THERE ARE OBSERVABLE METABOLIC SIGNATURE PATTERNS IN *C. ELEGANS*: SPECIFICALLY FOR DIFFERENT LIFE STAGES GROWN WITH AND WITHOUT THE ADDED ANTIOXIDANTS VITAMIN C AND VITAMIN E?

by

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There are Observable Metabolic Signature Patterns in *C. elegans*: Specifically for Different Life Stages Grown with and without the Added Antioxidants Vitamin C and Vitamin E?

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at George Mason University.

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DEDICATION

This work is dedicated to my family (husband (Raj) and children (Samantha and Arienna), mother and father, brother and wife, sister and husband, nieces and nephews), and my advisor and mentor Dr. James D. Willett. I want to also thank all those that supported and encouraged me in this long road, a dream come true. With the completion of this degree a new window opens, with God's help.

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LIST OF ABBREVIATIONS

5-HT	-	Serotonin, 5-hydroxytryptamine
5-HTP	-	5-hydroxy-tryptophan
8-OH-guanine	-	8-hydorxy-guanine
ATP	-	Adenosine Triphosphate
ATD	-	Alzheimer-type domentia
AVG	-	Average
BSA	-	Bovin Serum Albumin
Ca^{+}	-	Calcium
CAT	-	Catalase
CEAS	-	Coulometric Electrochemical Detector Array System
C. elegans	-	*Caenorhabditis elegans*
CGMP	-	cyclic guanosine momophosphase
COMT	-	Catechol-O-methyl-transferase
Cu/Zn SOD	-	Copper/Zinc SOD
DDC	-	DOPA Decarboxylase
E. Coli	-	*Escherichia coli*
Epi	-	Epinephrine
Fe^{+}	-	Iron
Guan	-	Guanine
Guano	-	Guanosine
GSSG	-	GSH disulfide
GST	-	Glutathione Transferase
GPx	-	Glutathione peroxidase
HLE	-	Heated Liver Extract
H_2O_2	-	Hydrogen peroxide
HPA	-	Hypothalamo-pituitary-adrenal
HPLC	-	High Performance Liquid Chromatography
Hx	-	Hypoxanthine
IGF	-	Insulin-like Growth Factors
IIS	-	Insulin/IGE-/signaling
ILP	-	Insulin-like peptide
K^{+}	-	Potassium
L1	-	Larval stage 1
L2	-	Larval stage 2
L3	-	Larval stage 3

L4	-	Larval stage 4
$L^{\cdot}$	-	carbon-centered lipid radical
LCEC	-	HPLC with electrochemical detection
LH	-	phospholipid
$LO^{\cdot}$	-	alkoxy radical
LOOH	-	Lipid hydroperoxide
M	-	Reproductive *C. elegans* Population
MPA	-	Mobile phase A
MPB	-	Mobile phase B
NAD	-	Nicotinamide Adenine Dinucleotide
NADH	-	Nicotinamide Adenine Dinucleotide Phosphate
NA-5-HT	-	N-Acetyl Serotonin
$NO^{\cdot}$	-	Nitric oxide radical
Norepi	-	Norepinephrine
$^{\cdot}O_2$	-	Singlet oxygen
$O_2^{\cdot -}$	-	Superoxide anions
O	-	Old *C. elegans* Population
$OH^{\cdot}$	-	Hydroxyl radical
$ONOO^{-}$	-	peroxynitrile
OS	-	Oxidative stress
ORFs	-	Open reading frames
PG	-	Prostaglandins
PK-C	-	Protein Kinase C
PUFA	-	Polyunsaturated fatty acids
RNS	-	Reactive nitrogen species
$ROO^{\cdot}$	-	Peroxyl radicals
ROI	-	Reactive Oxygen Intermediates
ROS	-	Reactive oxygen species
S	-	Sulphur
SIR2	-	Silencing information regulator 2
TH	-	Tyrosine Hydroxylase
Tpp	-	Tea polyphenols
Trp	-	Tryptophan
Tyr	-	Tyrosine
SDS	-	Sodium Dodecyl Sulphate
sd	-	Standard Deviation
sGC	-	Soluble guanylate cyclase
SOD	-	Superoxide Dismutase
UV	-	Ultraviolet
Xan	-	Xanthine
Xantho	-	Xanthosine
Y	-	Young *C. elegans* Population

ABSTRACT

THERE ARE OBSERVABLE METABOLIC SIGNATURE PATTERNS IN *C. ELEGANS*: SPECIFICALLY FOR DIFFERENT LIFE STAGES GROWN WITH AND WITHOUT ADDED ANTIOXIDANTS VITAMN C AND VITAMIN E?

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Aging, a complex phenomenon, experienced by all living organisms, continues to intrigue mankind. Characteristics of aging in animals include a decline in motility and strength, reduction of memory retention, weakening of the immune system, decrease or cease of the ability to reproduce, pigmentation, and decrease in skin, ligament and muscle elasticity. The most common theories on aging are: the Gene Regulation theory, the Free Radical theory, the Oxygen Stress-Mitochondrial Mutation theory, Programmed Cell Death theory, Evolution Theory and Neuroendocrine and Immune (System-Based) theory.

This research investigates oxidative stress using Vitamin C and/or E at each life stage of the laboratory-cultured axenic nematode *C. elegans*. Size and response to cold temperature stress (functions of age) were used to separate the *C. elegans* into pre-

reproductive, reproductive and post-reproductive life stages. Redox responses for electrogenic compounds in the *C. elegans*, separated using reverse phase high performance liquid chromatography (CEAS-HLPC), and sixteen electrodes set between 0 and 900 mv at 60 mv intervals, were measured and saved as chromatographs for each life stage/treatment. Within these chromatographs one can identify and acquire quantitative data for the electrogenic analytes. Tryptophan, tyrosine, serotonin, norepinephrine, epinephrine, 8-Hydroxyguanine and the purine catabolites (guanosine, guanine, hypoxanthine, xanthosine, xanthine and uric acid) were identified and measured.

Tryptophan and tyrosine are small molecules that are conserved throughout evolution. Products of their metabolism include the neurotransmitters and hormones serotonin, norepinephrine and epinephrine that are involved in the aging process. Epinephrine and norepinephrine control responses to stress and increase heart rate. Serotonin is associated with behavioral responses. 8-Hydroxyguanine is a marker of DNA damage. The purine catabolites are formed from the breakdown of nucleotides and reflect alterations in nucleotide synthesis and salvage. The analysis of this set of metabolites provides one means of investigating effects of antioxidants on levels of cellular homeostasis dependent upon these ligands and their end products.

The population distributions are altered in *C. elegans*, cultured for 10 days in response to doses of Vitamin C and/or E at levels of 0.1, 0.5 and 1.0 mg/ml of media. The cultures with the Vitamin treatment had observable differences in population profiles, life stage distributions and analyte profiles at the end of their incubation period when compared to the untreated population. An increase of pre-reproductive *C. elegans*

in all Vitamin treated populations was observed. This effect is likely due the antioxidant properties of the Vitamins, resulting in a delay of the aging process.

A marker of DNA damage repair, 8-Hydroxyguanine was not found in any of the populations treated with Vitamin E and C in combination, but was found in all post-reproductive stages in populations derived from controls, or those treated with Vitamin C or Vitamin E alone. All of the purine metabolites were found in greater concentrations in the pre-reproductive worms treated with the Vitamin C and E combinations, compared to the Vitamin C, Vitamin E or untreated pre-reproductive worms. This implies that these antioxidants are capable of affecting purine metabolism, possibly through a reduction of oxidative damage to DNA and RNA.

There are substantial observed alterations in the trp/tyr ratios, that can affect the production of a host of key metabolites derived from these amino acids, that serve as important factors, ligands, neurotransmitters and hormones, that are key to the establishment and maintenance of cellular and physiologic homeostasis, in these, and other organisms. Serotonin was found in the post-reproductive *C. elegans*, where the concentrations in the Vitamin C & E combination experiments were the least. Epinephrine existed in measurable quantities in the pre-reproductive life stage, with the smallest concentration in the Vitamin C & E combination experiments. These are all indication that homeostasis is better maintained in the Vitamin C & E treatments, since there is minimal damage to the control systems due to ROS.

In summary the biochemical patterns from CEAS-HPLC are altered in the respective age groups where *C. elegans* is grown in the presence of the known oxidative

suppressors, Vitamin C and E. Higher concentrations of added Vitamin C and E do not seem to have negative effects on the biochemistry of the nematode, as measured with this analytic system but the combination of Vitamin C and E treatment produces the most dramatic effect. This study provides data that is supportive of the current theories of aging that involve ROS such as the Free Radical theory. However, the effects of the Vitamins on the organism can be explained by the nueroendocrine theory since the neurotransmitters concentrations changed with the addition of Vitamin C & E. It also implies Vitamin C and /or E increases longevity, with their antioxidant properties.

1. INTRODUCTION

This research came about because of the controversy as to whether antioxidants slow down the aging process and if they do, how much antioxidant is required to be effective in helping to slow down the aging process Also, at the time the study was designed there was concern that excessive amounts of antioxidants could have a negative impact on biological systems' functions. Vitamin C and E antioxidants were selected for this study as they are very common to everyone's diet, readily available over the counter and present in many food sources.

This research was performed on the nematode *C. elegans*. This organism is readily available, easy to culture in the laboratory, has a short life span, and vast knowledge is known of this nematode. Its entire genome and neurological map is known. It's an excellent model for age related studies and there are several age mutants. There are several web based sources of information on the nematode, *C. elegans*: WormBase (http://www.WormBase.org), Caenorhabditis Genetics Center (CGC) (www.cbs.umn.edu/CGC/), the National Bioresourse Project (www.shigen.nig.ac.jp/c.elegans/), the Wormbook website (www.wormbook.org), Wormatlas (www.wormatlas.org/index.htm) and Wormimage (www.wormimage.org).

2. BACKGROUND AND SIGNIFICANCE

THEORIES ON AGING

Aging (senescence) is a phenomenon that intrigues not only scientists, but society as a whole. The lengthening of the human life span, the desire of some to live longer, the increased percentage of elderly in the population and the medical related expenditures of the elderly are some of the factors that stimulate aging research (*Weinert B T & Timiras P S, 2003*). This remarkable phenomenon is not yet fully understood. It is a multi-factorial process that occurs at cellular, organ and intact organism levels. Aging is commonly referred to as a process that increases vulnerability of an organism to challenges during the lifetime, thereby increasing the potential of death, mostly after the organism attains its maximum reproductive competence (*Massoro E D, 1993; Jan Vijg and Yousin Suh, 2005*). Such characteristics of aging in animals include a decrease in motility and strength, reduction in memory retention, weakening of the immune system and, decrease in skin, ligament and muscle elasticity, with advancement in age. In biological systems deterioration is not inexorable, the systems can respond to their environments and use their own energy and try to defend and repair themselves.

Longevity is the property of being long-lived, approaching maximum life span for that species. The oldest observed age of death in a species is the maximum life span for that species. It is not greatly influenced by environmental conditions. Among the many

conditions that limit longevity (such as accidents, famine and violence), aging is probably the only one that has a biological limit to life. The wide variation in longevity among species points towards genetic factors that specify an organism's potential to reach old age. There are markers associated with senescence that occur regularly and without exception. They do not, of course, determine onset of death. There is evidence that points toward the existence of a universal genetic regulatory network that controls survival and aging rate through the wear and tear processes and cellular defense systems, the Gene Regulation theory of Aging *(Jan Vijg and Yousin Suh, 2005*).

There are several hypothesis and theories on aging, all with evidence to support them. The different theories of aging are not mutually exclusive since aging is considered to be an extremely complex, multifactorial process. They may adequately describe some or all features of the normal aging process alone or in a combination with other theories. Some the most common theories are; 'the gene regulation theory', 'the free radical theory of aging' (*Harman D, 1992*), 'oxygen stress-mitochondrial mutation theory of aging' (*Miquel J, 1998*), 'programmed cell death theory' (*Bargmann C I, 1991*), 'evolutionary theory on aging' (*Jazwinski S M, 1996*), and, the 'neuroendocrine and immune theories on aging' ['Shock's hypothesis' (*Dannon D et. al., 1986*)]. These theories can be broadly categorizes into either the evolutionary and mechanistic theories of aging (*Hughes K A & Reynolds R M, 2005)*, or the programmed and error theories of aging *(Weinert B T & Timiras P S, 2003*).

GENE REGULATION THEORY OF AGING. The Gene Regulation Theory of aging, a molecular theory of aging, proposes that senescence results from changes in gene expression. Many recently discovered genes, such as *daf-2, pit-1, amp-1, clk-1, p66Shc and SIR2* have been found to affect stress resistance and lifespan in laboratory organisms. This suggests that they can be part of a fundamental mechanism for surviving adversity. The conserved insulin and insulin-like molecules signal transduction pathway appears to negatively regulate life span in many species, including yeast, *C. elegans* and possibly mammals. These mechanisms in which mutations in the pathway increase longevity normally result in sterility, thereby reducing metabolic costs associated with reproduction, that interact with oxidative-stress resistance mechanisms *(Tatar M et. al., 2001)*. Insulin-signal related mutations that increase longevity in worms and flies typically increase the expression of antioxidant genes, increasing the resistance to oxidative stress. These mutations can also induce increased expression of heat shock proteins and increase thermotolerance in the flies and worms *(Hughes K A & Reynolds R M, 2005)*.

FREE RADICAL THEORY OF AGING. The "free radical theory" postulated by Harman in 1956, considers aging as a single common process, modifiable by genetic and environmental factors, responsible for aging and death in all living things (*Harman D, 1992*). This theory, a cellular theory, postulates that aging is caused by free radical reactions, i.e., nonspecific, essentially irreversible reactions that produce the cellular and molecular changes associated with the intrinsic aging process. Partially reduced oxygen

species such as superoxide anions (O_2^{-}) and hydroxyl radicals (OH^{-}) are believed to be the main source of age-related cell injury in aerobic organisms. These free radicals arise during irradiation or as byproducts of aerobic metabolism. However, cells possess an elaborate defense system to destroy these reactive species, including enzymes such as catalase, superoxide dismutase and glutathione peroxidase and, antioxidants. Cell injury by these free radicals includes DNA damage, damaged proteins and oxidized lipids, which are found in aged organisms. Since enzymes are proteins, such damage can result in alteration or termination of their activity (*Avery L et. al., 1990; Cohn J P, 1987; Harman D, 1992*). Strong evidence in support of the free radical theory came from life span experiments in flies and worms *(Larsen PL, 1993)*. Dietary or calorie restriction (DR or CR) has been found to increase the life span in yeast, nematodes, flies and mammals. It could be that dietary restriction reduces the generation of ROS *(Longo VD, Finch CE. 2003).*

OXYGEN STRESS-MITOCHONDRIAL MUTATION THEORY OF AGING.

The "oxygen stress-mitochondrial mutation theory" of aging assumes that cell senescence derives from mitochondrial membrane and genetic damage caused by oxygen radicals (*Miquel J, 1998*). This theory stems from the free radical theory of aging, but differs in that it focuses exclusively on a particular organelle, the mitochondria, and the type of damage. It incorporates the mitochondrial theory of aging: "The vicious cycle of oxidative damage to mtDNA by ROS (reactive oxygen species) and free radicals may be considered as the basis for this aging theory" (*Wei Yau-Huei, 1998*). The oxygen stress-

mitochondrial mutation theory of aging suggests that age-related mitochondrial damage occurs mainly, if not exclusively, in the differentiated somatic cells, and especially in neurons and other fixed postmitotic cells because they cannot regenerate their mitochondria as effectively as proliferating cells, which renew their organelles each time they engage in the mitochondrial division that accompanies mitosis (*Miquel J*, 1998).

PROGRAMMED CELL DEATH THEORY OF AGING. The "programmed cell death" theory is based on the observation that certain cells possess mechanisms involving specific gene products are designed to kill the cell in response to appropriate physiological signals. Because these cell deaths all involve the same sequence of morphological changes and require the activities of the same genes, programmed cell death can be regarded as an expression of a specific cell fate (*Bargmann C I, 1991*). Programmed cell death occurs normally during the development of all animals. Studies have shown cells that die by physiological mechanisms often undergo characteristic changes termed “apoptosis” or “programmed cell death”. There are two classes of genes that are involved in programmed cell deaths: (i) “determination genes” that decide which cells die, and (ii) “differentiation genes” which are necessary to express the fate of programmed cell death (*Vaux D L et. al., 1992*). It is indicated that the primary cell-death pathway is conserved throughout much of evolution. It appears to function in morphogenesis, metamorphosis and tissue homeostasis, as well as in developmental processes such as the generation of neuronal specificity and sexual dimorphism, eliminating unwanted cells (*Cohn J P, 1987; Driscoll M J, 1992; Jacobson M D, 1997*).

Therefore the role for apoptosis in the life span of individual cells (including T cells of the immune system) suggests that the same process may contribute to determining the life span of the whole organism. Relatively low rates of immune cell apoptosis appear to correlate with long life expectancy (*Slater A F G & Orrenius S, Oxidative stress and apoptosis,* in *Oxidative Stress and Aging, Cutler RG et. al., 1995*). Therefore, this theory can imply that aging is genetically controlled since programmed cell death generally refers to any cell death that is mediated by the intracellular death program, no matter what triggers it and whether or not it displays all of the characteristic features of apoptosis (*Jacobson M D, 1997*).

EVOLUTION THEORY OF AGING. The "evolution (or evolutionary) theory" which originated in the 1940s, assumes a powerful genetic basis of aging. Emerging from this theory is the concept that aging is not likely to be regulated in the 'same programmed' way as are early life processes such as development, it is the effect of gene variants that have escaped the force of natural selection *(Jan Vijg and Yousin Suh, 2005*). In the natural world organisms die from a wide array of extrinsic as well as intrinsic causes. In most species, few individuals live long enough to show obvious signs of senescence. Therefore, natural selection cannot plausibly explain the evolution of a temporal control system or "aging clock" whose primary function is to bring about senescence and death in a few survivors within a species, when the action is detrimental to the individuals. Nonetheless, some genes do influence aging and longevity and many of these are genes that regulate the processes of somatic maintenance and repair, such as

the stress-response systems (*Jazwinski S M, 1996; Ltihgow G J & Kirkwood T B L, 1996*).

Within the evolutionary theory of aging there are multiple routes by which senescence can evolve. Mutation accumulation (MA), antagonistic pleiotropy (AP), and disposable soma are some of the routes of the model that make different assumptions about patterns of age-specific effects of mutations (*Hughes K A & Reynolds R M, 2005)*. There is scant experimental evidence to support the MA theory. However, this can explain the familiar forms of Alzheimer's and Huntington's diseases. The genes involve (called private aging genes) originate in accidental germ line mutations. They are neutral in early age but start exerting their adverse effects at later ages. The detrimental, late-acting mutations may accumulate in the population and ultimately lead to pathology and senescence *(Perls T, et. al., 2002, Hughes K A & Reynolds R M, 2005)*.

Public aging genes involve the concept of antagonistic pleiotropy. They are mutations with the opposite pattern of effects, beneficial late and deleterious early. An example is the role of p53-dependant apoptosis in preventing cancer and possibly causing aging. During aging there is the progressive loss of cells by apoptosis that may lead to reduced organ function. However, in early age the cancer surveillance by p53 is critical to reach reproductive maturity *(Campisi J, 2003, Hughes K A & Reynolds R M, 2005)*. The disposable soma theory of aging can be considered a special case of antagonistic pleiotropy, where the basic idea argues that the somatic organism is effectively maintained only for reproductive success; afterwards it is disposable. Somatic maintenance and repair are metabolically costly, in other words, longevity, has a cost; the

balance of resources invested in longevity vs. reproductive fitness determines the life span (*Hughes K A & Reynolds R M, 2005; Weinert B T & Timiras P S, 2003; Mukhopadhyay A & Tissenbaum HA, 2007)*. Evolutionary tradeoff is an essential concept in both the Disposable Soma Theory and antagonistic pleiotropy. The disposable soma theory does not postulate the specific cause of aging, whereas the antagonistic pleiotropy theory suggests that some genes may be selected for beneficial effects early in life but deleterious effects with age, thereby contributing directly to senescence. There are experimental results that support antagonism between reproduction and longevity. For example limiting reproduction by destroying germ line cells can result in extended life span in both *Drosophila* and *Caenorhabditis elegans (Arantes-Oliveira N et. al., 2002, Sgro C M and Partridge L, 1999)*.

SYSTEM-BASED THEORIES OF AGING. In the system-based theories of aging (neuroendocrine and immune theories) the aging process is related to the decline of the organ systems essential for (a) the control and maintenance of other systems within an organism and, (b) the ability of the organism to communicate and adapt to the environment in which it lives *(Weinert B T & Timiras P S, 2003*). This can also be explained in the "Shock's hypothesis" of aging, which considers the aging process as the initiation of a breakdown in control systems that regulate an organisms' complex physiological functions - a breakdown in homeostasis (*Dannon D et. al., 1986*). This hypothesis originated from studies of changes in endocrine organ control functions with age. It suggested that specific organ systems such as the brain, the immune system or the

neuroendocrine system, serve as master controllers of the body determining development and aging rates.

The neuroendocrine theory proposes that aging is due to changes in the neural and endocrine functions that are crucial for (a) coordinating communication and responsiveness of all body systems with the external environment; (b) programming physiological responses to environmental stimuli; and (c) maintaining an optimal functional state for reproduction and survival while responding to environmental demands. These changes not only selectively affect the neurons and hormones that regulate significant functions such as growth, development and aging, but also affect those that regulate survival through adaptation to stress. These regulators are "biological clocks". In this theory the hypothalamo-pituitary-adrenal (HPA) axis is the master regulator, it signals the onset and termination of each life stage, in humans. One of the major functions of the HPA is the maintenance of internal "homoeostasis", despite changes in the environment. Chronic exposure to severe stress from a multitude of physical, biological and or emotional stimuli may exhaust or weaken the capacity to adapt, leading to diseases and death. Aging would then be the result of a decreasing ability to survive stress *(McEwen BS, 2002)*. It's one of the many definitions of aging that suggests a close relationship between stress and longevity *(Weinert B T & Timiras P S, 2003*).

Integration of responses to environmental stimuli is carried out by the hypothalamus. The hypothalamus regulates several important nervous functions (such as sympathetic and parasympathetic visceral functions), behaviors (which includes sexual

and eating behaviors, rage and fear), and endocrine functions. It produces and secrets hypophysiotropic hormones that stimulate or inhibit hormone released from the pituitary gland. The pituitary gland regulation occurs by releasing hormones, or by stimulating a peripheral endocrine gland such as the adrenal cortex, thyroid or gonads. The catecholamines epinephrine and norepinephrine are the major hormones of the adrenal cortex in humans, yet they exist in *C. elegans* that have simple nervous systems. They function as neurotransmitters for the sympathetic division of the autonomic nervous system, where they respond rapidly to any external or internal stress through circulatory (increased blood pressure) and metabolic adjustments. With aging there is a reduction in sympathetic responsiveness. It's characterized by a decreased number of catecholamine receptors in peripheral target tissues, a decline of heat shock proteins that increase stress resistance in many animal species, decreasing the capability of catecholamines to induce these heat shock proteins *(Udelsman R et. al., 1993)*. The hormones of the adrenal cortex are glucocorticoids, mineralocorticoids, and sex hormones. Glucocorticoids regulate lipid, protein and carbohydrate metabolism. Mineralocorticoids regulate water and electrolytes. The sex hormone dehydroepiandrosterone decreases with aging. Glucocorticoids and other steroid hormones are regulated by positive and negative feedback between the target hormones and their central control, the pituitary and hypothalamus. With aging and the response to continual and severe stress, the feedback mechanisms may be impaired. For example, the glucocorticoids may become toxic to neural cells, disrupting feedback control and hormonal cyclicity *(Sapolsky RM, 1992; Sapolsky RM, 1998)*. The discovery of the insulin/IGF pathway controlling stress and

longevity in some animal species support the neuroendocrine theory of aging *(Kawano T, et. al., 2000*).

Another system-based theory of aging is the *Neuroendocrine-immuno theory*, an extension of "Shocks hypothesis of aging". In animals with multisystem regulation, there is significant interaction and integration between the neuroendocrine and immune systems *(Weinert B T & Timiras P S, 2003*). These interactions occur through neuropeptides, cytokines and, hormones. Parallel to neuroendocrine interactions, the immune system has several essential functions such as the control and elimination of foreign organisms and substances in the host body while, at the same time recognizing and sparing its own molecules from destruction. Generally, in elderly humans immunosenescence is characterized by a decrease resistance to infectious diseases, a decreased protection against cancer, and an increased failure to recognize its molecules *(Ginaldi L and Sternberg H, 2003)*. The thymus is one of the most important immune organs in humans. Both the neuroendocrine and immune systems have the ability to modify their function according to demand. Their plasticity is most efficient at early ages.

Results form experiments involving caloric restriction have shown that caloric restriction is the most potent and reproducible environmental variable capable of extending the life span in a variety of animals from worms to rats. The many benefits of caloric restriction are accompanied by a number of negative effects that may prevent its applicability in humans and other animals, such as stunted growth and the failure of sexual maturation *(Merry BJ, 1999)*. It is believed that caloric restriction reduces the

amount of ROS in the system hence reduces the stress involved in maintaining a homeostasis system.

COMPARISON OF THE COMMON THEORIES OF AGING. The mitochondrial theory of aging encompasses the free radical theory of aging since they both involve reactions and damages caused by free radicals. This theory (the mitochondrial theory of aging), singles out the central senescence-causing factor as oxy-radicals but, fails to explain the reasons for the lack of repair and regeneration mechanisms to counteract the injurious effects of the radicals. It does not postulate the existence of *specific* senescence-programming genes, but stresses the importance of the genetically controlled process of cell differentiation as the basis of the oxygen-caused mitochondrial injury that triggers cellular and organismic aging. The programmed cell death theory of aging is completely at odds with the free radical and oxidative stress theories of aging. It also differs from the gene theory of aging, in that apoptosis genes are not involved in oxidative stress resistance.

The "evolutionary theory" of aging does not support the programmed cell death theory of aging but does accept that certain genes influence aging and longevity - stress response and tolerance genes. These genes can enhance repair systems for damages created by ROS. The system-based theories on aging assume that aging is regulated by "biological clocks", such as changes in endocrine organ control functions, which contradicts the gene regulation and programmed cell theories of aging. Oxidative stress created by ROS can trigger changes in the endocrine organ control functions. It is

possible that ROS reactions can trigger the breakdown of homeostasis producing alterations in the robust control systems, leading to degradation in repair and replacement processes. Therefore, some aspects of these several aging theories (system-based theories on aging [which include enzyme and Shock's hypotheses of aging] together with the free radical, mitochondrial and the oxygen stress-mitochondrial mutation theory of aging) are incorporated within each other due to the involvement of ROS.

Since oxidative stress is a key determinant factor in the aging process, examination of oxidative suppressor and stressors can reveal important information on the aging process as a whole. Means of increasing longevity, prolonging arrival at the organismal status defining senescence in animals would reduce diseases of the aged and provide a higher quality of life in the aged.

OXIDATIVE STRESS AND SUPPRESSORS

In an aerobic environment organismal exposure to reactive oxygen intermediates (ROI), including molecular oxygen, superoxide, hydrogen peroxide, and hydroxyl radicals, occurs. Aerobic organisms have adapted oxygen dependent oxidation - reduction reactions to drive key metabolic and regulatory pathways essential to normal cell growth. As an accessory to these processes, a number of defense mechanisms to control the level of ROI have evolved. Oxidative stress results when ROI and other oxidizing species exceed the cellular antioxidant capacity, resulting in oxidative damage to lipids, proteins and DNA (*Scandalios J G, 1997*).

There have been studies on oxidative stress in sick patients. For example, recently there was a study to determine oxidative stress in patients with acute and chronic viral hepatitis B. It was found that patients with both acute and chronic viral hepatitis B were under the influence of increased oxidative stress, which was associated with a decrease of the antioxidants measured (beta-carotene and whole blood reduced glutathione) (*Dikici I et. al, 2005*).

Oxidative suppressors, commonly known as antioxidants, are compounds that retard oxidative stress. "An antioxidant has been defined as 'any substance that, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate' [*(2, 4) Paoletti R et. al., p3, 1998*]." The human diet contains an array of dietary antioxidants with the most common being ascorbate (Vitamin C), tocopherols (Vitamin E), carotenoids and flavoniods. Within the body there are enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione transferase (GST) and glutathione peroxidase (GPx), which detoxify ROIs and thus provide protection against oxidative damage.

Vitamin C is a water-soluble vitamin, found naturally in many food sources such as citrus fruits and green vegetables. It structure was identified by Szent-Gyorgyi in the early 1900s (*Paoletti R et. al., 1998*). Most animal species can make Vitamin C from glucose, with the exception of humans, primates and guinea pigs. In humans vitamin C exists in two biologically active forms, ascorbic acid and dehydroascorbic acid. The ready interconversion of these two forms gives vitamin C its antioxidant capabilities. Vitamin C is used in preventive medicine treatments such as in helping to prevent and

fight the common cold and flu. Also, it is known to be involved in the metabolism of several amino acids and essential neurotransmitters, such as the formation of hydroxyproline, hydroxylysine, norepinephrine, epinephrine, serotonin, homogentistic acid, and carnitine (*Wolinsky I & Driskell J A, 1997; Davies M & Austin J, 1991*).

Vitamin E is an essential fat-soluble vitamin. It includes a group of eight naturally occurring compounds in two classes designated as tocopherols and tocotrienols with different biological activities. This group of compounds is highly lipophilic and readily partitions into lipoproteins and membranes. The highest biological antioxidant activity is found in α-tocopherol. It is the most available form of Vitamin E in food. The richest source of vitamin E in the diet is found in vegetable oils and products made from these oils, such as wheat germ, nuts and, some leafy green vegetables. The most widely accepted biological function of vitamin E is its antioxidant property in preventing lipid peroxidation of polyunsaturated fatty acids (PUFA). Vitamin E is the most effective chain-breaking, lipid-soluble antioxidant in the biological membranes where it contributes to membrane stability, regulates fluidity, and protects critical cellular structures against damage from reactive oxygen species (ROS) and other free radicals (*Wolinsky I & Driskell J A, p122*, 1997, *Ye H et. al.,* 2008).

OXIDANTS

An oxidant removes electrons from another substance, and is thus reduced itself. The generation of ROS (reactive oxygen species) is associated with aerobic life. Reactive oxygen species are responsible for the oxidative damage of biological target

molecules. They include peroxyl radicals ($ROO^{\cdot}$), the nitric oxide radical ($NO^{\cdot}$), the superoxide anion radical ($O_2^{\cdot -}$), the hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$), peroxynitrite ($ONOO^-$), and hydrogen peroxide (H_2O_2). Humans and *C. elegans* have several pathways for the generation of ROS, which are similar, the most common being in the mitochondria. The superoxide radical appears to play a central role, generated by enzymatic one-electron reduction of oxygen from xanthine oxidase, NADPH oxidase, by leakage of the respiratory chain, or by redox-cycling mechanisms. The most reactive species is the hydroxyl radical, which has no practicable defense in terms of interception, within the cell. The peroxyl radical is a relatively long-lived species. It can be generated in lipid peroxidation. Further products of lipid peroxidation are alkyoxyl radicals and organic hydroperoxides. The organic hydroperoxides can rearrange themselves to form endoperoxide intermediates, which decompose to form aldehydes. These aldehydes can then react with amine groups modifying the protein part of lipoproteins. Preferential targets for the chemical reaction of singlet molecular oxygen are double bonds, such as in polyunsaturated fatty acids and, in DNA, bases like guanine. The nitric oxide radical is a signaling compound formed enzymatically from arginine. It is also produced by activated macrophages, where in excess it is cytotoxic. Nitric oxide reacts directly with superoxide forming peroxynitrite. Peroxynitrite is capable of oxidizing guanines in DNA, generating strand breaks and, inducing lipid peroxidation. The nitric oxide radical, relaxes smooth muscle cells in blood vessel walls thereby lowering blood pressure and, interferes with cellular signaling pathways by nitrating tyrosine residues in proteins (*Lancaster J, 1997; Paoletti R et. al., 1998*).

SIGNAL TRANSDUCTION BY OXIDANTS

In biological systems a diversity of antioxidant defense systems are operative to counteract the peroxidant load. The concentrations of individual ROS formed by cells and tissues, is usually tightly controlled by specific metabolizing and scavenging systems, since ROS in excess usually results in cytotoxic effects. Thus, to prevent cytotoxic effects, there are interactions of individual oxidant species with cell-control mechanisms involved in signaling processes. The signaling systems most sensitive to oxidants appear to be linked to the function of the metabolic pathways. In low oxidant concentrations prostaglandins (PG) are synthesized and, soluble guanylate cyclase (sGC) and cyclic guanosine monophosphase (cGMP) are activated. Other signaling systems appear to be activated at higher levels of oxidants, which suggest roles for these mechanisms in pathophysiological processes and cellular responses to oxidant stress. Moderate concentration of ROS species result in tyrosine phosphorylation, protein kinase C (PK-C) activation, catecholamine oxidation, S-thiolation and S-nitrosation (disulfide formation of protein thiols and amino groups), alteration of K+ (potassium) and Ca^{2+} (calcium) channels, alteration in gene regulation and, proliferation. High concentrations of ROS result in apotosis and necrosis (*Scandalios J G, 1998*). Most of these compounds affected by the ROS act as second messengers in signaling, resulting in great amplification of the signal. They exist in both humans and *C. elegans*.

From several studies it is concluded that peroxides appear to be essential cofactors for the cyclooxygenase reaction and potent stimuli for the production of prostaglandins by tissues (*Wei et. al., 1995*). However, prostaglandin production is not altered by

increased tissue levels of the superoxide anion, resulting in the inhibition of Cu/ZnSOD activity (*Omar et. al., 1991*). It was later revealed that peroxides appear to oxidize the heme of this enzyme from its basal Fe^{3+} state to a Fe^{4+} oxidation state, explaining the Cu/ZnSOD inactivity (*Wei et. al, 1995*). The release of the precursor of prostaglanins appears to be tightly controlled by the activity of phospholipases, which seems to be directly or indirectly stimulated by peroxides (*Boyer et. al., 1995; Rao et. al., 1995*). Prostaglandin production is very sensitive to stimulation by low levels of peroxides, dominated by the production of one specific prostaglandin. However, exposure to increased peroxide levels can either change the profile and actions of the prostaglandins produced or can impair prostaglandin signaling, which may be associated with the expression of regulation by other pathways of arachidonic acid metabolism (*Tsai et. al., 1992; Gurtner & Burke-Wolin, 1991*).

Soluble guanylate cyclase (sGC) synthesis is stimulated by hydrogen peroxide metabolism with catalase and the NO ROS. However, the superoxide anion seems to inhibit both the hydrogen peroxide and NO reaction to form sGC. The formation of cGMP is controlled by the formation of sGC. Therefore, reactive oxygen species regulate sGC activity or signaling thought to be mediated by cGMP in intact cells and tissues (*Wolin M S, 1996*).

The oxidative metabolism of NO, $ONOO^-$, and resulting thiol nitrosation interactions have the potential to regulate systems that could participate in signaling-like processes. An appreciable level of scavenging of NO (nanomolar levels) by the superoxide anion results in an attenuation of NO signaling mechanisms, such as those

mediated through cGMP (*Pryor W A & Squadrito G L, 1995*). Signaling-like actions of large concentrations of exogenous $ONOO^-$ that might originate include vascular relaxation (*Liu S et. al., 1994; Wu M et. al, 1994*), the uncoupling of receptor action (*Lipton S A et. al., 1993*), the inhibition of mitochondrial respiration (*Radi R et. al., 1994; Xie Y & Wolin M S, 1995*), and the inhibition of glyceraldelyde-3-phosphate dehydrogenase (*Mohr S et. al., 1994*).

There is accumulating evidence suggesting that certain tyrosine kinases or phosphates are potential systems whose function is directly controlled by specific ROS or RNS (reactive nitrogen species) (*Fialkow L et. al., 1993; Schultze-Osthoff K et. al., 1995; Brumell J H et. al., 1996*). A study suggests that inhibition of the phosphates readily occurs in the presence of ROS which in turn results in the activation of tyrosine kinases which autophosphorylate themselves, associated with stimulation of the activity of the kinase in its phosphorylated state (*Brumell J H et. al., 1996*). Multiple signaling mechanisms including phospholipases, gene expression, and proliferation may involve events initiated by oxidant-elicited stimulation of tyrosine kinases (*Natarajan V 1995; Schultze-Osthoff K et. al., 1995*). Multiple signaling mechanisms sensitive to the actions of oxidants may be important in the processes that control K^+ channels, protein kinase C activity, and cellular control of Ca^{2+} homeostasis (*Scandalios J G, 1997*).

Above demonstrates the complex signaling systems developed through evolution to control and breakdown ROS. The neuroendocrine theory of aging focuses on the breakdown of this complex signaling system that comprises of primary and secondary messengers. Some of these messengers are neurotransmitters and hormones that are

produced by metabolites of evolutionary compounds such as tyrosine, tryptophan and purine catabolites. These mentioned metabolites exist in both humans and *C. elegans* and most of the enzymes involved in their breakdown are common to them both.

ANTIOXIDANTS

The antioxidant defense systems include enzymatic and nonenzymatic antioxidants. Superoxide dismutase scavenges the superoxide anion while, catalase and GSH peroxidases reduce hydrogen peroxide and organic hydroperoxides, respectively. Indirect antioxidant functions are provided by enzymes that restore endogenous antioxidant levels, such as the replenishment of GSH levels upon the reduction of GSH disulfide (GSSG) by GSH reductase. Reactive intermediates produced in reactions of peroxidants and biological molecules are conjugated by phase-II detoxification enzymes (such as GSH-*S*-transferases, also know as glutathione transferase [GST]) which favor their excretions. The glutathione transferases inactivate endogenous α,β-unsaturated aldehydes, quinines, epoxides and hydroperoxides formed as secondary metabolites during oxidative stress. They are also involved in the biosynthesis of leukotrienes, prostaglandins, testosterone, and progesterone and, the degradation of tyrosine *(Hayes J et. al., 2005)*. The control of the levels of free transition metals such as iron and copper ions can help in preventing ROS formation. They are located in metal binding proteins such as ferritin, transferritin, and ceruloplasmin. A variety of endogenous, low-molecular-weight compounds are also involved in antioxidant defense such as GSH,

ubiquinol-10 and, bilirubin. GSH serves as a cofactor of several detoxifying enzyme and is also involved in the reduction of protein disulfides.

The most common dietary antioxidants are ascorbate, tocopherols, carotenoids and flavonoids. There functions overlap to some degree and, many of them are tightly interlinked in interlocking cycles of regeneration and recycling (**Figure 1**). Vitamin C (ascorbate) scavenges superoxide and other free radicals. It can regenerate tocopherol from the tocopheroxyl radical, which is formed when tocopherol reacts with a lipid peroxyl radical (*Packer J E, et. al., 1979*). Vitamin E (α-tocopherol) is the major lipid chain-breaking antioxidant in lipid domains. Carotenoids are singlet oxygen scavengers.

The major natural antioxidants can be biologically compartmentalized depending on where they are found. Vitamin E, ubiquinol and carotenoids are found in intracellular systems membranes. Vitamin E is a poor antioxidant outside a membrane bilayer but extremely effective when incorporated into the membrane (*Gutteridge J M C, 1978*). Vitamin C, glutathione, alpha lipoate, superoxide dismutase, catalase, glutathione peroxidase, glutathione-transferase and ferritin are found in the cytosol. Vitamin C, uric acid, bilirubin, caeruloplasmin, transferrin, lactoferrin, albumin, haptoglobin and superoxide dismutase are found in extracellular fluids. Finally, α, γ- tocopherols, tocotrienols, ubiquinol-10 and carotenoids (β-carotene, lycopene, phytofluene) are found in lipoproteins (*Packer L in Oxidative Stress and Aging, 1995*). GSH is vital to antioxidant defenses because it serves as a substrate in the GSH-PX reaction; acts as a free radical scavenger and helps to regulate the thiol disulfide concentration of a number of glcolytic enzymes and Ca-ATPase, indirectly preserving intracellular Ca^{2+}

homeostasis; and, it regenerates other scavengers and antioxidants like α-tocopherol (*Benzi G. & Moretti A, 1995*).

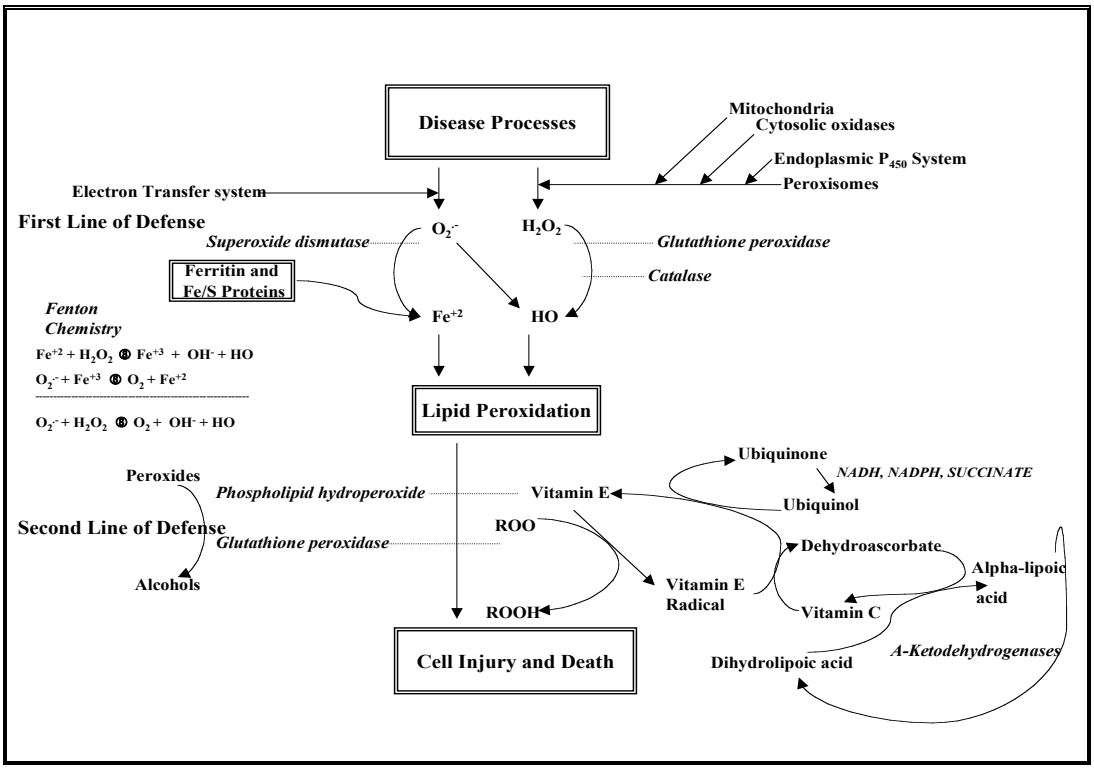


Figure 1: Effects of superoxide anion and hydrogen peroxide production in the cell, together with some defense mechanisms.

Vitamin C is the most effective, least toxic, water-soluble dietary antioxidant "free radical scavenging" vitamin known. It has been found to be important in the prevention of scurvy, in the processes of collagen synthesis, norepinephrine synthesis, amidation of peptide hormones, and the regeneration of vitamin E and providing protection against oxidative damage in a variety of tissues. It is found in high concentrations in many tissues such as human plasma. Vitamin C level varies from tissue

to tissue, and species to species according to function or tissue requirement. Upon reaction with ROS, it is oxidized in two one-electron steps to dehydroascorbate by dehydroascorbate reductases. Compared to vitamin C, dehydroascorbate is found in low levels. Dehydroascorbate (DHAA) is potentially toxic. It is a highly reactive, unstable compound that has been shown to accumulate in some disease states and is associated with cellular damage, including disruption of membranes, islet cell dysfunction and neurotoxicity. Ascorbate has been shown to be affective against superoxide anion, hydrogen peroxide, the hydroxyl radical, peroxynitrite, and singlet oxygen. Important sources of vitamin C include fresh fruits and green vegetables (*Paoletti R, 1998*).

Ascorbic acid is an essential factor in many hydroxylation reactions. Hydroxyproline and hydroxylysine are constituents of collagen, the main protein of fibrous connective tissue in animals. Collagen contributes to nearly one-third of the total mammalian body protein, occurring in tendons, ligaments, skin, bone, teeth, cartilage, heart valves, intervertebral disks, cornea, and lens in addition to its general tissue distribution as extra-cellular framework. There are no codons for hydroxyproline and hydroxylysine. When collagen is synthesized, proline and lysine are hydroxylated post-translationally on the growing polypeptide chain. The proline and lysine hydroxylase requires ascorbic acid, iron (II), and α-ketoglutarate as the co-oxidant. Collagen formed in the absence of ascorbic acid is unable to form proper fibers, resulting in skin lesions and blood vessel fragility characteristic of scurvy. The plasma complement protein that constitutes an important part of the guinea-pig's defense against pathogens, contains hydroxyproline, which also depends on Vitamin C (*Davies M B & Austin J, 1991*).

The copper- containing enzyme 4-hydroxyphenylpyruvate dioxygenase, which requires vitamin C for its activity, is involved in the formation of fumaric acid and acetoacetic acid in animals, from the catabolic processing of the amino acids phenylalanine and tyrosine. Tyrosine is the precursor for the synthesis of the hormone and neurotransmitter norepinephrine. This is formed from dopamine (3,4-dihydroxyphenylethanolamine) by the vitamin C-requiring dopamine β-mono-oxygenase, in the adrenal medulla of animals. In the pineal gland, tryptophan undergoes hydroxylation to form the vasoconstrictor and neurotransmitter 5-hydroxytryptamine (serotonin). The initial hydroxylation step catalyzed by tryptophan-5-mono-oxygenase requires vitamin C (*Davies M B & Austin J, 1991*).

Lysine is converted into carnitine through a chain reaction driven at two points by hydroxylases that act on γ-butyrobetaine and trimethyl-lysine. These hydroxylases require vitamin C for full activity. Carnitine is essential for the transport of energy-rich fatty acids from the cytoplasm to the mitochondria matrix. Vitamin C is also required in the stepwise conversion of cholesterol to bile acid, cholic acid, via 7α-hydroxycholesterol, 3α,7α-dihydroxycoprostane and 3α,7α,12α-trihydroxycoprostane. In lipid metabolism, conventional fatty acids with even number of carbon atoms are α-oxidized by a mono-oxygenase and subsequently decarboxylated to form an odd-numbered carbon derivative. Both these steps appear to require ascorbic acid (*Davies M B & Austin J, 1991*).

Vitamin C also has reducing properties. In aqueous parts of the cell, it assists another water-soluble anti-oxidant, the tripeptide glutathione, in mopping up potentially

dangerous oxidizing radicals. Its reducing property also 'assists' another vitamin, folic acid. For folic acid to be in its functional state, it must be in its most reduced tetrahydrofolate form. This is brought about and/or maintained by ascorbic acid. In red blood cells there is the tendency of the aggressive superoxide free radical to produce non-functional methaemoglobin (metHb), by oxidizing the haem iron from iron (II) to iron (III). This particular process is reversed by metHb reductase, which involves cytochrome b_5 and ascorbic acid (*Davies M B & Austin J, 1991*).

