regulatory mechanism of DHA-

induced anti-stress reaction in rats. *Brain Res*. 2003;964(1):136-143. doi:10.1016/S0006-8993(02)04113-6

43. Vinot N, Jouin M, Lhomme-Duchadeuil A, Guesnet P, Alessandri J-M, Aujard F, Pifferi F. Omega-3 Fatty Acids from Fish Oil Lower Anxiety, Improve Cognitive Functions and Reduce Spontaneous Locomotor Activity in a Non-Human Primate. Izquierdo I, ed. *PLoS One*. 2011;6(6):e20491. doi:10.1371/journal.pone.0020491

44. Ferraz AC, Delattre AM, Almendra RG, Sonagli M, Borges C, Araujo P, Andersen ML, Tufik S, Lima MMS. Chronic ω-3 fatty acids supplementation promotes beneficial effects on anxiety, cognitive and depressive-like behaviors in rats subjected to a restraint stress protocol. *Behav Brain Res*. 2011;219(1):116-122. doi:10.1016/j.bbr.2010.12.028

45. Sun GY, Simonyi A, Fritsche KL, Chuang DY, Hannink M, Gu Z, Greenlief CM, Yao JK, Lee JC, Beversdorf DQ. Docosahexaenoic acid (DHA): An essential nutrient and a nutraceutical for brain health and diseases. *Prostaglandins Leukot Essent Fat Acids*. 2018;136:3-13. doi:10.1016/j.plefa.2017.03.006

46. Tang M, Zhang M, Wang L, Li H, Cai H, Dang R, Jiang P, Liu Y, Xue Y, Wu Y. Maternal dietary of n-3 polyunsaturated fatty acids affects the neurogenesis and neurochemical in female rat at weaning. *Prostaglandins Leukot Essent Fat Acids*. 2018;128(January 2017):11-20. doi:10.1016/j.plefa.2017.11.001

47. Mazahery H, Stonehouse W, Delshad M, Kruger M, Conlon C, Beck K, von Hurst P. Relationship between Long Chain n-3 Polyunsaturated Fatty Acids and Autism Spectrum Disorder: Systematic Review and Meta-Analysis of Case-Control and Randomised Controlled Trials. *Nutrients*. 2017;9(2):155. doi:10.3390/nu9020155

48. Matsui F, Hecht P, Yoshimoto K, Watanabe Y, Morimoto M, Fritsche K, Will M, Beversdorf D. DHA Mitigates Autistic Behaviors Accompanied by Dopaminergic Change in a Gene/Prenatal Stress Mouse Model. *Neuroscience*. 2018;371:407-419. doi:10.1016/j.neuroscience.2017.12.029

49. James S, Montgomery P, Williams K. Omega-3 fatty acids supplementation for autism spectrum disorders (ASD). *Cochrane Database Syst Rev*. 2011;(11). doi:10.1002/14651858.cd007992.pub2

50. Mazahery H, Conlon CA, Beck KL, Mugridge O, Kruger MC, Stonehouse W, Camargo CA, Meyer BJ, Jones B, von Hurst PR. A randomised controlled trial of vitamin D and omega-3 long chain polyunsaturated fatty acids in the treatment of irritability and hyperactivity among children with autism spectrum disorder. *J Steroid Biochem Mol Biol*. 2019;187:9-16. doi:10.1016/j.jsbmb.2018.10.017

51. Horvath A, Łukasik J, Szajewska H. ω-3 Fatty Acid Supplementation Does Not Affect Autism Spectrum Disorder in Children: A Systematic Review and Meta-Analysis. *J Nutr*. 2017;147(3):367-376. doi:10.3945/jn.116.242354

52. De Crescenzo F, D'Alò GL, Morgano GP, Minozzi S, Mitrova Z, Saulle R, Cruciani F, Fulceri F, Davoli M, Scattoni ML, Nardocci F, Schünemann HJ, Amato L, Nardocci F. Impact of polyunsaturated fatty acids on patient-important outcomes in children and adolescents with autism spectrum disorder: A systematic review. *Health Qual Life Outcomes*. 2020;18(1):1-12. doi:10.1186/s12955-020-01284-5

53. Politi P, Cena H, Comelli M, Marrone G, Allegri C, Emanuele E, Ucelli di Nemi S. Behavioral Effects of Omega-3 Fatty Acid Supplementation in Young Adults

with Severe Autism: An Open Label Study. *Arch Med Res*. 2008;39(7):682-685. doi:10.1016/j.arcmed.2008.06.005

54. Mankad D, Dupuis A, Smile S, Roberts W, Brain J, Lui T, Genore L, Zaghloul D, Iaboni A, Marcon PMA, Anagnostou E. A randomized, placebo controlled trial of omega-3 fatty acids in the treatment of young children with autism. *Mol Autism*. 2015;6(1):1-11. doi:10.1186/s13229-015-0010-7

55. Feng Z, Zou X, Jia H, Li X, Zhu Z, Liu X, Bucheli P, Ballevre O, Hou Y, Zhang W, Wang J, Chen Y, Liu J. Maternal Docosahexaenoic Acid Feeding Protects Against Impairment of Learning and Memory and Oxidative Stress in Prenatally Stressed Rats: Possible Role of Neuronal Mitochondria Metabolism. *Antioxid Redox Signal*. 2012;16(3):275-289. doi:10.1089/ars.2010.3750

56. Gao J, Wu H, Cao Y, Liang S, Sun C, Wang P, Wang J, Sun H, Wu L. Maternal DHA supplementation protects rat offspring against impairment of learning and memory following prenatal exposure to valproic acid. *J Nutr Biochem*. 2016;35:87-95. doi:10.1016/j.jnutbio.2016.07.003

57. Yui K, Koshiba M, Nakamura S, Kobayashi Y. Effects of Large Doses of Arachidonic Acid Added to Docosahexaenoic Acid on Social Impairment in Individuals With Autism Spectrum Disorders. *J Clin Psychopharmacol*. 2012;32(2):200-206. doi:10.1097/JCP.0b013e3182485791

58. Yang B, Fritsche KL, Beversdorf DQ, Gu Z, Lee JC, Folk WR, Greenlief CM, Sun GY. Yin-yang mechanisms regulating lipid peroxidation of docosahexaenoic acid and arachidonic acid in the central nervous system. *Front Neurol*. 2019;10(JUN):642. doi:10.3389/fneur.2019.00642

59. Yang B, Li R, Michael Greenlief C, Fritsche KL, Gu Z, Cui J, Lee JC, Beversdorf DQ, Sun GY. Unveiling anti-oxidative and anti-inflammatory effects of docosahexaenoic acid and its lipid peroxidation product on lipopolysaccharide-stimulated BV-2 microglial cells. *J Neuroinflammation*. 2018;15(1):1-16. doi:10.1186/s12974-018-1232-3