It has been found that caspase proteases (ced-3 in the *C. elegans*) are a central component of an apoptotic program for programmed cell death. Vitamin C has been observed to prevent this type of apoptosis. The inhibition of superoxide in neutrophilic leukocytes occurs with both vitamin A and C (*Evans P H et. al.,* in *Oxidative stress and aging, Cutler RG et. el., 1995; Paoletti R, 1998*).

The generic term for all entities that exhibit the biological activity of alpha-tocopherol is vitamin E. Eight substances have been found in nature to exhibit vitamin E activity: d-α-, d-β-, d-γ-, and d-δ-tocopherol; and d-α-, d-β-, d-γ-, and d-δ-tocotrienol; together with their acetate and succinate derivatives and; synthetic tocopherols and their acetate and succinate derivatives. Each form of this vitamin E exhibits different biopotency. Only 12.5 % of synthetic vitamin E is the same as d-α-tocopherol (natural vitamin E), which is due to the presence and position of the methyl groups on the aromatic ring of the molecule and the configurations at the asymmetric carbons of the side chain. There is evidence that humans strongly discriminate between the naturally occurring RRR-α-tocopherol form in vitamin E and SRR-α-tocopherol, one of the eight

stereoisomers present in synthetic vitamin E. The discrimination does not seem to appear during absorption, but is a post-absorptive phenomenon in the liver. The acetate and succinate esters of vitamin E are often used as vitamin E sources in commercial supplements because they are more stable in the presence of oxygen than the free tocopherols. After ingestion of a vitamin E ester, almost all of it in the blood and tissue is in the unesterified tocopherol form. In animals, evidence indicates the mid gut is the site of greatest absorption of tocopherols and that bile is needed for normal absorption (*Pryor W A, 1994*).

Vitamin E is of particular importance in neurological function. Vitamin E has been shown to play an important role in the prevention of arteriosclerosis. It also inhibits protein kinase C and smooth muscle cell proliferation, which does seems to be due to its antioxidant properties (*Azzi A et. al.,* in *Oxidative stress and aging, 1995*). The inhibition of protein kinase C results in the inhibition of the production of NADPH oxidase-mediated superoxide anion by stimulated macrophages (*Evans P H et. al.,* in *Oxidative stress and aging, Cutler RG et. al., 1995*). It was shown in rats that exhausting exercise can increase the levels of protein oxidation in skeletal muscles. This increase of protein damage was alleviated when these animals were fed with vitamin E (*Reznick A Z et. al., 1992; Witt E H et. al., 1992*). Muscle damage due to immobilization may be reduced by the administration of vitamin E, since it was found to reduce the levels of both muscle atrophy and lipid peroxidation (*Kondo H, et. al., 1991*). This may be of utmost importance to aging persons lying immobilized for long periods of time. Also, vitamin E may function to stabilize membranes and to maintain cellular homeostasis. In humans it

has been reported that the plasma and tissue vitamin E is altered by heavy and unaccustomed exercise. It has been reported that alcoholics are treated with vitamin E, since they maintain low circulation levels of vitamin C, vitamin E, glutathione and selenium, and elevated concentrations of plasma lipid peroxides (*Meydani M* in *Oxidative stress and aging, Cutler RG et. al., 1995*).

A variety of antioxidants such as tea polyphenols (TPP), carotenoids, vitamin E and C are found in tea leave. Tea TPP includes catechin compounds. The antioxidative properties of TTP were found to be superior to those of vitamin E and C, both in *vitro* and *vivo*. This effect could be due to TPP dual solubility properties: soluble in water as well as in organic solvents, and its strong chelating ability. It was shown that tea catechins strongly inhibited the Cu^{2+} mediated oxidation of LDL obtained from porcine serum. Also, they were much more effective than vitamin C, E or BHT. TPP inhibited the oxidative modification of cholesterol ester and fragments of LDL (*Tomita I et. al.,* in *Oxidative stress and aging, Cutler RG et. al., 1995*).

AGING THEORIES THAT INCORPORATE OXIDATIVE STRESS

Evidence that supports both the free radical theory and oxygen stress-mitochondrial mutation theory of aging involve reactions and damages caused by free radicals. These theories do not involve the breakdown of "control" or homeostasis that is implicated in the neuroendcrine theory of aging, caused by ROS. The free radical theory of aging is supported by the observation that the mean life span of a species can be

increased by slowing the rate of initiation of random free radical reactions through dietary changes (*Cohn J P*, 1987).

Free radicals also appear to play a major role in many conditions such as heart disease, cancer, and neurodegenerative diseases, which is caused by the breakdown in control organs, which occurs more frequently in older people, which can be explained by the system-based theories on aging. Superoxide is a free radical compound with an odd number of electrons, derived from molecular oxygen by the addition of a single electron. Biologically reactions that produce superoxide occur under a very broad spectrum of pathological circumstances, including all infectious diseases, all inflammatory diseases, and all diseases that involve ischemia (restriction in blood supply, generally due to factors in the blood vessels) and reperfusion (caused by the absence of oxygen and nutrients from blood). The neurotransmitters norepinpehrine and epinephrine helps to control blood pressure in humans. Alterations in their concentrations due to ROS can result in diseases involving blood circulation. The superoxide radical is not a highly reactive species, but it's reactions show considerable versatility. This radical can serve as a mild oxidant, a fairly strong reductant, or as an initiator or terminator of free radical chain reactions (*McCord J M, 1998*).

Superoxide has been found to directly inactivate many key enzymes, such as catalase, creatine phosphokinase, glyceraldehyde-3-phosphate dehydrogenase, glutathione peroxidase, myofibrillar adenosine, and Ca^{2+}-Mg^{2+}-ATPase. These key enzymes are part of the first line of defense against ROS, which can cause diseases and death. Many believe that the most generally destructive action of the superoxide radical

may be the bringing about of the reductive release of iron (fenton chemistry) from ferritin (**Figure 1**). Iron is a necessary trace element, essential for life. Through evolution humans have about 10 % efficiency for absorbing iron but no mechanism for eliminating excess iron. Therefore, cells continuously store excess absorbed iron in a complex with the protein ferritin, which is found in all tissues. The hydroxyl radical formed in the reductive release of iron from ferritin, is an extremely powerful oxidizing species, with it's oxidizing potential second to atomic oxygen. It can attack all classes of biological macromolecules: depolymerize polysaccharides, cause DNA strand breaks, inactivate enzymes, and initiate lipid peroxidation (**Figure 1**). Since lipid peroxidation is a chain reaction that is geometrically amplified by redox-active iron, it is this action of the hydroxyl radical that probably have the greatest pathophysiological consequences in diseases such as ischemic heart disease and stroke (*McCord J M, 1998*). Lipid peroxidation is a function of age.

The hydroxyl radical is capable of reacting with a polyunsaturated fatty acid chain of a phospholipid molecule (LH), resulting in the free radical ($L^{\cdot}$, the carbon-centered lipid radical) that propagates the chain reaction. In vivo, the end result of one such chain reaction is the accumulation of 10 to 15 lipid hydroperoxide (LOOH) molecules. The ferrous iron is also capable of reducing the hydroperoxide to form a new radical, the alkoxy radical ($LO^{\cdot}$), which can initiate a new chain reaction (**Figure 1**). Therefore, the presence of both superoxide radical and redox-active iron can be devastating to the cell in terms of maintaining membrane structure and function, which is seen as a function of age. **Figure 1** shows two lines of defense mechanisms that act to remove these charges

ions from the cells and prevent cell death and injury. The antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase; act as a first line of defense, directly intercepting the active oxygen species. The antioxidant vitamins E and C together with the antioxidant enzyme phospholipid hydroperoxide glutathione peroxidase act as the second line of defense when the first is overwhelmed (*McCord J M, 1998*).

Certain damaged proteins accumulate during aging and with age related diseases (*Starke-Reed P E & Oliver C N, 1989*). Examples of such proteins are the proteases whose inactivity, decreased activity, or absence causes the accumulation of oxidized proteins. For those proteins (which is indicated by a loss of protein -SH groups, protein carbonylation, and loss of catalytic activity of enzymes) that are particularly susceptible to oxidative damage, there is an age-related two- to threefold exponential increase in their concentrations (*Sohal R S & Weindruch R, 1996*). One characteristic of aging in animals (which include *C. elegans* and humans) is the accumulation of lipofuscin in cells. Lipofuscin contains damaged proteins and oxidized lipids caused by free radical reactions. It is a product of molecular changes primarily to membranous material brought about by reactions with hydroxyl radicals. It is believed that lipofuscin is formed within secondary lysosomes due to the interplay of two processes, the production of partially reduced oxygen species by mitochondria and the oxygen species interaction with the autophagocytotic degradation products produced within secondary lysosomes. In young animals lysosomes show no accumulation of the "age" pigment while functioning to clear damaged cell components (*Adelman R C & Dekker E E, 1985; Brunk U T et. al., 1992*). Pigmentation is a function of age to both humans and *C. elegans*.

A characteristic of aging and oxidative stress damage on DNA is the production of 8-hydroxyguanine. This compound is a mutagen that alters DNA repair mechanisms. It has been identified in laboratory grown *C. elegans* media.

It was found that the level of antioxidant defenses provided by the activities of the enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione concentration neither uniformly declined with age nor corresponded to variations in maximum life span potential of different mammalian species (*Harman D, 1992*). A positive correlation has been found between tissue concentrations of the antioxidants superoxide dismutase, carotenoids, alpha-tocopherol, and uric acid, and life span in mammals (*Cutler R G, 1991*). Yu carried out a series of experiment with dietary antioxidants and found that vitamin E increased the mean life span of the rotifer, nematode and rat. When vitamin C was fed to the rotifer, there was no change in the mean life span (*Yu B P, 1994*). In *C. elegans* RNA and protein synthesis are age dependent, decreasing in the old animals, which could be a reflection of the degree of free radical reactions within the cells (*Sarkis G J, et. al., 1988)*, and/or association with reproductive functions, a likely correlation.

In contrast to data accumulated in studies in mammalian systems, the *C. elegans age-1* mutant was found to exhibit an increase in superoxide dismutase and catalase late in life. The observed increase in life span is postulated to occur as a direct consequence of these increased levels of the free-radical protection enzymes or, as an alteration in system controls maintaining a robust, responsive replacement and repair function within cells and tissues, an example of the neuroendocrine theory on aging.

Changes in oxygen concentration perturb the aging rate in both the wild type and mutants of *C. elegans*. Oxygen burden affecting the organism serves as a factor that contributes and affects the aging of the nematode (*Honda S & Matsuo M, 1992*). It has been proposed that free radicals, especially those of molecular oxygen accelerate aging processes in animals. However, "rate of living" is another explanation whereby increased metabolic rates speed the process of development thus, the time of arrival at “end stage" in the organism’s genetic program.

Experimental results support the prediction that caloric restriction would lower the steady-state level of oxidative stress, retard the age-associated accrual of oxidative damage, and increase metabolic potential (*Sohal R S & Weindruch R, 1996*). Gerontologists acknowledge that dietary restriction (reduced intake of calories) is the most powerful anti-aging intervention known in laboratory organisms. There is substantial evidence that the anti-aging effects of dietary restriction are not due to its ability to retard growth, deter obesity, reduce metabolic rate, or suppress the deleterious action of glucocorticoids. It is hypothesized that with dietary restriction there is a reduction of ROS, hence a slower process in the breakdown of homeostasis. However, it was found that dietary restriction exerts a diversified anti-aging action by limiting physiological dysfunction and by retarding or suppressing pathological process, thereby extending median, and more importantly, maximum life span (*Yu B P* in *Oxidative stress and aging, Cutler RG et. al., 1995*). Discussed below are examples where both hypotheses can be used to explain the findings.

The main point emerging from oxidative stress studies is that molecular oxidative damage during aging is ubiquitous, substantial, and likely enhances mortality rates which increase exponentially with age (*Sohal R S & Weindruch R, 1996*). The SOD activity of several organs was found to positively correlated with the maximum life span for various animal species, including primates. The postulation that oxygen is involved and acts as a life span determinant in the nematode can be used to explain the above observations, since it increases the concentration of ROS within the organism (*Honda S, et. al., 1993*).

Extended life span is seen as part of the natural life process of nematodes. There are several mutants that have the capability to alter the concentrations of enzymes used in reducing ROS, when compared to non-mutants, expanding their lives. The dauer larva state, the *age-1*and *daf-2* mutants in *C. elegans* are examples where life span is extended (*Friedman D B & Johnson T E, 1988; Johnson T E, et. al., 1988, Vijg J & Suh Y, 2005, Antebi A, 2007*). It was later discovered that the *age-1* mutant encodes a downstream component of a conserved P13-kinase/PDK/Akt pathway *(Morris JZ et. al., 1996)*. In the *daf-2* mutant the insulin/IGF-1 signaling (IIS) receptor homolog acts upstream in the pathway, increasing longevity in the worm through the transcription factor called DAF-16 *(Kenyon C et. al., 1993; Antebi A, 2007)*. IIS is best understood as a signaling pathway selected in evolution to regulate organismal survival (*Antebi A, 2007*).

Gene expression in terms of control mechanisms for ROS removal is very common in the many-studied *C. elegans* and their mutants. During larval development, DAF-16/FOXO specifies the dauer diapause, but can independently trigger longevity in adults (*Dillin A et. al., 2002*). This forkhead/winged helix accumulates in the nuclei of

many cell types and affects the activities of genes involved in many processes, including metabolism, stress response, and antimicrobial action *(Libina N et. al., 2003)*. The DAF-16 ability to detoxify ROS (stress resistance) could be the mechanism underlying their increased longevity *(Sampayo JN et. al., 2003)*. These *C. elegans* were tested for hyperresistance to cellular damage that may be relevant to aging. Relative to the parental strain, the *age-1* strain **TJ401** displayed hyperresistance to oxidative stress. The age dependent increase in the activities of SOD and catalase were parallel to the time course of hyperresistance in the mutant but, not the parent strain. This was consistent with the *age-1* gene product functioning as a negative regulator of SOD and catalase activities. Elevated levels of SOD activity but not of catalase activity were present when the *wild-type* and *age-1* dauer larvae were compared with young adults. The common increases in SOD activity prompted the cloning of the *C. elegans* Cu/Zn SOD gene. Its position on the physical map of the genome is in the region to which the *age-1* gene has been genetically mapped. However, it is unlikely that a mutation at the SOD locus confers the age phenotype. These results support the free radical theory of aging by suggesting that the increased resistance to oxidative stress may be among the causes of increased longevity in both strain **TJ401** and in the dauer larva (*Larsen P L, 1993; Vanfleteren J R, 1992*). Also, these mutants are supportive evidence for the gene regulation theory of aging but like the free radical theory of aging, does not explain how the aging process occur with ROS interaction and why ROS does not have the same effect on the pre-reproductive life stage. This gap in explanation comes from the neuroendrocrine theory

of aging, where after the genes are expressed it takes time for the control systems to breakdown, which occurs with age, causing death.

Experiments were carried out to investigate the adaptation to oxidative stress in life stages of the *C. elegans*. Lower initial activities of SOD and catalase were found in the older worms, as well as the failure of the middle-aged and old worms to induce SOD in response to hyperoxia (an excess of oxygen in tissues and organs), which could contribute to a higher mortality rate. However, the young worms did induce SOD in response to these oxidative stressors and suffered no loss of viability. Therefore, one may assume that the induction of SOD was a factor that protected the young worms, since the induction of catalase was barely apparent (*Darr D & Fridovich I, 1995*).

At the cellular level it was found that a large part of reducing activity generated in response to radical attack in some conditions is associated with protein bound DOPA (3,4-dihydroxy-phenylalanine). DOPA can convert readily into a quinone and undergo cyclic reactions in the presence of several biological reductants (such as glycine, which may act as a coreductant) [*Dean R T, et. al., 1992*]. Also, protein bound reactive species on histones or some other DNA binding protein, might be sufficiently close in apposition to DNA to generate local radical fluxes capable of damaging the DNA (*Sies H & Menck CFM, 1992; Sohal R S & Brunk U T, 1992*). In this way, protein damage by radicals could be linked to mutational damage, and DNA strand breakage and loss: features known to occur in aging. Such reactions might be particularly likely in organelles in which there is a substantial radical flux, such as the mitochondria, which are the sites of known age related losses of and alterations in DNA. Since aging does not seem to be

routinely associated with a deficiency of antioxidant systems, it may be that an enhanced accumulation of certain radical damaged molecules (proteins or otherwise) results partly from changes in the frequency with which radical damage occurs or, alterations in capacity to repair. Once individual proteins begin to accumulate molecules bearing reactive moieties, one can envisage that these in turn contribute to an increasing flux of radical mediated damaging events (*Harman D, 1992; Sohal R S & Brunk U T, 1992*). In young or robust adults such damages are repaired but, in the senescent organism the repair mechanisms are either stopped or cannot repair as fast as the damages are made, an example in the breakdown of homeostasis, as explained by the neuroendrocrine theory of aging.

Another set of genes involved in longevity in the nematodes is the CLK genes. These genes regulate physiological, developmental and behavioral clocks during the nematode life cycle, hence can be considered as supportive evidence for the evolution theory of aging. Four identified clk mutants have shown a moderate increase in life span *(Hekimi S & Guarente L, 2003)*. The CLK-1 that localize in the mitochondria of all somatic cells of the *C. elegans* is required for the biosynthesis of coenzyme Q9, which plays an essential role in the electron transport chain, in both nematode and mammals *(Burgess J et. al., 2003)*. It seems likely that the *daf-2* and the *clk* mitochondrial mutants affect some aspects of energy metabolism and/or stress response. There is controversial evidence that longevity in these mutants is caused by attenuating metabolic rate, which decrease molecular damage due to oxidative stress *(Van Voorhies WA & Ward S, 1999; Braeckman BP et. al., 2002)*. There are over 40 single-gene mutants in *C. elegans* that

display an increase in longevity, each with an increased ability to respond to a certain type of stress such as heat, ultraviolet radiation and ROS *(Johnson TE et. al., 2002)*. The discovery of the *SIR2* (silencing information regulator 2) gene in the nematode demonstrates the convergence of longevity and functional pathways of somatic maintenance. This gene was first discovered in the yeast. *SIR2* is an NAD-dependant histone deacetylase with a role in silencing the rDNA and telomeres, by removing acetyl tags from histones. It may be essential to transducer metabolic signals to the downstream regulatory events that increase longevity, by reducing somatic damage *(Lin SJ & Guarente L, 2003)*. It has been demonstrated that extra doses of *Sir2.1* in the nematode extend adult life span by 50% by negatively regulating the insulin signaling pathway, one of the evidence that suggests *Sir2.1* may work in parallel to IIS *(Tissenbaum HA & Guarente L, 2001; Berdichevsky A et. al., 2006; Wang Y et. al., 2006)*.

The expression of OLD-1 appears to be dependent on DAF-16, which is required for the life extension of *age-1* and *daf-2* mutants. The *old-1* gene is stress-inducible, it increases thermotolerance and longevity by its overexpression, as does the heat shock protein HSP70F *(Munoz MJ, 2003)*. These "age" mutants still undergo the same biological changes at senescence.

Sensory perception can influence life span. A large amount of *daf-16* dependant mutants, deficient in sensory neuron structure and function, have extended longevity (*Apfeld J & Kenyon C, 1999*). Those mutants cause DAF-16/FOXO to relocate to the nucleus (*Lin K, et. al., 2001*). The release of ILPs (insulin-like peptides) from sensory cells are thought to be regulated by components of sensory cell signal transduction, which

includes G-protein coupled receptors, G-proteins, cGMP channel subunits, ciliary proteins, Tubby, and proteins implicated in transmission. This influences the organismal physiology (*Apfeld J & Kenyon C, 1999; Alcedo J & Kenyon C, 2004; Ailion M et. al., 1999; Munoz MJ & Riddle DL, 2003; Mukhopadhyay A et. al., 2005; Lans H & Jansen G, 2007*). The exact sensory cues that govern ILP synthesis and release probably include nutrients, and various repellants or attractants. Supporting evidence was found in *Drosophilia* (*Libert S et. al.; 2007*). Serotonergic inputs may work upstream of ILS. In a *daf-16*/FOXO-dependant manner, mutants in the serotonergic receptor *ser-1* are longer lived (*Murakami H & Murakami S., 2007*). The ILS receptors can act antagonistically as in the cases of the *ser-4* serotonergic receptor mutant and *thp-1*, the serotonin production deficient stress resistant mutant. These mutants are short-lived (*Murakami H & Murakami S, 2007; Sze JY et. al., 2000; Liang B et. al., 2006*).

Calorie (or dietary) restriction (CR [or DR]) is the most consistent model of extended lifespan, which demonstrates the role of metabolism in the process *(Katic M & Kahn CR, 2005)*. Metabolism is not slowed down by CR, but is speed up and altered by the diet *(Sinclair D & Guarente L, 2006)*. It retards aging and extends median and maximal lifespan in yeast, worms, fish, flies, mice, rats, monkeys, and humans [suggested by data] *(Roth GS et. al., 2002; Mattison JA et. al., 2003; Bodkin NL et. al., 1995; Fontana L et. al., 2004)*. To figure out how DR extends lifespan in *C. elegans*, two hypotheses were tested: the rate of living hypothesis and the insulin/IGF-1 signaling *(Walker G et. al., 2005)*. Temperature strongly influences the lifespan of the *C. elegans*, the higher the temperature, the shorter the lifespan. The nutrient-responsive target of

rapamycin (TOR) kinase, an evolutionary conserved gatekeeper integrates nutrient and hormonal cues to modulate growth and longevity (*Kaeberlein M & Brian K, 2007*). When TOR activity is high, faster growth and cell division is favored because nutrients are abundant. With the reduction of nutrients, TOR activity is decreased, leading to reduced growth and enhanced resistance to stress and increased lifespan (*Kaeberlein M & Brian K, 2007*).

Sinclair and Guarente believe that CR is a biological stressor like natural food scarcity, where a defense response is initiated to boost the organism's chances of survival. In mammals this include changes in cellular defenses, repair, energy production and activation of programmed cell death. In yeast, CR affects two pathways that increase *Sir2* enzymatic activity in cells. CR turns on a gene call *PNC1* (in yeast), which produces an enzyme that rids cells of nicotinamide, which normally represses *Sir2*. The second pathway involves respiration where a mode of energy production creates NAD as a by-product, while lowering the levels of NADH. NADH is an inhibitor of *Sir2*. By altering the ratio of NAD/NADH, *Sir2* activity is influenced *(Sinclair D & Guarente L, 2006)*. *Sir2* and its siblings are collectively referred to as Sirtuins. The mammalian version of the yeast *Sir2* is *SIRT1*. They both have the same enzymatic activity. However, in more complex organisms, the pathway by which Sirtuins achieve their effect is much more complicated. For example, increased *Sirt1* in mice and rats allows some of the animals' cells to survive in the face of stress that would normally trigger programmed cell death. Recent research found that NAD levels rise in liver cells under fasting conditions, promoting *Sirt1* activity. *Sirt1* was found to act as a sensor of nutrient availability and a

regulator of the liver response. There is a possibility that *Sirt1* also regulates fat storage in response to diet and, helps control inflammation in the body. However, *Sirt1* does regulate production of insulin and insulin-like growth factor [IGF-1] *(Sinclair D & Guarente L, 2006)*.

With the *C. elegans*, a complex mechanism that includes the IGF-1 signaling pathway is suggested *(Walker G et. al., 2005)*. The insulin-like signaling cascade consists of proteins encoded by genes for insulin-like proteins (*ins-7* and multiple other insulin-like ligands), *daf-2* (the insulin/IGF-1 receptor homologue), *age-1*, *akl-2, daf-16* and *daf-18.* The *C. elegans* genome contains 37 'insulin-like' ligands which are expressed mainly in neurons but, are also found in intestine, muscle, epidermis and gonad *(Walker G et. al., 2005)*. Studies have shown that decreased *daf-2*signaling in germline and somatic gonads significantly increased the lifespan of the worm *(Patel MN et. al., 2002)* and, *daf-2* signaling in the nervous system is a critical regulator of longevity *(Wolkow CA, 2000)*. The *Daf-2* pathways are linked to increased resistance to oxidative stress, where they express high levels of antioxidative enzymes, such as catalase and SOD *(Honda Y & Honda S, 2002; Honda Y & Honda S, 1999)*. The *age-1* mutation partially prevents the age-associated decrease of catalase in adult worms *(Honda Y & Honda S, 2002; Honda Y & Honda S, 1999, Wolkow CA et. al., 2000)*. The *ctl-1* gene, which encodes a cytosolic catalase, a downstream target of *Daf* signaling, is required for the extension of adult lifespan by *daf-2 (Taub J et. al., 1999)*. The *ctl-1* and *ctl-2* catalase genes, *gst-4* (gluthatione-S-transferase) gene and small heat shock protein genes were all

increased in worms with reduced *Daf-2* activity and, decreased in worms with decreased *Daf-16* activity *(Honda Y & Honda S, 1999)*.

Recently PHA-4/FOXA1 and SKN-1/NRF transcription factors (genes) have been shown to be required for DR induced longevity (*Antebi A, 2007; Panowski SH, 2007; Bishop NA, 2007*). SKN-1 early role is in pharynx and gut specification, and later role is in response to oxidative stress in the gut. Neuronal SKN-1 probably regulates the organismal response to DR through a hormonal mechanism. Like SKN-1, PHA-4/FOXA1 has an early role in endo/mesoderm specification in worms and mammals. Later in mammalian life PHA-4/FOXA1 regulates glucose homeostasis. PHA-4 in *C. elegans* influences the life span of the entire organism, even though it is expressed in the adult gut, gonad and nervous system, implying endocrine control, another piece of supporting evidence for the neuroendocrine theory on aging (*Antebi A, 2007; Friedman JR & Kaestner KH, 2006*).

In human and animal cells mitochondria are actively involved in biological oxidation by channeling the catabolism-generated reduced co-enzymes into the membrane-located respiratory chain where oxygen is consumed under delicate control. However, a small fraction of the oxygen consumed is not fully reduced by the electron transport chain, resulting in ROS. The rate of production of superoxide anions and hydrogen peroxide increases with age in the mitochondria. There is evidence supporting respiratory chain function decline with age in human skeletal muscle, liver and brain. Of the respiratory chain electron transport complex, complex I appear to be the most affected. Complex IV activity also declines with time (*Schapira A H V* in *Oxidative*

stress and aging, Cutler RG et. al., 1995). The presence of divalent metal ions such as Fe^{2+} and Cu^{2+} with the ever-increasing ROS and other free radicals in the mitochondria, inevitably lead to enhance production of more vicious hydroxyl radicals. Thus, the naked inner-membrane-associated mtDNA is increasingly vulnerable to oxidative damage and mutations with age. In humans, the frequency of occurrence and relative proportion of the mutated or modified mtDNA has been shown to increase exponentially with age. As the mutant mtDNAs accumulate with age, the functions of the respiratory enzymes containing the mutant mtDNA-encoded protein subunits gradually decline in tissue cells, generating more ROS and free radicals, which further increase the oxidative stress and oxidative damages to mtDNA and other vital biomolecules in the cell, evidence for the oxygen-stress-mitochrondrial mutation theory of aging.

In humans, at least five different types of mtDNA mutations have been found to be age-related: large-scale deletion, point mutation, insertion, small tandem duplication in the D-loop region, and DNA rearrangement (*Wei Yau-Huei, 1998*). Studies have shown mtDNA mutation in brain tissues of patients with Parkinson's disease. The mitochondrial DNA may be unable to prevent the intrinsic mutagenesis caused by the byproducts of respiration because, in contrast to the nuclear genome, it lacks excision and recombination repair. Thus, the resulting mitochondrial impairment and concomitant cell bioenergic decline may cause the senescent loss of physiological performance and may play a key role in the pathogenesis of many age-related degenerative diseases (*Hans Joenje, 1992; Miquel J, 1998*).

Central nervous system (CNS) tissue contains high concentrations of mitochondria and undergoes a large, constant flux of oxygen, which inevitably produces free radicals during the process of electron transport. Parkinson's disease results from the loss of dopaminergic neurons in the substantia nigra, which are oxidative components. It is caused by a deficiency of the neurotransmitter dopamine in the nigrostriatal pathway. Dopamine and norepinephrine are oxidized, leading to neuronal degeneration and, resulting in products and symptoms characteristic of Parkinson's disease. Hydrogen peroxide is produced from dopamine metabolism. Patients of Parkinson's disease have increase level of ferric and total iron and, markers of lipid peroxidation such as malondialdehyde and lipid hydroperoxides, in their substantia nigra (*Ogawa N and Mori A: Parker L:* in *Oxidative Stress and Aging, Cutler RG et. al., 1995*).

The effects of calorie restriction common to both rodents and nonhuman primates include lower visceral fat mass, lower circulating insulin and IGF-1 concentrations, increased insulin sensitivity, lower body temperature, lower fat-free mass, lower sedentary energy expenditure, decreased levels of thyroid hormones and decreased oxidative stress *(Heilbronn LK et. al., 2003)*. Reduced metabolism and subsequently reduction in free-radical production is a possible explanation for the anti-aging effects of calorie restriction. Other effects of CR such as lower body temperature, increased insulin sensitivity, decreased insulin/IGF-1levels, sympathetic nervous system activity, and altered gene expression in muscle, heart and brain, have all been suggested to play a role in the effects of CR on longevity *(Katic M & Kahn CR, 2005)*. There has been some naturally occurring episodes of calorie restriction in human populations but their diet lack

proteins and micronutrients *(Heilbronn LK et. al., 2003)*. Here CR is associated with short stature, late reproductive maturation, lower gonadal steroid production in adults, suppressed ovarian function, impaired lactation performance, impaired fecundity and impaired immune function. CR is also highly effective in reducing the risk of atherosclerosis in humans *(Fontana L et. al., 2004)*. Insulin and insulin-like growth factors (IGF-1 and IGF-2) belong to a family of hormones/growth factors that regulate metabolism, growth, cell differentiation and survival of most tissues in mammals. Their effects are mediated by two closely related receptors, insulin and type 1 IGF receptors, which are expressed on the surface of most mammalian cells *(Katic M & Kahn CR, 2005)*.

In summary, all the theories on aging have supporting evidence as discussed above. However, the system-based theories on aging like the neuroendocrine theory on aging explain how the ROS are involved in the breakdown of homeostasis. Most of the aging studies done on the *C. elegans* are mutant related. The purpose of this study is to provide some biochemical evidence showing alterations in some molecules as the *C. elegans* age, which are also common to humans and have similar pathways.

METABOLITES OF INTEREST THAT SUPPORT SOME AGING THEORIES

All metabolic functions within a living organism are controlled by a complex set of regulatory control system and sensing systems which regulate and transmit signals associated with the changes in the environment. Physiological stimuli result in the activation of basic systems such as the neuromusculature network via the neurochemical

system, as also seen in the serotonin deficient male mutant *C. elegans*. Signals arising from metabolic alteration of metabolites fundamental to the biochemistries of all known organisms represent the "first level" of evolving signal systems. They are derived from the building blocks fundamental to all organisms, such as the amino acids tyrosine and tryptophan and the nucleotides, which are discussed in this study.

Neurotransmitters and hormones have widespread effects as chemical regulators of coordinate physiological activity throughout the body. The neurotransmitters such as the cateocholamines and their related compounds act as messengers of signals, hormones and key control enzymes. Tyrosine and tryptophan neurotransmitters are multi-geneproducts, where they provide signals with feedback capabilities to control genomic expressions. They have been assigned an important role in sensory processes, learning, memory, motivation, and motor coordination during development, maturity, and aging. Tyrosine is converted to the catecholamines hormones dopamine, norepinephrine and epinephrine. It occurs in proteins that are part of signal transduction processes. Also, the thyroid hormones triiodothyronine (T3) and thyroxine (T4) are derived from tyrosine. Tryptophan can breakdown to form serotonin, a brain neurotransmitter.

Figure 2 gives an outline for the synthesis and degradation of tyrosine (Tyr) and tryptophan (trp), which occurs in the cytosol of the *C. elegans*. The sites in the cell for the degradation and synthesis of amino acids vary taxonomically. Nicotinate derived from tryptophan serves as a precursor to NAD (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate). These nicotinamide-containing coenzymes functions as carriers of hydrogen atoms and electrons in intermediary

metabolites oxidation-reduction reactions. Other products of tryptophan metabolism include acetyl- CoA, acetoacetyl CoA, serotonin and indoleacetate. Some of the products of tyrosine metabolism include fumarase, dopamine and acetoacetyl CoA.

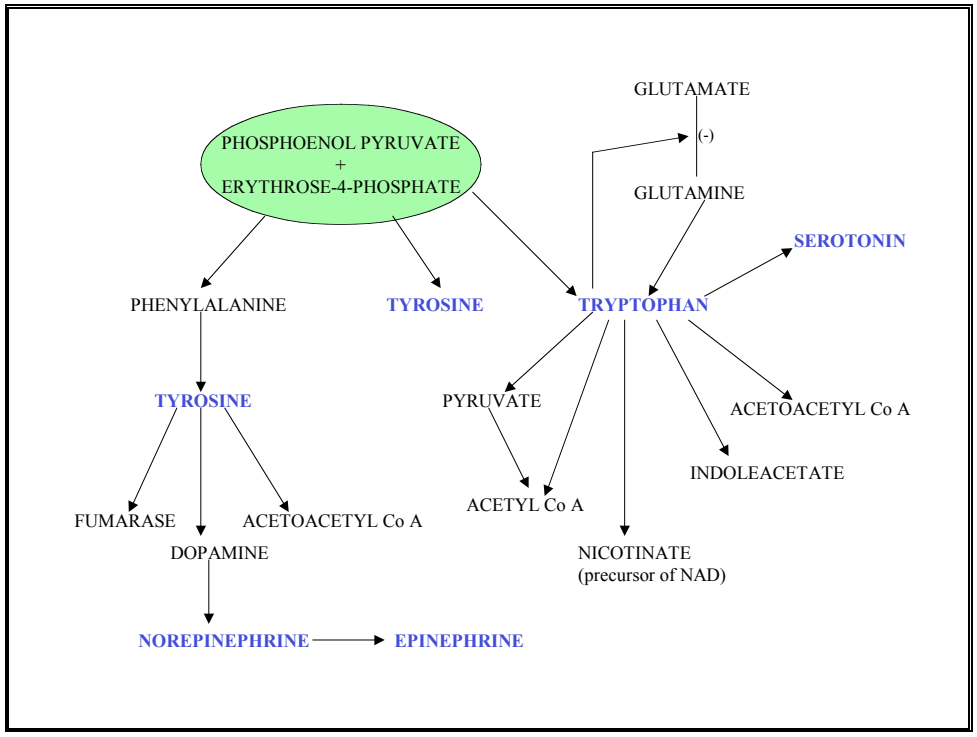


Figure 2: Outline of tyrosine and tryptophan metabolism.

The neurotransmitters derived from tyrosine and tryptophan is either primary or secondary amines. They have been proposed as key physiological regulators in specific neuronal and general nervous system functions. Tyrosine gives rise to a family of catecholamines including that of dopamine, norepinephrine and epinephrine. The reactions involved in the formation of these catecholamines from tyrosine include oxidation, decarboxylation, dehydration, hydroxylation and methylation, all of which are

taking place in the cytosol of the cell. Examples of known effects associated with the catecholamines are stated below where these neurochemical signaling agents are effectors of the highest levels of neurologic function in man. Changes in systems responsive to catecholamine neurotransmitter's physiologic states are known in a host of organisms. The neurological disorder Parkinson's disease in humans is associated with an underproduction of dopamine, and has been treated by administering L-dopa. An overproduction of dopamine in the brain is associated with psychological disorders such as schizopheria, since levels of catecholamines are correlated with blood pressure in vertebrates. In humans abnormal blood pressure normally occurs in (aging) adults.

Dopamine is converted to norepinephrine and the latter to epinephrine, by the enzyme dopamine-β-hydroxylase. It is concentrated within storage vesicles that are present in high concentrations within the nerve terminals. The two enzymes that are primarily responsible for the catabolic inactivation of dopamine are mono amine oxidase (MAO) and catechol-O-methyl-transferase (COME). MAO oxidatively reduces catecholamines to their corresponding aldehydes which, are subsequently converted to their analogous acids by aldehyde dehydrogenase. Because of MAO's intracellular location (cytosol), it plays an important role in the breakdown of catecholamines. COME is primarily located on the outer plasma membranes of all cells acting on extracellular dopamine. It methylates virtually any catechol compound regardless of the R group (Kegg web page). Homovanillic acid (HVA) is also a product of dopamine oxidation, a major metabolite of brain dopamine.

The oxidative metabolism of tryptophan is the most complex of all the oxidative pathways of amino acid metabolism (Lehninger A L, 1993). This pathway generates the indolealkyl amine neurotransmitters. Like the tyrosine degradative pathway, the reactions involve oxidation, hydroxylation, decarboxylation, methylation and acetylation reactions. These reactions also take place in the cytosol.

Serotonin is derived from tryptophan in a two-step pathway. Its biosynthesis is initiated by an active uptake of dietary tryptophan into the serotonergic neurons. The enzyme tryptophan hydroxylase that converts tryptophan to 5-hydroxytryptophan is normally not saturated with tryptophan in vivo. Thus, excess dietary tryptophan increases the serotonin levels and, deprivation of tryptophan coupled with competition with phenylalanine result in depressed serotonin levels. Serotonin serves as a neurotransmitter binding to receptors on the surface of cell membranes (the synaptic membrane). The level of serotonin has been found to be associated with levels of behavioral responses. *C. elegans* concentrates its food, bacteria, by pharyngeal pumping which is stimulated by serotonin (*Wood, 1988*). *C. elegans* mutants lacking immunocytochemically detectable serotonin entirely or from neuronal processes exhibit reduced male mating efficiency (*Wood, 1988*).

Indoleamine 2,3-dioxygenase catalyzes the formation of kynurenine from tryptophan. Its activity controls the levels of free tryptophan, and thus the serotonin levels also. 5-Hydroxyindoleacetic acid (5-HIAA) is formed from serotonin, by the enzyme monoamine oxidase (MAO). This is the most important catabolic enzyme of 5-HT. In some animals (or cells), serotonin could be converted to N-acetyl-5-HT (NA-

5HT), by the enzyme N-acetyl transferase, and then to melatonin. Serotonin serves as a neurotransmitter binding to receptors on the surface of cell membranes (the synaptic membrane).

Studies have shown that the plasma levels of norepinephrine (norepi) vary rhythmically in humans and other animals with an ultradian (shorter than one day) periodicity. Such spontaneous oscillations in plasma norepi levels reflecting alterations in sympathetic nervous activity are suggested as influencing resistance to bloods' flow through vesicles. It was also found that depressed patients excrete disproportionately greater amounts of Norepi than their controls.

The effect of tyrosine on mood, performance, heart rate and blood pressure of some healthy young subjects was assessed. It was found that excess tyrosine had no effect on mood, systolic blood pressure and heart rate. Changes in both serotonin and catecholamines have an effect on depression. Acute exposure to cold stress has been shown to impair short-term, or working memory. This was found to be related to the reduction in or disruption of sustained release of brain catecholamines. Administering a supplemental dose of the catecholamine precursor tyrosine was found to be affective in protecting against the effects of cold stress on delayed matching-to-sample performance, possibly by preventing cold stress-induced reduction in brain catecholamine levels (*Shurtleff D, et. al., 1992*). In diabetes patients, serotonin synthesis is reduced but, the presence of carbohydrates increases its synthesis. The above examples document the importance of the neurotransmitters derived from tryptophan and tyrosine affecting/controlling complex biosystem functions in organisms as diverse as humans and

the nematode *C. elegans*. Changes in their concentrations and or ratios may provide supportive evidence for the neuroendocrine theory of aging.

Investigations in humans show neurotransmitter changes in intact cellular networks are more profound in early-onset Alzheimer-type dementia (ATD) groups than in late-onset ATD patients (*Shurtleff D et. al., 1992*). In particular, cholinergic, serotonergic and noradrenergic changes were more severe in the early-onset ATD groups. Of the neurotransmitter changes found in ATD brain, the most marked was the serotonergic deficits in which not only the concentrations of serotonin and 5-HIAA but also activity of the synthesizing enzyme, tryptophan hydroxylase, was severely depleted.

Figure 3 is of purine nucleotide catabolism. Xanthine oxidase is a flavoenzyme, with an atom of molybdenum and four iron-sulfer centers in its prosthetic group. It oxidizes hypoxanthine and xanthine to uric acid, where molecular oxygen is the electron acceptor in the complex reaction. Guanine undergoes hydrolytic removal of its amino group to yield xanthine. Guanine is targeted for oxidation due to its chemical composition. Purine nucleotides degrade to free purines, which are salvaged and used again to make nucleotides. This should decrease with age since RNA and protein synthesis decrease with age in *C. elegans*. Cancer cells grow more rapidly than cells of most normal tissues hence, the greater requirements for nucleotides as precursors to DNA and RNA synthesis. ROS also attack the purine nucleotides, affecting DNA and RNA.

8-Hydroxyguanine is one of the compounds produced and accumulated from oxidative damage of cellular DNA lesions, caused by ROS. The presence of 8-hydoxyguanine may lead to mutagenesis, resulting in a direct link between 8-

hydroxyguanine and carcinogenesis. Damage caused by ROS will lead to the accumulation of 8-hyrdoxyguanine.

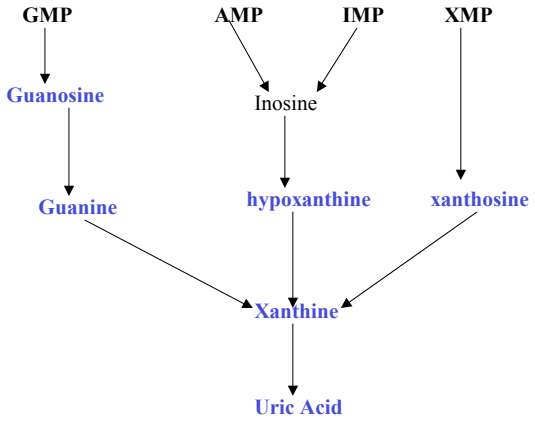


Figure 3: Outine of the purine nucleotide catabolism in *C. elegans*

BACKGROUND ON THE *NEMATODE CAENORHABDITIS ELEGANS*

Caenorhabditis elegans (*C. elegans*) is a small (less than 1.5 cm in length), free living, non-parasitic, soil nematode, belonging to the phylum Nematoda, class Secernentea, order Rhabditida, family Rhabditidae and genus Caenorhabditis (*Wood W B, 1988*). The organism feeds primarily on bacteria. There are two sexes, hermaphrodites and males (whose occurrence is less than 0.5 % of a total population). Under optimal conditions, hermaphrodites reproduce with a life cycle of about 3 days. There are extensive differences between both sexes of *C. elegans* (**Appendix 1**). A large part of the animal shows sexual specializations of one kind or another but there are some structural similarities. The hermaphrodite has 959 somatic nuclei, while the adult male has 1031.

About 650 of the somatic nuclei appear to be sexually indifferent, the remainder being sexually specialized. The germ line develops differently in the two sexes. The haploid genome size is 8 x 10^7 nucleotide pairs, about one-half that of the fruit fly. In *C. elegans* a cuticle exoskeleton covers the outermost surfaces of all hypodermal cells, the pharynx, and the rectum.

A hermaphrodite *C. elegans* that has not mated lays about 300 eggs during its reproductive life span. It undergoes internal fertilization of its eggs, as the mature oocytes pass through the spematheca into the uterus. Normally, eggs are held in the uterus for the first few cleavages and are then deposited through the vulva, at about the time of gastrulation, approximately 3 hours after fertilization. Embryogenesis occurs directly after fertilization, it normally requires about 14 hours to be completed. It can be divided into two phases: cell proliferation and organogenesis, and morphogenesis. The first larval stage (L1), 250 μm in length consisting of 558 cells in the hermaphrodite and 560 in the male, hatches from the egg about 14 hours after fertilization. Larval development proceeds over the next 50 hours through three additional stages, L2, L3, and L4. In extreme external conditions, the L2 transforms into the alternative dauer larvae, and then reverts into the L4 larval stage when conditions improve. The dauer larvae can survive at least 2 months under such extreme conditions. The L4 finally molts giving rise to the mature reproductive adult *C. elegans* [**Appendix 2**] (*Wood W B, 1988; Corsi A K, 2006*).

Three morphological phylogenies hypotheses are that the pseudocoelomate nematodes arose early in animal evolution, (A) - as part of a radiation of "aschelminth"

phyla, (B & C) - that split into protostome groups (annelids, anthropods, mollusks, and others) and deuterostome groups (chordates, brachiopods, and others). This scheme implies that nematodes are equally distant from both arthropods and vertebrates. However, results of studies indicate that nematodes branched off before the arthropod-vertebrate split. A new high-level taxon of animals that shed a cuticle by ecdysis (the Ecdysozoa) is proposed. The Ecdysozoa include arthropods, nematodes, and their allies (*Baxter M, 1998*).

THE USE OF *C. ELEGANS* AS A MODEL IN AGE RELATED STUDIES

The genome sequence of *C. elegans* was completed in December 1998. It's the first for a multicellular organism. Genetic sequence comparisons were done between *Saccharomyces cerevisiae* (yeast) and *C. elegans*, with surprising results (*Chervitz S A et. al., 1998*). Orthologous pairs were observed: the set of highly conserved proteins encoded by a minority of the open reading frames (ORFs) in each organism (~ 40% of yeast and ~ 20% of worm). These conserved proteins carry out the core biological processes shared by these two eukaryotes, such as intermediary metabolism, DNA and RNA metabolism, protein folding, trafficking, and degradation. The second result was that the worm has a number of specialized, committed cell types with distinct and coordinated programs of gene expression, unlike yeast. Most of the signaling and regulatory genes known, or expected to be involved in multicellularity, have no yeast orthologs. The basic assumption that the so-called "model organisms" will provide reliable functional annotation for the human DNA sequence is supported by the following

observations. The sum of the biology of worm and yeast can be obtained efficiently by studying core functions largely in yeast and signal transduction largely in the worm, with practically no overlap. Since the evolutionary distance between the yeast and worm did not interfere with the finding of orthologs and shared domains, it is likely that a robust chain of annotation is possible through all eukaryotes (*Chervitz S A et. al., 1998*).

The key attributes of *C. elegans* as an experimental system for biologic studies are its simplicity, ease of cultivating in the laboratory, short life cycle, transparency, suitability for genetic analysis, and small genome size, when compared to other animals including mammals (*Johnson T E et. al., 1991, Corsi A K, 2006*).

Quantitative genetic analysis indicates a substantial genetic component that determines the life span. Long-lived strains can be obtained by interstrain breeding or by isolation of apparent single-gene mutants. The *age-1* mutant was the first gene mutant in any organism to demonstrate an increase in life span (*FriedmN DB and Johnson TE, 1988)*. Since most of the genome has been studied and identified in *C. elegans*, it is considered a useful model for studying some aspects of aging.

The complete anatomy of the *C. elegans* is known at the electron microscope resolution level. A genomic description of the animal, including detailed genetic mapping, and physical mapping of the entire genome is at hand. The complete wiring diagram of the nervous system is available from earlier anatomical work. Some knowledge of neurophysiological function has been gained from neurotransmitter analysis and electrophysiologial study of the homologous nervous system in the large nematode *Ascaris suum*.

The biology of digestion in the *C. elegans* is still somewhat of a blind spot, where little is known. There are two major surfaces that act as interfaces with the nutrient environment. One surface is at the chemosensory and olfactory neurons of the amphids. The other surface is the apical surface of intestinal cells. *C. elegans* feeds on the *E. coli* bacillus which is about 2 μM long and 1 μM thick *(Walker G et. al., 2005)*.

Nematodes and humans have some common signaling pathways such as the insulin/IGF pathways and receptors. Serotonin receptors occur in both animals. Tyrosine kinase activity is found in both animals. Tyrosine, tryptophan, and purine metabolisms are similar. The neurotransmitters norepinephrine, epinephrine and serotonin are common to them. Products of ROS activity are common to both animals such as DNA damage accumulation, protein carbonylation, lipofuscin and glycosylation end products *(Collins JJ. et. al., 2008)*.

Therefore, as a model for the analysis of the aging process, *C. elegans* offers significant advantages: a 20 - day life span, a 3 - day life cycle, excellent genetics, identification of several age-related mutants, the occurrence of drugs and environmental factors that alters their life span, an absence of inbreeding depression, the ease of separating the life stages, indefinite cryogenic storage of stocks, and the existence of more than 50 other laboratories working on this organism (*Larsen P L, 1993; Hartman P S et. al., 1988, Corsi A K, 2006, Collins JJ et. al., 2008*).

SEPARATION TECHNIQUE

The separation technique to be used in this research is HPLC (high performance liquid chromatography) with electrochemical detection. This analytical technique has become one of the most popular methods for the determination of the catecholamines and their related compounds, in both clinical laboratories and physiological research. It separates and detects compounds that are electrogenic. Such compounds when subjected to a potential difference undergo molecular rearrangement at the working electrodes' surface, with the loss (oxidation) or gain (reduction) of electrons. Therefore, this research focuses on small soluble proteins and amino acids, and other electrogenic compounds that are detectable by CEAS-HPLC, from the *C. elegans*.

The first practical liquid chromatography/electrochemistry experiments were carried out in 1972. The concept of electrochemical detection in flowing liquids comprises a wide spectrum of techniques provided by modern electrochemistry, ranging from conductometric and high-frequency impedance measurement, through potentiometry, polarography, voltammetry and coulometry, to the monitoring of electrokinetic phenomena. However, polarographic, voltammetric and coulometric measurements are the most important and thus the term 'electrochemical detection' is considered as a synonym for these methods (*Krstulovic A M, 1986*).

Reversed phase chromatography with alkyl sulfate added (in a liquid phase) is used to provide some element of charge=charge interaction, which the separation depends on together with the long chain C18 tail. The polar portion of these chains is what bonds the chain to the solid support material, in the C18-column. The separation is primarily

based upon partition coefficients of the analytes extracted and the liquid phase characteristic of the support material. It is the long chain alkyl sulfates, where the long alkyl chain associates with the C18 tails to which the mobile phase is exposed. The affinity of each compound for the stationary phase is affected by pH and the concentration of other salt ions that may affect the partition coefficient with the C18 chains of the solid phase in the column by associating with the molecule. Separation of charged compounds can be optimized by gradually changing the pH and/or salt concentration of the solution being passed through the column, so as to create a pH or salt gradient, which is done with the methods created to run through the carbon-18 cloumn and electrochemical detectors. A modern enhancement of this and other chromatographic techniques is called **high-performance liquid chromatography (HPLC)**. This takes advantage of stronger solid support material and, improved apparatus designed to permit chromatography at high pressures (using pumps A and B at different flow rates), allowing better separations in shorter time.

Column chromatography has several depending factors, such as the materials packed (the nature of the solid support and the chemical modifiers of the surfaces of these supports) in the column, which determines the types of molecules absorb and the strength of absorption (partition coefficients of the analytes in the mobile phase). Weakly absorbed molecules are eluted first where as, the most strongly absorbed molecules are eluted last. In addition to the column factor, the composition of the buffer solution (in this case mobile phase A and B) and its' flow rate and pressure are also determinants of elution. With reversed phase chromatography for small molecules (proteins) and charged

compounds separation, the pH of the mobile-phase is used to manipulate the charge on the compound. Depending on the pI of the small protein, the partition coefficient of the analytes with the stationary phase is affected by alteration of the charge state through changes in the pH of the mobile phase. Once the molecules pass through the column and are separated, they are detected. The two main types of detections are: electrochemical detection, which is dependent upon the redox potential of the molecules, and ultraviolet (UV) light detection which detects the UV radiation absorption of the molecules.

HPLC with electrochemical detection (LCEC) has become one of the most popular methods for the determination of catecholamines and related compounds, in both clinical laboratories and physiological research. A fully automated in-line extraction reversed-phase HPLC method with chemiluminescence detection has been developed. The method involves reversed- phase liquid chromatography with postcolumn fluorescence derivation involving a periodate oxidation. This is followed by reaction with meso-1,2-diphenylethylenediamine in the presence of hexacyanoferate (III). The detection limit varies from 13 to 570 fmol on column, depending on the compounds (*Nohta H et. al., 1994*).

The coulometric electrochemical-detector array system (CEAS) HPLC is from ESA, Inc.. It has two pumps that can be used for gradient flow of the two buffers used in this experiment. There are four cells with sixteen electrochemical detectors that were set from 0 to 900, incrementing in 60mVolts. After sample injection (volume of 30 μl), the sample is pushed through the column by the dual pumps, where the analytes are separated via reverse phase chromatography. As the separated analytes passes through the

electrochemical cells, they are oxidized at specific current. This signals produced from the cells (vary upon the pi of the compound and its concentration) are saved in chromatograms. In the chromatograms the retention time represents the time when that particular sample is eluted and, the channels represent electrochemical potential at which the sample is detected, in this case oxidized as it is being eluted. The system is fully automated. Detection involves redox reactions occurring at the voltage of the channel. The instrument is sensitive in detecting signals at picoAmps ranges. (fentogram or fentomol concentration levels).

The buffers used for elution are the Mobile Phases. They contain electrolytes and organic solvents (but are predominantly aqueous). In this study, the CEAS system uses dual mobile phases A and B, water and methanol phases, which run together in a gradient flow to enhance elution and separation. A lithium salt buffer solution is the major buffer in this mobile phase, with the organic solvent being methanol.

There are many general advantages for the CEAS-HPLC: high sensitivity combined with good precision with regard to quantitation, even at very low solute concentrations; a wide linear dynamic range and a selectivity of detection that can be varied to a certain extent by variation of the electrochemical detection; many background substances present in the samples do not interfere and thus the sample pretreatment is often simpler than with other methods. CEAS-HPLC is also a simple, rapid and inexpensive method. It uses very small sample sizes, ranging from 10 μl to 50 μl. It can measure analytes with soluble concentrations from femtogram to microgram range.

Imbedded in the software is method to perform a calibration curve for the known. The data produced, after analysis with the incorporated software can be exported into Excel.

Catecholamines and their metabolites are ideally suited for electrochemical detection, due to their redox potential and rapid electrochemical reactions. Large signals are obtained even at low working electrode potentials, where the background current and noise are low (giving high signal-to-noise ratio) and where very few other substances interfere electrochemically.

CEAS-HPLC has its shortcomings. The most serious lies in the fact that the sensor, the working electrode, is in direct contact with the test solution (the column and sensors get clog [dirty]). Another drawback is the dependence of the electrochemical signal on the liquid flow-rate, which makes the detection susceptible to fluctuations in the pumping rate (the data experience an accordion effect of about a minute at most since it is gradient flow). The electrochemical signal also depends on temperature, but not critically. As the number of samples analyzed increases and there are changes to the environment, such as temperature, the retention time of samples may shift forward or backward creating a window of error. Therefore, it is important to run the knowns frequently during a run (about every nine to 12 sample, as controls), use the same batch of mobile phases and, to use the same column for all the analyses. All of these variables affect where the analyte is detected in the time line of the chromatogram, but do not affect which electrodes set at which potentials will see the molecule and respond. The response is always dependent upon the molecular state for the entity, its lowest lying

molecular orbitals, and as such, is nearly invariant with respect to the protocol employed in the separations protocol.

The CEAS-HPLC software is capable of monitoring and controlling up to 16 independent channels of data, controlling hardware (such as pumps and autosamplers), performing data integration, ratio calculations, and report generation. The CoulArray detector (ESA, Inc.) has gain autoranging, which permits the measurement of low (fentomole on column) to high levels (micromole on column) of compounds simultaneously. The detector is the only ECD that can be routinely used with aggressive HPLC gradients. Generally, changes in the organic concentration of the mobile phase during a gradient cause the detector baseline to shift. Data are lost when then gain range is saturated for significant baseline shift. However, the CoulArray detector software combines autoranging with a baseline drift correction algorithm so that the use with gradient HPLC is practical (*Acworth I N & Gamache P H, 1996*).

3. RESEARCH DESIGN AND METHODS

This research is segregated into four (4) subsections: (a) sample preparation; (b) metabolite separation and identification; (c) data analysis and, (d) discussion of results (which will be discussed in the next section).

The organism used in this research is the nematode *C. elegans*. There are four (4) sets of experiments: (i) control experiment; (ii) Vitamin C experiment; (iii) Vitamin E experiment and, (iv) Vitamin C & E experiment. The *C. elegans* are grown under laboratory conditions in an axenic sterile liquid media.

To be measured are the population distributions (pre-reproductive, reproductive and post-reproductive *C. elegans*) at the start and end of the experiment. If there is a change in the distribution at the end of the experiment for the Vitamin treatment from the untreated, one may assume that the addition of the Vitamin(s) has affected the growth rate and aging process. Measuring and recording signals (voltage) from electrogenic compounds from the nematodes, when oxidized from CEAS-HPLC will give unique signal pattern (chromatograms) for each age group and/or experimental categories. From these chromatograms tyrosine, tryptophan and their neurotransmitters (epinephrine, norepinephrine, serotonin) concentrations are measured and analyzed as a function of life stage and Vitamin treatment. Changes in these concentrations and their respective ratios when comparing the untreated to the treated nematode, and the different life stage will

show the effectiveness of the Vitamins on the aging process and/or change in homeostasis, evidence to support the neuroendocrine theory of aging. Identifying unique signal (peak/analyte) using a subtraction algorithm will demonstrate signature patterns for that treatment/life stage. Clustering of these chromatograms, if separated for life stage/treatment will confirm the biology and the presence of unique patterns if they exist.

SAMPLE PREPARATION

CULTIVATION OF *C. ELEGANS.* To a 250 mls conical flask 8 gms of bacto peptone (ordered from DIFCO laboratories) and 6 gms of yeast extract (ordered from DIFCO laboratories) are dissolved in 180 mls of distilled water (using LABCONCO water prodigy 11 system), to form the liquid media. Two liquid media flasks are made. These media flasks along with forty-four (44) empty 25 ml conical flasks (Pyrex) are then stoppered with non-absorbent cotton (from Fisher) and covered with foil, to be sterilized. They are sterilized in an autoclave (Amsco Scientific Autoclave), at 121 ^{o}C for 20 m inutes (using LIQUID 1 cycle). The sterilized media are then placed under a sterile biological hood (Class 11 A/B3 Biological Safety Cabinet from Forma Scientific Inc.) to cool before the addition of 5.4 ml heated liver extract (HLE) to each of media flasks. (The protocol for culturing *C. elegans* and the preparation of the HLE can be obtained in *Sudama G, p38*). The media is now called HLE-media. Aliquots of 4 mls media-HLE are pipetted (using Fisher Serological pipettes) into the sterile 25 mls Erlenmeyer flasks.

Control Experiment: To each of three (3) 25 mls Erlenmeyer flasks with media-HLE, 0.4 mls of *C. elegans* (using culture dated March 3rd 2000) is used to inoculate each flask. Sterile distilled water is used to make the volume of the cultures to 5 mls. These flasks with *C. elegans* are labeled P1, P2 and P3. P represents positive controls. Negative controls are the flasks with media-HLE, distill water and/or Vitamin E/Vitamin C, without *C. elegans*. They are labeled N, NC, NE, NCE, where the N represents negative control, C for Vitamin C, E for Vitamin E and H for Vitamin C & E. All the control flasks are then stoppered again with their original cotton and foil paper. They are then placed in a dark incubator (Precision low temperature incubator 815), at approximately 21.8 ^{o}C for 10 days. The pH of the media is measured for each flask at the end of the incubation period.

Sample population counts are done on each culture periodically. This is done by removing 10 μl of culture, diluting to 100 μl with water, and counting under the light microscope (Olympus Tokyo CK Light Microscope). During the culture growth only the reproductive and post-reproductive *C. elegans* counts were recorded. At the start and end of the culture growth period total population counts were taken. Pre-reproductive (young) *C. elegans* are less than 600 μm in length and are comprised of L1, L2 and L3 lava stages. The reproductive (middle age) *C. elegans* are between 600 and 900 μm in length and are mainly the L4 and some young adults. The post-reproductive (old) *C. elegans* are greater than 900 μm in length.

Vitamin C Experiment: The vitamin C experiment is carried out using the same procedure as the control experiment with the difference being the addition of Vitamin C at concentrations of 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml to the inoculated culture, at the time of preparation. Fifty (50) ml of vitamin C (stock concentration 25 mg/ml, from Sigma, lot# 46H02965) is made by dissolving 1.25 grams of vitamin C in distilled water, to the 50 ml miniscus in a 50 ml volumetric flask. Sterile filtration of the vitamin C solution is done through a 0.20 μm filter (Nalgene ^{TM} Disposable Filterware 150 ml, Nagle Company), under vacuum filtration. Each experiment is done in triplicates. The negative control (NC) does not have *C. elegans*, while the positive control (P) does not have vitamin C. To three of the media-HLE-*C. elegans* flasks (a volume of 4.4mls), 0.02 mls of the stock sterile Vitamin C and 0.58 mls of sterile water are added to each flask which are then labeled CA1, CA2 and CA3. These are the 0.1 mg/ml Vitamin C grown *C. elegans*. The same process is repeated in another three media-HLE-*C. elegans* flasks, with 0.1 mls of the stock sterile Vitamin C and 0.5 mls of sterile water added to each flask which are then labeled CB1, CB2 and CB3. These are the 0.5 mg/ml Vitamin C grown *C. elegans*. The 1.0 mg/ml Vitamin C grown *C. elegans* have 0.2 mls Vitamin C and 0.4 mls of water added to the three media-HLE-*C. elegans* flasks. They are labeled CC1, CC2 and CC3. The pH of the media is measured for each flask at the beginning and end of the incubation period.

Vitamin E Experiment: The procedure for the vitamin E experiment is identical to the vitamin C experiment with exceptions. Instead to vitamin C being added to the

sterile media-HLE-*C. elegans*, concentrations of 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml of sterile vitamin E is used. Vitamin E solution was first attempted by trying to dissolve 1.25 grams of Vitamin E (d-alpha-Tocopherol, from Sigma, lot#117H0337) in distilled water, making to the 50 ml miniscus in a 50 ml volumetric flask, which was then sterile filter like the Vitamin C. The flasks with the 0.1 mg/ml Vitamin E are labeled EA1, EA2 and EA3. EB1, EB2 and EB3 are the flasks with the 0.5 mg/ml Vitamin E. The flasks with 1.0 mg/ml Vitamin E are labeled EC1, EC2 and EC3. This experiment was set up on March 23, 2000. The pH of the media is measured for each flask at the start and end of the incubation period.

Another Vitamin E experiment was set up on November 15, 2000 where the Vitamin E is made soluble by dissolving 1.25 grams of Vitamin E (d-alpha-Tocopherol, from Sigma, lot#117H0337) in 20 ml TWEEN 80 (from Acros, Lot#A010105102), adding distilled water to the 50 ml miniscus. This is then sterile filtered, like Vitamin C stock solution. The flasks with the 0.1 mg/ml Vitamin E are labeled 1E1, 2E1 and 3E1. 1E2, 2E2 and 3E2 are the flasks with the 0.5 mg/ml Vitamin E. The flasks with 1.0 mg/ml Vitamin E are labeled 1E3, 2E3 and 3E3. Of TWEEN 20, 60 and 80, TWEEN 80 dissolved the Vitamin E completely.

Vitamin C & E Experiment: In March a Vitamin C and E experiment was also set up where the concentrations of Vitamin C and E were 0.1 mg/ml each in three Erlenmeyer flasks, labeled HA1, HA2 and HA3. Flasks labeled HB1, HB2 and HB3 had concentrations of Vitamin C of 0.5 mg/ml and Vitamin E of 0.1 mg/ml, labels of HC1,

HC2 and HC3 are of Vitamin C and E concentrations of 0.5 mg/ml each. Finally flasks labeled HD1, HD2 and HD3 were of concentrations 0.1 mg/ml Vitamin C and 0.5 mg/ml Vitamin E. The pH of the media is measured for each flask at the start and end of the incubation period.

HARVESTING AND AGE FRACTIONATION OF *C.*ELEGANS POPULATIONS. The ten days old cultures are transferred into test-tubes and are washed three times with 5 mls aliquots of 0.03 % sterile saline solution, followed by sterile distilled water, under sterile conditions. The majority of the liquid is separated from the *C. elegans* by the process of centrifugation, from each wash. The contents are centrifuge at 2500 RPM for 10 minutes (using a Sorvall GLC -2B centrifuge), where the supernatant is pipetted out (with sterile serological pipettes) and discarded. The pellet is the *C. elegans.* This washing process is called harvesting.

The harvested *C. elegans* are now age separated using temperature stress. To the washed *C. elegans*, 1 ml sterile distilled water is added before placing in an ice bath for 10 minutes. Resulting from the cold temperature is the separation of the old nematodes (pellet) from the rest of the population (the supernatant). Mobility decreased with an increase in age, when cold temperature is applied to the *C. elegans*. The young and middle-aged *C. elegans* are pipetted out from the old *C. elegans* and placed in eppendorf tubes. They are again placed in the ice bath for another seven (7) minutes, which results in the separation of the young and middle-aged nematodes. This process is repeated one more time to reduce spillover of the population age categories. Therefore the last fraction

is used as the young *C. elegans* and the first is used as the old *C. elegans* in this research. Sample count and size for each separated population is carried out using a light microscope (Olympus Tokyo CK Light Microscope). Age fractionation is now completed and the samples (*C. elegans* in 0.5 ml sterile saline solution [0.3 %]) are stored in eppendorf tubes, at -70 ^{o}C, until needed for analysis. This process of the repeated cold temperature stress separates the pre-reproductive (L2 and L3 whose size is less than 600 nm) from the reproductive (L4, size between 600 and 900 nm) and, from the post-reproductive (adult, size > 900 nm). Some spill over from one population category to the other does occur.

METABOLITE SEPARATION AND IDENTIFICATION.

The technique of high performance liquid chromatography (HPLC), specifically Coulochem Electrode Array System (CEAS) is used to separate the metabolites of interest. This separation system was made by ESA Inc. (which includes Solvent Delivery Module Model 582, ESA Model 540 Autosampler, CoulArray Detector with Sensor(s) Model 6210 and, CoulArray for Windows Applications Software). Methods are created to separate the metabolites based mainly on their solubility, partition coefficient using C-18 reversed phase chromatography, oxidation and reduction potentials and size, depending on the mobile phases constituents to be used. The Mobile phases A and B (MPA and MPB) that are made, samples and standards are all prepared according to HPLC standards (using HPLC grade reagents, and filtering with 0.2 μm nylon filter).

These samples and standards are then analyzed using the appropriate method(s) and, the data is collected for analysis.

The methods developed and used in the research are listed in **Appendix 3**. A solvent gradient with fluctuating flow rate is used to elute the metabolites. The methods were tweaked to get the best separation of the analytes, based on their elution time (also referred to as retention time). A semi solvent wash is included in the method, which helps to keep the system clean. The cells potentials are set in increments of 60 mV, starting with 0 mV in channel 1 (or cell 1) and ending with 900 mV in channel 16 (or cell 16). A Carbon18 column (HR-80 {RP-C18}, 4.6 mm ID, from ESA Inc.) is used in separating the metabolites, which contributes to the method development. Flow rates are dependent on mobile phase composition, column packing, and temperature. With this system, the C-18 column is in a temperature-monitored oven. The temperature chamber was set at 25 ^{o} C, in developing separation method for a complex system of about 2000 analytes in the post-reproductive nematodes. The polarity of the metabolites to be separated plays a key factor in the mobile phases choice and the method development.

A lithiun stock buffer solution is made, before the preparation of MPA and MPB. Under the fume hood, 300 ml of double distilled water (using an ElgaStat Maxima Ultra Pure Water System, with the water purified at 18.2 ohms) is added to 84 g of lithiun hydroxide (Fisher Scientific, L128-500), in a 1liter beaker. While swirling (using a Therolyne Nuova II Stir Plate) with a magnetic stirrer, 160 ml of phosphoric acid (o-Phosphoric acid 85 %, A365-4, from Fisher Scientific) is slowly added to dissolve the lithium hydroxide. Double distilled water is added to reach the 900 ml miniscus. The pH

is adjusted to 3.0 with phosphoric acid using a pH meter (Beckman ϕ720 pH meter) and left over night before checking the pH again. Lithium stock buffer solution pH should be in the range of 3.0 +- 0.3. Vacuum filtration of the lithium stock with a 0.2 μm filter paper (Whatman GF filter paper, catalog no. 1001 090) is carried out before it is used to make the mobile phases.

MPA (one [1] liter) is made by dissolving 10 mg of lauryl sulfate (dodecyl lithium sulfate from Sigma, L-4632) in 40 ml of lithium stock buffer before adding double distilled water (approximately 960 ml) to the one (1) liter miniscus of the beaker. The acceptable pH for MPA is between 4.3 and 4.4. MPB is made by dissolving 100 mg lauryl sulfate in 40 ml of lithium stock buffer and 360 ml double distilled water before adding methanol (approximately 600 ml, HPLC grade from Fisher Scientific, A452-4) fill to the 1 liter miniscus of the beaker. MPB pH falls between 4.6 and 4.7. Both MPA and MPB are vacuum filtered with 0.2 μm nylon filter paper.

Standards, which are individual known compounds of interest, are prepared. Stock solutions of 1 mg/ml are prepared by dissolving 1 mg of the standard in 1 ml of MPA. Cocktails with each compound of interest concentration of 1 μg/ml are made from these stock solutions, which are used for HPLC analysis. The stored frozen nematode samples are homogenized (using a 60 Sonic Dismembrator from Fisher Scientific), heated at 100 ^{o} C for 5 minutes and centrifuged for 15 minutes at 14,000 rpm (Eppendorf Centrifuge 5415 C, Brinkmann), where the supernatant is removed for analysis. Dilutions of the sample supernatant using MPA were done depending on the number and age of nematodes in each sample. These diluted samples are then filtered (with Cameo

3N Syringe Filter, Nylon, 0.45 Micron, from Micron Separations Inc.) before pipetting 100 μl of the sample into HPLC vials (0.25 ml vials from Sun Inc.) for analysis. Diluted standards are also pipetted into these HPLC vials for analysis.

The vials with the samples are then placed onto the autosampler tray. Methods and series are programmed on the autosampler before the autosampler is programmed to run the samples. The 'method' within the autosampler controls the volume of the sample to be pipetted for analysis. A 25 μl sample size was selected for analysis because it gives highly reproducible results. Controls were set to wash the pipettor with methanol solution after every new sample is injected into the system. Each "series" covers one method type. An experiment can be programmed with several series which incorporates several methods. Within the "method" one determines how many injections from the sample are needed. After the autosampler is programmed, the software method developed is then loaded and run from the Coularry software, on the computer. This software method controls and communicates with the autosampler. The results are saved as files that can be viewed in the form of chromatograms. These chromatograms are the raw data from the experiment, which are saved onto a study within the software.

Under the section, "work with collected data", the data is stored and analyzed. After the data is imported into a study created, by following the instructions on importing the data (has to be followed or the data will not be imported in the correct format), it goes through a series of analysis. An active filter method is chosen first where baseline subtraction of the raw data is carried out. The baseline is a chromatogram with no compounds present. There are four choices, none, low, medium and high. The medium

data subtraction filtration method is used based on the software manual suggestion for the gradient separated data. An active peak detection method is then created (or you can use the default) where you decide limits for the base width, minimum shoulder, the minimum peak height, the minimum half width and maximum half width. Here the minimum peak height is set to 2.0 nA. After the active filter method (which are statistical formulas to remove noise and smooth the peaks) and the active peak detection methods have been applied to the data, a peak name table has to be created. Using the known analytes and the cocktail analytes, each known analyte is identified by their retention time and voltage (channel) of oxidation, in the standard cocktail. The steps to create the peak name table are followed using the wizard within the software. An active calibration method is then developed. This uses the analyzed standard cocktails at various known concentrations that are then analyzed at these various concentrations, and represented as levels during the creation of the calibration method. Again the software wizard is followed here. Finally, the chromatograms are analyzed when using the file sets that were created and made active. The results are the identified and unknown peaks, within the data set from the chromatogram, which can then be exported into excel, depending on ones interest.

PROTEIN ASSAY. In order to provide standardization for the HPLC analysis chromatogram metabolites, the soluble protein content for each sample was determined using the Bio-Rad Protein Assay. The Bio-Rad Protein Assay is based on the method of Bradford, a simple and accurate procedure for determining concentration of solubilized protein. It is a dye-binding assay in which a differential color change of the dye,

Coomassie Brilliant Blue G-250, occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of the dye shifts from 465 nm to 595 nm when binding to protein occurs. The Coomassie blue dye binds to primarily basic and aromatic amino acid residues. Interferences may be caused by chemical-protein and/or chemical-dye interactions (e.g., basic buffer conditions and detergents).

The samples in the MPA were analyzed for their soluble protein content, using the standard Microassay Procedure (*Bio-Rad, 1069).* The standard curve was done using know concentrations of Bovin Serum Albumin (BSA), diluted in MPA. The procedure was carried out in 96 well ELSIA plate and their absorbencies were measured at 595 nm, using a spectrophotomer plate reader (μQuant Biotek Instrument, Inc.).

DATA ANALYSIS.

Data analysis has three (3) sections: evaluating and displaying the treated population ratios (y/m and y/o) for all the experimental categories, collection and processing of chromatographic data using CoulArray for Windows application software and, external data manipulation of the unprocessed chromatographic data using a generated subtraction algorithm, to identify unique patterns and analytes, and applying statistical software for data clustering.

The population counts (percents) for the life stages are examined and discussed in terms of the Vitamin treatment effects on life stage ratios (y/m and y/o). The percents for the three life stages, in all experimental category replicates, are shown using Barycentric plots (ternary plot on three variables which sum to a constant). These illustrate the

variability of the replicates as well the treatment effects. These plots were generated by a program written in the software "R", with the aid of Dr. Daniel Carr. He also suggested the analysis of variance portions of the statistics that were done in R.

A combination of Student's *t* test and analysis of variance was done on the y/m and y/o ratios and log (base 2) of the y/m and y/o ratios. A p-value of <0.05 from both the *t* tests and the F statistics in the analysis are taken as evident that comparisons are statistically significant. The analysis of variance was a two-way factorial analysis involving all the dose combination of 0.5 mg/ml or less. There were no interaction experiments for the 1.0 mg/ml dose groups. Separate one-way analyses Viatimin C and Vitamin E included the 1.0 mg/ml dose groups.

Nomral QQ plots were also generated using the residuals from the means of the dose group replicates for the y/m and y/o ratios and log base 2 y/m and y/o ratios. An approximate straight line fit indicates the residual distribution is similar to a normal distribution. This is a key assumption in the analysis of variance and important for t-test as well when the number of replicates is small.

CoulArray for Windows is a Windows application that is used with the ESA CoulArray Coulometric Electrode Array Detector. It is an integrated program that provides system control, as well as the collection, processing and reporting of electrochemical data obtained from an HPLC system that includes the CoulArray detector. Modes of operation of the program can be used to configure instrument hardware; view chromatographic data; control system functions; generate method(s) for the collection and processing of chromatographic data; collect process and report

chromatographic data; and, to transfer processed chromatographic data to external data manipulation programs. Within the CoulArray software, the processed chromatograms undergo noise reduction by baseline and sample reduction noise, all mathematical formulas built into the software. 'Peak name table and calibration curves' are created with the know standards in leu of identifying these compounds in the samples. The elution time of each compound measured is based on its electrochemical potential and thus channels providing signal, size and the system pH gradient. The calibration curve allows one to quantify the compound against the known standard, which is set up by the concentration/response peak height/area from the standard curves. These metabolites are then normalized using the protein concentration data.

Involved in the external data manipulation is the conversion of the collected unprocessed chromatographic data set to matrix data by first importing the data into CEAS512 software, which has the capabilities of transferring the chromatographs into text files, in matrix format. This data is in a tabulated matrix format, of 16 columns and over 9000 rows. **Appendix 11** shows a sample of part of the matrix generated by the CEAS512 software. Each column represents a data channel, the voltage at which the measurement is made. There are 16 channels with voltage increments of 60 mVolts. The captured chromatographic data is done approximately every 0.008 minute. The time line is the row. This matrix data is then read and processed by the subtraction algorithm (**Appendix 10**). Embedded in the subtraction algorithm is a normalization factor, which uses the soluble protein data (from the protein assay) for each sample to normalize the chromatographic matrix data against gram of soluble protein. Also, Dr. Kinser wrote the

section to remove the wobbling (in time) of the data since it is gradient obtained data. The purpose of the subtraction algorithm is to find out if there are unique compounds with certain conditions, hence metabolic signature patterns per condition. The results of the subtraction data are then viewed in Excel as shown in the graphs, in the results and discussion chapter.

In the subtracting algorithm the two text (matrix A and B) files are read, then normalized. The wobbling is removed and then subtraction is performed. The subtracted data is stored into file C in the matrix format. Finally one decides if to capture data for certain channel(s) and time frame and save all three files (A, B and C) in one file. This file can then be viewed in Microsoft Excel.

Finally the text data are brought into software called PowerArray (www.niss.org) where principle component analysis is performed on the studies. The data of interest are converted into 1-dimenion matrix and all the files are on separate columns. A design file is also created with the name of each file (column). Both files are imported into PowerArray. PowerArray is a stastical package developed by Jack Liu from the national institute of Statistical Sciences (www.nist.org). With the principle component analysis section data clustering is viewed, as demonstrated and shown in the results and discussion section.

4. RESULTS AND DISCUSSION

Several questions concerning antioxidants and life span served as the basis for the experimental design employed in this research. First and foremost is the visual. Are there any changes in the population distributions, at the end of the treatment period when comparing the worms treated with Vitamin C and/or E, with the untreated populations since both Vitamin C and E are antioxidants? Antioxidants are supposed to reduce the amount of oxidative damage within the organism, as a whole, according to the literature. Are there changes in the biochemistry of worms at various treatment levels? Are there any dose response relationships with the concentrations of the antioxidants use? Is it better to use both the combination of Vitamin C and E or does using one alone have the same impact as the other or together?

Since this study deals with aging, it will be appropriate to analyze and discuss the results in relation to the three age categories, pre-reproductive (or young [y]), reproductive (or middle aged [m]) and post-reproductive (or old [o]) *C. elegans*. As mentioned in chapter 3, the pre-reproductive *C. elegans* are the L1, L2 and L3 larval stages (less than 600 nm in length), the L4 and young adults are considered the reproductive *C. elegans* (between 600 and 900 nm in length) and, post-reproductive are the adults whose length are greater than 900 nm. In the pre-reproductive phase cell proliferation followed by organogenesis and morphogenesis occurs. In the reproductive

phase, mostly reproduction occurs. In the post-reproductive worms some reproduction occurs, in addition to senescence.

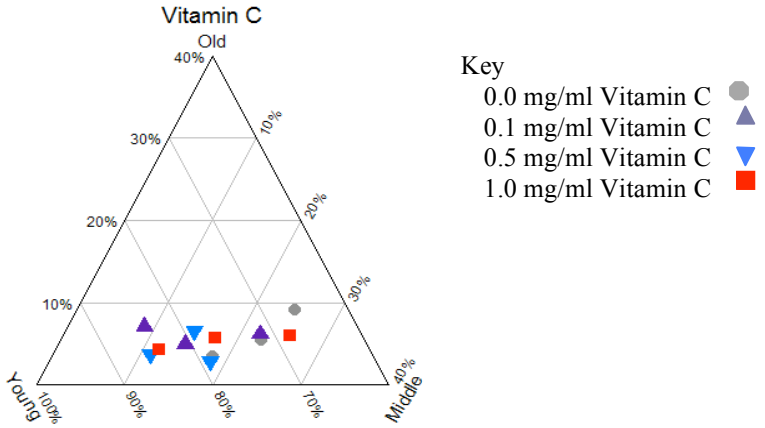
Tyrsoine, tryptophan, serotonin, epinephrine, norepinephrine, 8-Hydroxyguanine and the purine catabolites were identified and measured. The breakdown products of the purine pathway and 8-hydroxy guanine were measured as indicators of the status of DNA synthesis and repair. Does the addition of the Vitamin C and/or E reduce oxidative stress enough to alter the relative amounts of measurable purine catabolites and 8-hydroxy guanine, a product of damaged DNA excision repair? Does the antioxidants alter the concentrations/ratios of tryptophan, tyrosine and their neurotransmitters, thereby altering the control system involve in the aging process by reducing oxidative stress.

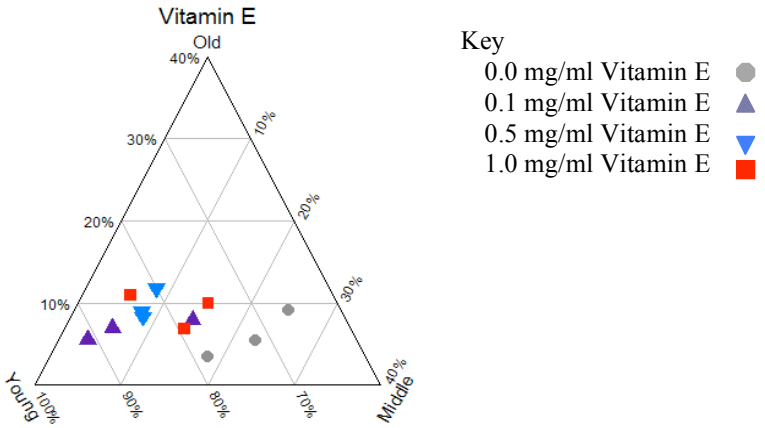
A protein assay was run on each sample to determine the soluble protein content, which is used as a normalization factor for the analytes measured, per μg soluble protein, in the HPLC data. This allows representation of the data relative to viable cell mass. Unique metabolites in an age group/experimental category provide a signature pattern characteristic of the life stage examined. Data clustering utilizing Principle Component Analysis is run on the raw chromatographic data, as a separate tool to verify that the data for each age/experimental group is different/unique.

POPULATION DISTRIBUTION RATIOS OF *C.* ELEGANS GROWN WITH VITAMIN C AND/OR E SUPPLEMENTS UNDER AXENIC MEDIA CONDITIONS.

C. elegans counts were done at the start and end of the incubation period, as outlined in chapter 3. The populations were divided into the three age groups as defined.

Each experiment was done in replicates of three and the average for each experimental category life stage ratios are displayed for the start and end of the experiment incubation period. Included in **Appendix 4** is a photograph of a harvested untreated population. The data obtained in these studies is examined and is discussed in terms of how it correlates to/with the individual life stages, and associated changes in percentage or ratios of one life stage to the other. Another means of displaying the data is via the barycentric plots **(Figure 4)** where distinct patterns were obtained for each treatment category.





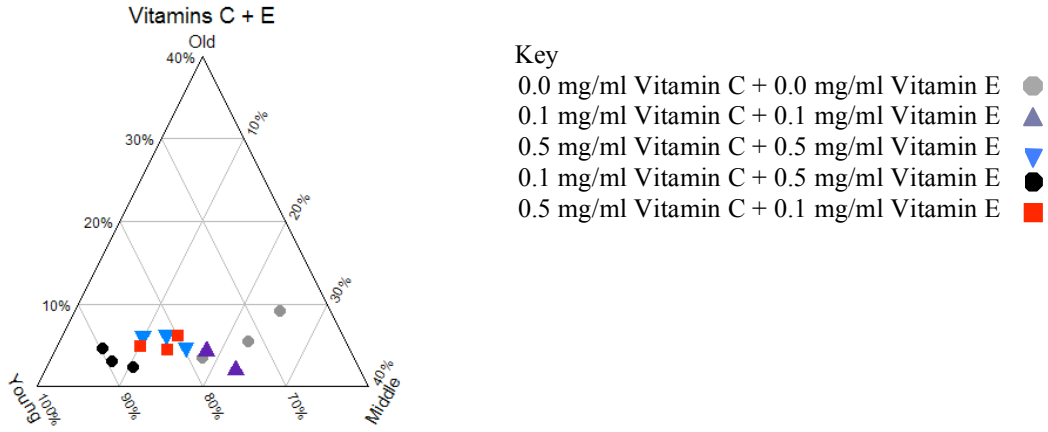


Figure 4: Barycentric plots displaying the population life stages for Vitamin C and/or E supplement treatments. These plots are focused on the lower left corner of the full barycentric plots. The full plots extend to Old with 100 % and Middle with 100 %. The grid lines for the components Young, Middle, and Old in the Figures above have percents with the same angular orientation and component label. For example, with the Vitamin C plot the Young axis labels run along the bottom. They start at 100 % and labels go down to 70%. The red square dot on the right has less than 70 % young. The 60% at the end is omitting to avoid over plotting. The Old axis labels start at 40% at the top vertex and decrease down the left side to 10 %. The top gray dot has a little less than 10% Old. The 0% label is suppressed to avoid over plotting. The Middle axis labels down the right side. The different symbols are between 20% and 30%.

Student's *t* tests were performed (using Microsoft Excel) on the log base 2 of the y/m and y/o life stage ratios. There are statistically significant differences in the population profiles based on the treatment categories. The log y/m ratios for the treatments resulted in p-values <=0.5, with the exception of the 0.1 mg/ml Vitamin C and the 0.1 mg/ml Vitamin C & 0.1 mg/ml Vitamin E combination treatments. For the log y/o ratios the p-values are >0.05 for all the treatments.

The two-way factorial analyis using vitamin C and/or E dose groups of 0.5 mg/ml or less provide a balance 3 x 3 design with three replicated per cell. Based on the normal

QQplots **(Appendix 4H)** of replicate residuals from the cell means, the log transformation of the ratio provides residual close to those expected from a normal distribution. There is one outlier in the y/m residuals QQ plot. The anova results are reported just for the log ratios. The choice log base 2 is simply for the easy of interpretation given the modest range of the ratios. Note that the log of count ratios is the same as the difference of the log counts for the two groups being compared. The two-way log ratio y/m anova had an overall F test with p-value =0.004. The vitiamin C effect was not statistically significant. The vitamin E effect is statistically significant with p=0.006. The vitamin C and E interaction is also significant with p=0.006, therefore the vitamin C effect cannot be ignored.

Cells and margin effects for log (base 2) y/m

	C0	C0.1	C0.5	C Effect
C0	1.72	3.73	3.34	2.93
C0.1	2.48	1.96	3.53	2.65
C0.5	2.49	2.72	2.74	2.65
E Effect	2.23	2.80	3.20	2.74

Interactions for log y/m (departures from an additive main effects model)

	E0	E0.1	E0.5
C0	-0.69	0.74	-0.05
C0.1	0.34	-0.75	0.42
C0.5	0.35	0.01	-0.37

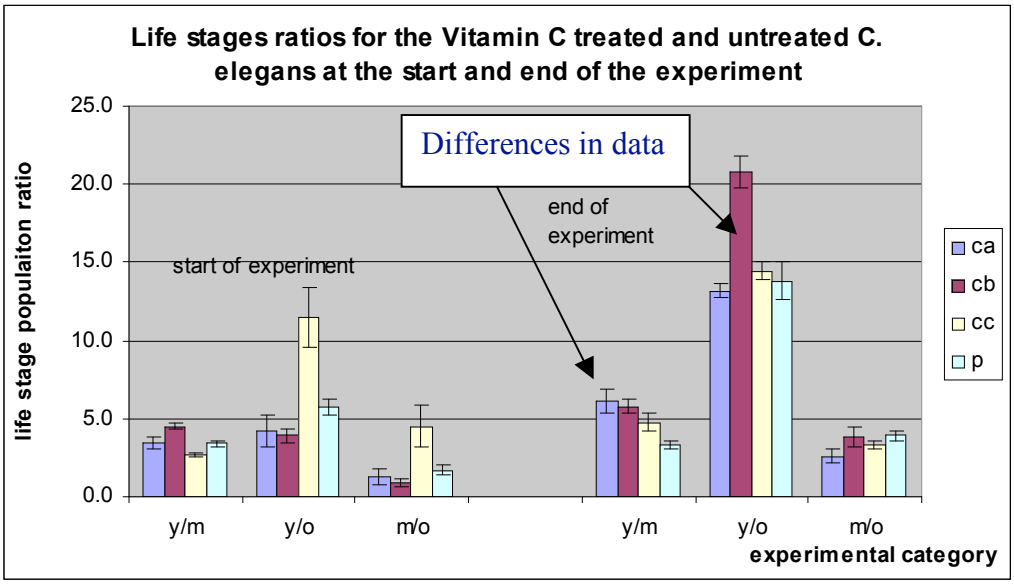
The C0E0.1 cell has the largest average log ratio but has one discordant replicate. A simple unequal variance t-test comparing it to the control group is not significant. The other high ratio comparison against the control group, C=0.1E0.5 and C=0E0.5 are signficant with p=0.007 and 0.012 respectively.

The one-way anova for C that include the C1.0 dose group is not signicant. The one-way anova for E that includes the e1.0 dose groups is very close to signifance with p=0.052. The diminished returns from both the baracentric plots and the one-way anovas suggest lower ratios signifinance and, that future experiments may not be needed to address doses higher than 1.0 mg/ml vitamin additive.

The two-way anova of the y/o ratio is not independent of the y/m assessment since the same numerator is involved. The overall F test is significant with p=0.012. Basically the C0.1E0.5 groups have a very low percent old and high percent young producing large ratio, hence stastical significance. The *t* test p-values for log y/o are all greater than 0.05, indicating no significance in the data.

Figure 5 shows the results for the Vitamin C population ratios averages. The y/o ratio for the 0.1 mg/ml Vitamin C treatment is different (greater than) from all the other experimental categories. This implies that the addition of 0.5 mg/ml Vitamin C, due to its antioxidant properties, reduced ROS stress, impacting development and/or reproduction. With the 0.5 mg/ml Vitamin C treatment, the transition through the separate life stages is slowed, thus ratios of young to old are greater than the untreated. The ratios of the y/m for the Vitamin treatments are greater than that of the untreated, displaying dose

response, but not by much. The error bars are the standard error of the mean for the ratio in all the population ratio data. An average of the three ratios are taken for each of the experimental categories.

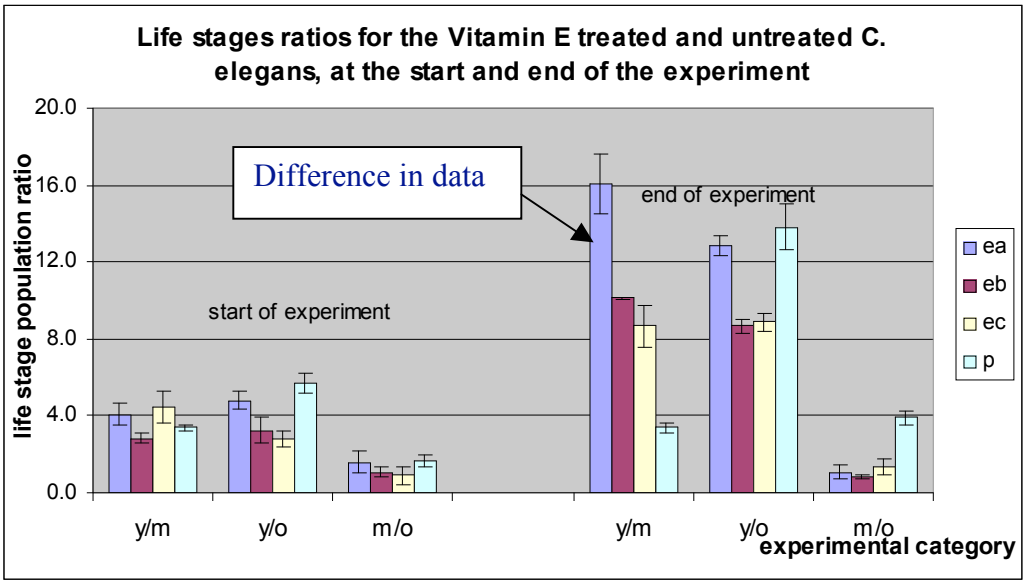


CA - 0.1 mg/ml Vitamin C treated *C. elegans*
CB - 0.5 mg/ml Vitamin C treated *C. elegans*
CC - 1.0 mg/ml Vitamin C treated *C. elegans*
P - untreated *C. elegans*

Figure 5: Graph showing the average life stages ratios for the Vitamin C treated and untreated *C. elegans* at the start and end of the experiment. The ratios are the pre-reproductive/reproductive (y/m), pre-reproductive/post-reproductive (y/o) and, reproductive/post-reproductive (m/o). Error bars are standard error of mean ratio.

Figure 6 shows the relationships of the life stages ratios for the various Vitamin E experimental categories. Here the ratios of the y/m is greatest for the 0.1 mg/ml Vitamin E treatment, when compared to all the y/m ratios in this experimental category. This result may be interpreted as Vitamin E changes the rate of development and reproduction.

The 0.1 mg/ml Vitamin E slowed down the development rate of the nematode in that there is a smaller number of reproductive *C. elegans*, when compared to the untreated populations.

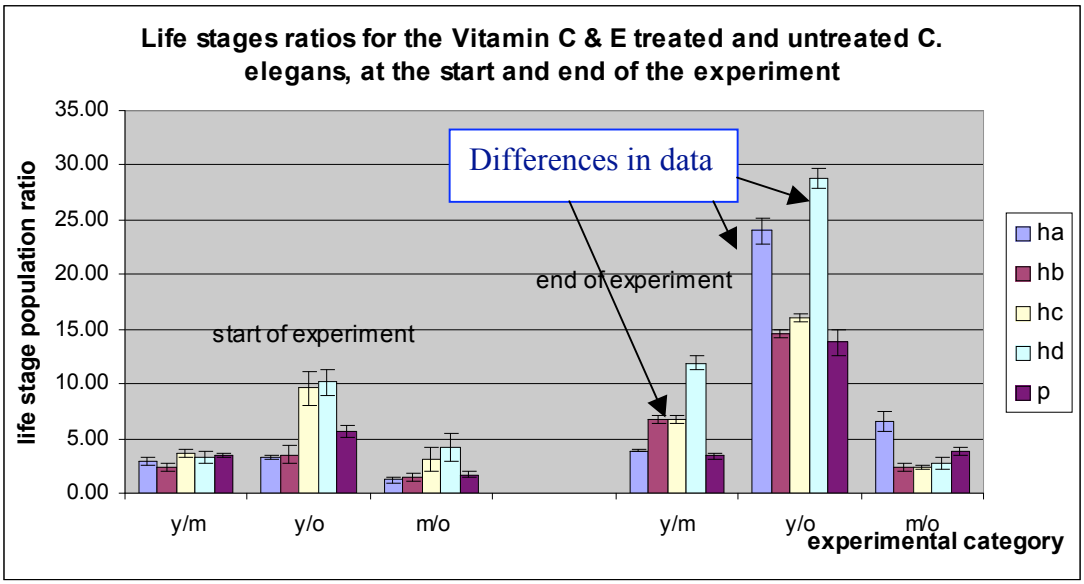


P - untreated *C. elegans*
EA - 0.1 mg/ml Vitamin E treated *C. elegans*
EB - 0.5 mg/ml Vitamin E treated *C. elegans*
EC - 1.0 mg/ml Vitamin E treated *C. elegans*

Figure 6: Graph of the average population distribution ratios for the Vitamin E dose treatment and the untreated *C. elegans* at the start and end of the experiment. The ratios are the pre-reproductive/reproductive (y/m), pre-reproductive/post-reproductive (y/o) and, reproductive/post-reproductive (m/o). Error bars are standard error of mean ratio.