60. Yang B, Li R, Woo T, Jr JDB, Song H, Gu Z, Cui J, Lee JC, Fritsche KL, Beversdorf DQ, Sun GY, Greenlief CM. Maternal Dietary Docosahexaenoic Acid Alters Lipid Peroxidation Products and (n-3)/(n-6) Fatty Acid Balance in Offspring Mice. Published online 2019:1-14. doi:10.3390/metabo9030040

61. Sun GY, Appenteng MK, Li R, et al. Docosahexaenoic Acid (DHA) Supplementation Alters Phospholipid Species and Lipid Peroxidation Products in Adult Mouse Brain, Heart, and Plasma. *NeuroMolecular Med*. 2020;1:3. doi:10.1007/s12017-020-08616-0

62. Zilkha N, Kuperman Y, Kimchi T. High-fat diet exacerbates cognitive rigidity and social deficiency in the BTBR mouse model of autism. *Neuroscience*. 2017;345:142-154. doi:10.1016/j.neuroscience.2016.01.070

63. Jones KL, Will MJ, Hecht PM, Parker CL, Beversdorf DQ. Maternal diet rich in omega-6 polyunsaturated fatty acids during gestation and lactation produces autistic-like sociability deficits in adult offspring. *Behav Brain Res*. 2013;238(1):193-199. doi:10.1016/j.bbr.2012.10.028

64. Hecht PM, Hudson M, Connors SL, Tilley MR, Liu X, Beversdorf DQ. Maternal serotonin transporter genotype affects risk for ASD with exposure to prenatal stress. *Autism Res*. 2016;9(11):1151-1160. doi:10.1002/aur.1629

CHAPTER 5: Future directions and extensions

5.1 DHA and myocardial function

The role of docosahexaenoic acid (DHA) in modulating myocardial function needs to be explored further. In an adult mouse study[1], a three-week supplementation of DHA in diet was sufficient to result in an almost twofold increase in 4-HHE ($p < 0.05$) as compared to the control group in heart with no significant change in 4-HNE levels as shown in Figure 3.3. Moreover, analysis of the phospholipid species in the heart tissue showed significant larger effects of DHA supplementation in all analyzed phospholipid classes (diacyl-PE, PC, PI and PS). The DHA-enriched group showed significant decreases in (n-6) species ($p < 0.001$), and significant increases in the (n-3) species ($p < 0.05$) in all classes examined as shown in Figure 3.6. Our previous pup study[2] also examined whether maternal DHA-enriched diet alters FA and peroxidation products in heart, plasma and brain in weanling pups. In this study, heart tissue had a large increase in DHA and decrease in ARA (Figure 5.1A and B), as well as increased levels of 4-hydroxyhexenal (4-HHE) (Figure 5.2A and B) in the DHA group. These results suggest possible effects of dietary DHA to moderate cardiovascular functions.

Heart attack and heart failure are the leading causes of death and are especially prevalent among the aged population.[3,4,5,6,7] Epidemiological studies suggest that consumption of (n-3) PUFAs and fish oil may have significant effects on cardiovascular diseases (CVD).[8,9,10,11] Large scale clinical and pharmacological studies additionally revealed that (n-3) PUFAs reduced the mortality of CVD.[12] However, mixed results from clinical trials indicated multiple factors, such as the quality and quantity of n-3 PUFA supplementation and cohort profiles (e.g., age, race, gender and educational status) affected

clinical outcomes.[13] The heart tissue is rich in mitochondria which are the energy-producing organelles important in maintaining cellular homeostasis and proper calcium concentrations.[14] DHA supplementation is known to alter key properties of cardiac mitochondria and attenuates development of ventricular dysfunction in pressure overload-induced heart failure.[15] Besides DHA, fatty acids (FA) in heart tissue are also rich in arachidonic acid (ARA, 20:4n-6), which is known to be involved in inflammatory responses.[16]

Considering the ability of dietary DHA to alter the FA and peroxidation products in the young pups and phospholipid species in adult mice, an important goal in future studies is to examine whether dietary DHA may impact FA and 4-HHE and offer beneficial effects and enhance mitochondrial function in adult and aged heart. Based on this background, it would be interesting to test the **hypothesis** that DHA supplementation leading to an increase in (n-3) and decrease in (n-6) fatty acids and increase in 4-HHE in heart tissue may imply changes in heart mitochondrial proteins and functions, and subsequently affect energy production and oxidative stress.

A well-designed study with adult (3-5 months old) and aged (15-18 months old) mice treated with dietary DHA for 6-8 weeks can be used to obtain information on DHA/ARA and 4-HHE/4-HNE ratios in young and old mitochondria. Results from this study may provide important information to answer whether dietary DHA may offer protective effects on heart failure/attack at old age. Moreover, findings from these studies will add to knowledge concerning mitochondrial proteomics and the possible relationships between proteins on FA and peroxidation products.

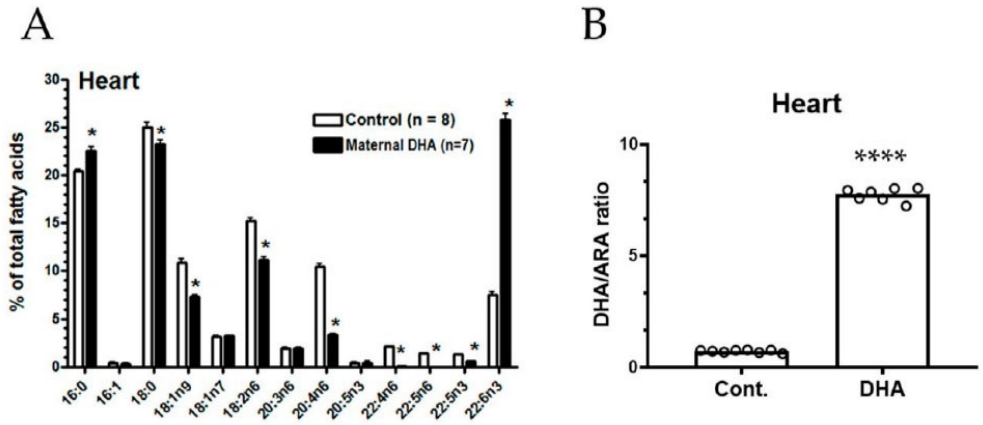

Figure 5.1. A and B: Fatty acids in the heart tissue from pups nursed by mothers fed control and DHA-enriched diets. Pups nursed by DHA mothers showed a large increase in 22:6 (n-3) (from 7.5 to 25.8%) and decrease in 20:4 (n-6) (from 10.5 to 3.3%) as compared to controls (Figure 5.1A). In addition to 20:4 (n-6), significant decreases in other (n-6) FA, such as 22:4 (n-6) and 22:5 (n-6) are also observed (Figure 1A). The DHA/ARA ratios in DHA and control pups were 7.8 versus 0.7, indicating an 11.2-fold increase (Figure 5.1B). Analysis of FA using one way-ANOVA indicated significance between control and 1% DHA diet group. * $p < 0.05$. Analysis of DHA/ARA ratios using two-tail unpaired t-test indicated significance between DHA group and controls. **** $p < 0.0001$. Figure is reproduced from reference [2].