The combination of Vitamin C and E impacted the growth rate in a different pattern from the Vitamin C or E alone treatment. There is a reduction in the growth rate of the population, when compared to the untreated worms (**Appendix 4**). The greatest

decline in the population size was seen in the combination of 0.5 mg/ml Vitamin C with 0.1 mg/ml Vitamin E, followed by 0.5 mg/ml Vitamin C with 0.5 mg/ml Vitamin E.



HA - 0.1 mg/ml Vitamin E and Vitamin C treated *C. elegans*
HB - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated *C. elegans*
HC - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated *C. elegans*
HD - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated *C. elegans*
P- untreated C. elegans

Figure 7: Graph showing the life stage ratios for the Vitamin C & E treatment and the untreated *C. elegans* at the start and end of the ten days incubation period. The ratios are the pre-reproductive/reproductive (y/m), pre-reproductive/post-reproductive (y/o) and reproductive/post-reproductive life stages for each experimental category. Error bars are standard error of mean ratio

Figure 7 shows the ratios of the various life stages at the start and end of the experiment, for the Vitamin C & E experiment. The ratio of y/m for the 0.1 mg/ml Vitamin C & 0.5 mg/ml Vitamin E (HA) is about 3 times that of the untreated, implying that there are less reproductive nematodes in this treatment compared to the others. Also,

the y/o ratios for the 0.1 mg/ml Vitamin C & 0.1 mg/ml Vitamin E (HA) and the 0.1 mg/ml Vitamin C & 0.5 mg/ml Vitamin E (HD) are larger than the untreated, implying that there are less post-reproductive than the untreated cultures.

In conclusion, small concentrations (0.1 mg/ml) of Vitamin E treatment alone seems to decrease the rate at which the pre-reproductive *C. elegans* is converted to the reproductive *C. elegans* when compared to all the experimental categories. Vitamin C alone treatment has the least significant impact on the life stage distributions throughout the time to which these populations were exposed. The rate of conversion of the pre-reproductive to the reproductive life-stage, are a little slower to that observed for untreated populations. The 0.1 mg/ml Vitamin C with the 0.5 mg/ml Vitamin E combination has the greatest impact in delaying the rate at which the pre-reproductive is converted to the reproductive and then the post-reproductive *C. elegans*, of all the experimental categories. It seems to slow overall passage through the developmental cycle. The data may also indicate that specific life stages respond differently to the addition of these antioxidants.

This may explain why more pre-reproductive *C. elegans* are observed in the Vitamin E treated cultures as opposed to the untreated. The same result was observed in the Tween dissolved Vitamin E data. Another way of looking at the data and getting the same end result, is to retain the reproductive function, by increasing life span of an individual, or increase reproductive success per individual, ie, one would not remove the old, but would increase the yield of progeny, thus numbers of young per number of old would increase. In any instance, the effect of the Vitamins on the system is real and

significant, increasing the life span of the individual nematodes, with the Vitamin C & E combination being the most effective.

The pH data is tabulated in Appendix 4F. The media with the most acidic pH (6.29) at the start of the experiment was that with the 1.0 mg/ml Vitamin C. The least acidic media at the start of the experiment was that of the 0.1 mg/ml added Vitamin C, a value of 6.39. The cultures became slightly basic with the growth of the *C. elegans*. The least basic media was that from the CC (1.0 mg/ml Vitamin C) population, with an average pH of 7.88, followed by the control population media with an average pH of 7.89, at the end of the experiment. The *C. elegans* grown in 0.1 mg/ml Vitamin C and 0.5 mg/ml Vitamin E combination produced the most basic media, an average pH of 8.19. This change in the pH in each experimental category is due to metabolite end products and their concentrations, which varies from the untreated. This change in the pH may have affected the growth rates in each experiment category. However, the normal pH range for this organism in the wild, soil conditions, range from weakly acidic, ~ 6 to weakly basic, ~8. The gut pH of the nematode runs at 8.6 normally.

TRYPTOPHAN AND TYROSINE CONCENTRATIONS AND RATIO.

Both tryptophan and tyrosine degradative pathways involve the formation of neurotransmitters which are involve in signaling. These neurotransmitters are multi-gene products, providing signal and feedback capabilities to control genomic expressions. Tyrosine degrades to a family of catecholamines, including dopamine, norepinephrine and epinephrine, important neurotransmitters, useful in maintaining homeostasis. In this

section tryptophan/tyrosine (trp/tyr) ratios for the various experimental categories are examined from the perspective of relevance to individual life stages. Does the trp/tyr ratio vary with life stage and does the addition of Vitamin C and/or E change the ratios? This question is asked since a change is ratio will show modifications in homeostasis.

The tyrosine and tryptophan concentrations increased with age in the untreated, Vitamin C and Vitamin E experiments (**Figure 8, 9, 10, 11**). With the Vitamin C & E combination treatment the tyrosine and tryptophan concentrations decreased about a 5-7 fold from the pre-reproductive life stage to the reproductive life stage and resumed their concentrations in the post-reproductive life stage. In the pre-reproductive life phase the Vitamin C & E treatment resulted in tryptophan and tyrosine concentrations being the largest of all the experimental categories. This was reversed in the reproductive life stage, where the tryptophan and tyrosine concentrations decreased for the Vitamin C & E treatment.

The ratio of trp/tyr did not fluctuate significantly with age for the Vitamin C & E treated nematodes. With the untreated nematodes trp/tyr concentration dropped slightly in the reproductive phase and increased significantly in the post-reproductive phase, implying more tryptophan is being measured in the post-reproductive life phase. Similar patterns are seen for the Vitamin C and Vitamin E (alone) experiments.

The data implies that Vitamin C or E alone is not as effective as Vitamin C and E together in fighting oxidative stress (since trp/tyr ratio did not change significantly with life stage, in the Vitamin C & E data). In the reproductive life phase, there is an increase in metabolism and hence an increase in ROS since a lot of energy is needed for

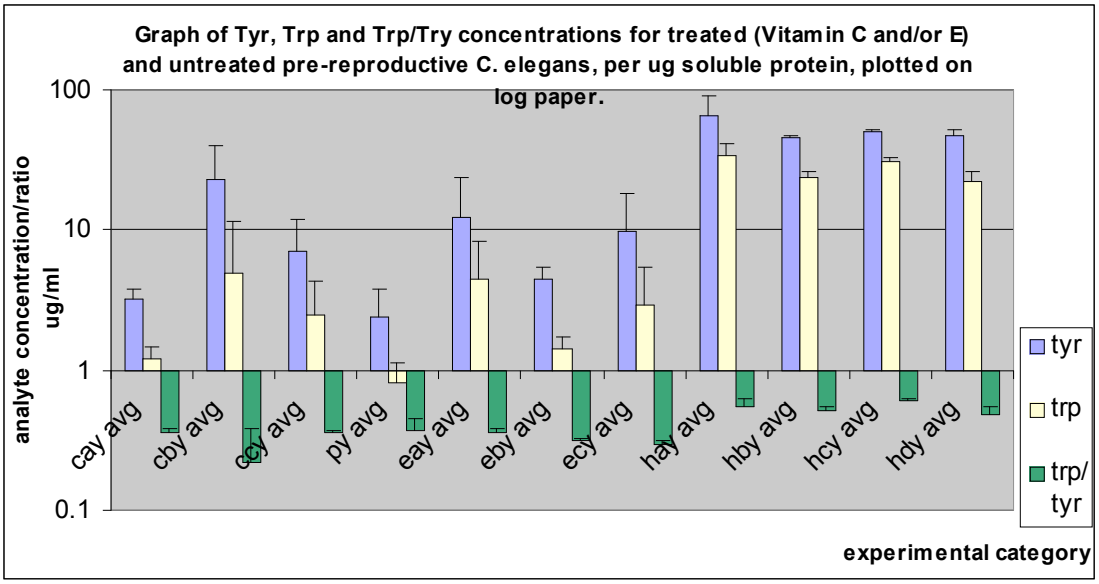
reproduction. In this reproductive life stage reproduction mostly occurs so there is DNA formation in the eggs. Since the concentrations of tyrosine and tryptophan is the same in the media in all the experimental categories and are available in surplus, the change in the tyrosine and tryptophan concentrations and ratios imply that they are being used for the formation of other products such as NAD (for energy formation), serotonin (needed for rhythmic locomotion) and epinephrine and norepinephrine (needed for circulatory control).

The differences seen with the tryptophan, tyrosine and trp/tyr ratios for the different Vitamin treatments, at different life stage, can imply that these are important analytes in maintaining homeostasis. These differences, when correlated with the experimental categories and their concurrent life stages can be interpreted as effectors of the population distribution changes observed in the treated and untreated nematodes. The trp/tyr ratios data is compatible with the theory that aging is initiated by the breakdown of control systems regulating complex physiological functions (the neuroendocrine theory of aging). Free radical reactions may trigger the breakdown of homeostasis. Signals arising from metabolic alteration of metabolites (which include tryptophan, tyrosine and their degradative products), fundamental to the biochemistries of all organisms represent the "first level" of evolving signal systems.

Figures 8 & **9** shows the tyrosine and tryptophan concentrations, and trp/tyr ratios for the pre-reproductive *C. elegans* in all the experimental categories. The untreated pre-reproductive *C. elegans* had the least amounts of tyrosine and tryptophan, of all the pre-reproductive worms. Of the Vitamin C dose response category, cby (0.5 mg/ml treated

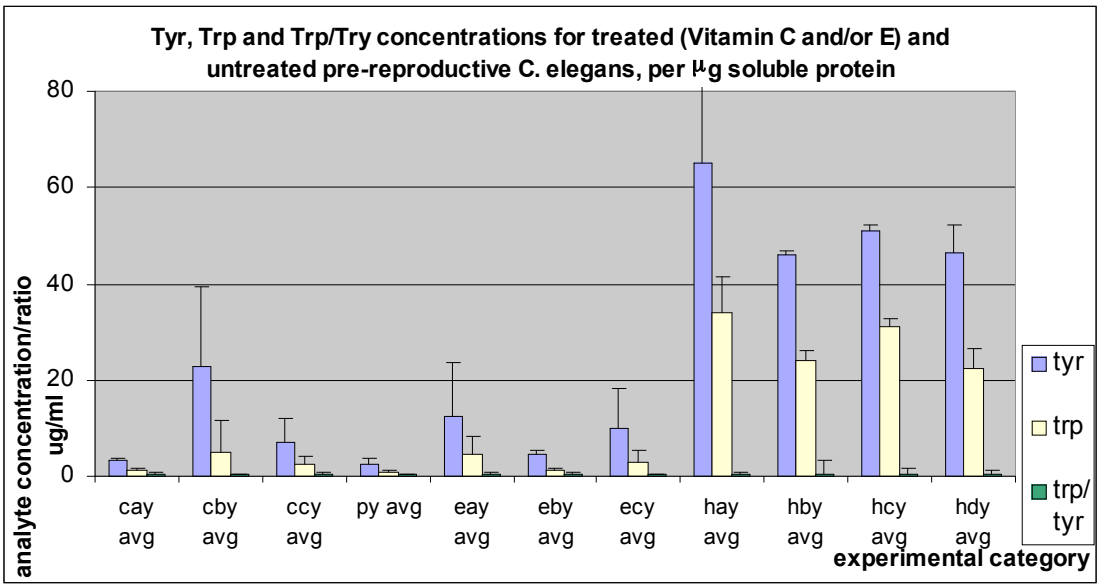
Vitamin C) had the most amounts of measurable tyrosine and tryptophan and cay (0.1 mg/ml) had the least amounts of measurable tyrosine and tryptophan. However, the trp/tyr ratio is the least in the cby category if the Vitamin C experiment and the other two Vitamin C experimental categories have similar trp/tyr ratios to the untreated pre-reproductive *C. elegans*. Of the Vitamin E populations the reverse is found in the eb (0.5 mg/ml added) Vitamin E populations. Here the measurable tyrosine and tryptophan concentrations are much less than the ea (0.1 mg/ml Vitamin E treated) and ec (1.0 mg/ml Vitamin E treated) populations. The trp/tyr ratios for the eb and ec populations are similar, but smaller than the untreated pre-reproductive *C. elegans*. The trp/tyr ratios observed in Vitamin C and E treated worms are larger than the ratios observed in the Vitamin C treated, Vitamin E treated and the untreated worms. The raw data used to generate **Figures 8** and **9** is found in **Appendix 5**.

With the reproductive life stage the ratio of trp/tyr is dose dependent for both the Vitamin C and Vitamin E treatments, decreasing as the concentrations of the Vitamin C and Vitamin E increased. The opposite is true for the Vitamin C and E combination treatments the hcm (0.5 mg/ml Vitamin C and 0.5 mg/ml Vitamin E) trp/tyr ratio is the greatest in this experimental category, 0.662 (**Appendix 5**). The untreated reproductive organisms had the lowest trp/tyr ratio, 0.267.



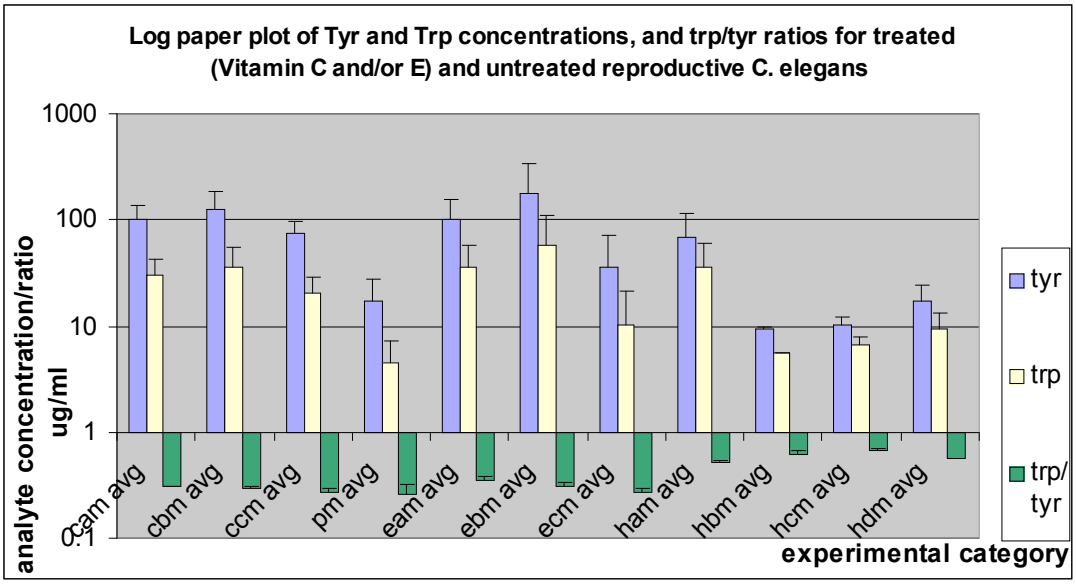
cay - 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*
cby - 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
ccy - 1.0 mg/ml Vitamin C treated pre-reproductive *C. elegans*
py - untreated pre-reproductive *C. elegans*
eay - 0.1 mg/ml Vitamin E treated pre-reproductive *C. elegans*
eby - 0.5 mg/ml Vitamin E treated pre-reproductive *C. elegans*
ecy - 1.0 mg/ml Vitamin E treated pre-reproductive *C. elegans*
hay - 0.1 mg/ml Vitamin E and Vitamin C treated pre-reproductive *C. elegans*
hby - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hcy - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hdy - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*

Figure 8: Graph of Tyrosine (tyr), Tryptophan (trp) and trp/tyr ratios for the treated and untreated pre-reproductive *C. elegans*, per μg soluble protein. This plot is done on log paper. Error bars are 1SD.



cay - 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*
cby - 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
ccy - 1.0 mg/ml Vitamin C treated pre-reproductive *C. elegans*
py - untreated pre-reproductive *C. elegans*
eay - 0.1 mg/ml Vitamin E treated pre-reproductive *C. elegans*
eby - 0.5 mg/ml Vitamin E treated pre-reproductive *C. elegans*
ecy - 1.0 mg/ml Vitamin E treated pre-reproductive *C. elegans*
hay - 0.1 mg/ml Vitamin E and Vitamin C treated pre-reproductive *C. elegans*
hby - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hcy - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hdy - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*

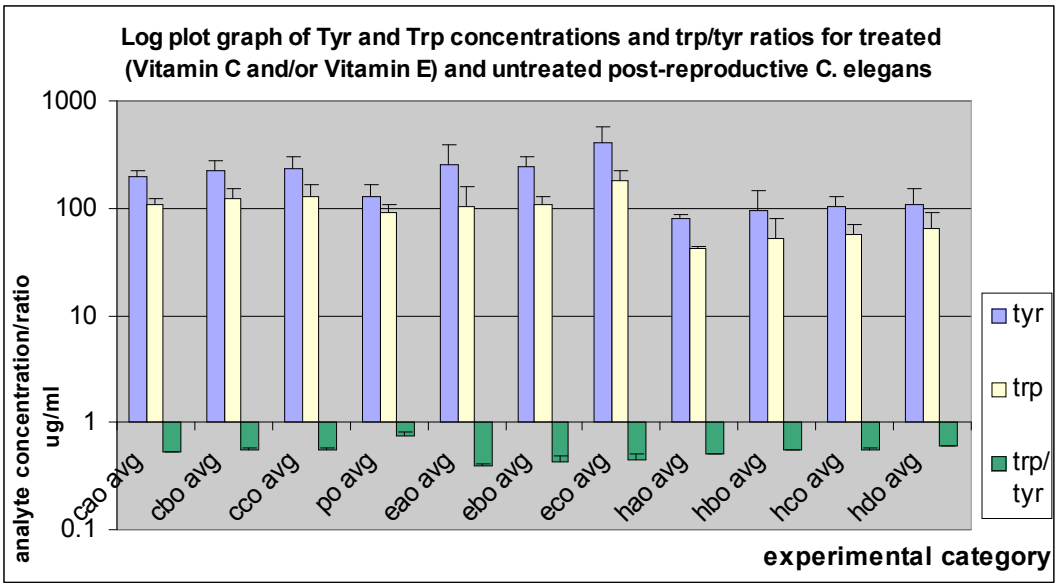
Figure 9: Graph showing average Tyrosine (Tyr), Tryptophan (Trp) concentrations and Trp/Tyr ratios for treated (Vitamin C and/or E) and untreated pre-reproductive *C. elegans*, per µg soluble protein. Error bars are 1SD.



cam - 0.1 mg/ml Vitamin C treated reproductive *C. elegans*
cbm - 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
ccm - 1.0 mg/ml Vitamin C treated reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
eam - 0.1 mg/ml Vitamin E treated reproductive *C. elegans*
ebm - 0.5 mg/ml Vitamin E treated reproductive *C. elegans*
ecm - 1.0 mg/ml Vitamin E treated reproductive *C. elegans*
ham - 0.1 mg/ml Vitamin E and Vitamin C treated reproductive *C. elegans*
hbm - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hcm - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hdm - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated reproductive *C. elegans*

Figure 10: A log paper plot of Tyrosine and Tryptophan concentrations and trp/tyr (tryptophan/tyrosine) ratios for the treated (Vitamin C and/or E) and untreated reproductive *C. elegans*, per μg soluble protein. The raw data is found in Appendix 5. Error bars are 1SD.

The trp/tyr ratio is the largest for the untreated post-reproductive *C. elegans*. Dose responses of Vitamin C and/or E treatments in relation to tyrosine and tryptophan concentrations are also found in the post-reproductive nematodes, like the reproductive life stage. The data for the post-reproductive worms is found in **Appendix 5.**



cao - 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*
cbo - 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
cco - 1.0 mg/ml Vitamin C treated post-reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
eao - 0.1 mg/ml Vitamin E treated post-reproductive *C. elegans*
ebo - 0.5 mg/ml Vitamin E treated post-reproductive *C. elegans*
eco - 1.0 mg/ml Vitamin E treated post-reproductive *C. elegans*
hao - 0.1 mg/ml Vitamin E and Vitamin C treated post-reproductive *C. elegans*
hbo - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hco - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hdo - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*

Figure 11: A log paper plot of Tyrosine and Tryptophan concentrations and trp/tyr (tryptophan/tyrosine) ratios for the treated (Vitamin C and/or E) and untreated post-reproductive *C. elegans*, per μg soluble protein. The raw data is found in Appendix 7. Error bars are 1SD.

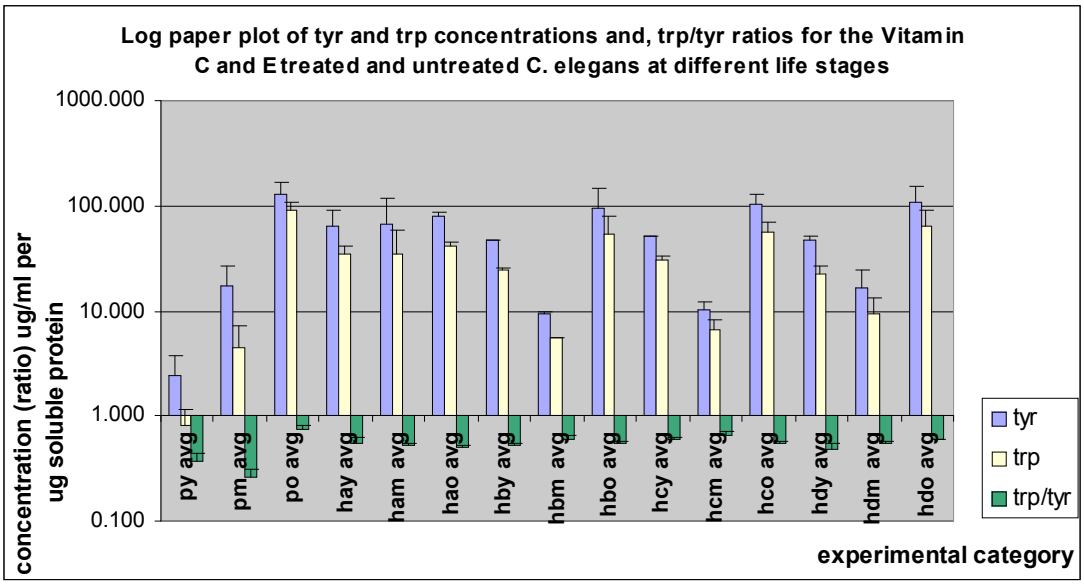
In viewing the results in terms of life stages the greatest differences are seen in the Vitamin C and E combination treated worms (**Figure 12**), when compared to the untreated worms. With the untreated *C. elegans*, the concentrations of tyrosine and tryptophan increased with age and the trp/tyr ratios varied, being the greatest (0.718) in

post-reproductive life stage and the least (0.267) in the reproductive life stage. The trp/tyr ratio in the untreated pre-reproductive life stage is 0.346. In all the Vitamin C and E combination treatments the pre-reproductive life stages, have tyrosine and tryptophan concentrations and ratios that are greater than those of the untreated pre-reproductive life stages. In the 0.1 mg/ml treated Vitamin C and E (ha) worms the concentrations of tyrosine and tryptophan increased with life stage, but the ratios of trp/tyr have a different pattern from the untreated worms, fairly stable throughout the life stages (pre-reproductive life stage 0.523 and post-reproductive life stage 0.512). The pattern changed in all the other different dose treatments of Vitamin C and E, where the reproductive stages in hbm and hcm had the largest trp/tyr ratios (0.599 and 0.662 respectively), when compared to the pre-reproductive and post-reproductive life stages.

With the Vitamin C dose treatment, the concentrations of tyrosine and tryptopan increased with age, similar to the untreated nematode. The trp/tyr ratio fluctuated with life stages, like the untreated nematode. With the 0.5 mg/ml Vitamin C treatment (cb) the pre-reproductive *C. elegans* had the lowest trp/tyr ratio (0.219). This difference in the ratio of trp/tyr may have triggered several other metabolic and pathway changes, affecting the time it takes for the larval development and reproduction to be completed (**Appendix 5**), as demonstrated in my data of the life stages.

There is a different pattern in the trp/tyr ratios for the Vitamin E treated worms, from the untreated worms. The trp/tyr ratios are very close to each other in the pre-reproductive and reproductive Vitamin E treated worms (in the 0.5 mg/ml Vitamin E experimental category the trp/tyr ratio is 0.320 for the pre-reproductive worms and 0.319

for the reproductive worms), being the smallest in the pre-reproductive life stage (**Appendix 5**) of the 0.1 mg/ml (0.355) and 1.0 mg/ml Vitamin E treatments (0.297).



py - untreated pre-reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
hay - 0.1 mg/ml Vitamin E and Vitamin C treated pre-reproductive *C. elegans*
ham - 0.1 mg/ml Vitamin E and Vitamin C treated reproductive *C. elegans*
hao - 0.1 mg/ml Vitamin E and Vitamin C treated post-reproductive *C. elegans*
hby - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hbm - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hbo - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hcy - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hcm - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hco - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hdy - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hdm - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated reproductive *C. elegans*
hdo - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*

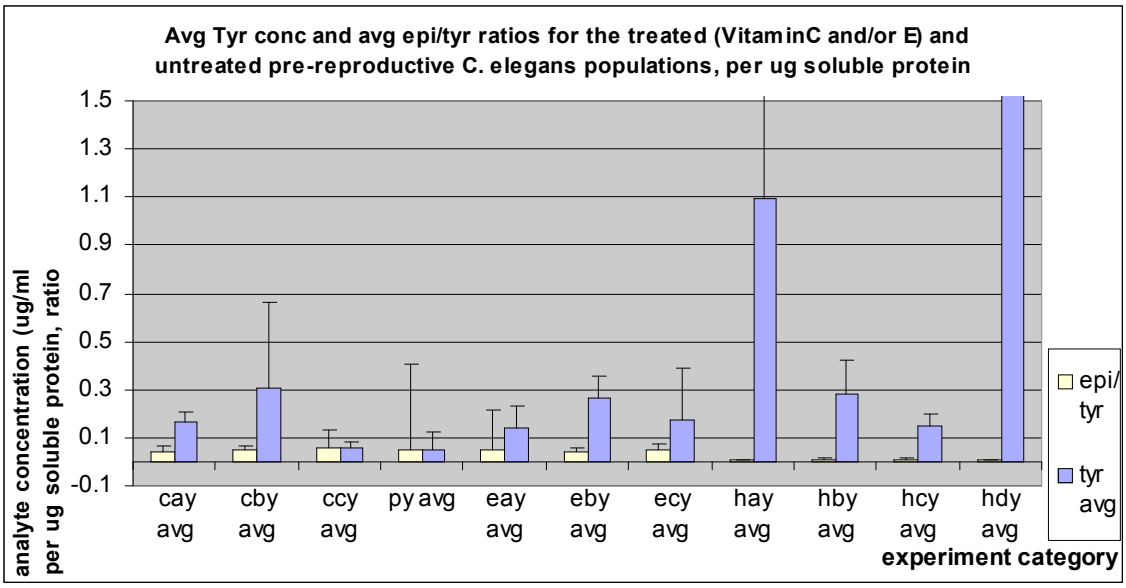
Figure 12: Log paper plot of tyrosine and tryptophan concentrations and tryptophan/tyrosine ratios for the Vitamin C and E treated and untreated *C. elegans* at different life stages. Error bars are 1SD.

TYROSINE, NOREPINEPHRINE AND EPINEPHRINE CONCENTRATIONS AND RATIOS.

Tyrosine, epinephrine and norepinephrine concentrations and the ratio of epi/tyr and norepi/tyr were measured. The neurotransmitters norepinephrine and epinephrine (catecholamines) are two oxidative degradation products of tyrosine. These catecholamines are effectors of the higest levels of neurologic function known in a host of organisms. Epinephrine and norepinephrine target the adrenal medulla of the brain in mammals, controling responses to stress and increases heart rate (which are age related functions), when acting as neurotransmitters. When these metabolites act as hormones, they regulate fuel metabolism in the liver and muscle. Hormones, given the nature of their function, normally occur in very small concentrations, in the micromolar to picomolar range.

No measurable amount of norepinephrine (Norepi) was recorded for all the pre-reproductive populations, the untreated and treated populations with the Vitamins. The instrument is sensitive to fentamoles. However, epinephrine (epi) was measured in the pre-reproductive populations. This can imply that all the norepinephrine formed is converted to epinephrine, maybe the rate-limiting step in forming epinephrine. **Figures 13, 14,** and **15** shows average epi/tyr (epinephrine/tyrosine) and norepi/tyr ratios for *C. elegans* grown in Vitamin C and/or E treated media, separated by their life stages. With the pre-reproductive nematodes the ratio of epi/tyr is much larger in the Vitamin C, Vitamin E or untreated worms than with them grown in the combination of Vitamin C and E at various concentration. The epi concentrations were relatively smaller in the

populations with the combination treatment than the untreated or treated with Vitamin C or E alone. The epinephrine concentrations are independent of the tyrosine concentrations in the pre-reproductive organisms; since the Vitamin C and E treated worms had the largest measurable tyrosine but not the largest measurable epinephrine, of all the experimental groups (as shown in Figure13).



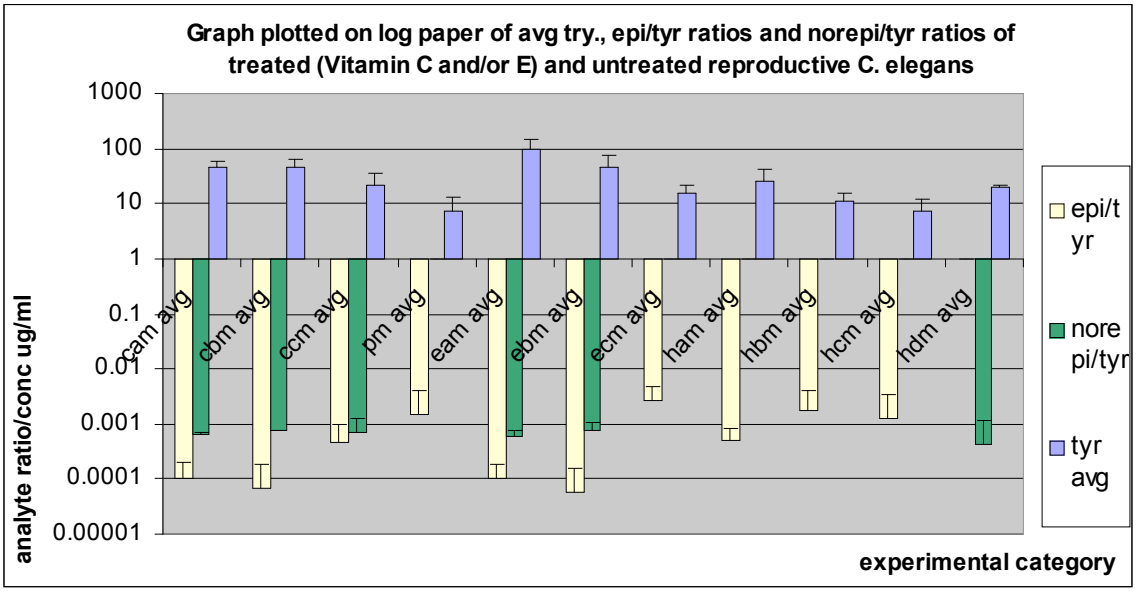
cay - 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*
cby - 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
ccy - 1.0 mg/ml Vitamin C treated pre-reproductive *C. elegans*
py - untreated pre-reproductive *C. elegans*
eay - 0.1 mg/ml Vitamin E treated pre-reproductive *C. elegans*
eby - 0.5 mg/ml Vitamin E treated pre-reproductive *C. elegans*
ecy - 1.0 mg/ml Vitamin E treated pre-reproductive *C. elegans*
hay - 0.1 mg/ml Vitamin E and Vitamin C treated pre-reproductive *C. elegans*
hby - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hcy - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hdy - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*

Figure 13: Graph displaying the average tyrosine (tyr) and epinephrine/tyroisine (epi/tyr) ratio for the treated and untreated pre-reproductive *C. elegans*, for all the experimental categories. The raw data used to generate this graph is found in Appendix 6. The concentration is per μg soluble protein.

Measurable amounts of norepi were recorded in some of the reproductive populations; in all the Vitamin C treated populations, in the 0.1 and 0.5 mg/ml Vitamin E treated populations and, in the combination 0.1 mg/ml Vitamin C with 0.5 mg/ml Vitamin E. No norepi was recorded in the untreated reproductive *C. elegans* populations. The ratio of epi/tyr changed in the reproductive and post-reproductive populations (**Figures 14 and 15**) when compared to the pre-reproductive populations. It is much lower for all the experimental categories. In the post-reproductive life stage norepi occurred in measurable amounts in all the experimental categories except the 0.1 mg/ml Vitamin E treated worms. Norepi and epi concentrations are supposed to decrease with increase of age. My data shows epi concentrations decreasing with age. Also, tyrosine concentrations are supposed to decrease with an increase of ROS, which is supported by **Figure 14**. The treated populations have higher concentrations of tyrosine than the untreated populations, since Vitamin C and E are acting as antioxidants, reducing the concentration of ROS in the organisms, supportive of the theories on aging involving ROS and explained by the neuroendocrine theory on aging.

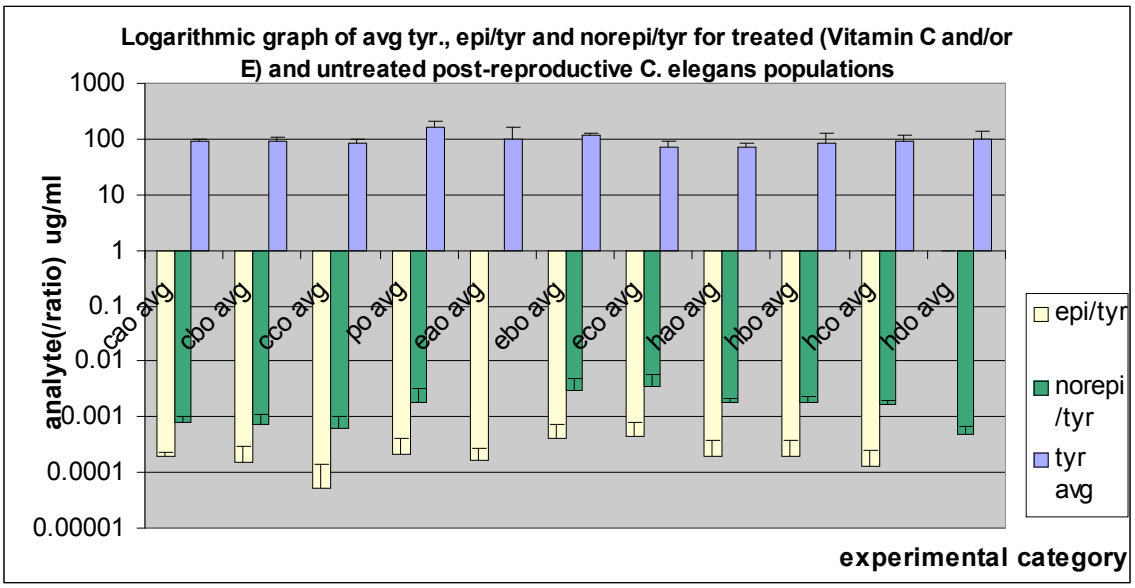
Tyrosine is an amino acid that can be synthesized (**Figure 3**). However, this is provided in the medium nutrient, like tryptophan. In the pre-rpeorductive nematode tyrosine occurs in very small concentrations, per μg soluble protein and the concentration increased in the reproductive worms and again in the post-reproductive worms. What is interesting in my data is that tyrosine concentrations varied with the experimental categories and life stages until the post-reproductive life stage where the concentrations are about the same for all the experimental categories. The measurable concentration in

the nematode may be dependent on its conversion to and from other products, like its degradative products. The smaller epi/tyr ratios in the pre-reproductive life stage Vitamin C and E combination treatment (compared to the reproductive and post-reproductive life stages) support greater concentration of tyrosine is needed in the pre-reproductive life stage. The Vitamins help to reduce ROS.



cam - 0.1 mg/ml Vitamin C treated reproductive *C. elegans*
cbm - 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
ccm - 1.0 mg/ml Vitamin C treated reproductive *C. elegans*
Pm - untreated reproductive *C. elegans*
eam - 0.1 mg/ml Vitamin E treated reproductive *C. elegans*
ebm - 0.5 mg/ml Vitamin E treated reproductive *C. elegans*
ecm - 1.0 mg/ml Vitamin E treated reproductive *C. elegans*
ham - 0.1 mg/ml Vitamin E and Vitamin C treated reproductive *C. elegans*
hbm - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hcm - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hdm - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated reproductive *C. elegans*

Figure 14: Graph plotted on log paper of the tyrosine concentrations, the epi/tyr and norepi/tyr ratios for the treated and untreated reproductive *C. elegans*. The concentration is per μg soluble protein. Errors bars are 1 SD.



cao - 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*
cbo - 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
cco - 1.0 mg/ml Vitamin C treated post-reproductive *C. elegans*
Po - untreated post-reproductive *C. elegans*
eao - 0.1 mg/ml Vitamin E treated post-reproductive *C. elegans*
ebo - 0.5 mg/ml Vitamin E treated post-reproductive *C. elegans*
eco - 1.0 mg/ml Vitamin E treated post-reproductive *C. elegans*
hao - 0.1 mg/ml Vitamin E and Vitamin C treated post-reproductive *C. elegans*
hbo - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hco - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hdo - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*

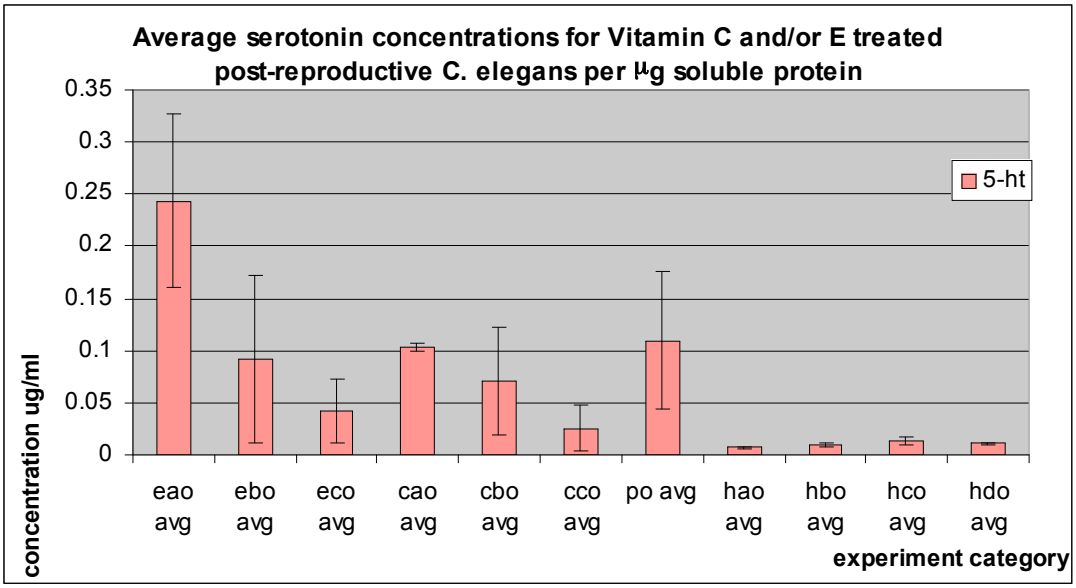
Figure 15: Graph done on log paper of the average tyrosine concentrations, epi/tyr ratios and norepi/tyr ratios for the treated and untreated post-reproductive *C. elegans*. The concentration is per μg soluble protein. Error bars are 1SD.

TRYPTOPHAN AND SEROTONIN CONCENTRATONS AND RATIO.

One of the catabolic products of tryptophan is the indolealkyl amine neurotransmitter, serotonin. As mentioned earlier, section 2, its biosynthesis is initiated by an active uptake of dietary tryptophan into the serotonergic neurons. In the *C. elegans*, the level of serotonin has been found to be associated with levels of behavioral

responses, such as pharyngeal pumping. No measurable serotonin (5-hydroxy tryptamine) was found in the pre-reproductive and reproductive *C. elegans*, in any of the experiments. The sensitivity level of the instrument is in fentomoles. The minimum peak detection height was set to 2.0 nAmps, when analyzing the results in CEAS-HPLC. However, serotonin was detected in the post-reproductive stages of all the experiments. In the *C. elegans* pharyngeal pumping and locomotion are functions of life stage, decreasing in post-reproductive life stage. A decrease in serotonin levels in the Vitamin C & E treated post-reproductive life stage may imply less serotonin is needed to carry out the above functions, since the serotonin receptors are the least damaged of all the post-reproductive nematodes by ROS. This is an example ROS damage ligands and proteins, altering in the neurotransmitter serotonin concentration and subsequently a breakdown in homeostasis. This data is supportive of the neuroendocrine theory of aging.

Figure 16 shows the average normalized (per μg soluble protein) serotonin levels for the treated and untreated post-reproductive nematode populations. The measurable serotonin level decreased in populations with increase level of Vitamin C or E. When compared to the average untreated population and population grown in Vitamin C or E only, the populations grown in the combination of Vitamin C and E had a significant decrease in measurable serotonin. The data for **Figure 16** is in **Appendix 7**.



cao - 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*
cbo - 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
cco - 1.0 mg/ml Vitamin C treated post-reproductive *C. elegans*
Po - untreated post-reproductive *C. elegans*
eao - 0.1 mg/ml Vitamin E treated post-reproductive *C. elegans*
ebo - 0.5 mg/ml Vitamin E treated post-reproductive *C. elegans*
eco - 1.0 mg/ml Vitamin E treated post-reproductive *C. elegans*
hao - 0.1 mg/ml Vitamin E and Vitamin C treated post-reproductive *C. elegans*
hbo - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hco - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hdo - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*

Figure 16: A plot of the average serotonin concentrations for the Vitamin C and/or E treated and untreated post-reproductive (o) *C. elegans*, per µg soluble protein. Error bars are 1SD.

Appendix 7 (Graph 6a) is a plot done on log paper. This format allows display of both the small data points and larger data points on one graph, when the dynamic range of both differs significantly. One can see the significance of all the data. Throughout the data display the errors bars are representative of 1 sd (one standard deviation) for the collective data points. This incorporates all errors involved in obtaining the data points;

population spills, pipetting errors and, instrumentation errors.

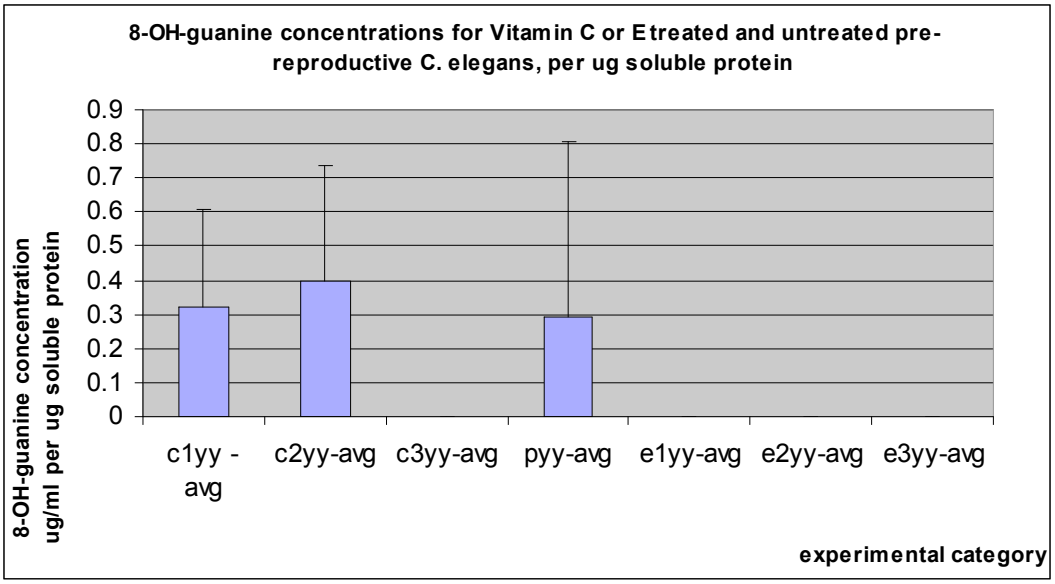
The ratio of tryptophan/serotonin was computed in the post-reproductive *C. elegans*, to see if there are alterations in homeostasis with the addition of Vitamin C and/or E. The tryptophan/tyrosine ratio was the greatest in the Vitamin C and E grown nematodes populations (**Appendix 7, Graph 6a**), at all four concentrations combinations, which implies that less serotonin is produced in relation to tryptophan as in the other treatment categories. Since serotonin is oxygen dependent, the presence of Vitamin C and E should reduce the availability of oxygen, leading to a lower concentration of serotonin in the presence of increase antioxidants, independent of tryptophan. Data presented in **Appendix 7** supports this premise. Tryptophan is an essential amino acid, present in the dietary nutrient in the medium, in which the organism is grown. Within the medium there is enough dietary supplement for the culture to grow for 21 days and these experiments are complete in ten days. The data (graph 6a) reflects the fact that serotonin biosynthesis is initiated by an active uptake of dietary tryptophan into the organism where uptake into serotonergic neurons could occur were this the only site of generation of serotonin in these organisms. Another interpretation is the fact that since serotonin is formed via an oxygen dependant, oxidation reaction, it could as well indicate an inhibition or reduction in this level of enzymatic activity, or a reduction of same, which could produce same result observed, in the Vitamin C & E experiments. Since serotonin is a neurotransmitter, a cellular signal, a change in its concentration results in shifts in homeostasis (compared to the untreated organism) produced by the antioxidants. This

results supports the aging theories associated with oxidative stress and is best explained by the neuroendocrine theory of aging.

8-HYDROXY GUANINE CONCENTRATIONS

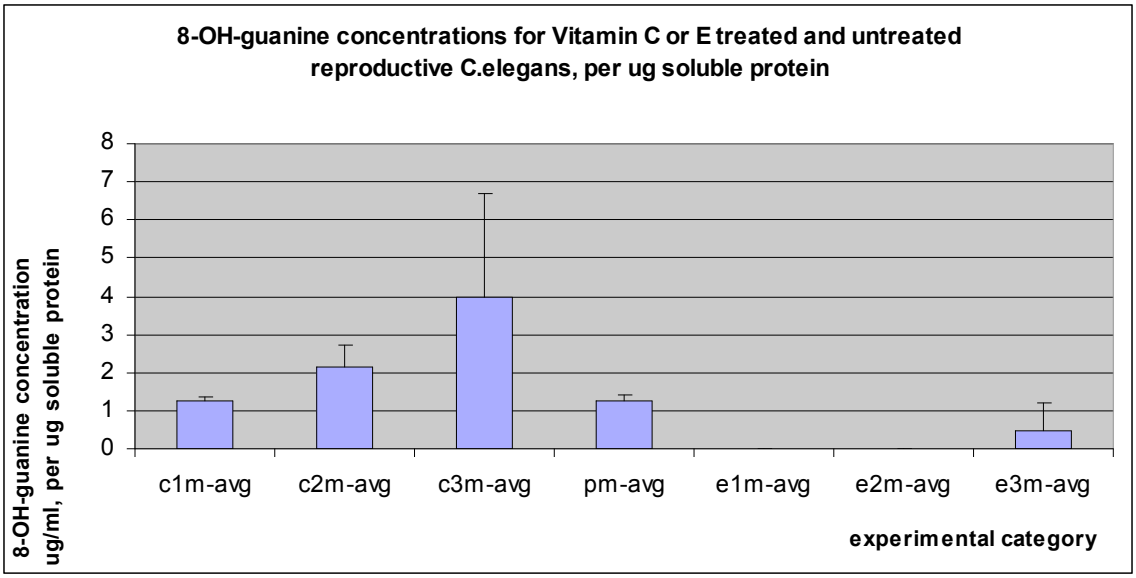
8-Hydroxy guanine, which is a DNA repair marker, is measured. This compound is found with and accumulates with DNA damage. It also causes mutagenesis if the DNA is replicated before it is repaired. The quantity of 8-Hydroxy guanine determines the extent of the DNA damage, caused by ROS.

The *C. elegans* grown in the combination of Vitamin C and E in all life stages showed no measurable amounts of separated 8-OH-guanine. Since the antioxidants are supposed to reduce oxidative stress, one would expect lower concentrations of 8-OH-guanine (if present) to be found in the youngest of the worm populations, as demonstrated in the data (**Figures 17 and 18, and Appendix 8**). Also, the increase in Vitamin C concentrations correlates with the results where no measurable amount of 8-OH-guanine is found in the 1.0 mg/ml Vitamin C treated pre-reproductive *C. elegans.* It is expected for 8-OH-guanine to appear in the reproductive life stages, as shown in the results, since reproduction occurs in this life stage. Also, Vitamin E seems to have a greater capacity of reducing the quantity of ROS that creates 8-OH-guanine from cellular DNA lesions. The combination of Vitamin C and E seems to be most effective in preventing cellular DNA lesions at all life stages. This data provides strong evidence in support of the free radical theory of aging. It also implies that Vitamin C & E used together is a stronger antioxidant than Vitamin C or E alone.



c1yy – 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*
c2yy – 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
c3yy – 1.0 mg/ml Vitamin C treated pre-reproductive *C. elegans*
pyy - untreated pre-reproductive *C. elegans*
e1yy – 0.1 mg/ml Vitamin E treated pre-reproductive *C. elegans*
e2yy – 0.5 mg/ml Vitamin E treated pre-reproductive *C. elegans*
e3yy – 1.0 mg/ml Vitamin E treated pre-reproductive *C. elegans*

Figure 17: A plot of 8-OH-guanine concentrations for Vitamin C or E treated and untreated pre-reproductive *C. elegans*, per μg soluble protein. Error bars are 1SD.



c1m - 0.1 mg/ml Vitamin C treated reproductive *C. elegans*
c2m - 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
c3m - 1.0 mg/ml Vitamin C treated reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
e1m - 0.1 mg/ml Vitamin E treated reproductive *C. elegans*
e2m - 0.5 mg/ml Vitamin E treated reproductive *C. elegans*
e3m - 1.0 mg/ml Vitamin E treated reproductive *C. elegans*

Figure 18: A plot of 8-OH-guanine concentrations for Vitamin C or E treated and untreated reproductive *C. elegans*, per μg soluble protein. Error bars are 1SD.

PURINE NUCLEOTIDE CATABOLISM ANALYTES

In studying oxidative stress and antioxidants with respect to aging in *C.elegans*, it is useful to monitor the analytes of purine nucleotide catabolism at the different life stages of the *C. elegans,* as a means of tracking the impact of these antioxidants on general functions of DNA synthesis from the perspective of nucleotide availability and nucleotide recycling. Guanine, hypoxanthine, xanthosine, xanthine and uric acid

(analytes of purine nucleotide catabolism) were measured with their ratios. These free purines can be salvaged and reused to make nucleotides.

The concentrations of the purine metaobilites were the largest in the Vitamin C & E treated *C. elegans*, of all the pre-reproductive experimental categories, greater than the untreated by at least a 10 fold (**Appendix 9D**). In the reproductive life stage the purine metabolites concentrations increased for the untreated, Vitamin C and, Vitamin E treatments by at least a 5 fold (**Appendix 9F**). There was a decrease in these metabolites in most of the dose response Vitamin C & E treatment, when compared to their pre-reproductive life stage. This is expected since DNA and RNA is needed for reproduction. The purine metabolites continued to demonstrate increased concentrations in the post-reproductive life stage, in all the treatments (**Appendix 9H**). The addition of Vitamin C & E resulted in an environment with a constant flux the purine nucleotides thoughout the nematode life cycle, with the exception of guanine, whose concentration decreased in the reproductive life stage. In the untreated nematode the availability of the purine nucleotides increased with age by about 20 fold, with the exception of guanine whose concentration decreased with age. This may be due to the fact that DNA and RNA metabolism increases with reproduction, hence the need for more purine nucleotides. A continual increase of the free purine nucleotides could imply that the nematodes continue to breakdown these nucleotides but they are not needed in the post-reproductive life stage. Guanine is a highly reactive compound. As ROS concentrations increases with age, guanine concentration will decrease, as shown in the data.

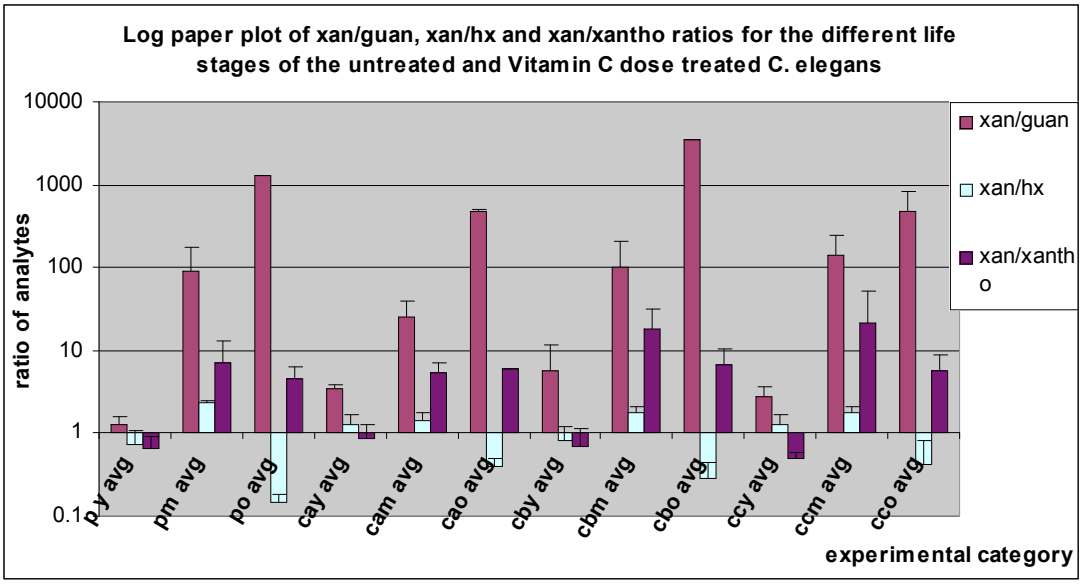
The data (**Appenidx 9**) can imply that the purine catabolitic metabolites are being reused in the untreated and the Vitamin C or E pre-reproductive nematodes to make nucleotides, needed for the reproductive phase since they are getting damaged by ROS. With the Vitamin C and E treated pre-reproductive nematodes the purine metabolites are converted to uric acid and excreted, hence their higher concentrations in the Vitamin C and E treatment compared to the untreated nematodes.

The ratios of xanthine/guanine (xan/guan), xanthine/hypoxanthine (xan/hx), and xanthine/xanthosine (xan/xantho) for the nematodes in the pre-reproductive, reproductive and post-reprdoctive life stages and all experimental categories are displayed in **Figures 19, 20, 21** and in **Appendix 9** (data and Graphs 24, 26 and 28). It was demonstrated that the ratios differs with treatment and life stage, between the analytes. These changes reflect altered flow of metabolites through the pathway caused by the antioxidant properties of the Vitamins, which may be due to modulation of the enzymes involved, may be due to oxidative damage.

The ratio of xanthine/xanthosine is the least (0.285) in the 0.5 mg/ml Vitamin C treated pre-reproductive (cby) *C. elegans*, followed by the 1.0 mg/ml (ccy), 0.1 mg/ml (cay) and untreated (py) pre-reproductive worms. The ratio of xanthine/guanine is the largest of all the three ratios, in each experimental category. In the untreated nematodes, the ratio of xanthine/hypoxanthine is the smallest of the three ratios monitered, implying that most of the xanthine formed comes from hypoxanthine, in the three life stages. In the Vitamin C treated pre-reproductive nematodes, the ratio of xanthine/xanthoxine is the smallest of the three ratios being monitored, implying that most of the xanthine formed

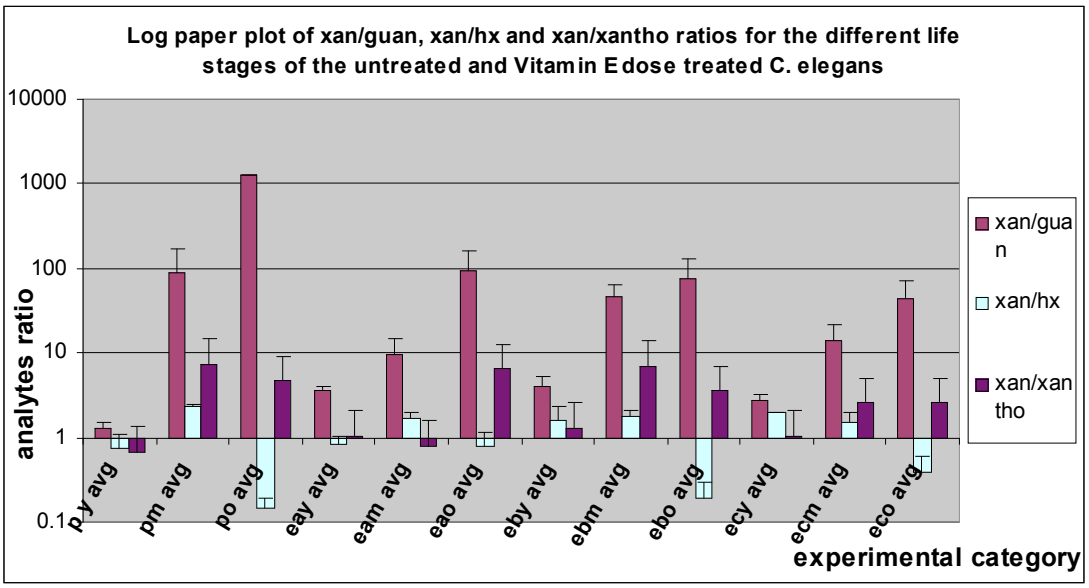
comes from xanthosine in the pre-reproductive Vitamin C treated nematodes, unlike the untreated pre-reproductive life stage, where it comes from hypoxanthine. Hypoxanthine is converted to most of the xanthine in the reproductive and post-reproducitve life stages in the Vitamin C dose treated nematodes, and the untreated nematodes (as displayed in **Figures 19, 20, 21 and Appendix 9 (Graphs 24, 26, and 28**). A somewhat similar impact is seen with the addition of 0.5 and 1.0 mg/ml Vitamin E. With the Vitamin C and E combination treatment there is a change in the reproductive and post-reproductive life stages on how the nematode forms xanthine, when compared to the untreated and Vitamin C or Vitamin E treated nematodes. In the populations with the Vitamin E and C combination xanthosine is of the high concentrations, implying most of the xanthine is made from xanthosine in pre-reproductive and reproductive life stages.

This change in the xanthine pathway formation is an example of the change in biology that is ongoing with the addition of anti-oxidants. This can relate to the population distribution observed at the end of the experiment, impacting aging. This data can be explained by the system- based theories on aging (aging is results from the breakdown in homeostasis), where there is a change in homeostasis due to the antioxidant properties of the Vitamin C and/or E.



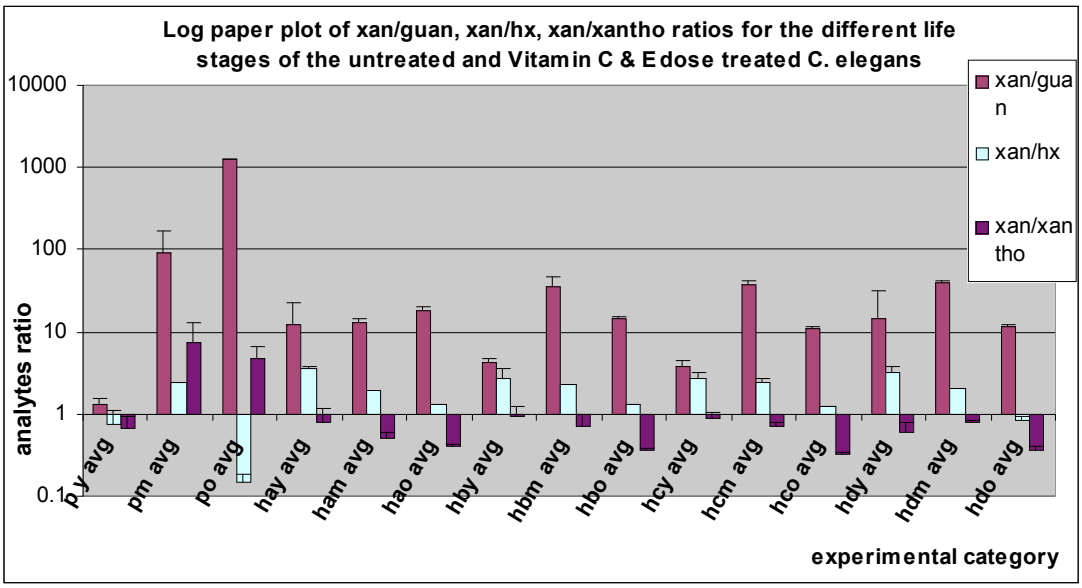
py - untreated pre-reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
cay - 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*
cam - 0.1 mg/ml Vitamin C treated reproductive *C. elegans*
cao - 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*
cby - 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
cbm - 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
cbo - 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
ccy - 1.0 mg/ml Vitamin C treated pre-reproductive *C. elegans*
ccm - 1.0 mg/ml Vitamin C treated reproductive *C. elegans*
cco - 1.0 mg/ml Vitamin C treated post-reproductive *C. elegans*

Figure 19: Log paper plot of purine metabolites ratios xanthine/guanine, xanthine/hypoxanthine and xanthine/xanthosine for the untreated and Vitamin C dose treated *C.elegans* at the pre-reproductive, reproductive and post-reproductive life stages. Error bars are 1 SD.



py - untreated pre-reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
eay - 0.1 mg/ml Vitamin E treated pre-reproductive *C. elegans*
eam - 0.1 mg/ml Vitamin E treated reproductive *C. elegans*
eao - 0.1 mg/ml Vitamin E treated post-reproductive *C. elegans*
eby - 0.5 mg/ml Vitamin E treated pre-reproductive *C. elegans*
ebm - 0.5 mg/ml Vitamin E treated reproductive *C. elegans*
ebo - 0.5 mg/ml Vitamin E treated post-reproductive *C. elegans*
ecy - 1.0 mg/ml Vitamin E treated pre-reproductive *C. elegans*
ecm - 1.0 mg/ml Vitamin E treated reproductive *C. elegans*
eco - 1.0 mg/ml Vitamin E treated post-reproductive *C. elegans*

Figure 20: Log paper plot of purine metabolites ratios xanthine/guanine, xanthine/hypoxanthine and xanthine/xanthosine for the untreated and Vitamin E dose treated *C.elegans* at the pre-reproductive, reproductive and post-reproductive life stages. Error bars are 1SD.



py - untreated pre-reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
hay - 0.1 mg/ml Vitamin E and Vitamin C treated pre-reproductive *C. elegans*
ham - 0.1 mg/ml Vitamin E and Vitamin C treated reproductive *C. elegans*
hao - 0.1 mg/ml Vitamin E and Vitamin C treated post-reproductive *C. elegans*
hby - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hbm - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hbo - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hcy - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hcm - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hco - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hdy - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hdm - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated reproductive *C. elegans*
hdo - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*

Figure 21: Log paper plot of purine metabolites ratios xanthine/guanine, xanthine/hypoxanthine and xanthine/xanthosine for the untreated and Vitamin C and E dose treated *C.elegans* at the pre-reproductive, reproductive and post-reproductive life stages. Error bars are 1SD.

DATA SUBSTRACTION

The chromatograms (electrogenic data) produced from the CEAS-HPLC analysis is used here to see if there are unique compounds with respect to life stage/treatment.

The program outputs differences in peak size and presence/absence of peaks. This will provide supporting data demonstrating observable signature patterns in the *C. elegans* at various life stages, with and without the antioxidants, Vitamin C and/or E. Unique peaks are found, together with changes in peak modulation data. This data supports the theory on aging that is explained by a change in control/homeostasis – the neuroendocrine theory on aging. However, the changes measured here are analytes and some of them are not neurotransmitters or hormones but may be products from changes in their concentraions.

The chromatograms produced in these experiments are quite complex. Some of them have reveal as many as 2000 analytes. An example of a chromatogram obtained from one of the standards used in the Coularry Data Analysis is shown in **Figure 22.** Examples of chromatographic data obtained from the pre-reproductive, reproductive and post-reproductive life stages are provided in **Figures 23 to 25**. The chromatograms' complexity increased with age. Software that has the capability to subtract one chromatogram from the other, after it is normalized per μg soluble protein was needed to simplify data analysis, and render clear, distinctive identification of components unique to an individual experimental protocol.

The CoulArray software does not have that capability. Since the data is of gradient flow, there is some shifting of the peaks along the chromatogram time line, (not more than 1 minute in retention time, an accordion effect), within the chromatograms when one is compared to the other. Writing a program to find the unique analytes reduces the time it would take do the same analysis by hand using the CoulArray

software. The CoulArray software does not remove the accordain effect in the data, hence, extracting the unique analytes observed, requires hands on data analysis by the observer, and is both labor and time intensive. One must track the response profile across the electrodes responding in a detailed fashion to assure accurate identification of each. It is then difficult to display the resultant in a fashion that is easy for an observer, unfamiliar with the instrumentation, to comprehend.

Dr. Jason Kinser, wrote a program in python (http://www.python.org), to remove the accordion effect of the chromatographic data, where the data was provided to him in a format applicable for the stretching algorithm. The subtracting, normalization and data storage section of the program was written by me (**Appendix 10)**. Outlined in the Research Design and Methods section is the process used in getting the chromatographic data converted into matrix format. Micorsoft Excel is used to display the results in chromatographic format. A portion of a chromatogram is displayed in the matrix format in **Appendix 11**. The number of rows is dependant on the CEAS-HPLC data collection time, set in the method created to run the instrument. The largest matrix has 10798 rows and 17 columns (where the first column is the time-line counter and the other sixteen are the 16 different voltages or channels), for a 90 minutes data collection period for one chromatogram.

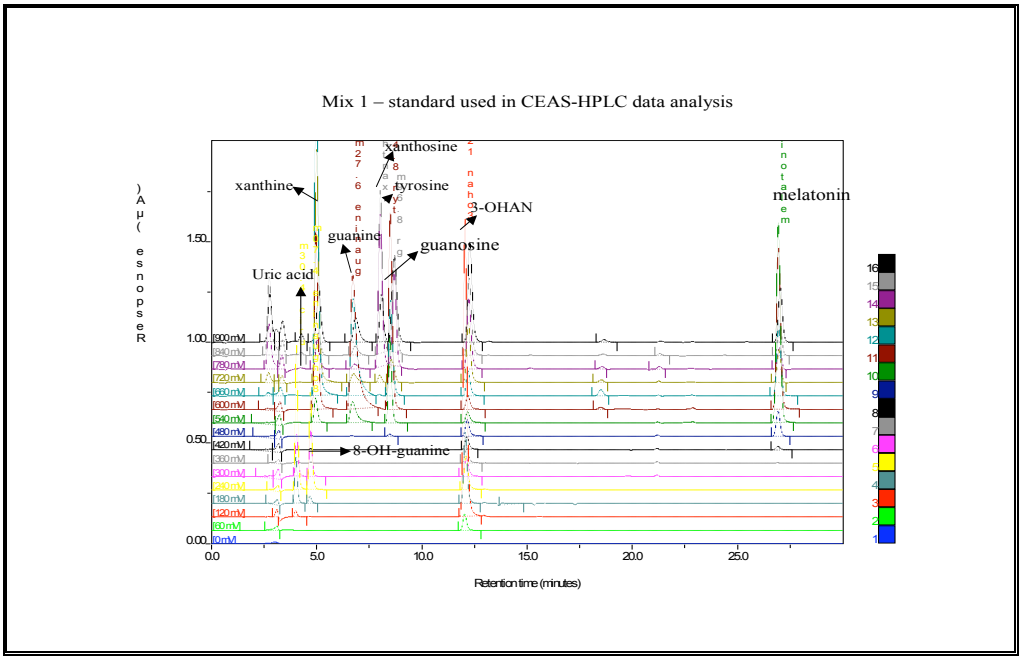


Figure 22: A chromatogram with 9 known analytes called Mix1 in this experiment. It is generated from the CoulArray data analysis software

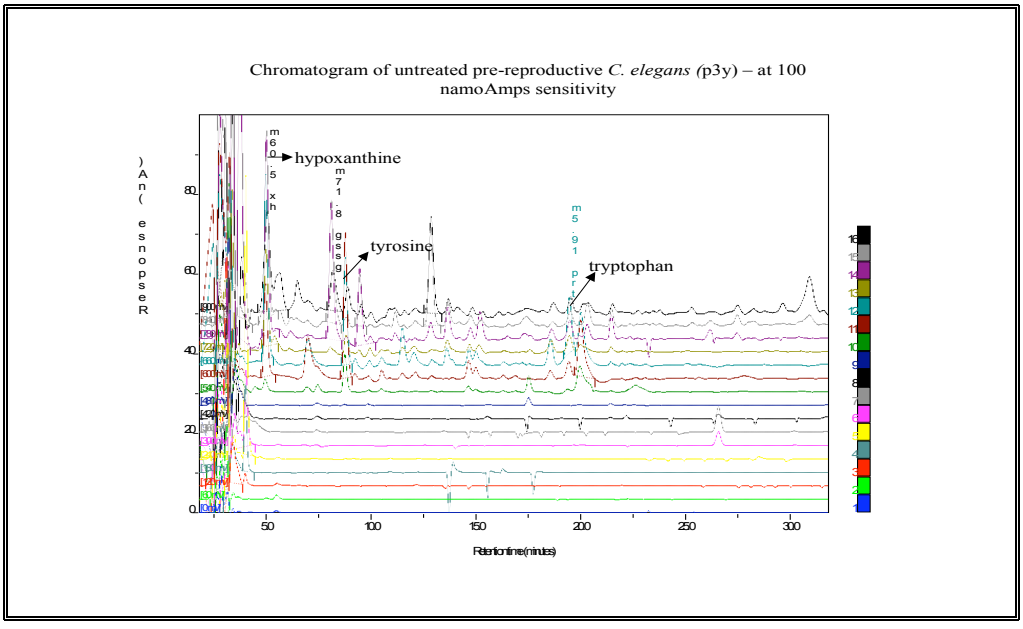


Figure 23: A chromatogram of an untreated pre-reproductive *C. elegans* sample, displayed from the CoulArray software. The sensitivity is 100 nanoAmps.

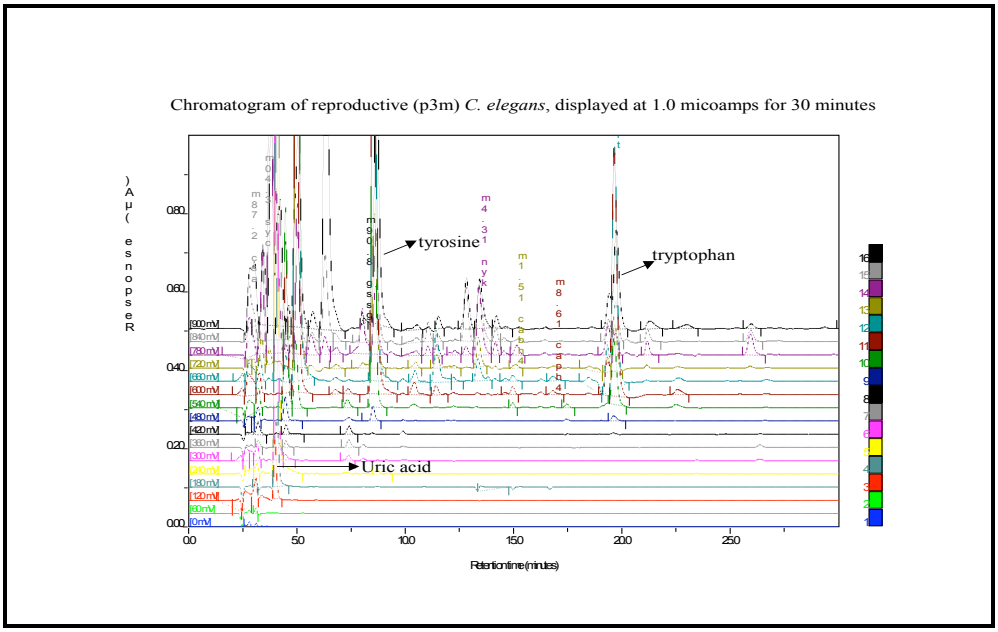


Figure 24: A chromatogram of an untreated reproductive *C. elegans* sample, displayed from the CoulArray software. The sensitivity is 1.0 microAmps.

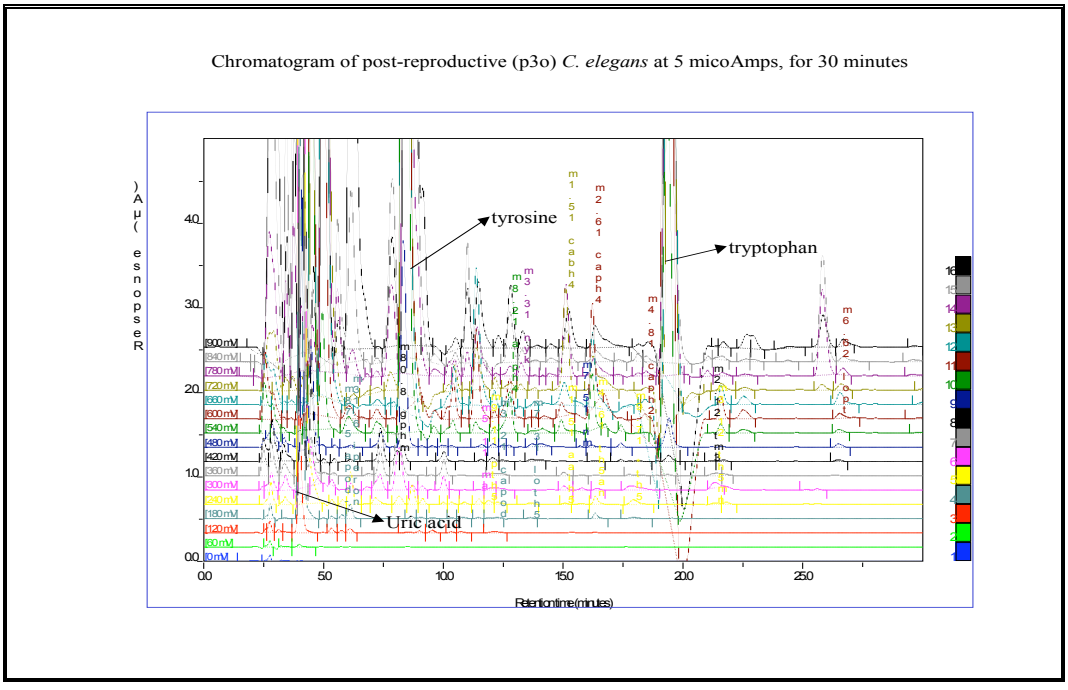


Figure 25: A chromatogram of an untreated post- reproductive *C. elegans* sample, displayed from the CoulArray software. The sensitivity is 5.0 microAmps.

From viewing the chromatograms in **Figures 23 to 25**, one can see that complexity of the chromatograms increases with age. The same is observed in the different life stages of all the treated populations. One can see that there are several compounds that are found in the reproductive and post-reproductive stage, that are simply not detected in the pre-reproductive stage (**Figure 26 to 28**). This is done by using the display wizard within the ColuArray for Windows software, where one can compare sections of chromatograms, to help the user to see any visual differences and similarities. Example of the pre-reproductive, reproductive and post-reproductive 1.0 mg/ml Vitamin C treated worms chromatograms are shown in **Figure 26 to 28**.

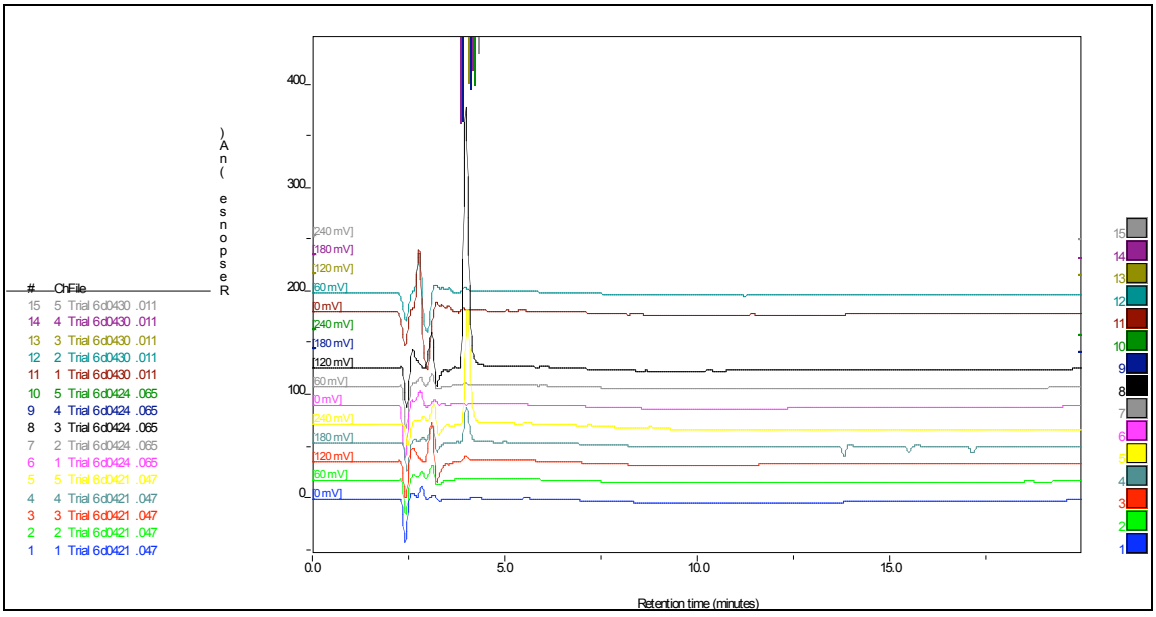


Figure 26: Metafile of a combination of cc2y, cc2m and cc2o chromatograms, for channels 1 to 5. Trial6d0421.047 represents cc2y (channels 1 to 5), 1.0 mg/ml treated with Vitamin C pre-reproductive population. Trial 6d0424.065 represents cc2m (channels 1 to 5), 1.0 mg/ml treated with Vitamin C reproductive population. Trial6d0430.011 represents cc2o (channels 1 to 5), 1.0 mg/ml treated with Vitamin C post-reproductive population.

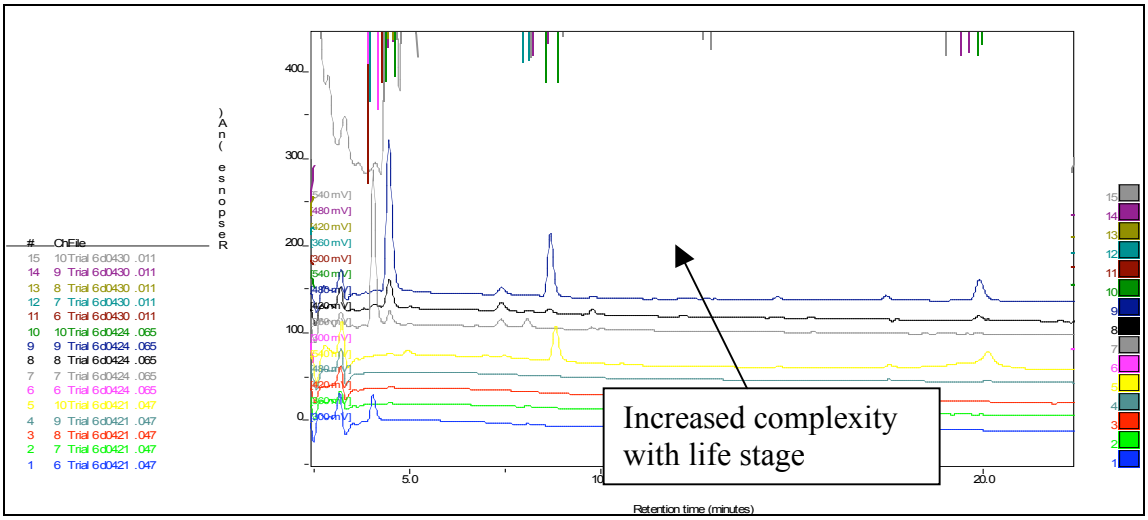


Figure 27: Metafile of a combination of cc2y, cc2m and cc2o chromatograms, for channels 6 to 10. Trial6d0421.047 represents cc2y (channels 6 to 10), 1.0 mg/ml treated with Vitamin C pre-reproductive population. Trial 6d0424.065 represents cc2m (channels 6 to 10), 1.0 mg/ml treated with Vitamin C reproductive population. Trial6d0430.011 represents cc2o (channels 6 to 10), 1.0 mg/ml treated with Vitamin C post-reproductive population. This figure has a different scale from Figure 29.

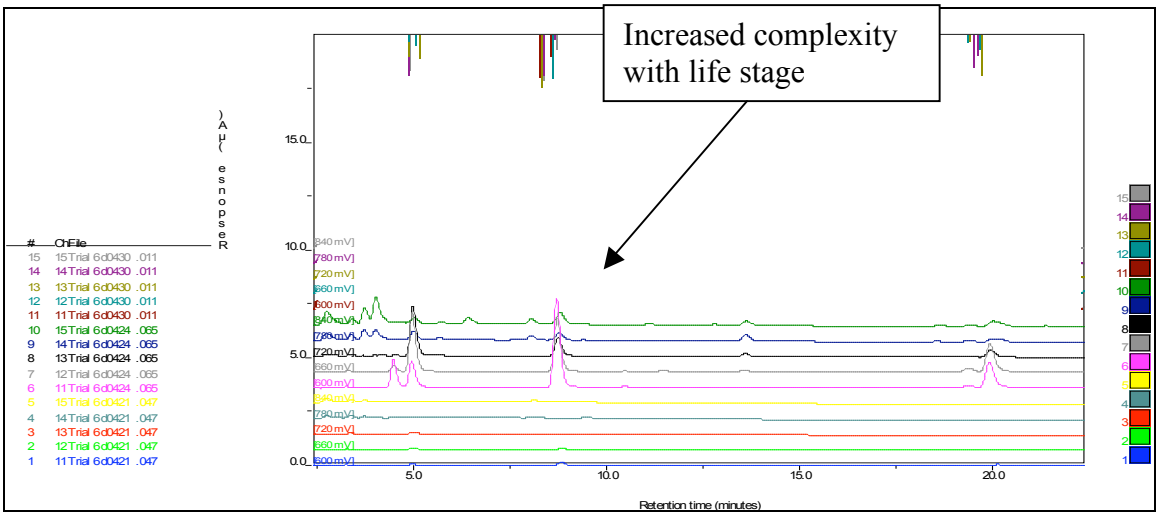


Figure 28: Metafile of a combination of cc2y, cc2m and cc2o chromatograms, for channels 11 to 15. Trial6d0421.047 represents cc2y (channels 11 to 15), 1.0 mg/ml treated with Vitamin C pre-reproductive population. Trial 6d0424.065 represents cc2m (channels 11 to 15), 1.0 mg/ml treated with Vitamin C reproductive population. Trial6d0430.011 represents cc2o (channels 11 to 15), 1.0 mg/ml treated with Vitamin C post-reproductive population. This figure has a different scale from Figures 29a and 29b.

Figures 29, 30, 31 and 32 (and **Appendix 12**) also outputs from the CoulArray multi-file display wizard. Here one can see the similarities and differences of the Vitamin C dose response populations (ca3y is 0.1 mg/ml Vitamin C treated, cb1y is 0.5 mg/ml Vitamin C treated and cc3y is 1.0 mg/ml Vitamin C treated) in relation to the untreated (p1y) and one combination of 0.1 mg/ml Vitamin C and E (ha3y). The first twenty minutes of the chromatograms are shown because that is the area where most of the peaks are eluted.

The only difference seen in **Figure 29** is the intensity of the peak observed at about 4 minutes on channel 3, which is very high in the vitamin C and E treated pre-reproductive *C. elegans*. With **Figure 30** the presence of the peaks at about 5.5 and 8 minutes in the Vitamin C and E treated pre-reproductive worms, and their absence in detectable quantities in the other chromatograms. These differences and all the others displayed in the other figures (**31**, **32** and **33**) is not the amount of nematode material that accounts for these unique differences. This fact is demonstrated with the application of the subtraction algorithm. With the subtraction algorithm the normalization factor will remove this doubt. In **Figure 32**, at channel 10 for ha3y, there is a fairly large peak at about 13 minutes and no trace in the other samples. Also the patterns present at about 20 minutes for all the chromatograms are the same except that of ha3y.

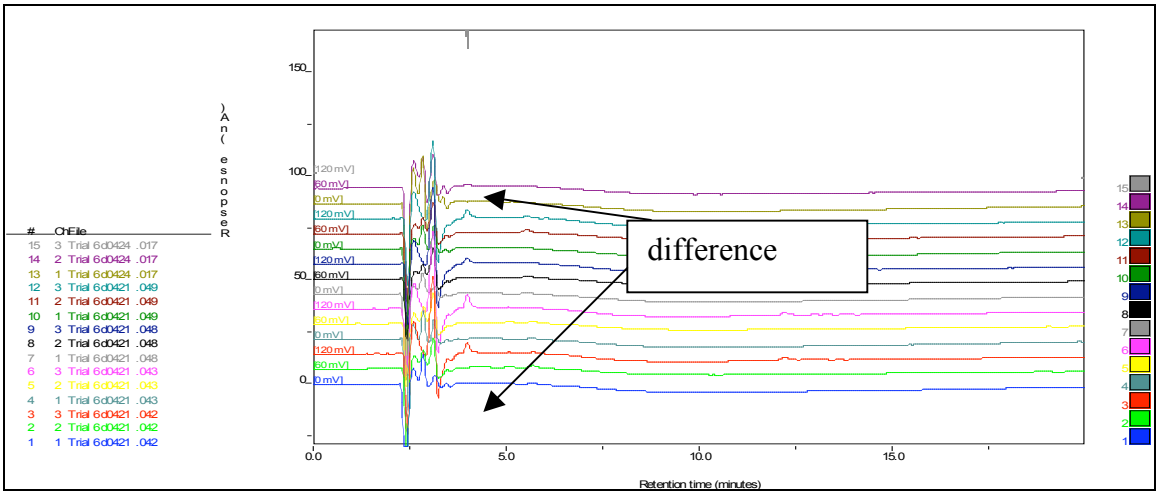


Figure 29: Metafile of a combination of ca3y, cb1y, cc3y, p1y and ha3y chromatograms, for channels 1 to 3 for each file. Trial6d0421.042 represents ca3y, 0.1 mg/ml treated with Vitamin C pre-reproductive population. Trial 6d0421.043 represents cb1y, 0.5 mg/ml treated with Vitamin C pre-reproductive population. Trial6d0421.048 represents cc3y, 1.0 mg/ml treated with Vitamin C pre-reproductive population. Trial6d0421.049 represents p1y, untreated pre-reproductive population. Trial6d0424.017 represents ha3y, 0.1 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E pre-reproductive population.

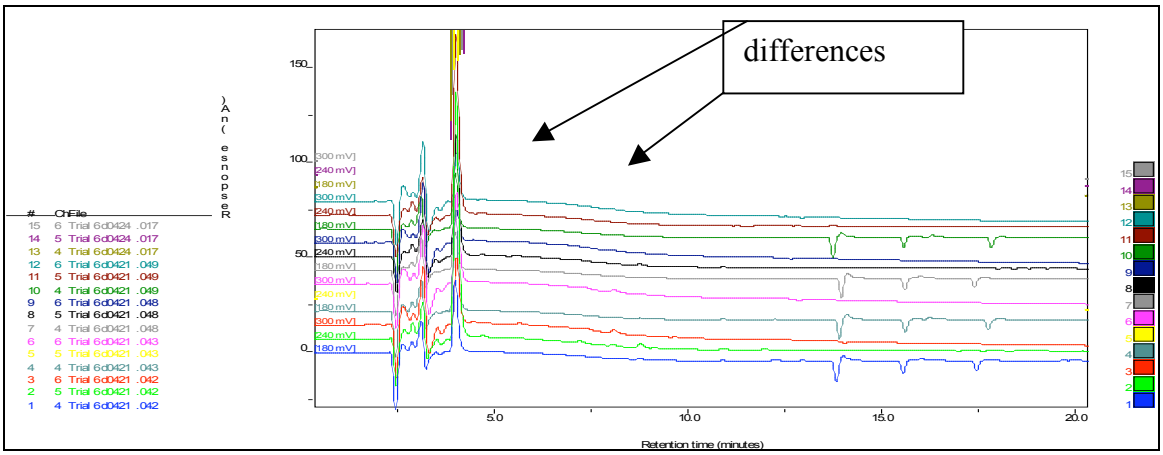


Figure 30: Metafile of a combination of ca3y, cb1y, cc3y, p1y and ha3y chromatograms, for channels 4 to 6. Trial6d0421.042 represents ca3y, pre-reproductive 0.1 mg/ml treated with Vitamin C population. Trial 6d0421.043 represents cb1y, pre-reproductive 0.5 mg/ml treated with Vitamin C population. Trial6d0421.048 represents cc3y, pre-reproductive 1.0 mg/ml treated with Vitamin C population. Trial6d0421.049 represents p1y, pre-reproductive untreated population. Trial6d0424.017 represents ha3y, pre-reproductive 0.1 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E population.

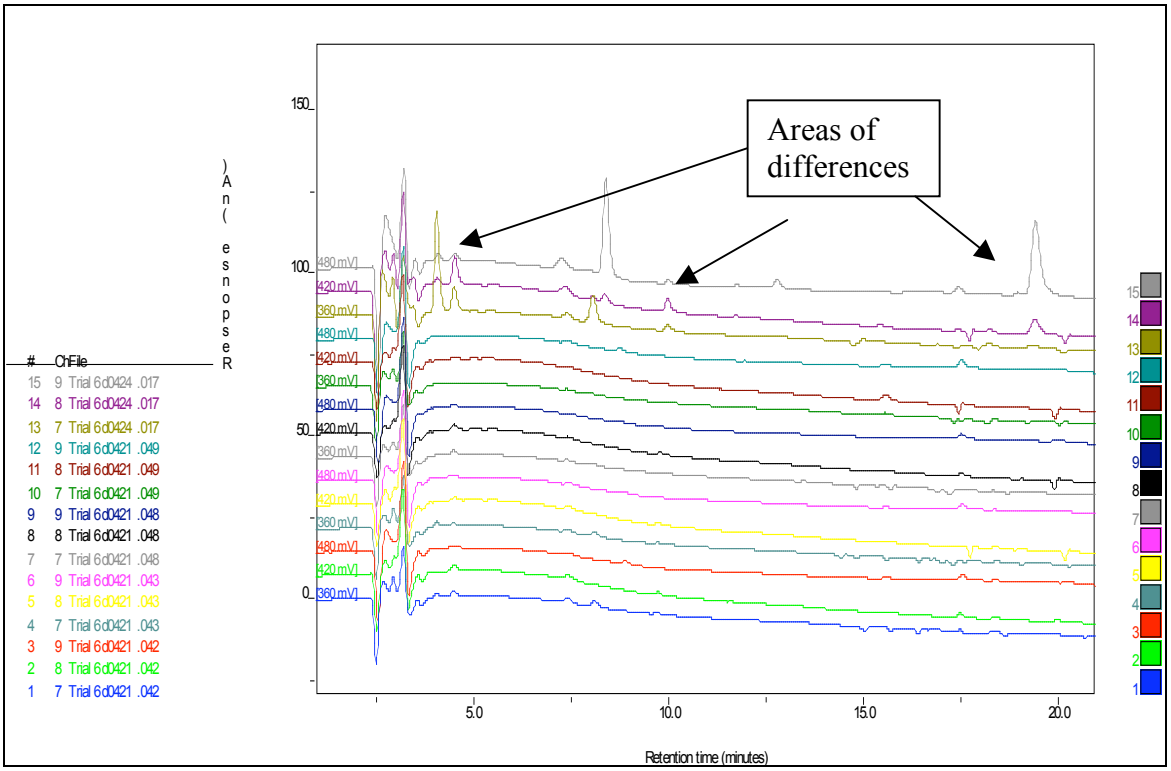


Figure 31: Metafile of a combination of ca3y, cb1y, cc3y, p1y and ha3y chromatograms, for channels 7 to 9. Trial6d0421.042 represents ca3y, pre-reproductive 0.1 mg/ml treated with Vitamin C population. Trial 6d0421.043 represents cb1y, pre-reproductive 0.5 mg/ml treated with Vitamin C population. Trial6d0421.048 represents cc3y, pre-reproductive 1.0 mg/ml treated with Vitamin C population. Trial6d0421.049 represents p1y, pre-reproductive untreated population. Trial6d0424.017 represents ha3y, pre-reproductive 0.1 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E population.