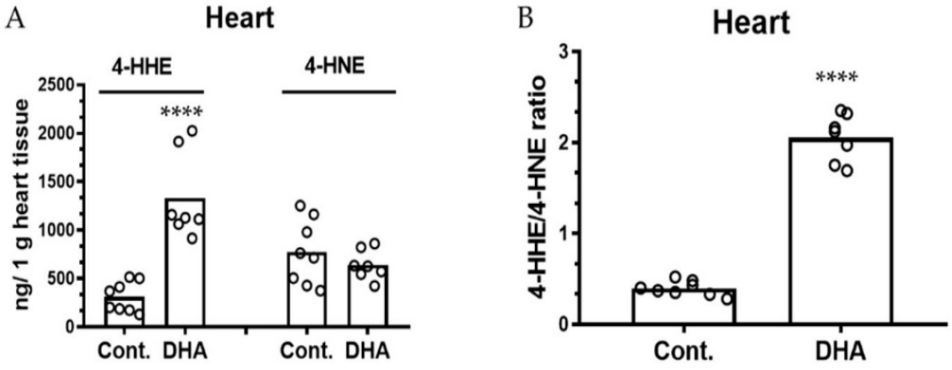

Figure 5.2. A and B: The levels of 4-HHE and 4-HNE in the heart tissue were 312 ± 55 and 774 ± 118 ng/g tissue, respectively, in the control group, and 1331 ± 168 and 642 ± 58 ng/g tissue, respectively, in the DHA pups (Figure 5.2A). These results led to a 4.3-fold increase in 4-HHE and a 5.2-fold increase of 4-HHE/4-HNE ratio in the heart when comparing the DHA pups with controls (Figure 5.2B). Analysis using two-tail unpaired t-test indicated significance between DHA group and controls. *** $p < 0.001$; **** $p < 0.0001$. Figure is reproduced from reference [2].

5.2 Changes in phospholipids species in DHA gene/stress model

Analysis of the phospholipid species and their specific polyunsaturated fatty acid (PUFA) profiles in the brain regions, heart, and plasma will be a useful extension of the maternal DHA gene/stress study. In the original study, investigations were carried out to ascertain whether prenatal supplementation of DHA alleviates autistic-like behaviors in a gene/stress mouse model and whether it alters lipid peroxidation activity in the brain. To achieve this, pregnant heterozygous serotonin transporter knockout-SERT-KO (HT) and wild-type (WT) mice were placed in either a non-stressed control condition or a chronic variable stress condition and fed either a control diet or a DHA-rich (1% by wt) diet. Male offspring from each group were assessed for anxiety and autism-associated behavior at post-natal day 60, including an open field test, elevated-plus maze test, repetitive behavior, and the 3-chamber social approach test. Our LC-MS-based method was used to follow changes in peroxidation product concentrations in mouse plasma, heart, and cerebral cortex.

Although the findings from the study confirm endogenous levels of 4-HHE and 4-HNE in all analyzed tissues, no significant variations in observed in 4-HHE levels for DHA supplement diet were shown to conclusively establish a trend in relation to the gene/stress effect (As shown in Figure 4.3 and 4.4 for heart and plasma, respectively). The values measured showed no apparent changes in 4-HHE levels between the control and 1% DHA diet conditions for both stressed and non-stressed WT and HT groups for both heart and plasma samples.

An important consideration, which may have accounted for less variation in results, may be the duration of supplementation. While the pups were weaned on postnatal day

(PD) 21 and sacrificed around post-natal day 70 in this study, supplementation in previous studies like the DHA adult mice[1] and maternal DHA pup study[2] lasted for 3 weeks. So different supplementation times can also be considered in future work (3, 6, 9 or 12weeks). Moreover, in the DHA adult mice study[1], an increase in (n-3) phospholipids due to DHA-enriched diet was associated with increases in 4-HHE but not 4-HNE as shown in Figures 3.6 and 3.3 respectively. This result shows a good correlation between 4-HHE and (n-3) phospholipids for DHA enriched diet. In a future study, the analysis of phospholipids species and specific PUFA profiles can be made and correlated with that from the lipid peroxidation products. This correlation could help bring some closure to conclusively relate the effect of DHA supplementation in gene/stress mouse model to autistic like behaviors.

5.3 References

1. Sun GY, Appenteng MK, Li R, et al. Docosahexaenoic Acid (DHA) Supplementation Alters Phospholipid Species and Lipid Peroxidation Products in Adult Mouse Brain, Heart, and Plasma. *NeuroMolecular Med*. 2020;1:3. doi:10.1007/s12017-020-08616-0
2. Yang B, Li R, Woo T, Browning J, Song H, Gu Z, Cui J, Lee J, Fritsche K, Beversdorf D, Sun G, Greenlief C. Maternal Dietary Docosahexaenoic Acid Alters Lipid Peroxidation Products and (n-3)/(n-6) Fatty Acid Balance in Offspring Mice. *Metabolites*. 2019;9(3):40. doi:10.3390/metabo9030040
3. Roser M, Ritchie H. Burden of Disease. *Our World Data*. Published online January 25, 2016. Accessed January 31, 2021. https://ourworldindata.org/burden-of-disease
4. Butrous H, Hummel SL. Heart Failure in Older Adults. *Can J Cardiol*. 2016;32(9):1140-1147. doi:10.1016/j.cjca.2016.05.005
5. Díez-Villanueva P, Alfonso F. Heart failure in the elderly. *J Geriatr Cardiol*. 2016;13(2):115-117. doi:10.11909/j.issn.1671-5411.2016.02.009
6. Dicker D, Nguyen G, Abate D, et al. Global, regional, and national age-sex-specific mortality and life expectancy, 1950-2017: A systematic analysis for the Global Burden of Disease Study 2017. *Lancet*. 2018;392(10159):1684-1735. doi:10.1016/S0140-6736(18)31891-9
7. Vasan RS, Sullivan LM, Roubenoff R, Dinarello CA, Harris T, Benjamin EJ, Sawyer DB, Levy D, Wilson PWF, D'Agostino RB. Inflammatory markers and risk of heart failure in elderly subjects without prior myocardial infarction: The

Framingham Heart Study. *Circulation*. 2003;107(11):1486-1491. doi:10.1161/01.CIR.0000057810.48709.F6

8. Marckmann P, Grønbæk M. Fish consumption and coronary heart disease mortality. A systematic review of prospective cohort studies. *Eur J Clin Nutr*. 1999;53(8):585-590. doi:10.1038/sj.ejcn.1600832

9. Marik PE, Varon J. Omega-3 dietary supplements and the risk of cardiovascular events: A systematic review. *Clin Cardiol*. 2009;32(7):365-372. doi:10.1002/clc.20604

10. Mozaffarian D, Psaty BM, Rimm EB, Lemaitre RN, Burke GL, Lyles MF, Lefkowitz D, Siscovick DS. Fish Intake and Risk of Incident Atrial Fibrillation. Published online 2004. doi:10.1161/01.CIR.0000138154.00779.A5

11. Schiano V, Laurenzano E, Brevetti G, De Maio JI, Lanero S, Scopacasa F, Chiariello M. Omega-3 polyunsaturated fatty acid in peripheral arterial disease: Effect on lipid pattern, disease severity, inflammation profile, and endothelial function. *Clin Nutr*. 2008;27(2):241-247. doi:10.1016/j.clnu.2007.11.007

12. Marchioli R. Dietary supplementation with N-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: Results of the GISSI-Prevenzione trial. *Lancet*. 1999;354(9177):447-455. doi:10.1016/S0140-6736(99)07072-5

13. Sakamoto A, Saotome M, Iguchi K, Maekawa Y. Marine-derived omega-3 polyunsaturated fatty acids and heart failure: Current understanding for basic to clinical relevance. *Int J Mol Sci*. 2019;20(16). doi:10.3390/ijms20164025

14. Sullivan EM, Pennington ER, Green WD, Beck MA, Brown DA, Shaikh SR. Mechanisms bywhich dietary fatty acids regulate mitochondrial structure-function

in health and disease. *Adv Nutr*. 2018;9(3):247-262. doi:10.1093/advances/nmy007

15. Dabkowski ER, O'Connell KA, Xu W, Ribeiro RF, Hecker PA, Shekar KC, Daneault C, Des Rosiers C, Stanley WC. Docosahexaenoic acid supplementation alters key properties of cardiac mitochondria and modestly attenuates development of left ventricular dysfunction in pressure overload-induced heart failure. *Cardiovasc Drugs Ther*. 2013;27(6):499-510. doi:10.1007/s10557-013-6487-4
16. Yang B, Fritsche KL, Beversdorf DQ, Gu Z, Lee JC, Folk WR, Greenlief CM, Sun GY. Ying-yang mechanisms regulating lipid peroxidation of docosahexaenoic acid and arachidonic acid in the central nervous system. *Front Neurol*. 2019;10(JUN):642. doi:10.3389/fneur.2019.00642

APPENDIX 1: List of abbreviations

ACN	Acetonitrile
AE	American elderberry
AH	Amygdalin hydrolase
AIN	Control
APCI	Atmospheric pressure chemical ionization
ARA	Arachidonic acid
ASD	Autism spectrum disorder
AUC	Area under curve
BW	Body weight
CDC	Centers for Disease Control and Prevention
CHD	1,3-cyclohexanedione
CNGs	Cyanogenic glycosides
CNS	Cyanogenic standards
COX	Cyclooxygenase
cPLA2	Cytosolic phospholipases A2
CTL	Control
CV	Coefficient of variation
CVD	Cardiovascular diseases
CVS	Chronic variable stress
DHA	Docosahexaenoic acid
dPE	Diacyl-phosphatidylethanolamine
DSHEA	Dietary Supplement Health and Education Act

EE	European elderberry
EI	Electron ionization
EPM	Elevated plus-maze
ESI	Electrospray ionization
FA	Formic acid
GC	Gas chromatography
HCN	Hydrogen cyanide
HHE	4-hydroxyhexenal
HNA	Heterozygous non-stressed control
HND	Heterozygous non-stressed DHA
HND	Heterozygous stressed DHA
HNE	4-hydroxynonenal
HO-1	Heme oxygenase-1
HPLC	High performance liquid chromatography
HPLC-DAD	High-performance liquid chromatography with photo diode array detectors
HSA	Heterozygous stressed control
HT	Heterozygous
iPLA2	Ca^{+2}-independent phospholipases A2
KO	Knock-out
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry

LIT-FT-ICR	Linear ion trap-Fourier transform ion cyclotron resonance mass spectrometer
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
LOX	Lipoxygenase
MDLI	Mandelonitrile lyase
ME	Matrix effect
ME	Matrix effect
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MUFA	Monounsaturated fatty acids
NBJ	Nutrition business journal
Nrf2	Nuclear factor erythroid 2-related factor 2
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PE-p1	Alkenylacyl-phosphatidylethanolamine or phosphatidylethanolamine plasmalogen
PH	Prunasin hydrolase
PI	Phosphatidylinositol
PL	Phospholipids
PLA2	Phospholipases A2
PM	Photomultipliers

PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
QqQ	Triple quadrupole
RCT	Randomized controlled trials
RE	Recovery
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Retention time
S/N	Signal to noise ration
SD	Standard deviation
SEM	Standard error of mean
SERT	Serotonin transporter
SFA	Saturated fatty acids
SIR	Selected ion recording
SPE	Solid-phase extraction
TCP	Total cyanogenic potential
TOF	Time-of-Flight
TQMS	Triple quadrupole mass spectrometer
U	Units
UHPLC	Ultra-high performance liquid chromatography
UHPLC-QqQ-MS/MS	Ultra-high performance liquid chromatography triple-quadrupole mass spectrometry
ULOQ	Upper limit of quantification

UV-Vis	Ultraviolet visible spectrophotometry
WNA	Wild type non-stressed control
WND	Wild type non-stressed DHA
WSA	Wild type tressed control
WSD	Wild type stressed DHA
WT	Wild type

APPENDIX 2: Chapter 2 - Picrate paper qualitative test results

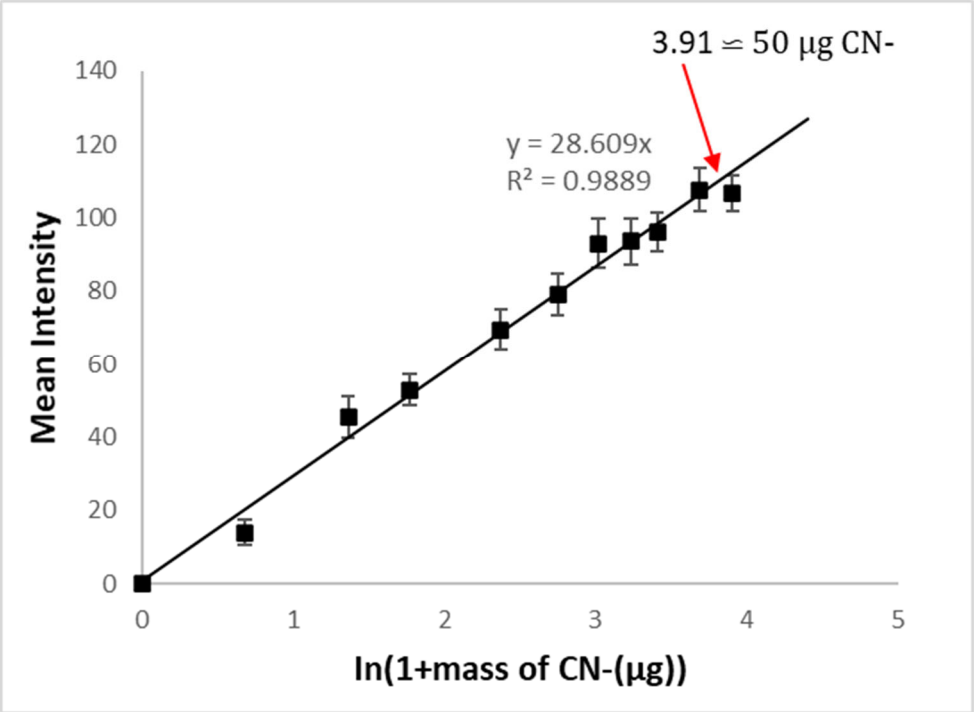

Figure A2.1. Calibration curve for CN^- eq. using amygdalin as the standard by the picrate-paper method and a camera-phone as the detector. The curve generated from a plot of mean intensity values verses the natural logarithm of cyanide equivalent amounts. An image the picrate-paper was converted from color to greyscale. Conversion to greyscale helped increase the upper limit of detection where color saturation became an issue. The conversion was done using Image J software (https://imagej.nih.gov/ij/index.html). The mean intensity values corresponding for each CN^- eq. were used to generate the calibration curve. The error bars represent the standard deviation of at least three replicate samples (n=3).