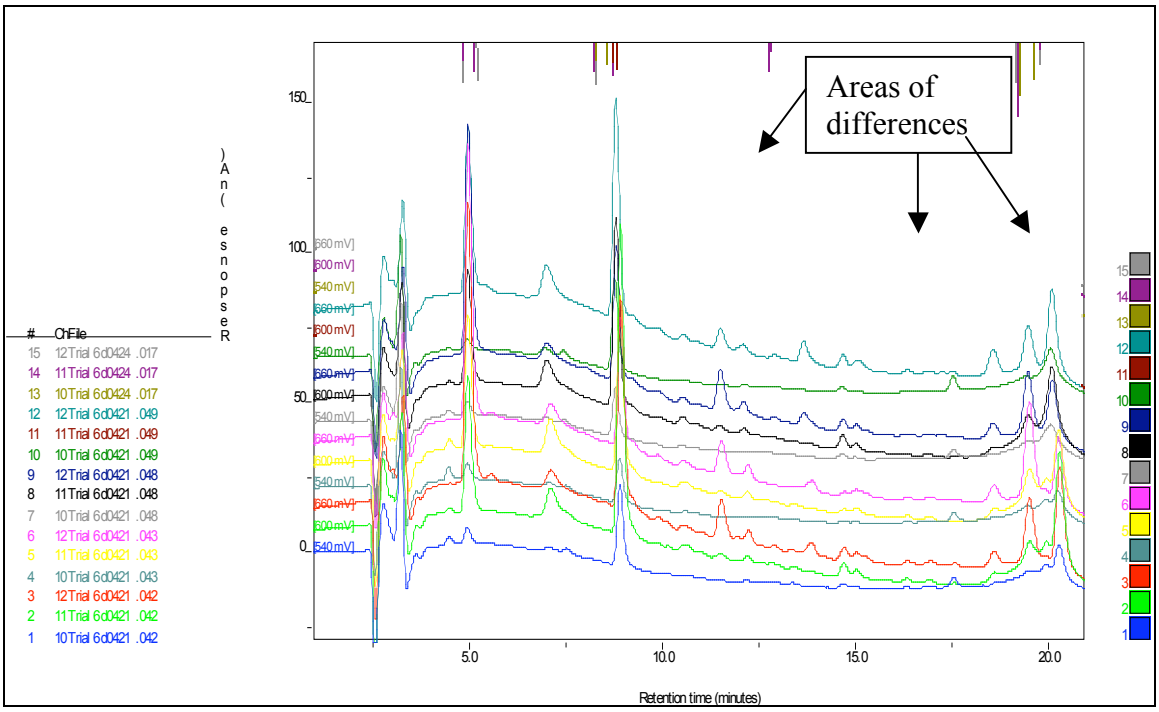


Figure 32: Metafile of a combination of ca3y, cb1y, cc3y, p1y and ha3y chromatograms, for channels 10 to 12. Trial6d0421.042 represents ca3y (channels 10 to 12), pre-reproductive 0.1 mg/ml treated with Vitamin C population. Trial 6d0421.043 represents cb1y (channels 10 to 12), pre-reproductive 0.5 mg/ml treated with Vitamin C population. Trial6d0421.048 represents cc3y (channels 10 to 12), pre-reproductive 1.0 mg/ml treated with Vitamin C population. Trial6d0421.049 represents p1y (channels 10 to 12), pre-reproductive untreated population. Trial6d0424.017 represents ha3y (channels 10 to 12), pre-reproductive 0.1 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E population.

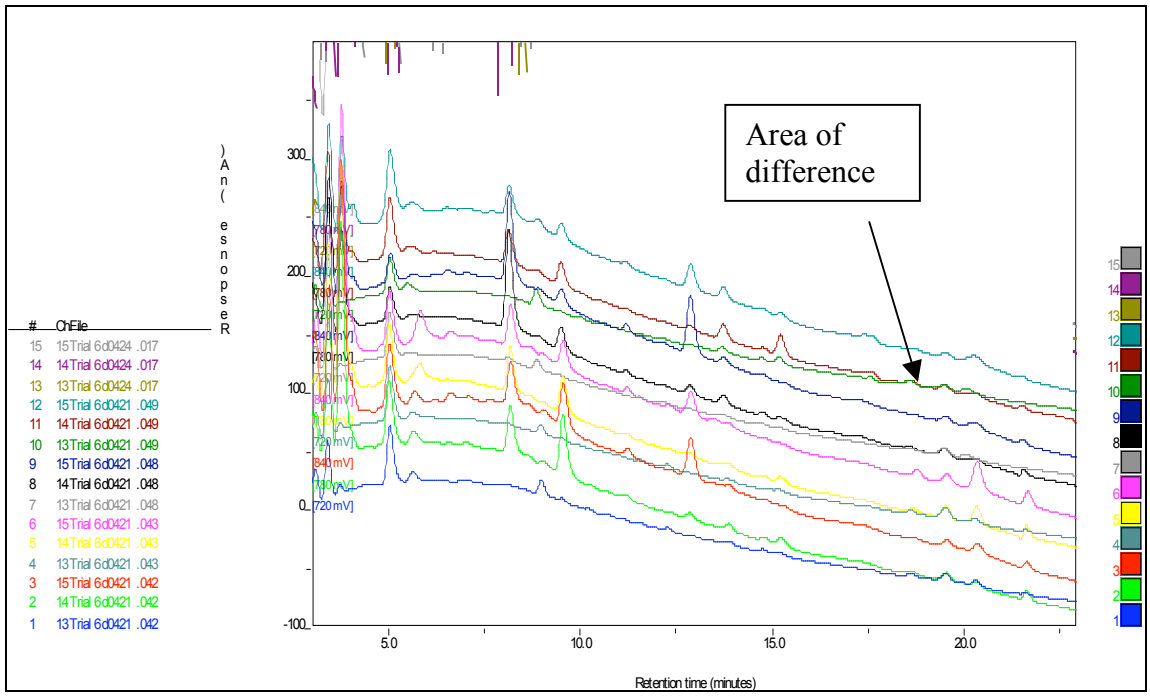


Figure 33: Metafile of a combination of ca3y, cb1y, cc3y, p1y and ha3y chromatograms, for channels 13 to 15. Trial6d0421.042 represents ca3y, pre-reproductive 0.1 mg/ml treated with Vitamin C population. Trial 6d0421.043 represents cb1y, pre-reproductive 0.5 mg/ml treated with Vitamin C population. Trial6d0421.048 represents cc3y, pre-reproductive 1.0 mg/ml treated with Vitamin C population. Trial6d0421.049 represents p1y, pre-reproductive untreated population. Trial6d0424.017 represents ha3y, pre-reproductive 0.1 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E population. The scale is different in this figure compared to 30a, 30b, 30c and 30d.

Data presented in **Appendix 13 and 14** show other examples of differences with dose treatment among the reproductive and post-reproductive populations. Data in Appendix 13 is of the reproductive *C. elegans* showing the Vitamin E dose response compared to the untreated and the 0.5 mg/ml Vitamin C with 0.1 mg/ml Vitamin E. Differences between the untreated and Vitamin C and E combination, between the Vitamin C and E combination and the dose treated Vitamin E and, the untreated and Vitamin E doses are seen for most of the channels, with the reproductive worms. With

the post-reproductive worms differences are seen with the Vitamin C treated compared with the untreated; the Vitamin E compared to the untreated; the Vitamin E compared to Vitamin C and, Vitamin C and E combination compared to the untreated and Vitamin C populations. The least difference is seen between the 0.5 mg/ml Vitamin C and the untreated post-reproductive *C. elegans*. This is due to the fact that Vitamin C is a less potent antioxidant when compared to Vitamin C & E combination treatment.

Results from the data subtraction algothrim for an untreated post-reproductive subtracting a pre-reproductive *C. elegans* population is shown is **Graph 32**. These are just a few examples where the differences can be seen for the normalized data.

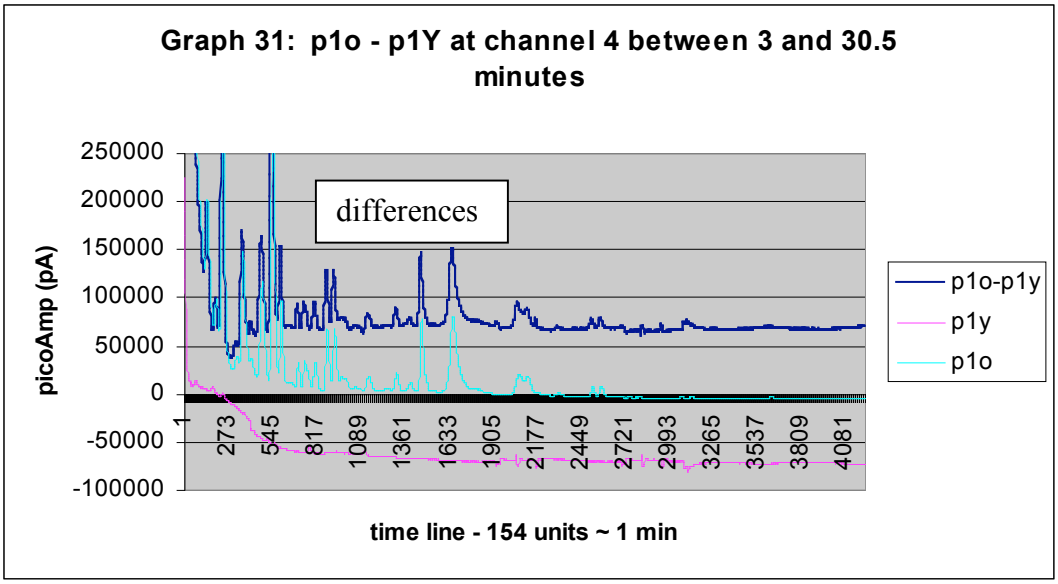


Figure 34: A plot (Graph 31) of post-reproductive nematodes subtracting pre-reproductive nematode chromatographic data. The subtracting algorithm is used, for channel 4. All the differences are seen in the peaks appearing in the post-reproductive *C.elegans*.

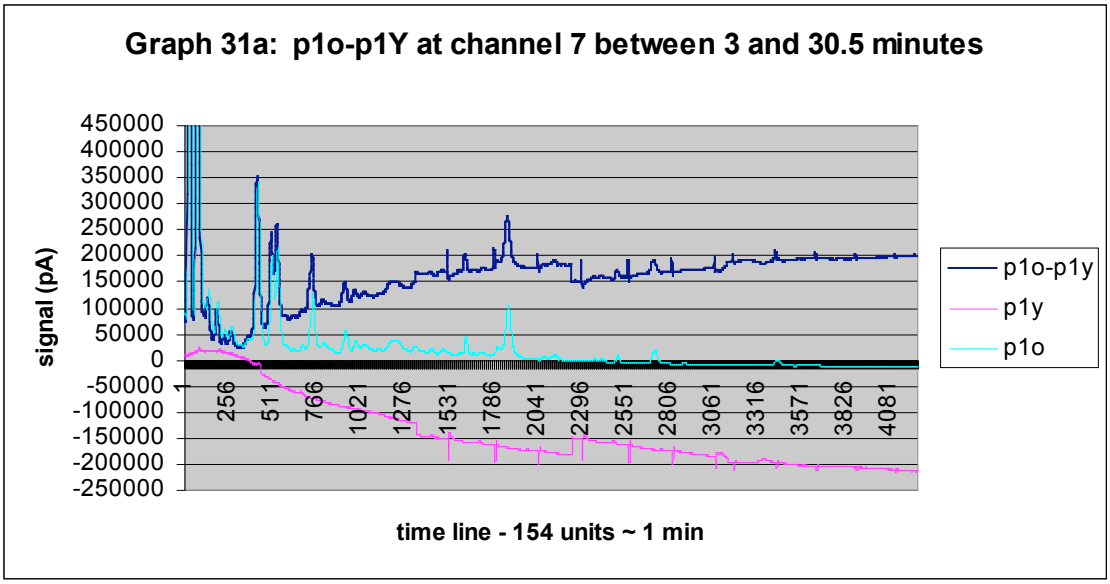


Figure 35: A plot of the untreated post-reproductive (p1o) subtracting the untreated pre-reproductive (p1y) *C. elegans* at channel 7. This plot shows the presence of peaks in the post-reproductive life stage that is not present in the pre-reproductive lifestage.

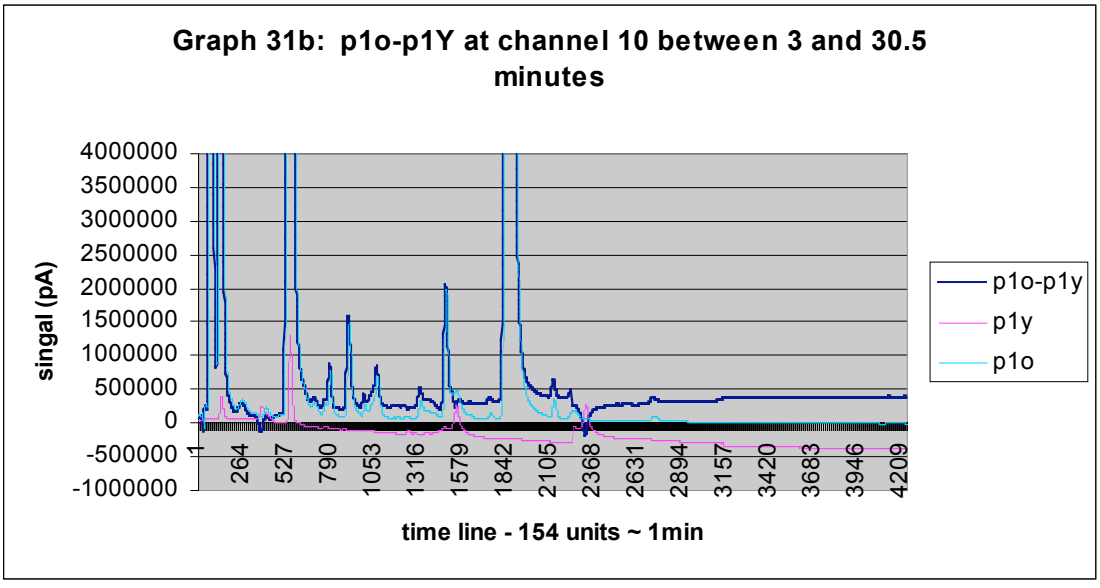


Figure 36: A plot of the untreated post-reproductive (p1o) subtracting the untreated pre-reproductive (p1y) *C. elegans* at channel 10. This plot shows the presence of peaks in the post-reproductive life stage that is not present in the pre-reproductive lifestage.

Graph 32 shows the difference between the untreated reproductive life stages p2m and p1m where the data was not normalized. The soluble protein (normalization factor) for p1m is 10.36 and 14.70 for p2m. It shows that the subtraction algrothim does work. The difference between the two chromatograms is small, which is expected for any signal data. The peaks do align themselves well, reducing the wobbling in the time and the very small peaks are noise since we are measuring picoAmps signals.

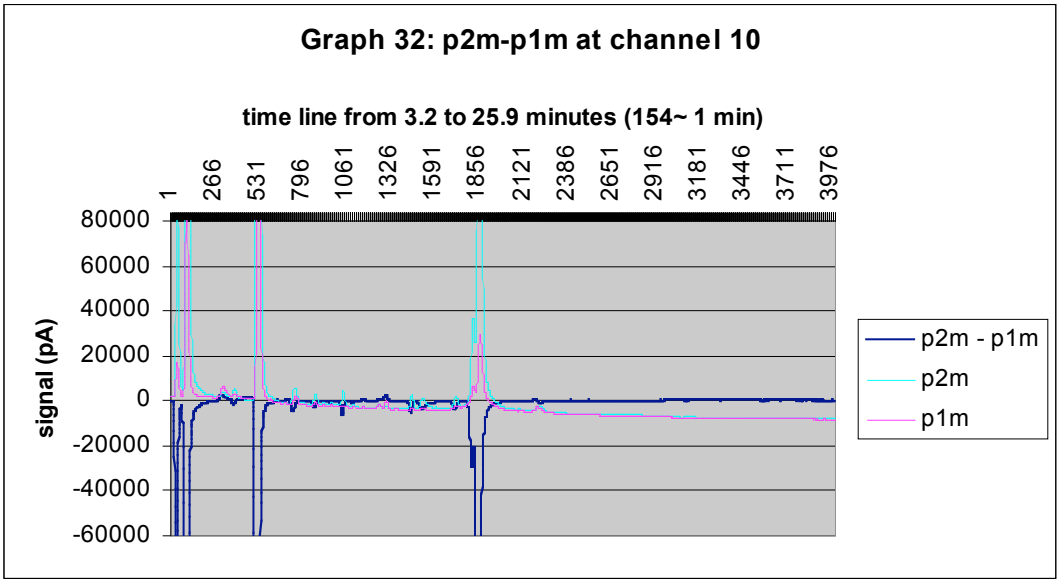


Figure 37: Graph 32 – results from the subtraction algorithm of two untreated reproductive life stage (p2m and p1m) *C. elegans*. This graph demonstrates that the algorithm does work.

Graph 33 shows the difference between the 0.1 mg/ml Vitamin E treated pre-reproductive organisms (ea2y) and the untreated pre-reproductive nematodes (p1y). There is a difference in the pattern between 1700 and 2000 time line. The ratio of those three peaks changed from untreated population when compared to the Vitamin E treated (0.1 mg/ml).

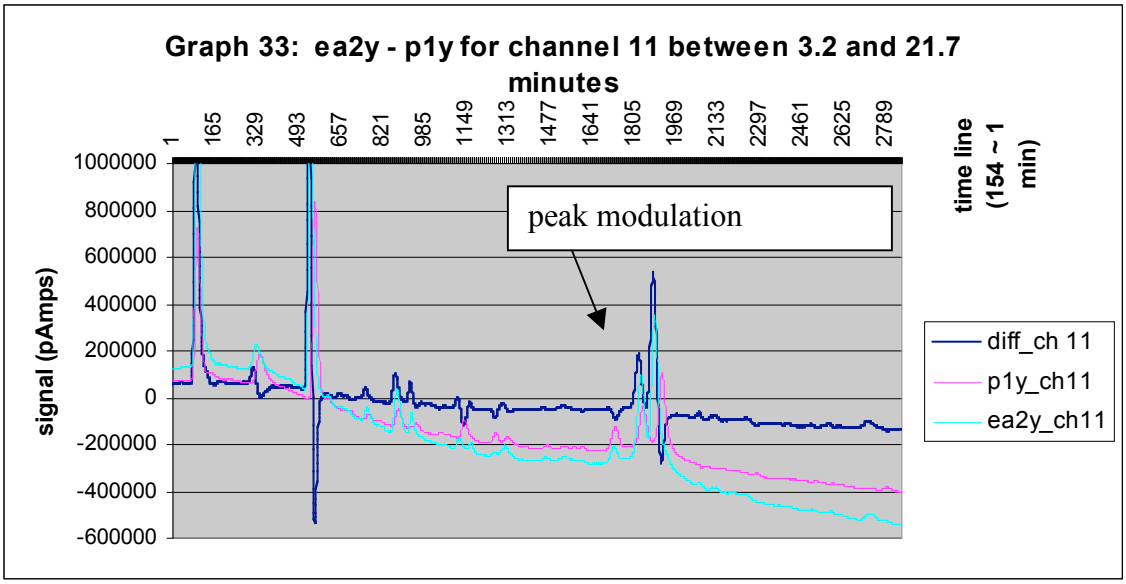


Figure 38: The difference between the 0.1 mg/ml Vitamin E treated pre-reproductive (ea2y) and the untreated pre-reproductive (p1y) *C. elegans*. This is for channel 11 between 3.2 and 21.7 minutes.

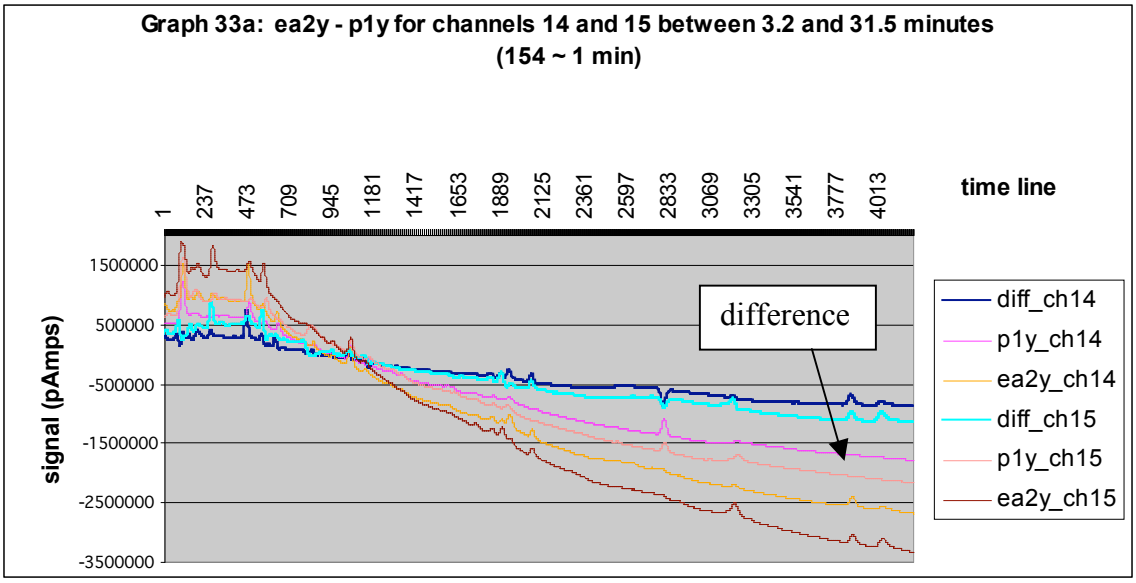


Figure 39: Difference between the 0.1 mg/ml Vitamin E treated pre-reproductive (ea2y) and the untreated pre-reproductive (p1y) *C. elegans*. Graph 33a is for channels 14 and 15 between 3.2 and 31.5 minutes. It shows the absence of peaks between 3700 and 4100 in the untreated (p1y) compared to the Vitamin E treated (ea2y – 0.1 mg/ml).

Graph 34, 34a, and 34b all show comparisons between ca1y – pre-reproductive 0.1 mg/ml treated Vitamin C and, the untreated pre-reproductive. The peak modulation present in Channel 11 between 1700 and 2050 time line does exits here, like the Vitamin E and untreated comparison, graph 33, with an increase in intensity for the Vitamin C compared to the Vitamin E, both at 0.1 mg/ml concentration, but in a different pattern. An example of a peak not present in the 0.1mg/ml Vitamin C treated pre-reproductive worms is shown in Graph 34b, at about 2750 time- line. This peak is present in the 0.1 mg/ml Vitamin E treated prereproductive *C. elegans* but, also absent in the 0.1 mg/ml Vitamin C and E combination treated pre-reproductive worms. The pattern found in c1y, in graph 34 is similar to those found in ha1y and ea2y.

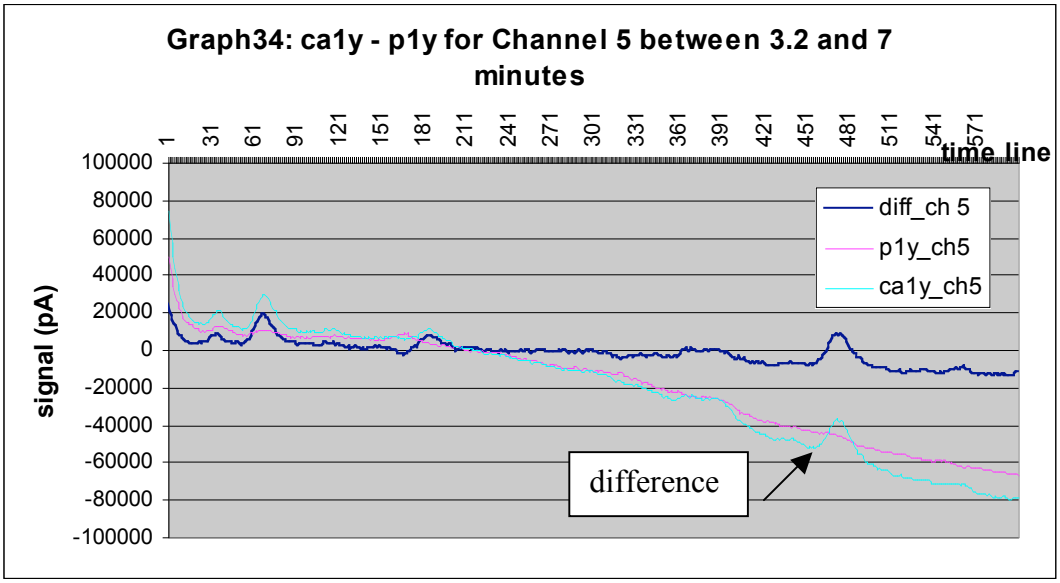


Figure 40: Data subtraction of 0.1 mg/ml Vitamin C treated pre-reproductive (ca1y) from untreated pre-reproductive (p1y) nematode, at channel 5. Graph 34: A peak is present in the Vitamin C treated worm but absent in the untreated, in the pre-reproductive life stage.

Graph 34a shows a change in the pattern from Graph 33, for the Vitamin C treated pre-reproductive *C. elegans* (ca1y), compared to ea2y and p1y, around 1700 to 2050 time line. The modulations of those peaks starting around 1700 time-line are different for the treated nematodes when compared to the untreated, pre-reproductive nematodes.

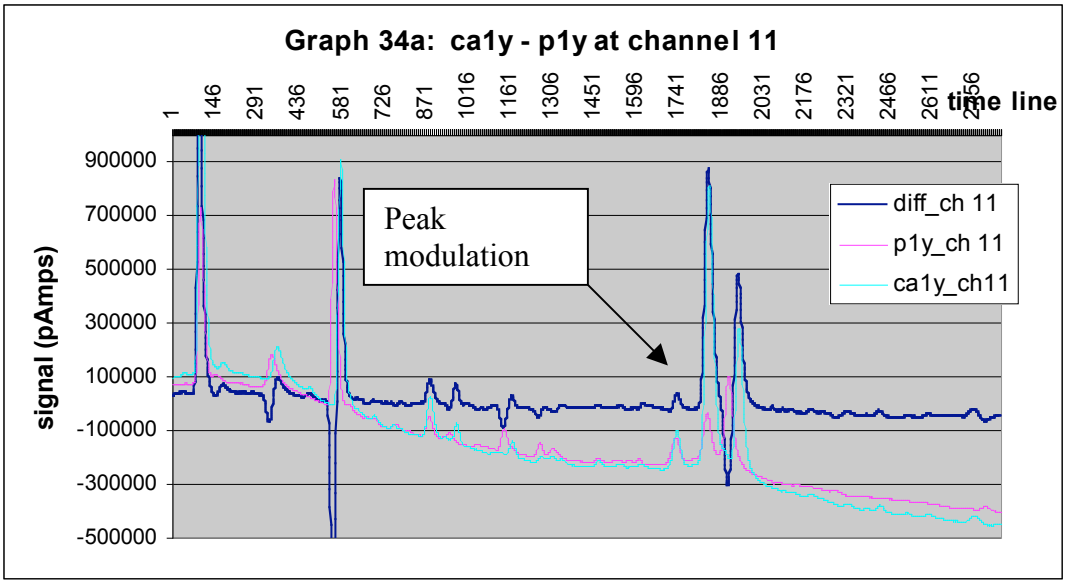


Figure 41: Data subtraction of 0.1 mg/ml Vitamin C treated pre-reproductive (ca1y) from the untreated pre-reproductive (p1y) nematode. Graph 34a is for channel 11.

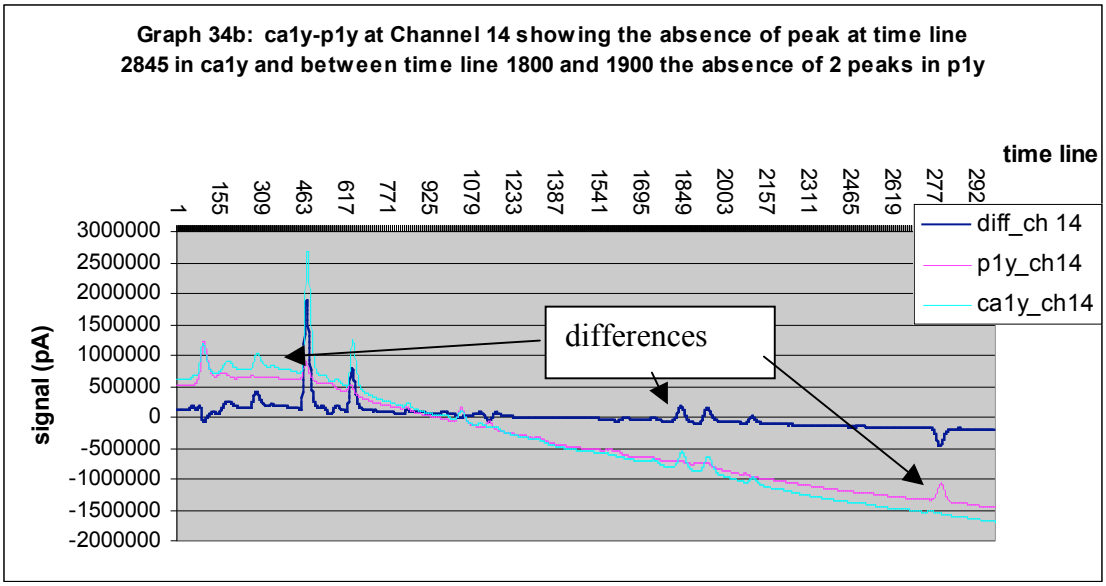


Figure 42: Data subtraction of 0.1 mg/ml Vitamin C treated pre-reproductive (ca1y) from the untreated pre-reproductive (p1y) nematode. Graph 34b is at channel 14, with several differences.

In comparing the chromatographic data for the 0.1 mg/ml Vitamin C and E combination (ha1y) pre-reproductive with the untreated pre-reproductive (p1y), there are a lot of differences. The peaks in ha1y are of much greater intensity than the untreated, after the normalization. In graph 35, two peaks with similar heights are present between the time line of 2950 and 3150 in ha1y that are not seen in p1y, ea2y or ca1y. This is expected since Vitamin C and E combination treatments had the most amount of biology changes.

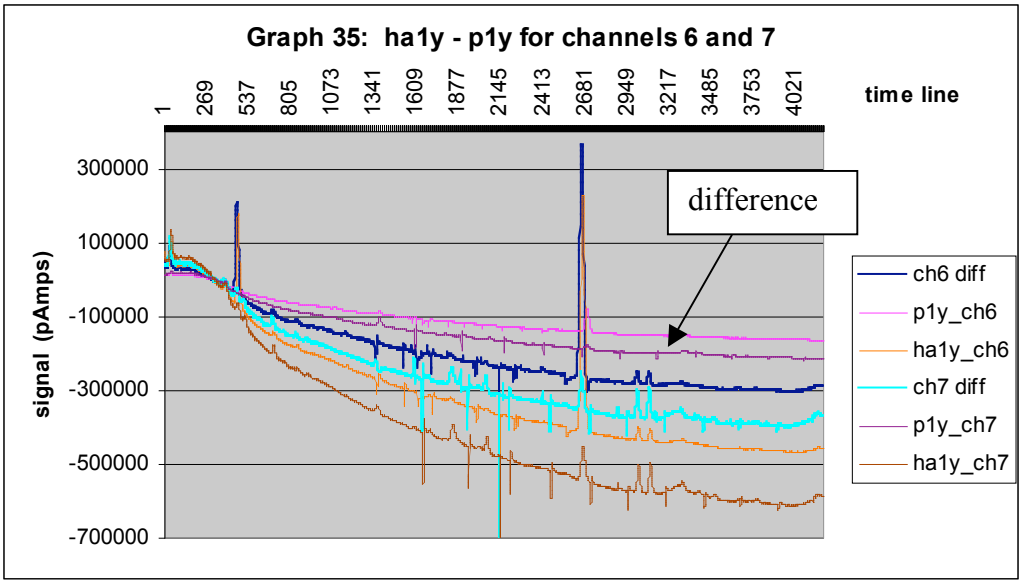


Figure 43: Data subtraction of 0.1 mg/ml Vitamin C & 0.1 mg/ml Vitamin E combination pre-reproductive (ha1y) subtracted from the untreated pre-reproductive nematode (p1y). Graph 35 is for channels 6 and 7.

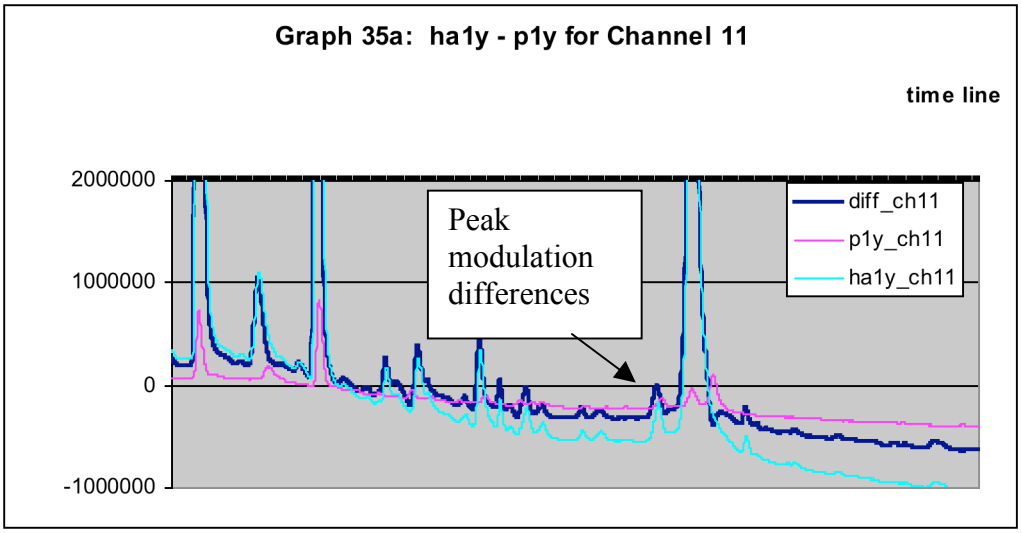


Figure 44: Data subtraction of 0.1 mg/ml Vitamin C & 0.1 mg/ml Vitamin E combination pre-reproductive (ha1y) subtracted from the untreated pre-reproductive nematode (p1y). Graph 35a is for channel 11.

Graph 35a a shows the difference for channel 11 for ha1y compared to p1y. Around 1700 to 2100 time line, the pattern is different from p1y, ea2y and ca1y.

Graph 36 shows the presence of a peak at about 2700 time line in greater intensity for pre-reproductive (cc2y) *C. elegans* than the reproductive (cc2m) *C. elegans*, treated with 0.5 mg/ml Vitamin C. This pattern is not seen in the post-reproductive *C. elegans*. A similar pattern is observed when comparing ca1y and ca1m, the pre-reproducitve and reproductive life stages of the 0.1 mg/ml treated worms with Vitamin C and, cc2y and cc2m, the pre-reproducitve and reproductive life stages of the 1.0 mg/ml treated worms with Vitamin C. With the untreated pre-reproductive and reproductive worms the compound exists in both life stages but in smaller concentrations in the reproductive phase. A different pattern is observed with the Vitamin E treated pre-reporductive and reproductive *C. elegans* (Graph 37). That compound does exist in the Vitamin C and E combination treatment but in much smaller quantities in the reproductive life stage.

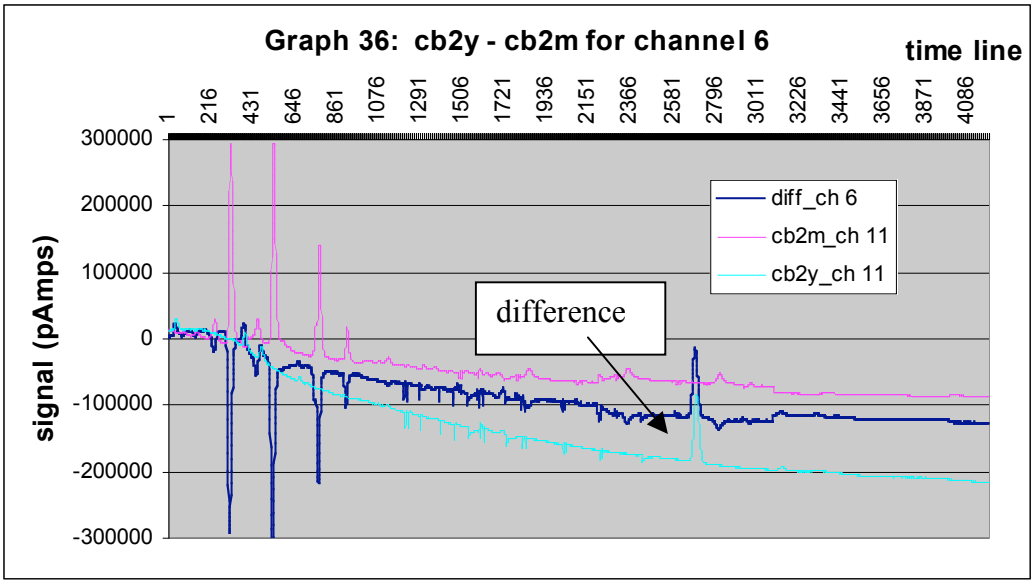


Figure 45: 0.5 mg/ml Vitamin C treated pre-reproductive (cb2y) subtracted from the 0.5 mg/ml Vitamin C reproductive (cb2m) *C. elegans*. Graph 36 is for channel 6.

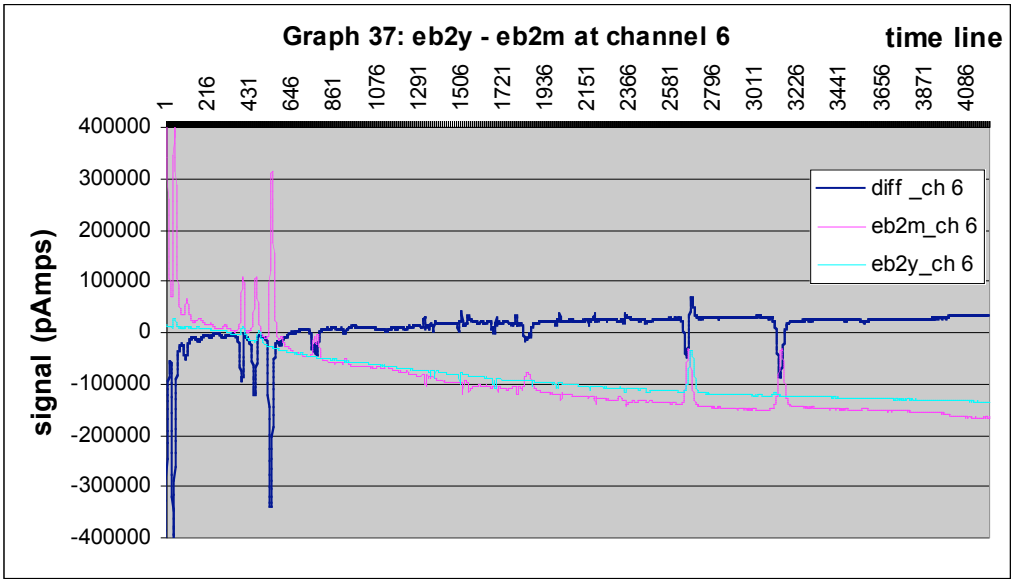


Figure 46: 0.5 mg/ml Vitamin E treated pre-reproductive (eb2y) subtracted from the 0.5 mg/ml Vitamin E reproductive (eb2m) *C. elegans*. Graph 37 is for channel 6.

Figures 34 through **46** demonstrate that there are specific signature patterns generated for the metabolites analyzed at each life stage (pre-reproductive, reproductive and post-reproductive) of C.elegans. It also provides evidence that there are unique patterns for each Vitamin treatment, whether it's Vitamin C, Vitamin E or Vitamin C and E combinations. The subtraction program written is of substantial value in determing those analytes that serve to define and identify the patterns unique to the experimental datasets described above. The information obtained can be used in a quantitative manner since it is normalized per μg soluble protein. However, the unique analyte patterns themselves, the qualitative aspect of the data, are sufficient to address aspects of the theories on aging that involve ROS and homeostatic contols.

PRINCIPLE COMPONENT ANALYSIS

A statistical software package was applied to the data to see if there is any clustering of the data. This is used to verify that there are differences in the biology and the data obtained from both the CoulArray software and the subtraction algorithm. Software called PowerArray, obtained from the National Institute of Statistical Sciences (www.niss.org), is used to perform the principle component analysis (PCA) on the data. First, each chromatographic data has to be converted into one column and depending on what PCA is being used; each of those chromatograms has to be placed into one matrix. A python program was written to format and normalize the text file chromatographic data (**Appendix 15)**. PCA was done on the untreated age separated data and the outcome is shown in graph 38.

The life stages data separated very well, where each life stage grouped together. There is a larger spread in the reproductive data that is due to spills from reproduction. Saw that the Vitamin C & E data separated from all the data, from the same life stage. This indicates that the biology is different with Vitamin C & E treatment compared to the Vitamin C, Vitamin E or untreated nematodes. This has been demonstrated in the biology.

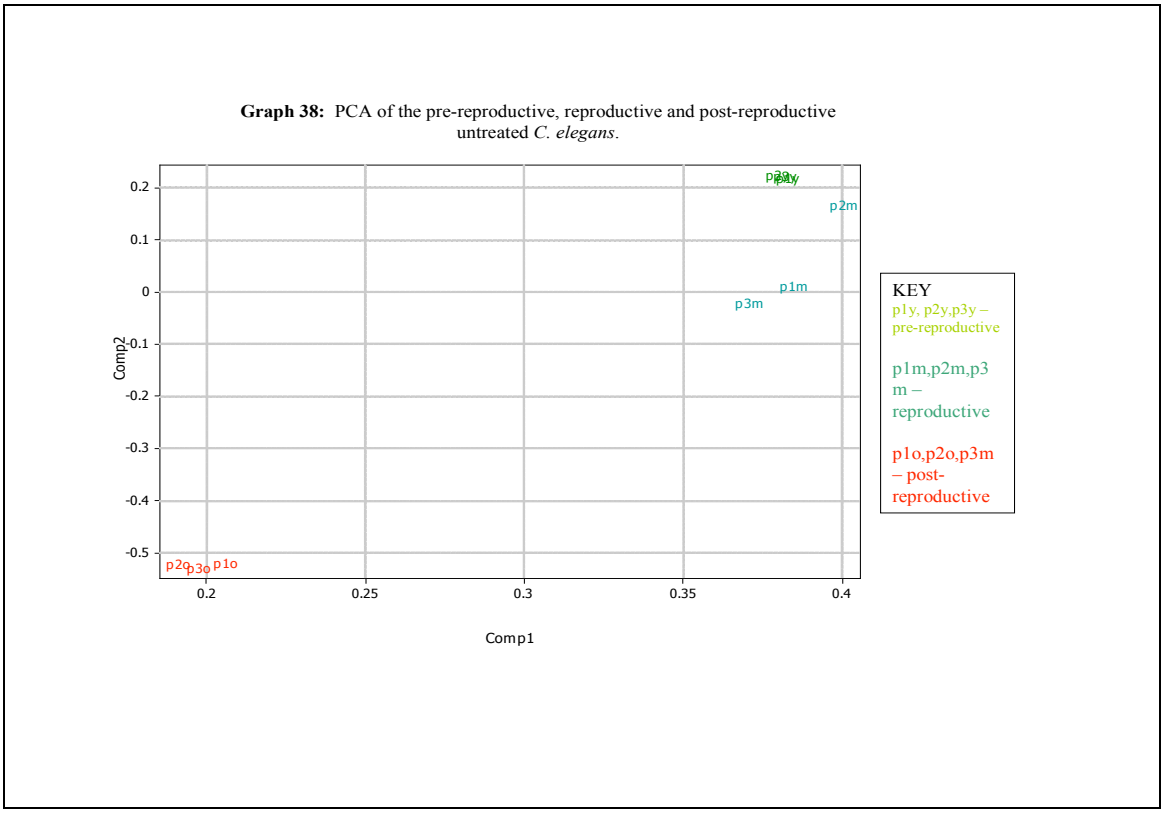


Figure 47: Graph 38 – PCA of the pre-reoprductive (py), reproductive (pm) and post-reproductive (po) untreated *C. elegans*, using PowerArray software.

Graph 39 shows the eigen values for Graph 38. The first two components

constitutes over 95 % of the total eign values among 10 principle components. This indicates that it is enough to capture the main variance information using only the first two principle components. From the score plot above (graph 38) the first component decrease with a change in life stages pre-reproductive and reproductive to post-reproductive. The score of the second principle decreases as the life stages changes from pre-reproductive to reproductive to post-reproductive. Components one and two separate out the post-reproductive from the pre-reproductive and reproductive *C. elegans*. The data for the pre-reproductive and post-reproductive nematodes separates out very well. There is a spread in the reproductive data that can be attributed by eggs within the worms and spill over from reproduction. The pairwise statistics confidence intervals for the data are: post-reproductive is 0.9544, reproductive is 0.8832 and pre-reproductive is 0.9991.

Graph 39: Plot of EigenValue for 9 components of PCA for the untreated pre-reproductive, reproductive and post-reproductive *C. elegans*

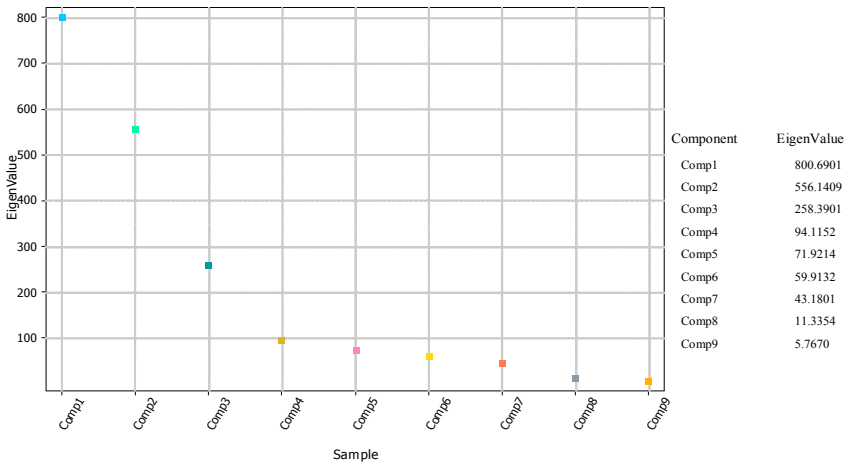


Figure 48: Plot of Eigen Values for 9 components of PCA for the untreated pre-reproductive (py), reproductive (pm) and post-reproductive (po) *C. elegans*. PowerArray software is used.

Graph 40 is the PCA score graph of all pre-reproductive *C. elegans* in the experiment. There are two outliers, **hc3y** and **ha3y** (Vitamin C & E combination pre-reproductive nematodes). With respect to component one, not taking in consideration the outliers, these nematodes are not separated from each other by the first component, unlike the second component, which seems to separate them into two sections. The untreated, the Vitamin C and the Vitamin E of various doses are all grouped together while the Vitamin C & E combination treatment are grouped together. There are no distinctions in

the dose treatment. Again component one and two makes up more than 95 % of the variation as shown in **Appendix 16** (the eigen value scores for the ten components). The pairwise statistics for the pre-reproductive data confidence levels are greater than 0.982.