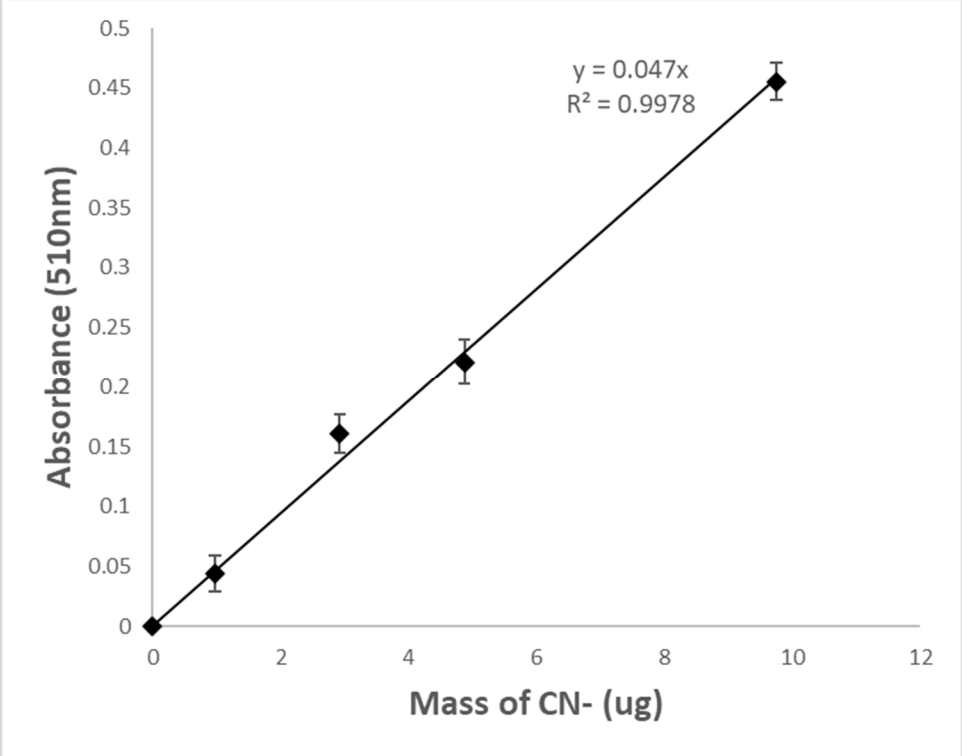

Figure A2.2. Calibration curve for CN^- eq. using amygdalin as the standard by the picrate-paper method and using a UV-Vis spectrophotometer as the detector (λ_{max} = 510 nm). The curve shows as expanded region at the lowest concentrations (0 to 10 µg). The curve generated from a plot of average absorbance verses cyanide equivalent amounts. The error bars represent the standard deviation of at least three replicate samples (n=3).

\

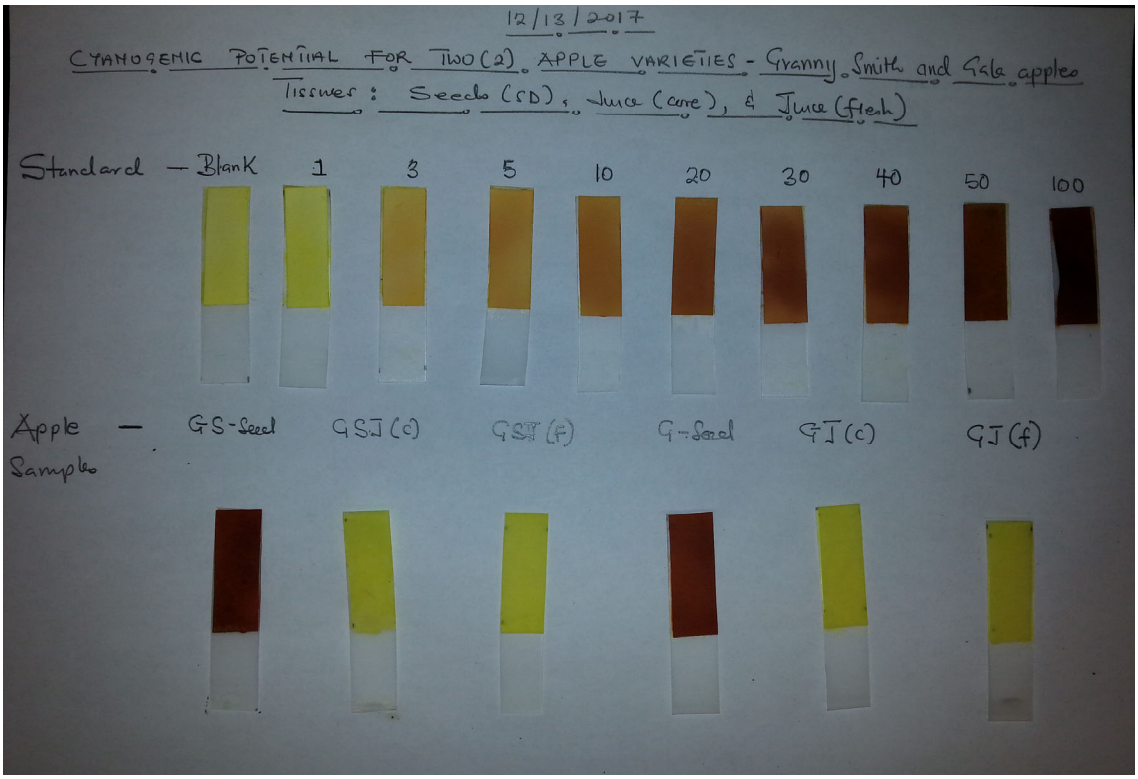

Figure A2.3. Picrate-paper results for results for apple seeds, core, flesh, and juice. Granny Smith (GS) and Galas (G) apples were used in the test. The top row of strips indicates the color changes for different concentrations of amygdalin (µg) as CN^- standard. The units of concentration for the CN^- standards is in µg. The bottom row shows picrate-paper results for apple samples. From left to right the samples are Granny Smith seeds (GS-seed), Granny Smith juice taken near the core (GSJ(c)), Granny Smith juice taken from the main part of the apple (GSJ(f)), Gala seeds (G-seed), Gala juice taken near the core (GJ(c)), and Gala juice taken from the main part of the apple (GJ(f)).

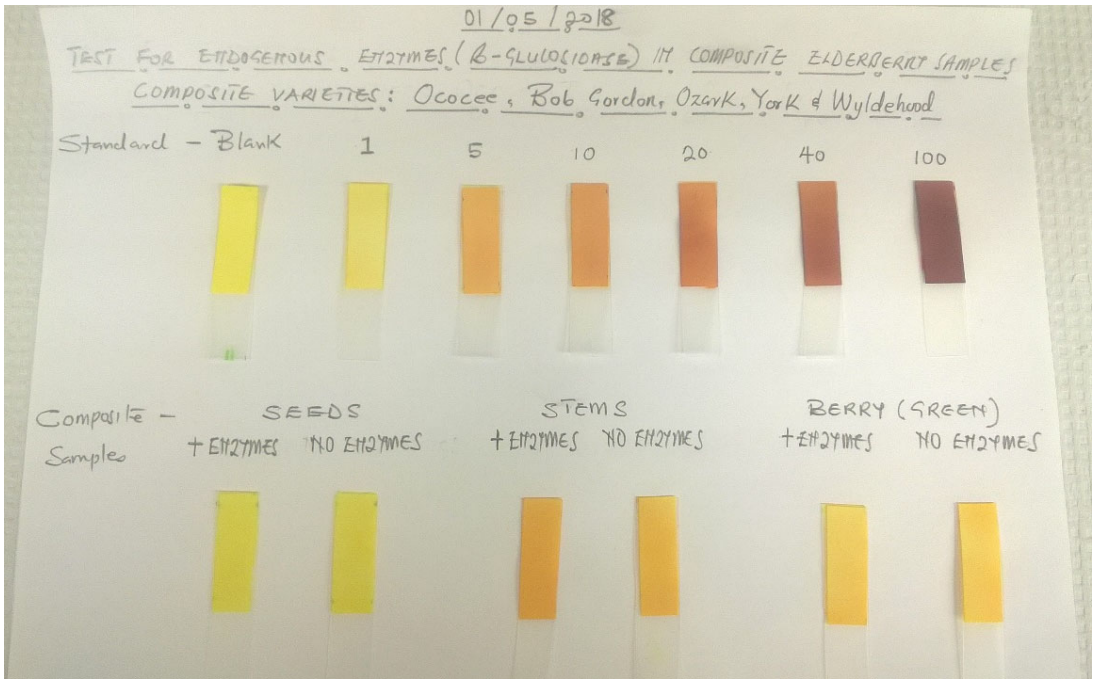

Figure A2.4. Picrate-paper results for different tissues (berries, seeds, and stems) of the pooled AE samples with and without the addition of endogenous enzymes. The pooled samples are a mixture of different AE genotypes. The top row of strips indicates the color changes for different concentrations of amygdalin (μg) as CN^- standard. The units of concentration for the CN^- standards is in μg.

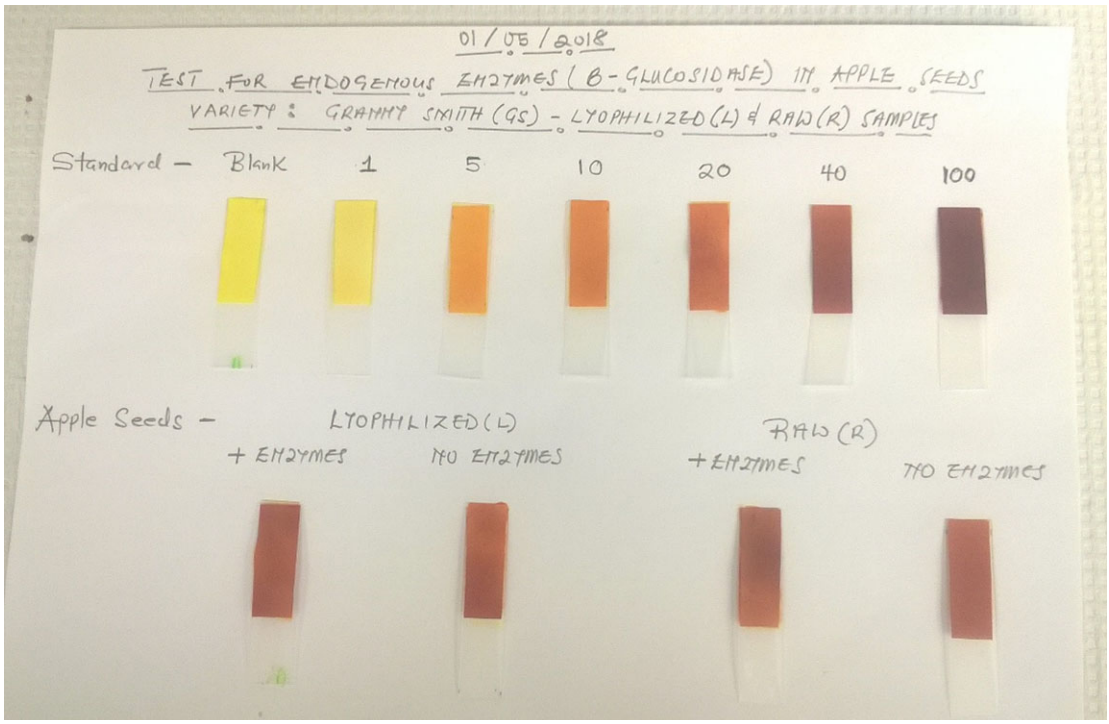

Figure A2.5. Picrate-paper results for endogenous enzymes test for raw (fresh) and lyophilized apple seeds. The top row of strips indicates the color changes for different concentrations of amygdalin (μg) as CN^- standard. The units of concentration for the CN^- standards is in μg.