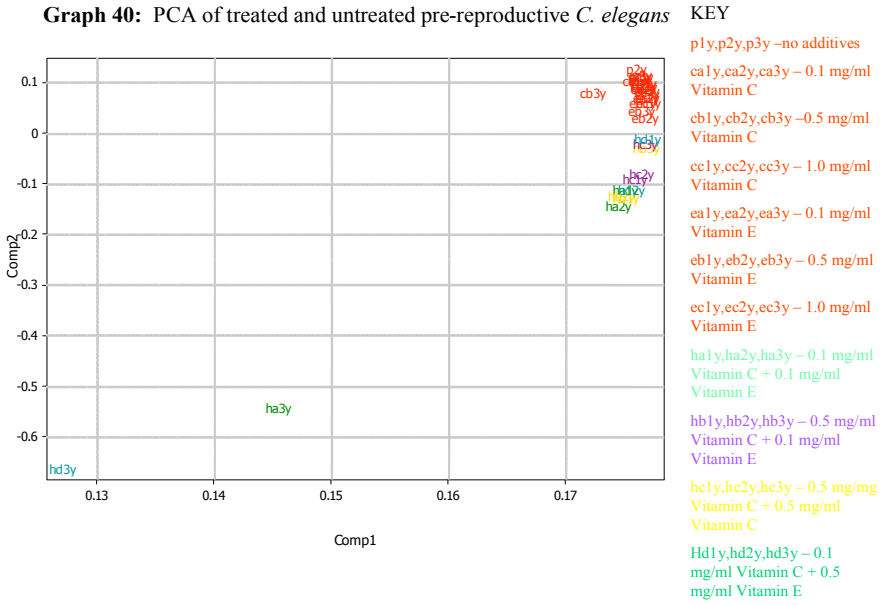


Figure 49: PCA component 1 and 2 of the pre-reoprductive nematodes of all the experimental categories *C. elegans*. Graph 40: – using PowerArray software.

Graph 41, shows the PCA for the reproductive *C. elegans*. Components one (1) and two (2) capture more than 90 % of the differences. **Appendix 17** shows the eigen values of the 10 components. Component one separates the 0.1 mg/ml Vitamin C and E treated worms (ha?m) from the rest. With an increase in Vitamin E concentration,

component one increase, as in the Vitamin C and E combination worms. The 1.0 mg/ml Vitamin C treated worms (cc*m) are also grouped together and separated from the rest by component one and two. The 0.1 and 0.5 mg/ml Vitamin C treated worms, together with the 0.1 and 0.5 mg/ml Vitamin E treated worms seems to clump together with components one and two. However, the untreated reproductive and the 1.0 mg/ml Vitamin E treated worms tend to group with the combination treatement with the exception of the 0.1 mg/ml Vitamin C and E treated worms. The pairwise statistics confidence levels for the reproductive data range from 0.81 to 0.99.

Graph 41: PCA of treated and untreated reproductive (m) *C. elegans*

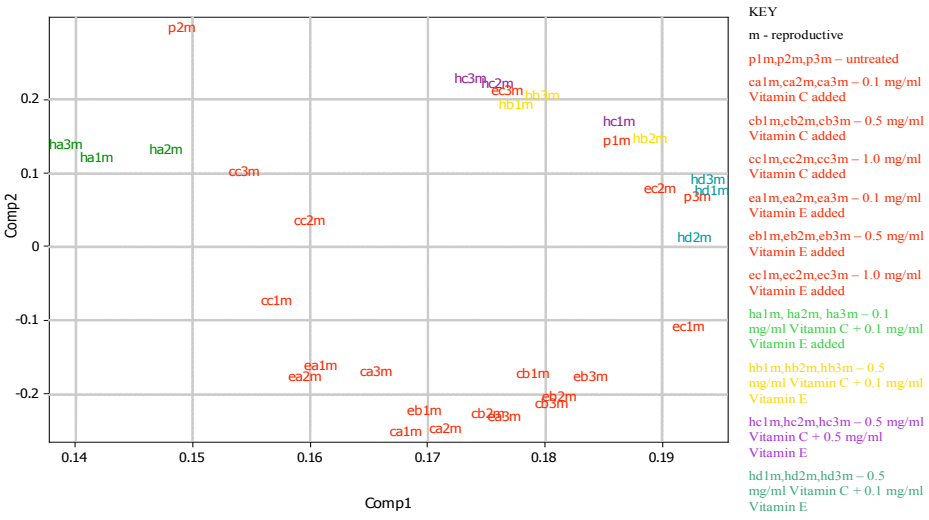


Figure 50: PCA component 1 and 2 of the reoprductive nematodes of all the experimental categories *C. elegans*. Graph 41: – using PowerArray software.

Appendix 18 shows the score plot of component one and three for the reproductive nematodes. Component three does not separate the worms based on treatment. The points are clumped together with the exception [ha*m] which is still separated from the rest of the worms.

Graph 42 shows the PCA score plot for the post-reproductive *C. elegans*. Components one and two capture more than 90 % of the difference. One can see that component one separate the Vitamin C and E combination by dose response. An increase in Vitamin C and E combination results in an increase in component one, 0.1 mg/ml Vitamin C and E has the smallest component one value and 0.1 mg/ml Vitamin C with 0.5 mg/ml Vitamin E resulting in the largest component one value. Component two also increase slowly with this treatment. Vitamin E treated post-reproductive worms also result in an increase in component one with the increase of Vitamin E. The reverse is the case for the Vitamin C treatment. Component two separates the untreated and Vitamin C, from the Vitamin E and that in turn from the Vitamin C and E combination treatment. **Appendix 19** shows the PCA EigenValue scores for the ten components. The pairwise statistics confidence levels range from 0.895 to 0.995, for the post-reproductive data.

Graph 42: PCA score plot of treated and untreated post-reproductive *C.elegans*

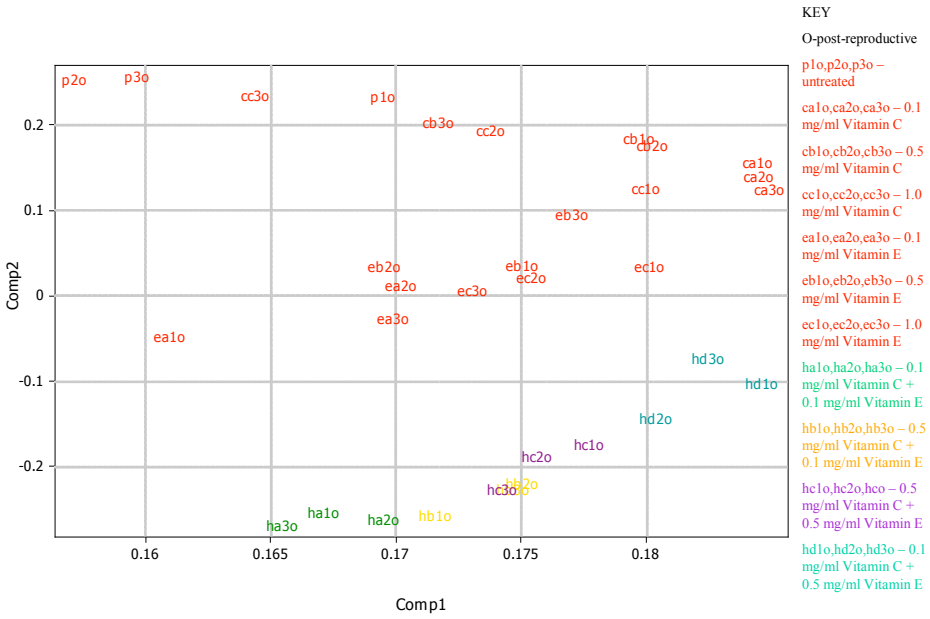


Figure 51: PCA component 1 and 2 of the post-reproductive nematodes of all the experimental categories *C. elegans*. Graph 42: – using PowerArray software.

5. CONCLUSIONS

There are the changes observed in the analytes studied that are important and are involed in homeostatic controls. Vitamin C and E treatment alters these analytes amounts, their relative ratios and thus, the overall pattern of these metabolites within the nematode system. The Vitamin treatments have affected the nematode metabolism in a fashion that reflected differences in phenotype know for this organism during the course of its reproduction, development and scenescence. There are significant and meaningful alterations in the metabolites identified and analyzed, those associated with and are representative of these stages, corresponding to treatment protocols. There are specific differences associated with the treatment of individual life stages as well, or, at least, that the effects of additional antioxidants from external sources on nematode growth and development, which are life stage dependent differences, as well as differnces in terms of dosage, and nature of the antioxidant vitamin to which the nematode is exposed. That the response appears nonlinear is interesting, that the effects, observed, when analyzesd via clustering algorithms are substantiated, and that there are unique analytes, within the metabolome surveyed, that are specific to each of the life stages and the antioxidant treatments.

In response to Vitamin C and E there are changes in the population distrubtion, the most pronounced observed in the 0.5 mg/ml treated Vitamin C, the 0.1 mg/ml

Vitamin E and, the 0.1 mg/ml Vitamin C + 0.1 mg/ml Vitamin E and 0.1 mg/ml Vitamin C + 0.5 mg/ml Vitamin E combination experimental categories. The pre-reproductive/post-reproductive (y/o) ratio increased with 0.5 mg/ml dosage Vitamin C experiment. In the Vitamin E treatment, a distinct difference was observed in the pre-reproductive/reproductive ratio (y/m) for the 0.1 mg/ml Vitamin E treatment, where the ratios for the Vitamin E treated nematodes were greated than the untreated nematodes. With the Vitamin C and E combination treatment the y/o ratios for the 0.1 mg/ml Vitamin C + 0.1 mg/ml Vitamin E and, 0.5 mg/ml Vitamin E + 0.1 mg/ml Vitamin C treatments had the greatest impact on the population, compared to the untreated. These differences in the ratios observed could mean that the Vitamins are having an impact on development and reproduction, taking longer period of time for the nematode to attain adulthood, compared to the untreated.

The concentration of serotonin in all the Vitamin C and E post-reproductive experiments were less than the untreated post-reproductive *C. elegans*. The ratio of tryptophan/serotonin for the Vitamin C and Vitamin E experiments were greater than that of the untreated post-reproductive. There are also dose response relationships for this ratio for the Vitamin C and Vitamin E experiments, where the tryptophan/serotonin ratio increased with the increase of the Vitamin concentration treatment. For the Vitamin C and E combination treatment trypotphan/serotonin ratios were also greater than the untreated, in the post-reproductive life stage. Serotonin concentrations were found to be independent of the tryptophan concentrations. Serotonin requires oxygen for its formation. Since Vitamin C and E combination is a more powerfull antioxidant, it may

explain why less serotonin is found in the Vitamin C and E combination treatment populations, compared to the untreated. This data implies that the addition of the antioxidants have affected the production of serotonin, a neurotransmitter, hence a potential change in the signaling system, which supports the theories on aging that supports ROS effects and homeostatis.

No norepinephrine (norepi) was found in any of the pre-reproductive *C. elegans* treatments. The tyrosine/epinephrine (tyr/epi) ratio for the Vitamin C and Vitamin E experiments were about the same as the untreated nematodes in the pre-reproductive life stage. The combination of Vitamin C and E treatment resulted in the tyr/epi ratios being less than the untreated, in the pre-reproductive life stage. Norepinephrine was measured in all of the Vitamin C reproductive experimental categories, but not in the untreated reproductive *C. elegans*. In the post-reproductive life stage norepi and epi are measured in the Vitamin C, Vitamin E, Vitamin C & E combination and, untreated experimental categories except in the 0.1 mg/ml Vitamin E (eao) treatment that had no norepi and the 0.5 mg/ml Vitamin E with 0.1 mg/ml Vitamin C (hdo) that had no epi. The differences observed in the tyr/epi ratios, the presence or absence of epi and norepi again supports the neuroendorince theories of aging. Here again the antioxidants treatments resulted in deviations from the untreated ratios and measurements in the life stages. This again suggests that ROS plays a very important role in aging.

Tryptophan (trp) and tyrosine are two very important amino acids that serve as precursors to the production of several neurotransmitters, which are key physiological regulatiors in specific neuronal and general nervous system functions. As mentioned

these neurotransmitters are muti-gene products. Therefore changes in their concentrations will have impact on the signaling systems and hence homeostasis in the organisms. In the pre-reproductive life stage the tyrosine and tryptophan concentrations varied with the Vitamin C, Vitamin E and untreated experiments. However, with the Vitamin C and E pre-reproductive combination their concentrations were greater than that of the untreated pre-reproductive nematodes. The trp/tyr ratio in the Vitamin C and E combination was less than the untreated, in the pre-reproductive life stage. In the reproductive life stage, the Vitamin C and Vitamin E treated worms had trp and tyr concentrations greater than that of the untreated worms. The Vitamin C and E combination treatment resulted in the trp and tyr concentrations to be less than that of the untreated, in the reproductive life stage. The ratio for trp/tyr was about the same for the Vitamin C, Vitamin E and untreated reproductive nematodes, but less than the Vitamin C and E combination treated reproductive nematodes. With the post-reproductive nematodes, the same pattern was observed for the trp and tyr concentrations. However, the trp/tyr ratios changed where the trp/tyr ratio for the untreated was greater than the Vitamin C, Vitamin E and Vitamin C and E combination treated post-reproductive nematodes. In viewing the results by life stages, the trp/tyr ratio fluctuates in the untreated, Vitamin C and Vitamin E treated worms, but it's relatively stable in the Vitamin C and E combination for the pre-reproductive, reproductive and post-reproductive life stages. This is significant, implying that Vitamin C and E combination has a big impact on the metabolites of trp and tyr, keeping their ratio stable hence slowering the breakdown of homeostatis – aging.

The DNA repair compound 8-OH-guanine is absent in all the Vitamin C and E combination treatment, unlike the untreated lifestages of the nematode. This suggests that the added antioxidants prevent the organisms DNA from getting damage with ROS. A reduction in ROS reduces stress applied to the system, slowering the process of aging and alteration of the signaling systems at the various life stages. Vitamin E is effective in preventing DNA damage in the pre-reproductive life stage, in dosages of 0.1 mg/ml to 1.0 mg/ml, unlike Vitamin C that is effective in the 1.0 mg/ml in the pre-rperoductive life phase. In the reproductive and post-reproductive life stages Vitamin C or E is not effective in preventing DNA damage but the Vitamin C and E combination is effective in preventing DNA damage in concentrations as low as 0.1mg/ml Vitamin C and 0.1 mg/ml Vitamin E.

In studying the purine catabolic products, I was able to see a change in the pathways for the formation of xanthine, with the addition of Vitamin C, Vitamin E and Vitamin C and E combination. Vitamin C an E had the greatest impact, where the formation of xanthine is changed from being primary hypoxanthine (in the untreated reproductive and post-reproductive life stages) to xanthosine in the reproductive and post-reproductive nematodes. Also, there is more measurable guanine in the Vitamin C, Vitamin E and Vitamin C and E combination in the post-reproductive worms when compared to the untreated post-reproductive, implying guanine is being excreted and not needed for nucleotide synthesis.

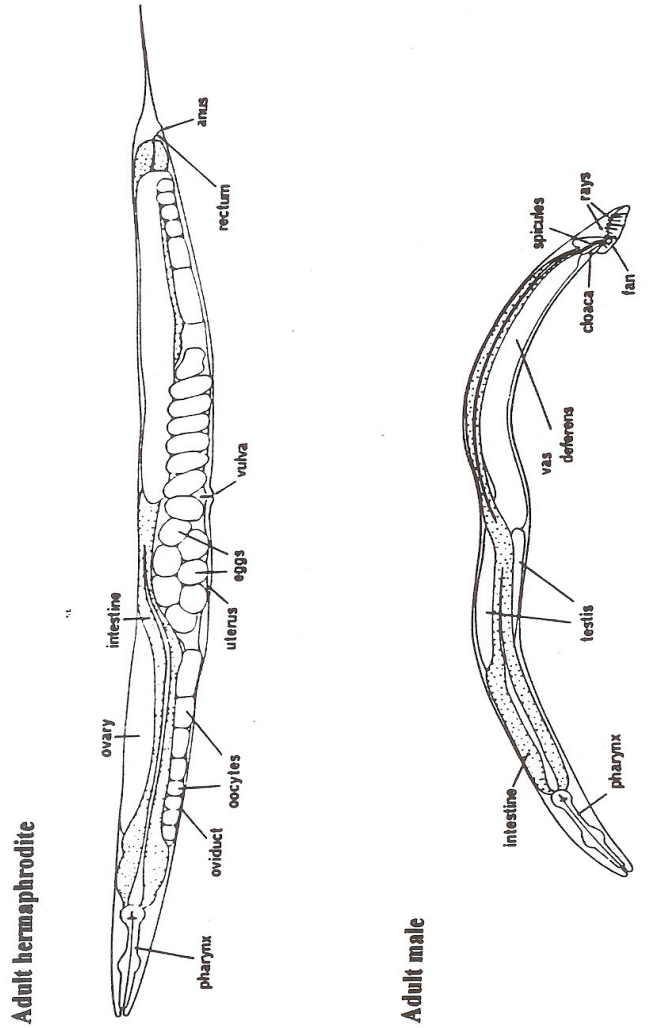
From the data subtraction and CoulArray software one can demonstrate unique patterns observed with the different life stages and also with different Vitamin treatment.

There is peak modulation around 17 to 20 minutes in channel 11, for different treatment types. Also, there are a few compounds that are present in one experiment category and not in the other, like the one present in Vitamin C for all the life stages, at channel 7, which is absent in the untreated worms. Also, at about 30 minutes there are two peaks that are present in channel 14 for the untreated *C. elegans* that are absent in the treated nematodes.

With the PowerArray software, data clustering using principle component analysis was observed. The pre-reproductive, reproductive and the post-reproductive data clustered in their own groups. This data alone implies that each life stage have unique patterns. The Vitamin C and E combination treatment was clustered, away from the other treatments, implying distinct difference in that data compared to the untreated, Vitamin C or Vitamin E data at the different life stages.

The data in this experiment all complemented each other, from the population distribution ratios (the visual aspect) to the biology. The results from the entire experiment support the theories that ROS are important in the onset of aging. It also implies that the tyrosine, tryptophan and their metabolites are important in the signaling system that control homeostatis and aging. It demonstrates that the combination of the antioxidants Vitamin C and E in small doses of 0.1 mg/ml each is very effective in reducing the ROS, hence slowering the aging process.

APPENDIX 1



Appendix 1: Diagram showing major anatomical features of C. elegans adult hermaphrodite and male

APPENDIX 2

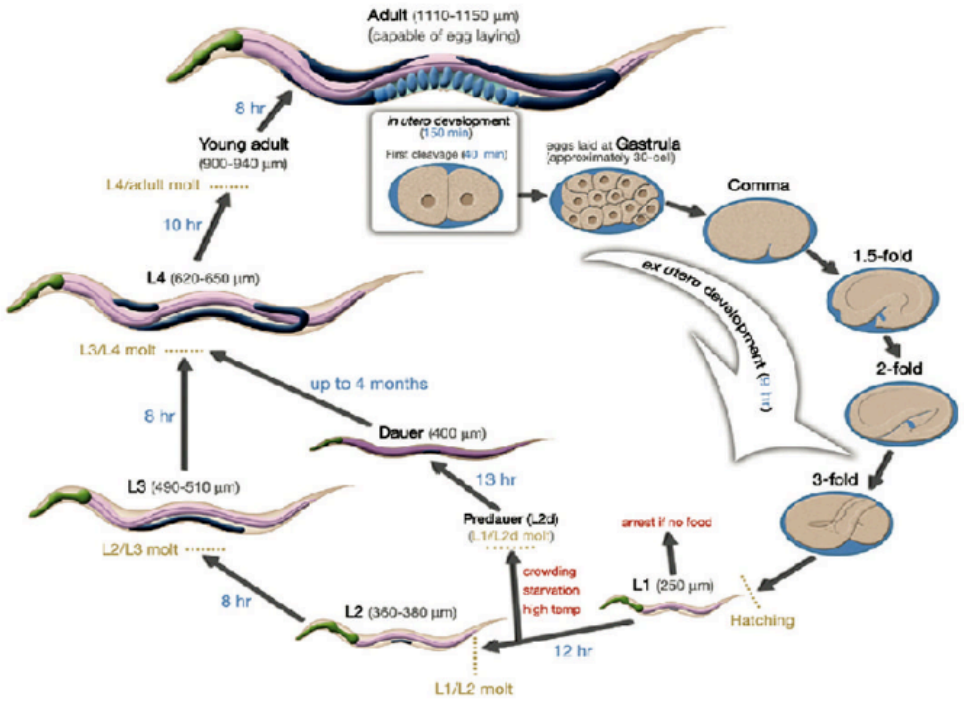


Figure 4.
Life cycle of the *C. elegans* hermaphrodite at 22°C. An adult hermaphrodite with developing oval-shaped embryos inside is shown at the top of the life cycle. Once the embryos reach the 28–30-cell stage, they are laid into the environment and continue to develop. When embryogenesis is complete, L1 larvae hatch out of the eggshell and begin post-embryonic development in the presence of adequate nutritional sources. The animal continues larval development through the L2–L4 stages until reaching adulthood. Note the dark-colored gonad that is expanding during this time period. Prior to the L1/L2 molt, if there is a lack of food or there are too many animals or the temperature is too high then the animals will enter the alternative life cycle and become dauer larvae until these unfavorable conditions improve. The size of the animals at each stage of development is indicated in parentheses, and the amount of time spent at each stage in development is indicated along the arrows.

Anal Biochem. Author manuscript; available in PMC 2007 April 24.

Appendix 2 – Life Cycle of the *C. elegans* hermaphrodite (Ann K. Corsi, *Anal Biochem, 2006*)

Line	Time (Min)	Activity	Value
1	0.00	FLOW %B	10 %B 0.30 ml/min
2	0.00	AUTOSAMPLER INJ	1.0 Sec
3	0.00	WAIT EXTERNAL	START 1
4	0.00	FILE	START
5	0.50	AUTO ZERO	ON
6	40.00	FLOW %B	70 %B 0.70 ml/min
7	55.00	FLOW %B	70 %B 0.90 ml/min
8	65.00	FLOW %B	70 %B 0.90 ml/min
9	75.00	FLOW %B	100 %B 0.90 ml/min
10	80.00	FLOW %B	0 %B 0.90 ml/min
11	85.00	FLOW %B	10 %B 0.30 ml/min
12	90.00	FILE	STOP

MAX Pressure 300 Bars Min Pressure 0 Bars

---------- CELLS POTENTIALS ----------

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
0	60	120	180	240	300	360	420	480	540	600	660	720	780	840	900

Appendix 3: Trial6d - CEAS-HPLC method designed to analyze samples

Line	Time (Min)	Activity	Value
1	0.00	FLOW %B	10 %B 0.30 ml/min
2	0.00	AUTOSAMPLER INJ	1.0 Sec
3	0.00	WAIT EXTERNAL	START 1
4	0.00	FILE	START
5	0.50	AUTO ZERO	ON
6	40.00	FLOW %B	70 %B 0.70 ml/min
7	55.00	FLOW %B	70 %B 0.90 ml/min
8	65.00	FLOW %B	70 %B 0.90 ml/min
9	75.00	FLOW %B	100 %B 0.90 ml/min
10	80.00	FLOW %B	0 %B 0.90 ml/min
11	85.00	FLOW %B	10 %B 0.30 ml/min
12	90.00	FILE	STOP

MAX Pressure 300 Bars Min Pressure 0 Bars

CELLS POTENTIALS

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
0	60	120	180	240	300	360	420	480	540	600	660	720	780	840	900

Appendix 3a: Trial6NN - CEAS-HPLC method designed to analyze samples

Appendix 3 contains the two methods created to analyze the samples based on the solvent front, the HPLC columns used and the type of compounds one wanted to identify. Trail6d is different from Trial6NN because the runtime per sample was shortened and the column was changed between both runs. In both methods there are gradient flow between mobile phases A and B and also pressure alterations to enhance the separation of the compounds of interest.

APPENDIX 4

average	start			end		
data	y/m	y/o	m/o	y/m	y/o	m/o
ca	3.4	4.2	1.2	6.1	13.2	2.6
cb	4.5	3.9	0.9	5.8	20.8	3.9
cc	2.7	11.5	4.5	4.8	14.5	3.3
p	3.4	5.7	1.7	9.4	14.8	2.7
	y/m	y/o	m/o	y/m	y/o	m/o
ca sd	1.157084	3.558893	0.950693	3.243524	2.619345	1.210223
cb sd	0.833333	1.382965	0.360555	1.712282	7.904523	2.180744
cc sd	0.386979	11.0492	4.787174	2.283799	4.066863	0.808664
p sd	0.52915	2.147889	0.698875	7.616773	11.32319	2.167271
	standard error of mean					
	y/m	y/o	m/o	y/m	y/o	m/o
ca sd	0.361035	0.997155	0.491717	0.755729	0.416524	0.435735
cb sd	0.226805	0.403739	0.219427	0.41094	1.001188	0.641281
cc sd	0.135751	1.882961	1.301298	0.604373	0.617148	0.257919
p sd	0.165683	0.519667	0.309974	1.432329	1.699774	0.758216

Appendix 4A: The average ratio data obtained from the Vitamin C and untreated nematodes populations, for each experimental category. The nematodes are separated into three age categories, pre-reproductive (y), reproductive (m) and post-reproductive (o) *C. elegans*. Also included are the ratios of y/m and y/o at the beginning and end of the experiments. This is graphed and discussed in the results and discussion, chapter 4.

average	start			end		
data	y/m	y/o	m/o	y/m	y/o	m/o
ea	4.1	4.8	1.6	16.1	12.9	1.1
eb	2.8	3.3	1.1	10.1	8.7	0.9
ec	4.4	2.8	0.9	8.7	8.9	1.3
p	3.4	5.7	1.7	9.4	14.8	2.7

	start			end		
	y/m	y/o	m/o	y/m	y/o	m/o
ea sd	2.03	1.95	1.30	10.78	3.21	0.64
eb sd	0.73	2.22	0.46	0.41	1.69	0.15
ec sd	3.10	1.23	0.79	5.71	2.27	0.76
p sd	0.53	2.15	0.70	7.62	11.32	2.17
	standard error of mean					
	y/m	y/o	m/o	y/m	y/o	m/o
ea sd	0.58	0.51	0.59	1.55	0.52	0.36
eb sd	0.25	0.71	0.25	0.07	0.33	0.10
ec sd	0.85	0.42	0.48	1.12	0.44	0.38
p sd	0.17	0.52	0.31	1.43	1.70	0.76

Appendix 4B – Table of average ratio population distribution data for the Vitamin E dose treatment nematode populations and the untreated populations, at the start and end of the experiment. The incubation period for the culture is 10 days. Also included is the average ratio of the pre-reproductive (y) life stage compared to the reproductive (m) and the post-reproductive (o) life stages, at the start and end of the experiment.

avg data	start				end		
	y/m	y/o	m/o		y/m	y/o	m/o
ha	2.90	3.25	1.24		3.91	24.00	6.51
hb	2.37	3.55	1.44		6.74	14.60	2.31
hc	3.57	9.58	3.08		6.79	16.08	2.42
hd	3.32	10.14	4.28		11.91	28.83	2.75
p	3.4	5.7	1.7		9.4	14.8	2.7

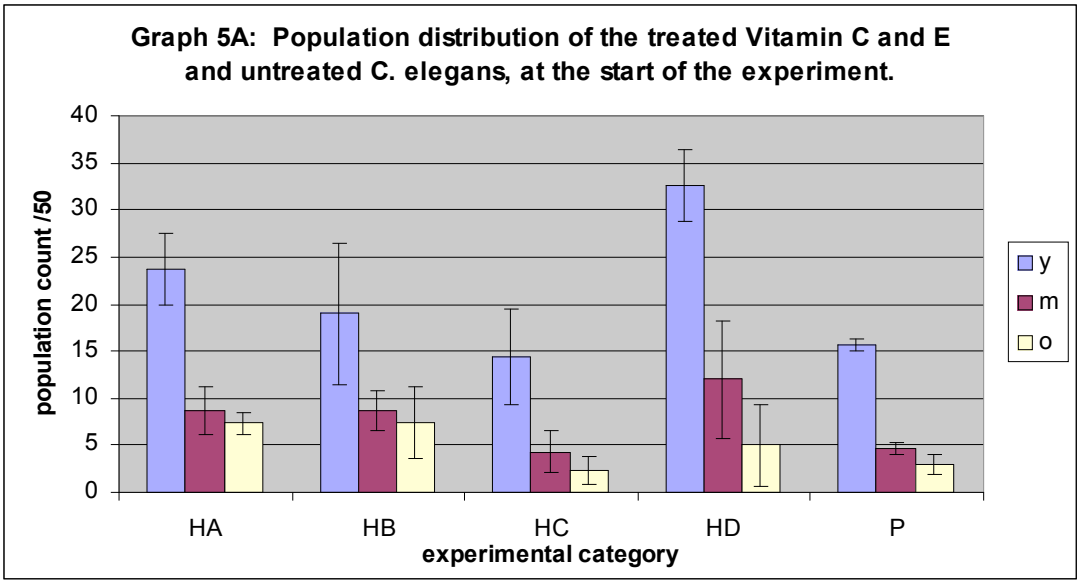
	y/m	y/o	m/o		y/m	y/o	m/o
ha sd	0.90	0.43	0.55		0.55	10.39	3.89
hb sd	1.20	2.84	0.78		1.77	2.12	0.91
hc sd	1.25	8.57	3.41		1.49	2.74	0.52
hd sd	1.86	6.24	4.57		3.63	9.02	1.64
p sd	0.53	2.15	0.70		7.62	11.32	2.17

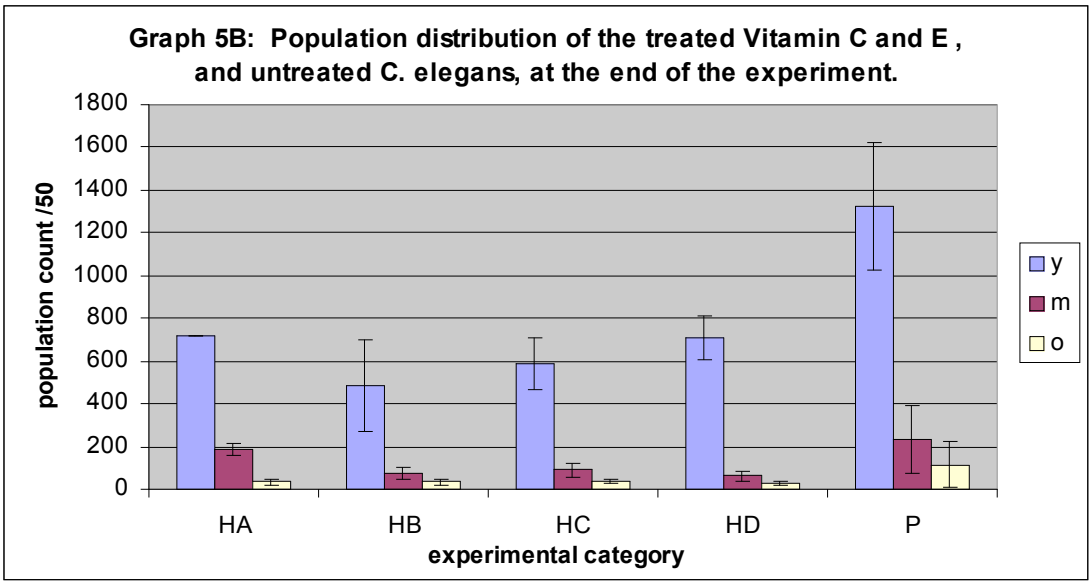
	standard error of mean						
	y/m	y/o	m/o		y/m	y/o	m/o
ha sd	0.30	0.14	0.29		0.16	1.22	0.88
hb sd	0.45	0.87	0.38		0.39	0.32	0.34
hc sd	0.38	1.60	1.12		0.33	0.39	0.19
hd sd	0.59	1.13	1.28		0.61	0.97	0.57
p sd	0.17	0.52	0.31		1.43	1.70	0.76

Appendix 4C – Average ratios for the population distribution of the dose response Vitamin C & E, and untreated *C. elegans*, at the start and end of the ten day experiment. Also included are the ratios of the pre-reproductive/reproductive (y/m) and the pre-reproductive/post-reproductive (y/o) *C. elegans*, at the various life stages.

Average nematode distribution for replicates of three populations for each experiment.						
start of experiment				end of experiment		
	y	m	o	Y	m	o
HA	23.66667	8.666667	7.333333	720	187	33.33333
HB	19	8.666667	7.333333	486.6667	73.33333	33.33333
HC	14.33333	4.333333	2.333333	586.6667	90	36.66667
HD	32.66667	12	5	706.6667	63.33333	26.66667
P	15.66667	4.666667	3	1323.333	234	113.3333
HA sd	3.785939	2.516611	1.154701	0	28.58321	11.54701
HB sd	7.549834	2.081666	3.785939	215.9475	30.5505	15.27525
HC sd	5.033223	2.309401	1.527525	124.231	30	5.773503
HD sd	3.785939	6.244998	4.358899	102.1437	23.09401	11.54701
P sd	0.57735	0.57735	1	295.0141	160.5864	106.9268
AVG	y/m-start	y/o-start		y/m-end	y/o-end	
HA	2.730769	3.227273		3.850267	21.6	
HB	2.192308	2.590909		6.636364	14.6	
HC	3.307692	6.142857		6.518519	16	
HD	2.722222	6.533333		11.15789	26.5	
P	3.357143	5.222222		5.655271	11.67647	
HA sd	1.50438	3.278719		0	0	
HB sd	3.626823	1.994178		7.068542	14.13708	
HC sd	2.179449	3.295018		4.141032	21.51743	
HD sd	0.606235	0.868554		4.422952	8.845903	
P sd	1	0.57735		1.837105	2.75903	

Appendix 4D – Tabulated average counts for the population distribution of the dose response Vitamin C & E, and untreated *C. elegans*, at the start and end of the ten day experiment. Also included are the ratios of the pre-reproductive/reproductive (y/m) and the pre-reproductive/post-reproductive (y/o) *C. elegans*, at the various life stages.

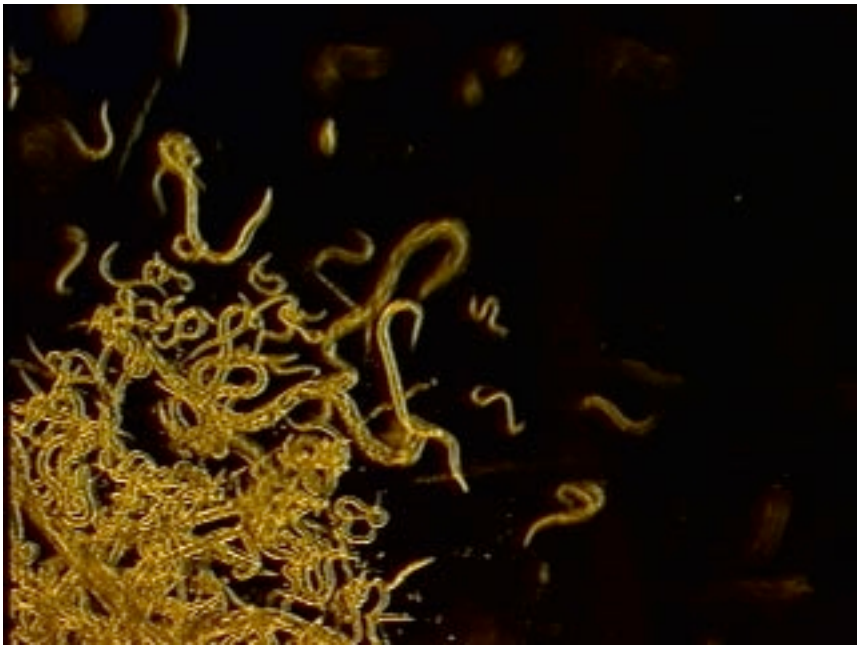




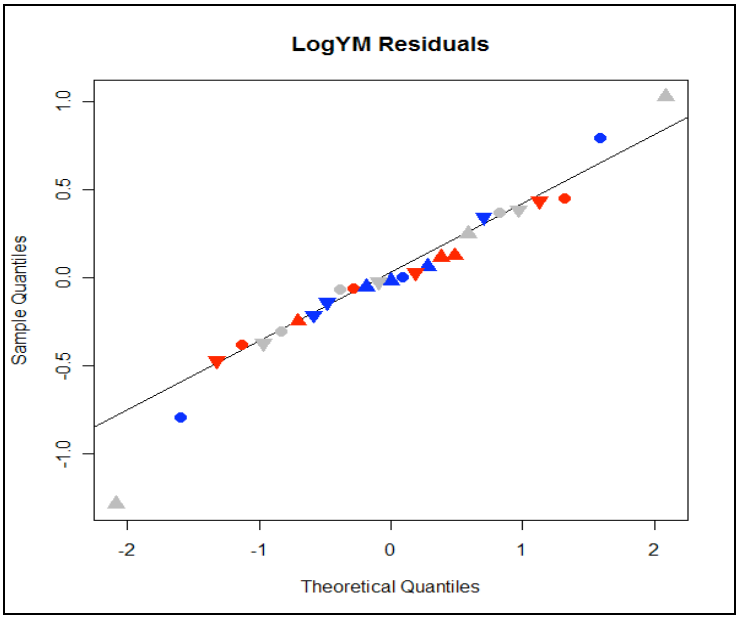
Appendix 4E – Graph 5A displays the population distribution of the various experimental categories for the Vitamin C & E dose response and untreated *C. elegans* at the start of the experiment. Graph 5B displays the population distribution of the various experimental categories for the Vitamin C & E dose response and untreated *C. elegans* at the end of the ten day incubation period.

pH avearges for each experimental group media at the start and end of the experiment.		
experimental group	pH average at start of experiment	pH average at end of experiment
p	6.38	7.89
ca	6.39	7.92
cb	6.33	7.94
cc	6.29	7.88
ea	6.34	7.91
eb	6.35	7.97
ec	6.34	7.92
ha	6.38	8.07
hb	6.37	8.07
hc	6.36	8.1
hd	6.36	8.19

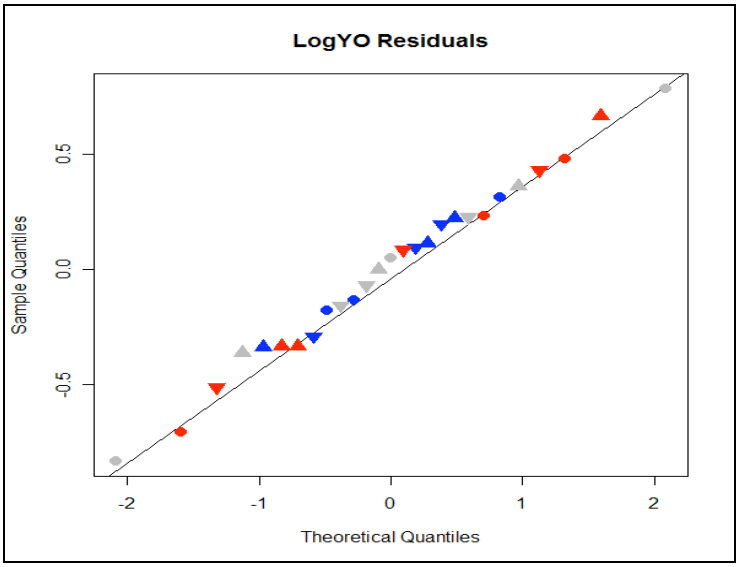
Appendix 4F – A table of the pH averages recorded at the start and end of each experimental category.



Appendix 4G – Photograph of untreated reproductive *C. elegans*, from the age fractionating process.



0.1 mg/ml Vitamin C
no Vitamin C/Vitamin E
0.1 mg/ml Vitamin E
0.5 mg/ml Vitamin C
0.5 mg/ml Vitamin E
0.1 mg/ml Vitamin C + 0.1 mg/ml Vitamin E
0.5 mg/ml Vitamin C + 0.1 mg/ml Vitamin E
0.5 mg/ml Vitamin C + 0.5 mg/ml Vitamin E
0.1 mg/ml Vitamin C + 0.5 mg/ml Vitamin E



Appendix 4H: QQ plots for log (base2) y/m and y/o population life state ratios.

APPENDIX 5

	cay avg	cby avg	ccy avg	py avg	eay avg	eby avg	ecy avg	hay avg	hby avg	hcy avg	hdy avg
tyr	3.264	22.820	6.940	2.382	12.490	4.497	9.825	64.927	46.200	50.848	46.365
trp	1.185	4.992	2.481	0.823	4.438	1.434	2.923	33.982	24.014	30.898	22.385
trp/tyr	0.363	0.219	0.357	0.346	0.355	0.319	0.297	0.523	0.520	0.608	0.483
tyr sd	0.543	16.584	4.987	1.351	11.074	0.973	8.450	26.197	0.714	1.563	5.885
trp sd	0.291	6.672	1.810	0.313	3.951	0.295	2.441	7.529	1.968	1.680	3.959
trp/tyr sd	0.536	0.402	0.363	0.232	0.357	0.303	0.289	0.287	2.756	1.075	0.673

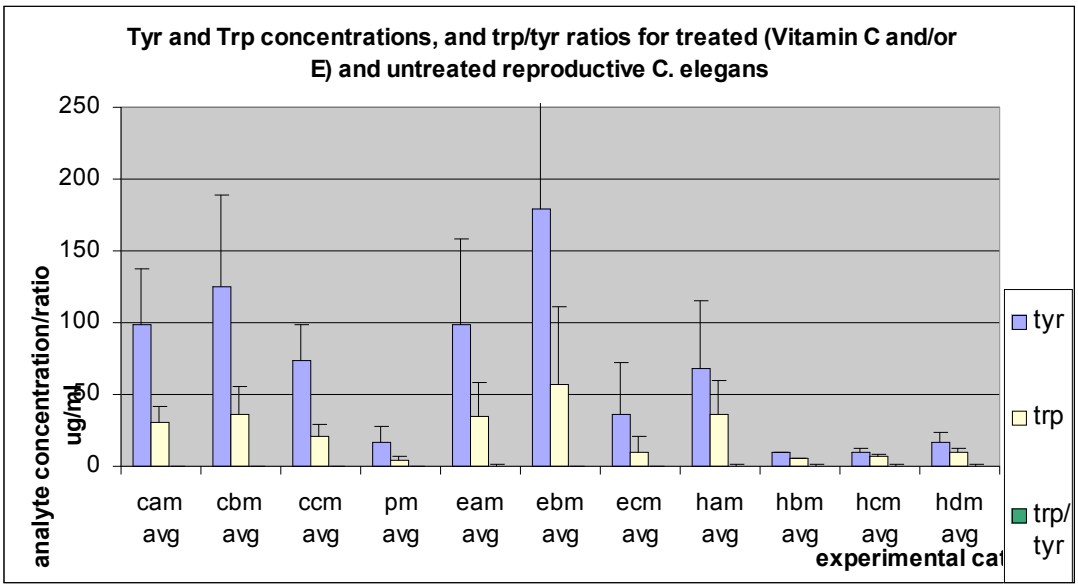
Appendix 5: Raw data generated from chromatographic data for the different experimental categories, of the pre-reproductive *C. elegans*. This data is plotted on Graphs, figure 7. This data was normalized with soluble protein data.

	Cam avg	cbm avg	ccm avg	pm avg	eam avg	ebm avg	ecm avg	ham avg	hbm avg	hcm avg	hdm avg
tyr	99.166	124.722	73.508	16.979	98.984	179.598	35.766	68.229	9.181	10.163	16.868
trp	30.206	36.534	20.586	4.525	35.355	57.498	10.070	35.461	5.500	6.729	9.300
trp/tyr	0.305	0.293	0.280	0.267	0.357	0.320	0.282	0.520	0.599	0.662	0.551
tyr sd	37.762	63.861	24.717	10.218	59.800	163.097	36.833	47.365	0.740	2.206	7.216
trp sd	11.731	19.190	8.179	2.748	23.257	53.276	10.970	23.719	0.142	1.323	3.818
trp/tyr sd	0.311	0.300	0.331	0.269	0.389	0.327	0.298	0.501	0.192	0.600	0.529

Appendix 5A: Raw data generated from chromatographic data for the different experimental categories, of the pre-reproductive *C. elegans*. This data is plotted on Graphs, figures 8 and 9. This data was normalized with soluble protein data.

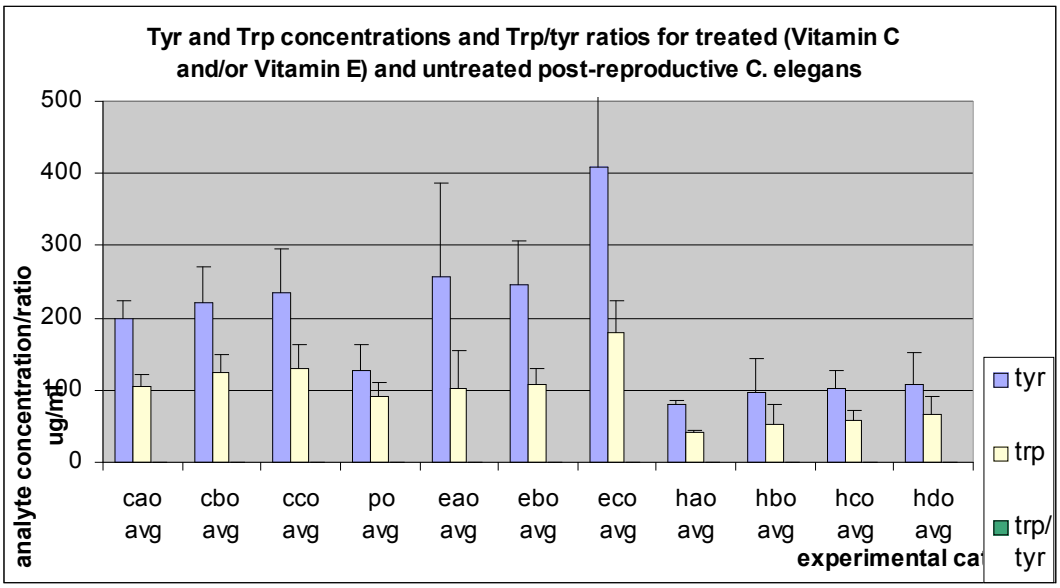
	Cao avg	cbo avg	cco avg	po avg	eao avg	ebo avg	eco avg	hao avg	hbo avg	hco avg	hdo avg
Tyr	198.688	222.051	235.518	127.393	256.224	245.370	408.943	80.920	95.598	101.858	108.291
Trp	105.750	124.938	129.517	91.520	101.744	106.592	180.493	41.392	52.698	56.945	65.277
trp/tyr	0.532	0.563	0.550	0.718	0.397	0.434	0.441	0.512	0.551	0.559	0.603
tyr sd	24.614	48.898	60.555	36.722	130.519	61.167	156.595	5.005	48.431	25.538	42.829
trp sd	14.866	25.052	33.825	18.529	53.642	23.884	43.207	3.097	27.432	13.785	25.599
trp/tyr sd	0.604	0.512	0.559	0.505	0.411	0.390	0.276	0.619	0.566	0.540	0.598

Appendix 5C: Raw data generated from chromatographic data for the different experimental categories, of the pre-reproductive *C. elegans*. This data is plotted on Graphs, figures 10 and 11. This data was normalized with soluble protein data.