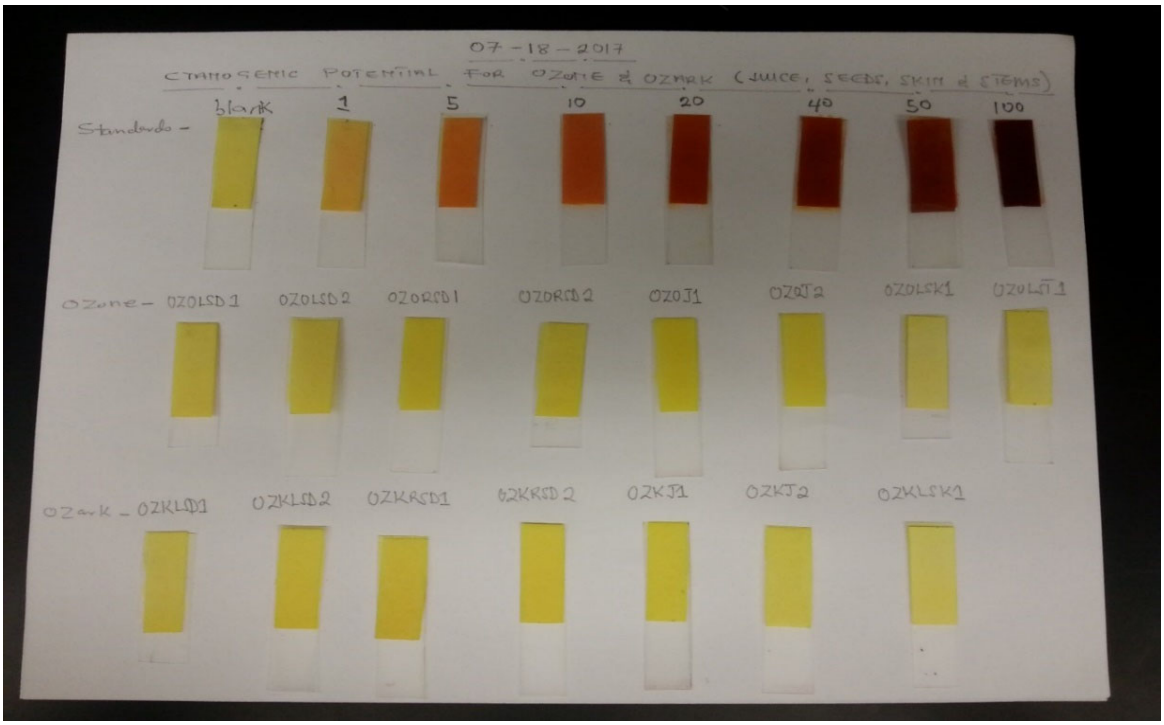

Figure A2.6. Picrate-paper results for different tissues (juice, seeds, stems, and skin) of Ozone and Ozark AE samples. The top row of strips indicates the color changes for different concentrations of amygdalin (μg) as CN^- standard. The units of concentration for the CN^- standards is in μg. The middle row from left to right are Ozone AE samples: OZOLSD1 and OZOLSD2 are lyophilized seeds, OZORS1 and OZORS2 are raw/fresh seeds, OZOJ1 and OZOJ2 are fresh juice, OZOLSK1 is lyophilized skin, and OZOLST1 is lyophilized stems. The bottom row from left to right are Ozark AE samples: OZKLSD1 and OZKLSD2 are lyophilized seeds, OZKRSD1 and OZKRSD2 are raw/fresh seeds, OZKJ1 and OZKJ2 are fresh juice, and OZKLSK1 is lyophilized skin

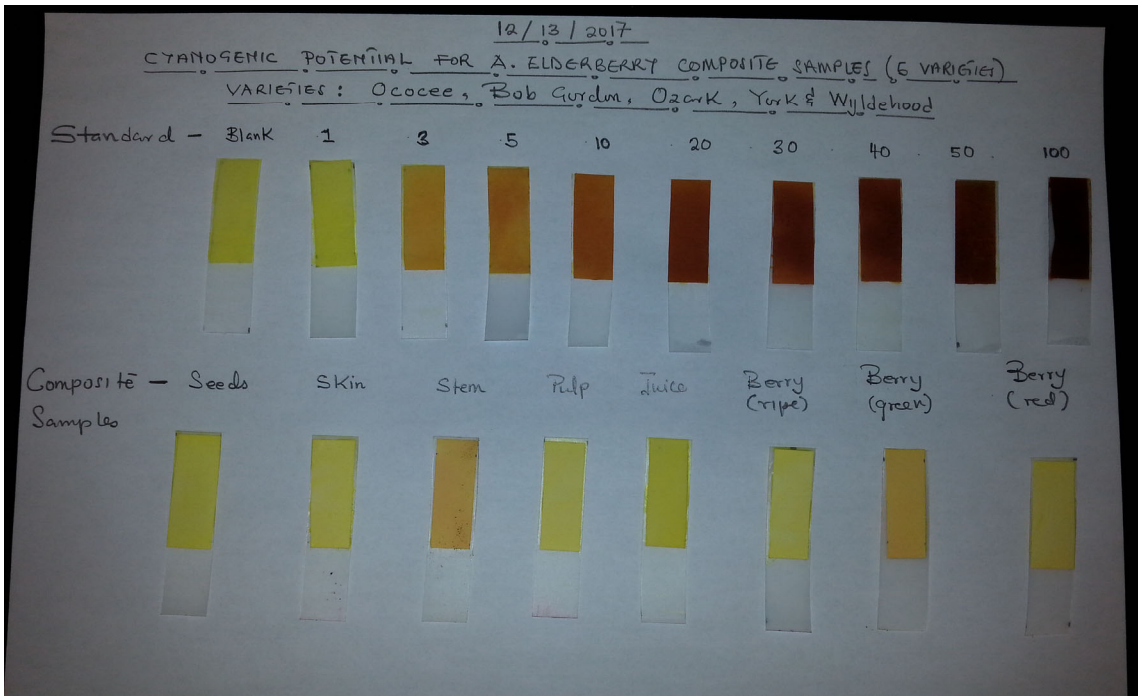

Figure A2.7. Picrate-paper results for different tissues (juice, seeds, stems, and skin) of the pooled AE samples. Elderberry pulp, fresh green, red and ripe berries are also analyzed. The pooled samples are a mixture of different AE genotypes. The top row of strips indicates the color changes for different concentrations of amygdalin (µg) as CN^- standard. The units of concentration for the CN^- standards is in µg.

APPENDIX 3: Chapter 3 – Mouse diet analyses and lipidomics

Table A3.1. Experimental Mouse Diets

Ingredients (g/kg)	CTL	DHA
cornstarch	397.486	397.486
casein	200	200
dextrin (dyetrose)	132	132
sucrose	100	100
fiber (alpha-cellulose)	50	50
mineral mix (AIN-93)	35	35
vitamin mix (AIN-93G)	10	10
L-cystine	3	3
choline bitartrate	2.5	2.5
soybean oil	50	0
corn oil	20	0
safflower oil	0	45
DHASCO algal oil*	0	25
t-butylhydroquinone (a synthetic anti-oxidant)	0.014	0.014
total	1000	1000

**a gift from DSM Nutritional Products, Columbia, MD, USA*

Table A3.2. Fatty acid composition of diets.

	Control diet	1.0% DHA diet
16:0	0.8[a]	0.4
18:0	0.2	0.2
18:1	1.8	1.0
18:2n6	3.9	4.0
18:3n3	0.4	0.1
20:5n3	nd[b]	0.1
22:5n3	nd	0.3
22:6n3 (DHA)	nd	1.0

[a] Values represent g/100 g of diet

[b] nd = not detected

Table A3.3. Lipidomic analysis of mouse cerebral cortex samples.

Phosphatidylethanolamine (PE) (nmol/mg protein)	MASS	DHA treatment #1	DHA treatment #2	DHA treatment #3	DHA treatment #4	Control #5	Control #6	Control #7	Control #8
P18:1-16:0/P16:0-18:1	922.53	2.82	3.12	2.92	2.49	3.16	2.11	3.43	3.02
P18:0-16:0/P16:0-18:0	924.54	1.16	1.23	0.93	0.96	1.73	1.19	0.95	1.27
D16:1-18:1	936.51	0.30	0.40	0.33	0.32	0.44	0.30	0.42	0.40
D16:0-18:1	938.52	2.66	2.51	2.29	2.12	2.44	2.31	2.41	2.43
P14:0-22:6	940.54	0.67	0.41	0.58	0.49	1.00	0.53	0.51	0.56
P16:0-20:4	944.51	1.74	1.25	2.34	1.62	2.83	2.98	2.48	1.69
P18:1-18:2	946.53	0.49	0.24	0.39	0.36	0.56	0.58	0.63	0.63
P18:1-18:1	948.54	4.14	4.49	4.38	4.06	4.37	3.33	5.50	5.98
P18:0-18:1	950.56	3.48	4.26	3.51	3.02	3.56	2.73	4.99	4.22
A18:0-18:1	952.58	0.37	0.17	0.25	0.23	0.56	0.10	0.28	0.44
D16:1-20:4	958.49	0.31	0.23	0.15	0.15	0.22	0.17	0.16	0.13
D16:0-20:4	960.51	1.16	1.31	1.65	1.52	2.48	2.11	1.86	1.81
D18:1-18:2	962.52	0.34	0.28	0.27	0.35	0.17	0.41	0.36	0.44
D18:0-18:2/D18:1-18:1	964.54	3.04	3.20	3.14	2.87	2.35	2.42	2.83	2.70
D18:0-18:1	966.55	2.74	2.65	2.32	2.34	3.43	2.48	3.21	3.70
P16:0-22:6	968.51	5.42	5.64	6.09	4.88	4.57	5.44	4.82	5.20
P18:2-20:4	968.51	4.44	4.61	4.98	4.00	3.74	4.45	3.94	4.26
P18:1-20:4	970.53	3.96	4.16	4.80	3.60	4.22	4.49	4.82	4.46
P18:0-20:4/P16:0-22:4	972.54	6.56	5.30	6.70	5.83	7.86	8.77	8.15	7.07
P18:0-20:3	974.56	0.71	0.74	1.09	0.85	1.53	1.15	1.11	1.10
P18:1-20:1	976.58	1.10	1.72	1.47	1.41	1.63	1.13	2.02	2.19
P18:1-20:0	978.59	0.92	0.81	1.02	0.91	1.38	0.90	1.42	1.37
P20:0-18:0/P18:0-20:0	980.48	0.24	0.18	0.09	0.14	0.21	0.12	0.13	0.16
D16:1-22:6	982.49	0.53	0.47	0.41	0.46	0.71	0.33	0.44	0.43
D16:0-22:6	984.51	9.71	9.71	9.52	7.76	9.37	7.88	8.20	8.48
D18:1-20:4	986.52	1.98	2.04	2.42	2.14	3.24	2.26	2.72	2.77
D18:0-20:4	988.54	13.80	12.30	14.32	12.07	18.75	17.96	14.65	13.65
D18:1-20:2	990.55	0.87	0.78	0.81	0.98	1.06	0.62	0.93	0.87
D18:1-20:1	992.57	0.21	0.34	0.16	0.26	0.27	0.30	0.31	0.37
D18:0-20:1	994.53	4.86	5.20	4.65	4.43	3.80	4.03	3.98	4.35
D18:0-20:0	996.54	15.92	16.22	14.55	13.70	14.55	13.01	14.69	15.08
P18:0-22:5/P18:1-22:4	998.56	3.33	3.40	2.52	2.47	3.74	2.47	2.93	2.99
P18:0-22:4	1000.58	2.30	2.40	3.00	2.00	3.82	2.98	3.08	2.75
A20:0-20:4	1002.59	0.24	0.06	0.45	0.28	0.48	0.41	0.42	0.44
P18:0-22:2	1004.61	0.14	0.12	0.15	0.21	0.51	0.14	0.24	0.25
D18:1-22:6	1010.52	2.89	2.36	2.42	2.21	2.18	2.32	2.23	2.45
D18:0-22:6	1012.54	23.08	22.61	21.00	19.31	21.21	18.34	20.32	21.58
D18:1-22:5	1012.54	4.73	4.63	4.30	3.96	4.34	3.76	4.16	4.42
D18:0-22:5/D18:1-22:4	1014.55	1.44	1.56	1.10	1.14	1.01	1.02	1.23	1.05
D20:0-20:4	1016.57	1.81	1.08	1.78	1.29	2.32	2.27	1.82	1.52
D18:0-22:0	1024.63	0.30	0.37	0.36	0.29	0.33	0.20	0.32	0.35
D20:6-22:6	1028.48	0.24	0.16	0.16	0.36	0.52	0.27	0.15	0.29
D20:4-22:6	1032.51	0.10	0.25	0.08	0.13	0.31	0.17	0.08	0.21
D22:6-22:6	1056.51	0.68	0.55	0.61	0.46	0.46	0.68	0.54	0.61
Total PE	Sum	137.91	135.53	136.45	120.41	147.44	131.62	139.90	140.17
Total Pepl	sum	43.61	44.07	46.95	39.29	50.43	45.49	51.16	49.18
dPE		94.30	91.46	89.50	81.12	97.01	86.13	88.74	90.99

[a]D - represents diacyl specis, [b]P - represents plasmalogen species (same as Pepl); [c]A - represents akylacyl species

VITA

Michael Kwame Appenteng was born and grew up in Accra, Ghana. He obtained his undergraduate degree (B.Sc.) in Chemistry from the University of Ghana, Legon in 2005. He subsequently obtained a Master's degree (M.Phil.) in Nuclear and Radiochemistry from 2009 to 2011 from the same University. He worked at the Ghana Atomic Energy Commission as an Assistant Research Scientist until his departure to the USA in August 2015 to start a PhD program in Chemistry at the University of Missouri, Columbia on full scholarship.

Under the supervision of Dr. C. Michael Greenlief, he conducted diverse research focused on the development and application of chromatography and mass spectrometry-based methods in the areas of phytochemicals and the impact on oxidative / inflammatory responses in mammalian systems. These methods were applied for quantitative analysis of cyanogenic glycosides in elderberry and lipid peroxidation products in mice. Moreover, Michael had the opportunity to serve as a graduate teaching and research assistant for the Chemistry Department and Gehrke Proteomics Center respectively at the University of Missouri, Columbia. He graduated from the University of Missouri-Columbia with a Ph.D. in analytical chemistry in May 2021.

Michael has accepted a Postdoctoral Associate position at the laboratory of Dr. Irina Stepanov, Masonic Cancer Center, University of Minnesota, beginning July 1, 2021. Research in the lab is focused on the application of analytical biochemistry approaches to the characterization of chemical carcinogen exposure, metabolism, and DNA damage. Michael is very excited to work and learn to broaden his knowledge in these areas.