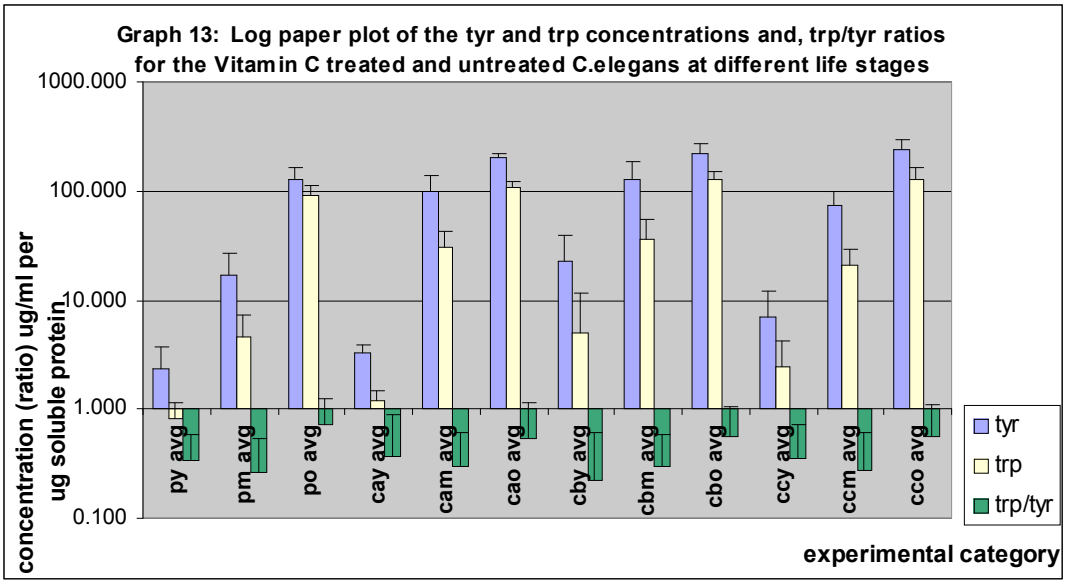
cam – 0.1 mg/ml Vitamin C treated reproductive *C. elegans*
cbm – 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
ccm – 1.0 mg/ml Vitamin C treated reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
eam – 0.1 mg/ml Vitamin E treated reproductive *C. elegans*
ebm – 0.5 mg/ml Vitamin E treated reproductive *C. elegans*
ecm – 1.0 mg/ml Vitamin E treated reproductive *C. elegans*
ham – 0.1 mg/ml Vitamin E and Vitamin C treated reproductive *C. elegans*
hbm – 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hcm - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hdm - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated reproductive *C. elegans*

Appendix 5C: A plot of Tyrosine and Tryptophan concentrations and trp/tyr ratios for the treated and untreated reproductive *C. elegans*, per μg soluble protein.



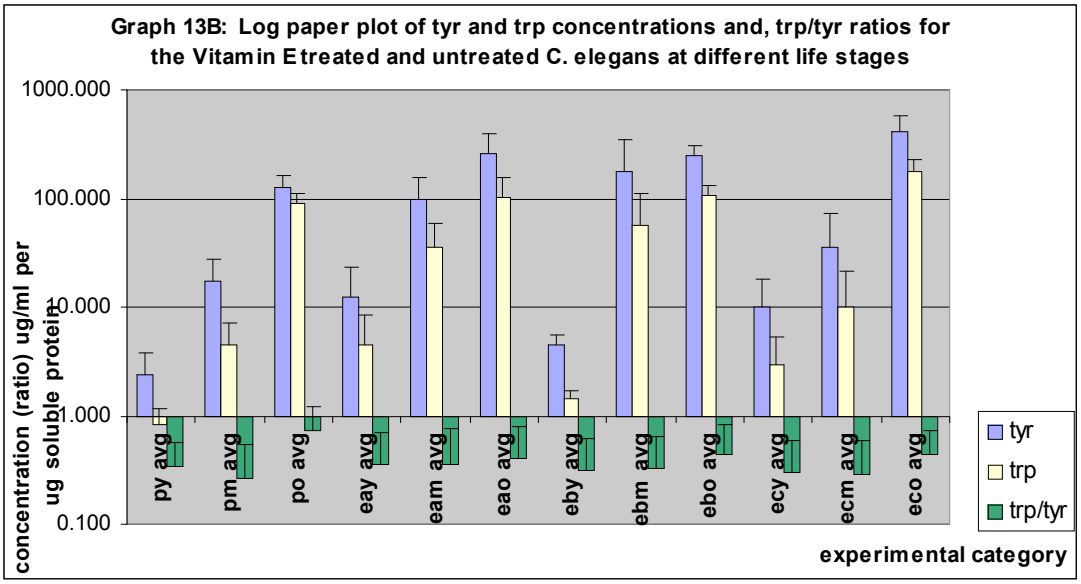
cao - 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*
cbo - 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
cco - 1.0 mg/ml Vitamin C treated post-reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
eao - 0.1 mg/ml Vitamin E treated post-reproductive *C. elegans*
ebo - 0.5 mg/ml Vitamin E treated post-reproductive *C. elegans*
eco - 1.0 mg/ml Vitamin E treated post-reproductive *C. elegans*
hao - 0.1 mg/ml Vitamin E and Vitamin C treated post-reproductive *C. elegans*
hbo - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hco - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hdo - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*

Appendix 5D: A plot of Tyrosine and Tryptophan concentrations and trp/tyr ratios for the treated and untreated post-reproductive *C. elegans*, per μg soluble protein.



py - untreated pre-reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
cay - 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*
cam - 0.1 mg/ml Vitamin C treated reproductive *C. elegans*
cao - 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*
cby - 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
cbm - 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
cbo - 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
ccy - 1.0 mg/ml Vitamin C treated pre-reproductive *C. elegans*
ccm - 1.0 mg/ml Vitamin C treated reproductive *C. elegans*
cco - 1.0 mg/ml Vitamin C treated post-reproductive *C. elegans*

Appendix 5E: Graph 13: A plot of Tyrosine and Tryptophan concentrations and trp/tyr ratios for the Vitamin C dose treated and untreated post-reproductive *C. elegans*, per μg soluble protein, on log paper.



py - untreated pre-reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
eay - 0.1 mg/ml Vitamin E treated pre-reproductive *C. elegans*
eam - 0.1 mg/ml Vitamin E treated reproductive *C. elegans*
eao - 0.1 mg/ml Vitamin E treated post-reproductive *C. elegans*
eby - 0.5 mg/ml Vitamin E treated pre-reproductive *C. elegans*
ebm - 0.5 mg/ml Vitamin E treated reproductive *C. elegans*
ebo - 0.5 mg/ml Vitamin E treated post-reproductive *C. elegans*
ecy - 1.0 mg/ml Vitamin E treated pre-reproductive *C. elegans*
ecm - 1.0 mg/ml Vitamin E treated reproductive *C. elegans*
eco - 1.0 mg/ml Vitamin E treated post-reproductive *C. elegans*

Appendix 5F: Graph 13B: A plot of Tyrosine and Tryptophan concentrations and trp/tyr ratios for the Vitamin E dose treated and untreated post-reproductive *C. elegans*, per μg soluble protein, on log paper.

APPENDIX 6

sample	cay avg	cby avg	ccy avg	py avg	eay avg	eby avg	ecy avg	hay avg	hby avg	hcy avg	hdy avg
tyr/norepi	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
tyr/epi	45.611	65.379	23.03	20.352	21.8441	51.09	37.893	543.399	201.221	182.779	659.621
epi/tyr	0.041109	0.0469	0.057	0.0498	0.04861	0.0406	0.0496	0.00489	0.00649	0.0073	0.00576
norepi/tyr	0	0	0	0	0	0	0	0	0	0	0
tyr avg	0.167281	0.307	0.058	0.0498	0.1364	0.2654	0.1731	1.09681	0.27758	0.15197	3.28958
norepi/epi av	#VALUE!	######	#####	######	#VALUE!	######	######	#VALUE!	#VALUE!	#VALUE!	#VALUE!
tyr sd	0.040387	0.3584	0.025	0.0776	0.09758	0.0868	0.2123	1.37839	0.14336	0.04434	5.36622
tyr/epi sd	44.99826	54.942	13.91	2.7906	5.93292	56.893	40.34	602.618	118.567	100.566	895.432
tyr/norepi sd	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
norepi/epi sd											
epi/tyr sd	0.022223	0.0182	0.072	0.3583	0.16855	0.0176	0.0248	0.00166	0.00843	0.00994	0.00112

cay - 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*
cby - 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
ccy - 1.0 mg/ml Vitamin C treated pre-reproductive *C. elegans*
py - untreated pre-reproductive *C. elegans*
eay - 0.1 mg/ml Vitamin E treated pre-reproductive *C. elegans*
eby - 0.5 mg/ml Vitamin E treated pre-reproductive *C. elegans*
ecy - 1.0 mg/ml Vitamin E treated pre-reproductive *C. elegans*
hay - 0.1 mg/ml Vitamin E and Vitamin C treated pre-reproductive *C. elegans*
hby - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hcy - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hdy - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*

Appendix 6A: Average raw data used to generate graph 7, Figure 14.

sample	cam avg	cbm avg	ccm avg	pm avg	eam avg	ebm avg	ecm avg	ham avg	hbm avg	hcm avg	hdm avg
tyr/norepi	1619.318	1357.5	#DIV/0!	#DIV/0!	1854.91	1561.7	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
tyr/epi	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	2467.98	#DIV/0!	#DIV/0!	#DIV/0!
epi/tyr	0.000106	7E-05	5E-04	0.0015	9.9E-05	6E-05	0.0026	0.0005	0.00178	0.00122	0
norepi/tyr	0.000622	0.0007	7E-04	0	0.00057	0.0007	0	0	0	0	0.00042
tyr avg	46.27175	46.392	22.19	7.5673	98.6357	45.504	14.885	26.4262	11.2489	7.23713	19.8497
norepi/epi av	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0	#DIV/0!	#DIV/0!	#DIV/0!
tyr sd	14.16251	18.416	14.23	5.7634	52.0435	29.19	6.9869	16.9497	3.78938	4.398	1.71248
tyr/epi sd											
tyr/norepi sd											
norepi/epi sd											
epi/tyr sd	9.21E-05	0.0001	5E-04	0.0027	8.7E-05	1E-04	0.0023	0.00031	0.00222	0.00212	0
norepi/tyr sd	5.97E-05	3E-05	6E-04	0	0.00018	0.0003	0	0	0	0	0.00073

cam - 0.1 mg/ml Vitamin C treated reproductive *C. elegans*
cbm - 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
ccm - 1.0 mg/ml Vitamin C treated reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
eam - 0.1 mg/ml Vitamin E treated reproductive *C. elegans*
ebm - 0.5 mg/ml Vitamin E treated reproductive *C. elegans*
ecm - 1.0 mg/ml Vitamin E treated reproductive *C. elegans*
ham - 0.1 mg/ml Vitamin E and Vitamin C treated reproductive *C. elegans*
hbm - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hcm - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hdm - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated reproductive *C. elegans*

Appendix 6B – Raw data used to generate Graph 8.

sample	cao avg	cbo avg	cco avg	po avg	eao avg	ebo avg	eco avg	hao avg	hbo avg	hco avg	hdo avg
tyr/norepi	1261.992	1698.4	1975	837.78	#DIV/0!	617.51	463.59	542.675	565.452	596.812	2396.84
tyr/epi	5169.387	#DIV/0!	#DIV/0!	#DIV/0!	8868.79	6365.7	5497	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
epi/tyr	0.000199	0.0002	5E-05	0.0002	0.00016	0.0004	0.0005	0.0002	0.00019	0.00013	0
norepi/tyr	0.000815	0.0007	6E-04	0.0018	0	0.003	0.0035	0.00187	0.00184	0.00171	0.00048
tyr avg	93.92781	91.759	85.12	166.22	103.122	113.81	69.687	73.7756	86.4122	93.6558	101.372
norepi/epi av	4.213502	#DIV/0!	#DIV/0!	#DIV/0!	0	18.96	19.113	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
tyr sd	6.465159	18.943	14.17	37.422	58.6637	16.757	19.831	11.4425	40.735	18.9105	39.6834
tyr/epi sd											
tyr/norepi sd											
norepi/epi sd											
epi/tyr sd	3.88E-05	0.0001	9E-05	0.0002	0.00012	0.0003	0.0003	0.00018	0.00018	0.00013	0
norepi/tyr sd	0.000168	0.0004	4E-04	0.0013	0	0.002	0.0024	0.00029	0.00043	0.00029	0.00022

cao - 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*
cbo - 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
cco - 1.0 mg/ml Vitamin C treated post-reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
eao - 0.1 mg/ml Vitamin E treated post-reproductive *C. elegans*
ebo - 0.5 mg/ml Vitamin E treated post-reproductive *C. elegans*
eco - 1.0 mg/ml Vitamin E treated post-reproductive *C. elegans*
hao - 0.1 mg/ml Vitamin E and Vitamin C treated post-reproductive *C. elegans*
hbo - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hco - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hdo - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*

Appendix 6C – Raw data used to generate graph 9.

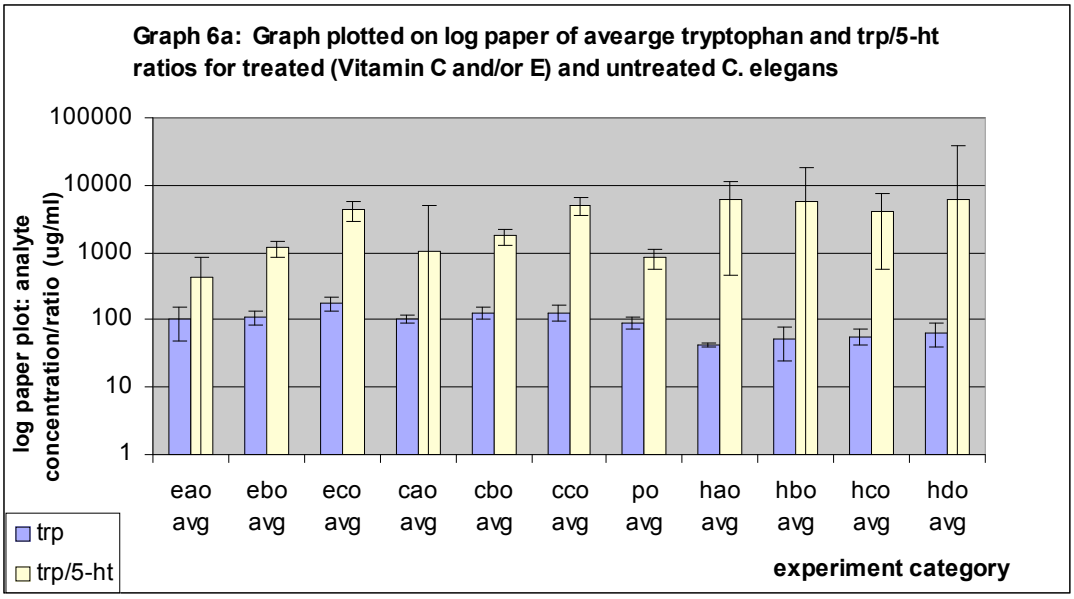
APPENDIX 7

sample	eao avg	ebo avg	eco avg	cao avg	cbo avg	cco avg
trp	101.7444	106.5923	180.4926	105.7498	124.9379	129.5172
5-ht	0.243333	0.091833	0.041717	0.102833	0.0715	0.025793
5-ht/trp	0.002392	0.000862	0.000231	0.000972	0.000572	0.000199
trp/5-ht	418.1277	1160.715	4326.631	1028.361	1747.383	5021.342
trp sd	53.64235	23.88435	43.20661	14.86634	25.05201	33.82485
5-ht sd	0.083291	0.08125	0.030644	0.003753	0.051728	0.021924
trp/5-ht sd	453.0029	293.9621	1409.934	3961.424	484.3024	1542.803
5-ht/trp sd	0.001553	0.003402	0.000709	0.000252	0.002065	0.000648

sample	po avg	hao avg	hbo avg	hco avg	hdo avg
trp	91.51967	41.39243	52.69815	56.94542	65.27679
5-ht	0.109933	0.006977	0.00907	0.0139	0.010933
5-ht/trp	0.001201	0.000169	0.000172	0.000244	0.000167
trp/5-ht	832.5015	5932.982	5810.159	4096.793	5970.439
trp sd	18.52851	3.097003	27.43174	13.7849	25.59888
5-ht sd	0.065247	0.000567	0.002278	0.003897	0.000777
trp/5-ht sd	283.9735	5462.56	12040.39	3536.915	32956.59
5-ht/trp sd	0.003521	0.000183	8.31E-05	0.000283	3.03E-05

eao - 0.1 mg/ml Vitamin E treated post-reproductive *C. elegans*
ebo - 0.5 mg/ml Vitamin E treated post-reproductive *C. elegans*
eco - 1.0 mg/ml Vitamin E treated post-reproductive *C. elegans*
cao - 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*
cbo - 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
cco - 1.0 mg/ml Vitamin C treated post-reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
hao - 0.1 mg/ml Vitamin E and Vitamin C treated post-reproductive *C. elegans*
hbo - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hco - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hdo - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*

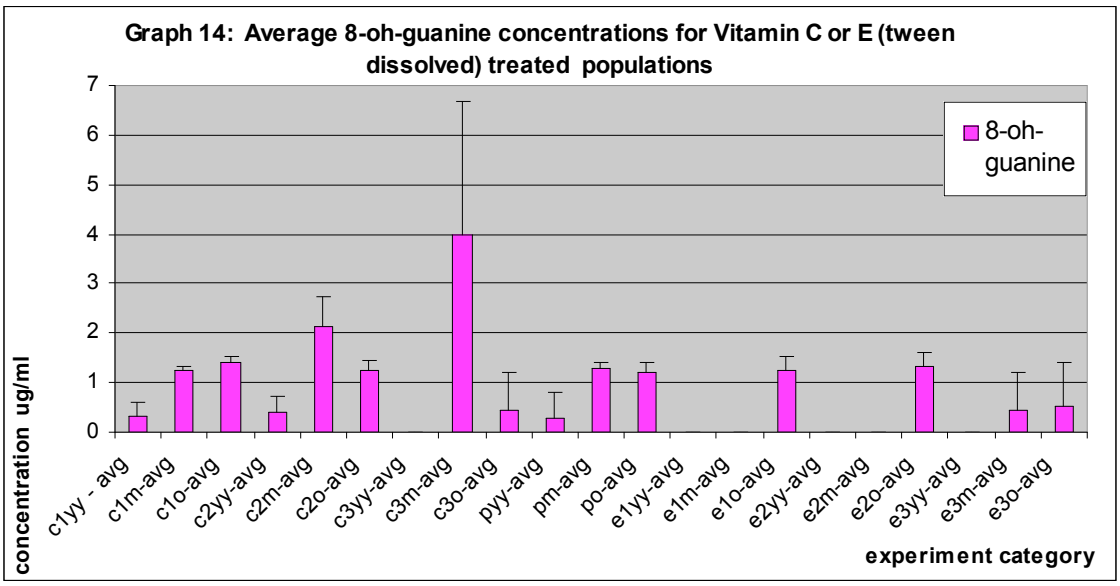
Appendix 7 – Average tryptophan (trp) and serotonin (5-ht) concentrations and, trp/5-ht ratios for all post-reproductive Vitamin C and/or E dose treated worms.



cao - 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*
cbo - 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
cco - 1.0 mg/ml Vitamin C treated post-reproductive *C. elegans*
Po - untreated post-reproductive *C. elegans*
eao - 0.1 mg/ml Vitamin E treated post-reproductive *C. elegans*
ebo - 0.5 mg/ml Vitamin E treated post-reproductive *C. elegans*
eco - 1.0 mg/ml Vitamin E treated post-reproductive *C. elegans*
hao - 0.1 mg/ml Vitamin E and Vitamin C treated post-reproductive *C. elegans*
hbo - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hco - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hdo - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*

Appendix 7B: Graph 6a - a plot on log paper of the average tryptophan (trp) concentrations and tryptophan/serotonin (trp/5-ht) ratios for the Vitamin C and/or E treated and untreated post-reproductive (o) *C. elegans*, per μg soluble protein.

APPENDIX 8



c1yy - 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*
c1m - 0.1 mg/ml Vitamin C treated reproductive *C. elegans*
c1o - 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*
c2yy - 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
c2m - 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
c2o - 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
c3yy - 1.0 mg/ml Vitamin C treated pre-reproductive *C. elegans*
c3m - 1.0 mg/ml Vitamin C treated reproductive *C. elegans*
c3o - 1.0 mg/ml Vitamin C treated post-reproductive *C. elegans*
pyy - untreated pre-reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
e1yy - 0.1 mg/ml Vitamin E treated pre-reproductive *C. elegans*
e1m - 0.1 mg/ml Vitamin E treated reproductive *C. elegans*
e1o - 0.1 mg/ml Vitamin E treated post-reproductive *C. elegans*
e2yy - 0.5 mg/ml Vitamin E treated pre-reproductive *C. elegans*
e2m - 0.5 mg/ml Vitamin E treated reproductive *C. elegans*
e2o - 0.5 mg/ml Vitamin E treated post-reproductive *C. elegans*
e3yy - 1.0 mg/ml Vitamin E treated pre-reproductive *C. elegans*
e3m - 1.0 mg/ml Vitamin E treated reproductive *C. elegans*
e3o - 1.0 mg/ml Vitamin E treated post-reproductive *C. elegans*

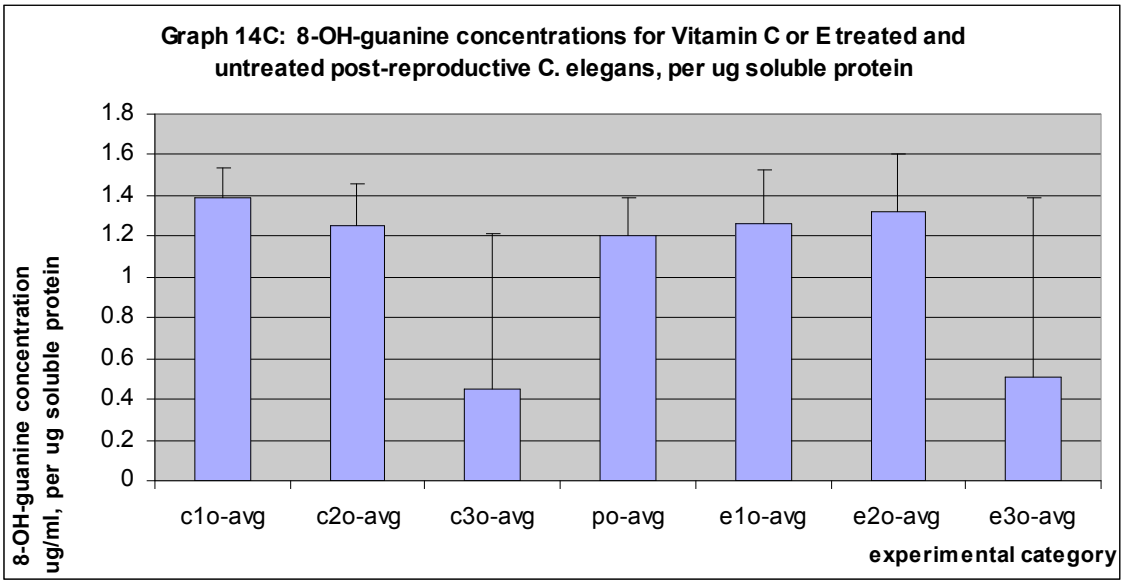
Appendix 8: Graph 14: A plot of 8-OH-guanine concentrations for the Vitamin C, and E dose treated and untreated *C. elegans* at different life stages, per μg soluble protein.

sample desc	c1yy - avg	c2yy-avg	c3yy-avg	pyy-avg	e1yy-avg	e2yy-avg	e3yy-avg
8-oh-guanine	0.321798	0.396114	0	0.294451	0	0	0
8-oh-guanine sd	0.285539	0.343115	0	0.510004	0	0	0

sample desc	c1m-avg	c2m-avg	c3m-avg	pm-avg	e1m-avg	e2m-avg	e3m-avg
8-oh-guanine	1.246949	2.129477	3.991021	1.280204	0	0	0.4448
8-oh-guanine sd	0.097314	0.603039	2.702752	0.137698	0	0	0.770415

sample desc	c1o-avg	c2o-avg	c3o-avg	po-avg	e1o-avg	e2o-avg	e3o-avg
8-oh-guanine	1.392729	1.251704	0.445728	1.203698	1.257228	1.315913	0.508838
8-oh-guanine sd	0.145336	0.201481	0.772024	0.186926	0.26565	0.288049	0.881333

Appendix 8A: Data used to generate Figures 16, 17, Appendix 8 and 8B. It's the 8-OH-Guanine concentrations for all the life stages of the various experimental categories (Vitamin C and E treated *C. elegans*).



c1o - 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*
c2o - 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
c3o - 1.0 mg/ml Vitamin C treated post-reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
e1o - 0.1 mg/ml Vitamin E treated post-reproductive *C. elegans*
e2o - 0.5 mg/ml Vitamin E treated post-reproductive *C. elegans*
e3o - 1.0 mg/ml Vitamin E treated post-reproductive *C. elegans*

Appendix 8B: Graph 14C – A plot of 8-OH-guanine concentrations for Vitamin C or E treated and untreated post-reproductive *C. elegans*, per μg soluble protein.

APPENDIX 9

	cay avg	cby avg	ccy avg	py avg	eay avg	eby avg	ecy avg	hay avg	hby avg	hcy avg	hdy avg
uric	6.63	7.61	8.61	3.98	3.98	106.21	78.79	49.45	172.95	78.11	121.84
	0.21	0.15	0.00	0.05	0.05	999.00	0.00	0.38	1.28	0.35	0.05
hx	1.49	3.97	2.27	1.72	1.72	6.85	8.84	17.52	27.33	9.87	20.29
xanthine	1.91	2.94	2.98	0.95	0.95	24.61	22.27	31.70	48.17	13.97	33.72
guanine	0.56	0.63	1.08	0.76	0.76	3.42	5.19	2.63	1.61	0.68	0.99
xanthonine	3.24	10.32	5.84	1.59	1.59	32.87	25.84	45.47	9.56	4.21	3.73
guanosine	3.04	2.67	5.15	0.68	0.68	11.55	6.73	35.91	49.88	19.36	30.96
uric sd	1.41	2.77	4.20	0.51	0.51	13.43	14.56	14.99	150.79	69.20	39.88
	0.09	0.14	0.00	0.08	0.08	0.00	0.00	0.29	1.93	0.27	0.08
hx sd	0.38	3.00	0.66	1.27	1.27	1.97	3.30	9.84	22.95	8.84	7.83
xanthine sd	0.78	1.81	1.38	0.07	0.07	6.87	4.44	20.18	50.34	10.18	9.92
guanine sd	0.27	0.19	0.20	0.19	0.19	2.71	0.71	3.50	1.01	0.23	0.86
xanthosine sd	3.29	14.16	1.85	0.71	0.71	7.33	7.11	70.35	5.93	1.19	2.16
guanosine sd	1.47	2.71	3.86	0.32	0.32	7.69	0.18	20.96	53.81	26.64	15.75
	cay avg	cby avg	ccy avg	p y avg	eay avg	eby avg	ecy avg	hay avg	hby avg	hcy avg	hdy avg
uric/xan	3.47	2.59	2.89	4.19	4.19	4.32	3.54	1.56	3.59	5.59	3.61
xan/guan	3.40	4.70	2.75	1.25	1.25	7.20	4.29	12.07	29.94	20.70	34.12
guan/guano	0.19	0.23	0.21	1.12	1.12	0.30	0.77	0.07	0.03	0.03	0.03
xan/hx	1.28	0.74	1.32	0.55	0.55	3.59	2.52	1.81	1.76	1.42	1.66
xan/xantho	0.59	0.29	0.51	0.60	0.60	0.75	0.86	0.70	5.04	3.32	9.03
guan/xantho	0.17	0.06	0.19	0.48	0.48	0.10	0.20	0.06	0.17	0.16	0.26
uric/xanthine sd	1.81	1.54	3.03	7.35	7.35	1.96	3.28	0.74	3.00	6.80	4.02
xanthine/guan sd	2.84	9.65	6.79	0.36	0.36	2.53	6.29	5.76	50.06	43.40	11.53
guan/guano sd	0.19	0.07	0.05	0.60	0.60	0.35	4.03	0.17	0.02	0.01	0.05
xanthine/hx sd	2.04	0.60	2.10	0.05	0.05	3.50	1.34	2.05	2.19	1.15	1.27
xanthine/xanthosine sd	0.24	0.13	0.75	0.10	0.10	0.94	0.62	0.29	8.49	8.55	4.60
guan/xanthosine sd	0.08	0.01	0.11	0.27	0.27	0.37	0.10	0.05	0.17	0.20	0.40

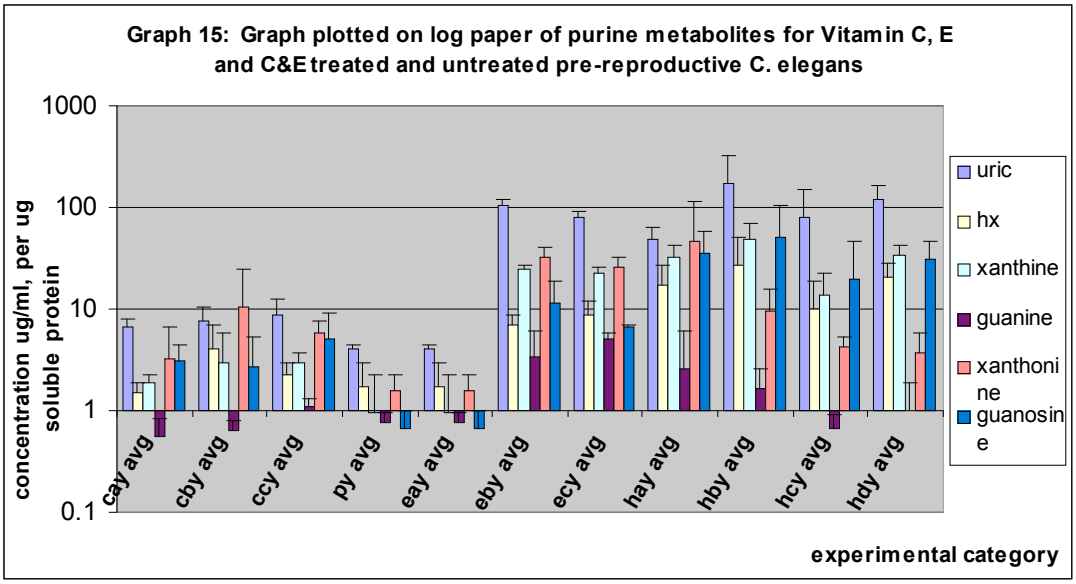
Appendix 9A: Data obtained from experiment that is used to generate Graphs 15 to 29 in this section. It's data for the purine catabolic metabolites for the pre-reproductive nematodes.

	cam avg	cbm avg	ccm avg	pm avg	eam avg	ebm avg	ecm avg	ham avg	hbm avg	hcm avg	hdm avg
uric	146.76	155.88	80.16	27.39	47.55	162.42	60.64	75.87	12.50	14.04	25.46
		900.00									
hx	20.10	27.19	21.04	5.21	20.56	28.62	9.50	18.21	2.48	2.60	6.11
xanthine	27.38	46.34	36.69	12.09	37.75	54.37	14.16	34.51	5.57	6.35	12.28
guanine	1.23	0.96	0.39	0.20	5.85	1.20	1.00	2.77	0.16	0.17	0.33
xanthonine	5.15	4.30	5.69	2.07	109.89	7.47	6.09	77.15	8.16	8.84	16.24
guanosine	39.99	40.19	20.23	3.77	44.25	57.79	10.02	19.17	2.02	2.14	4.20
uric sd	5.17	102.72	58.26	16.60	11.69	159.46	43.56	46.91	2.59	2.00	9.39
	0.12	0.81	0.77	0.11	0.28	1.82	0.29	0.00	0.00	0.00	0.00
hx sd	7.84	10.31	8.73	3.03	13.03	22.32	8.24	12.06	0.45	0.33	2.37
xanthine sd	6.71	9.50	10.99	6.80	26.88	46.98	10.45	22.75	0.90	0.84	4.66
guanine sd	0.58	0.89	0.29	0.11	4.95	1.08	0.78	2.05	0.03	0.04	0.17
xanthosine sd	0.44	4.00	4.72	1.57	97.75	6.18	4.41	62.16	1.87	1.88	7.27
guanosine sd	16.55	24.00	7.88	2.57	29.88	49.21	10.67	13.43	0.21	0.43	1.97
	cam avg	cbm avg	ccm avg	pm avg	eam avg	ebm avg	ecm avg	ham avg	hbm avg	hcm avg	hdm avg
uric/xan	5.36	3.36	2.18	2.27	1.26	2.99	4.28	2.20	2.24	2.21	2.07
xan/guan	22.20	48.13	93.66	59.66	6.46	45.23	14.19	12.44	34.53	36.33	37.14
guan/guano	0.03	0.02	0.02	0.05	0.13	0.02	0.10	0.14	0.08	0.08	0.08
xan/hx	1.36	1.70	1.74	2.32	1.84	1.90	1.49	1.89	2.25	2.44	2.01
xan/xantho	5.31	10.77	6.44	5.84	0.34	7.28	2.33	0.45	0.68	0.72	0.76
guan/xantho	0.24	0.22	0.07	0.10	0.05	0.16	0.16	0.04	0.02	0.02	0.02
uric/xanthine sd	0.77	10.81	5.30	2.44	0.44	3.39	4.17	2.06	2.89	2.39	2.02
xanthine/guan sd	11.66	10.64	38.38	60.21	5.43	43.59	13.40	11.11	27.13	20.05	28.22
guan/guano sd	0.03	0.04	0.04	0.04	0.17	0.02	0.07	0.15	0.16	0.10	0.08
xanthine/hx sd	0.86	0.92	1.26	2.25	2.06	2.11	1.27	1.89	1.97	2.50	1.96
xanthine/xanthosine sd	15.38	2.37	2.33	4.34	0.27	7.60	2.37	0.37	0.48	0.45	0.64
guan/xanthosine sd	1.32	0.22	0.06	0.07	0.05	0.17	0.18	0.03	0.02	0.02	0.02

Appendix 9B: Data obtained from experiment that is used to generate Graphs 15 to 29 in this section. It's data for the purine catabolic metabolites, the reproductive life stage of the nematode *C. elegans*.

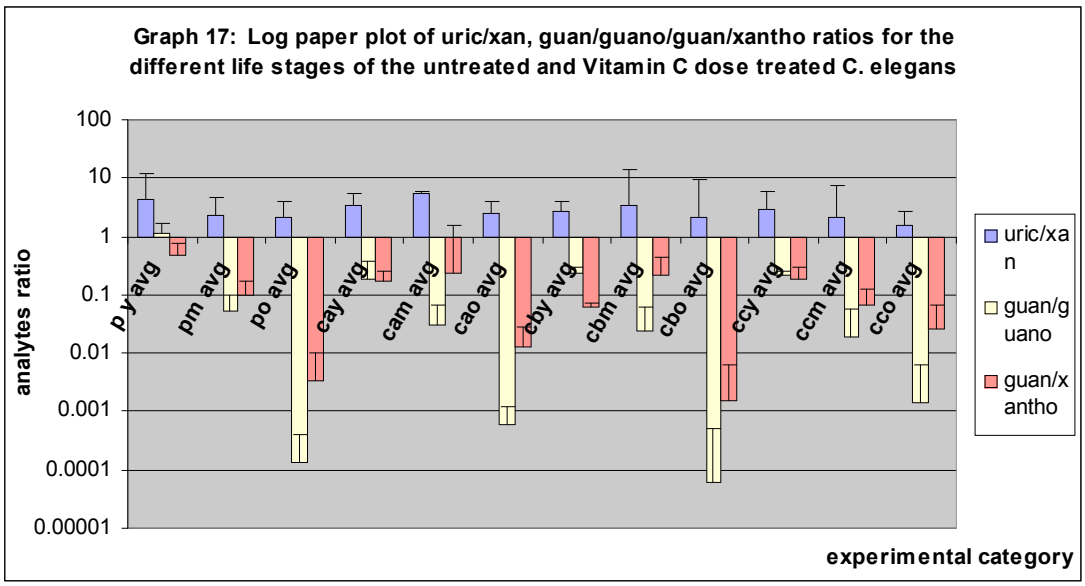
	cao avg	cbo avg	cco avg	po avg	eao avg	ebo avg	eco avg	hao avg	hbo avg	hco avg	hdo avg
uric	202.05	193.28	152.09	117.29	256.51	191.13	308.18	70.22	91.11	90.95	107.97
					1000.00						
hx	230.82	388.31	506.22	400.93	166.31	614.38	581.25	34.53	38.94	41.93	67.32
xanthine	84.05	91.40	98.31	53.43	112.01	108.30	167.05	44.28	49.67	50.16	57.74
guanine	0.18	0.03	0.71	0.04	3.04	2.08	7.41	2.45	3.41	4.64	4.92
xanthonine	14.26	17.18	26.61	12.44	36.94	37.90	128.71	111.80	134.78	153.50	158.79
guanosine	302.81	416.41	504.84	310.66	396.31	621.99	1114.16	37.18	47.59	62.70	75.61
uric sd	19.04	91.56	33.13	30.16	125.92	101.04	30.41	7.19	54.18	19.66	43.51
	0.00	0.00	0.89	0.00	2.77	2.39	4.59	0.00	0.00	0.00	0.00
hx sd	94.20	222.00	414.00	207.46	119.88	220.52	480.04	1.99	19.79	11.57	18.57
xanthine sd	12.09	12.33	28.66	16.15	50.57	14.85	66.57	1.83	25.28	12.74	22.19
guanine sd	0.03	0.04	1.02	0.04	3.87	1.44	9.06	0.34	1.71	1.45	2.08
xanthosine sd	2.06	9.38	25.73	5.52	44.28	20.69	157.91	7.65	67.78	40.16	71.42
guanosine sd	50.83	97.46	213.60	140.17	290.88	214.61	516.01	4.08	24.32	16.46	26.51
	cao avg	cbo avg	cco avg	po avg	eao avg	ebo avg	eco avg	hao avg	hbo avg	hco avg	hdo avg
uric/xan	2.40	2.11	1.55	2.20	2.29	1.76	1.84	1.59	1.83	1.81	1.87
xan/guan	459.77	3577.57	137.58	1282.02	36.85	52.01	22.53	18.05	14.56	10.82	11.74
guan/guano	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.07	0.07	0.07	0.07
xan/hx	0.36	0.24	0.19	0.13	0.67	0.18	0.29	1.28	1.28	1.20	0.86
xan/xantho	5.89	5.32	3.69	4.30	3.03	2.86	1.30	0.40	0.37	0.33	0.36
guan/xantho	0.01	0.00	0.03	0.00	0.08	0.05	0.06	0.02	0.03	0.03	0.03
uric/xanthine sd	1.57	7.43	1.16	1.87	2.49	6.81	0.46	3.94	2.14	1.54	1.96
xanthine/guan sd	363.61	278.64	28.00	439.00	13.07	10.32	7.34	5.37	14.80	8.80	10.68
guan/guano sd	0.00	0.00	0.00	0.00	0.01	0.01	0.02	0.08	0.07	0.09	0.08
xanthine/hx sd	0.13	0.06	0.07	0.08	0.42	0.07	0.14	0.92	1.28	1.10	1.19
xanthine/xanthosine sd	5.87	1.31	1.11	2.93	1.14	0.72	0.42	0.24	0.37	0.32	0.31
guan/xanthosine sd	0.02	0.00	0.04	0.01	0.09	0.07	0.06	0.04	0.03	0.04	0.03

Appendix 9C: Data obtained from experiment that is used to generate Graphs 15 to 29 in this section. It's data for the purine catabolic metabolites, the post-reproductive life stage of the nematode *C. elegans*.



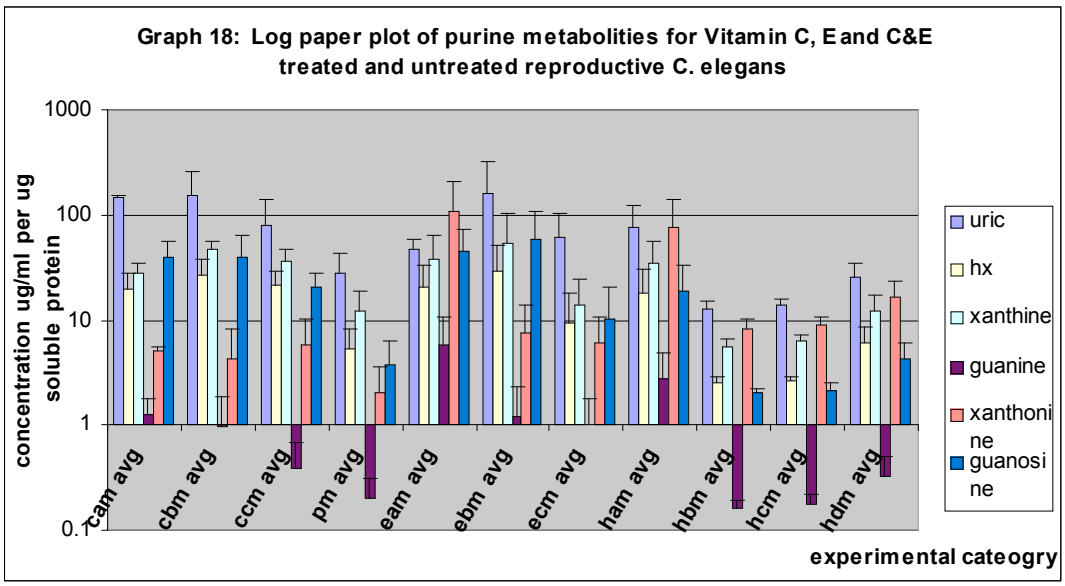
cay - 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*
cby - 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
ccy - 1.0 mg/ml Vitamin C treated pre-reproductive *C. elegans*
py - untreated pre-reproductive *C. elegans*
eay - 0.1 mg/ml Vitamin E treated pre-reproductive *C. elegans*
eby - 0.5 mg/ml Vitamin E treated pre-reproductive *C. elegans*
ecy - 1.0 mg/ml Vitamin E treated pre-reproductive *C. elegans*
hay - 0.1 mg/ml Vitamin E and Vitamin C treated pre-reproductive *C. elegans*
hby - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hcy - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hdy - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*

Appendix 9D: Graph 15 - Log paper plot of purine catabolic metabolites in the pre-reproductive life stages of all the experimental categories for the *C. elegans.* The data is per μg soluble protein.



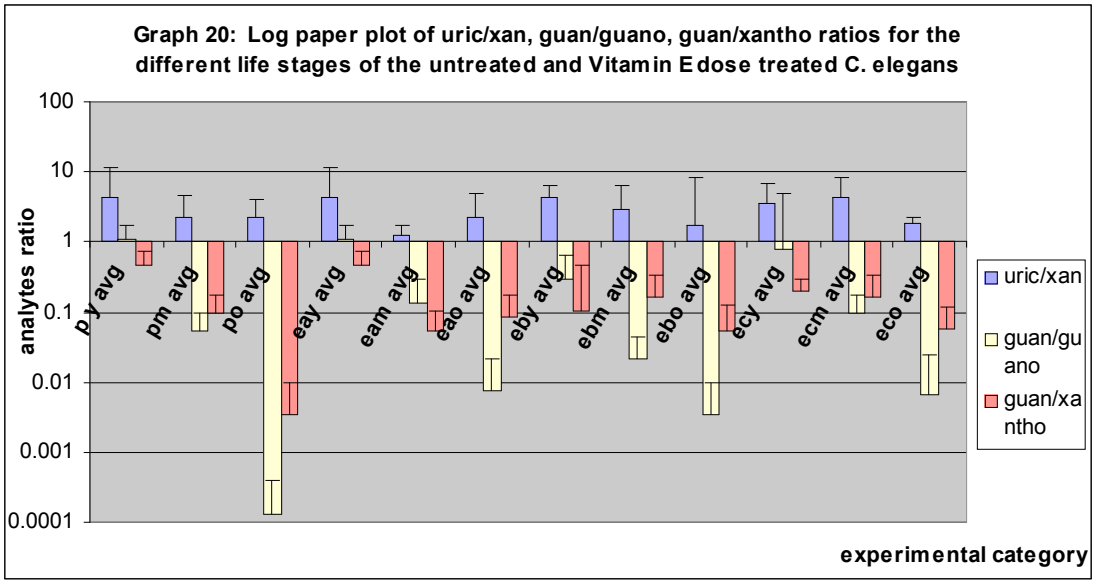
py - untreated pre-reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
cay - 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*
cam - 0.1 mg/ml Vitamin C treated reproductive *C. elegans*
cao - 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*
cby - 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
cbm - 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
cbo - 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
ccy - 1.0 mg/ml Vitamin C treated pre-reproductive *C. elegans*
ccm - 1.0 mg/ml Vitamin C treated reproductive *C. elegans*
cco - 1.0 mg/ml Vitamin C treated post-reproductive *C. elegans*

Appendix 9E: Graph 17 – Log paper plot of uric acid/xanthine, guanine/guanosine, guanine/xanthosine ratios for the Vitamin C dose treatment and untreated *C. elegans* in the pre-reproductive, reproductive and post-reproductive life stages.



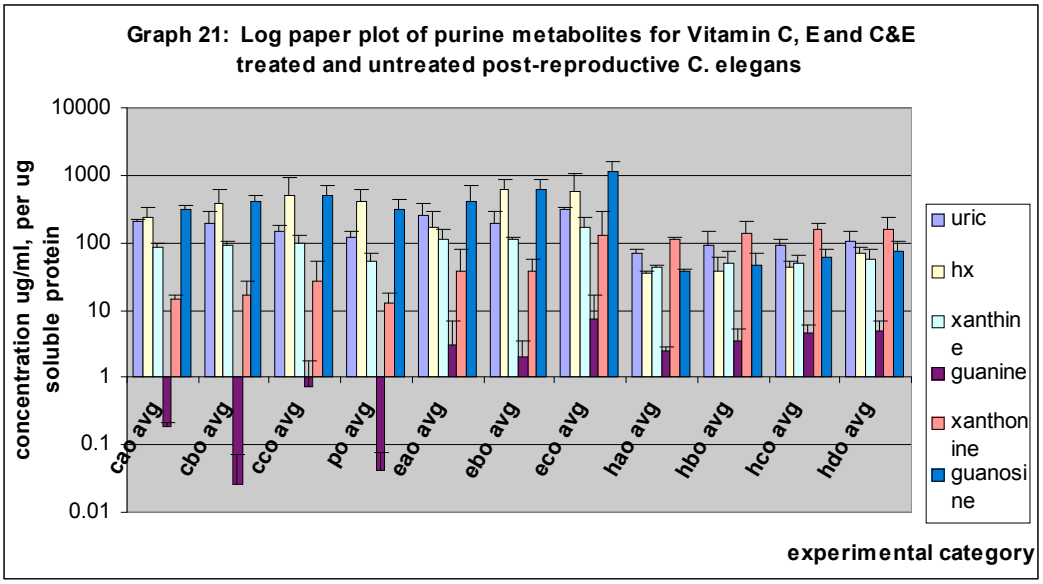
cam - 0.1 mg/ml Vitamin C treated reproductive *C. elegans*
cbm - 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
ccm - 1.0 mg/ml Vitamin C treated reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
eam - 0.1 mg/ml Vitamin E treated reproductive *C. elegans*
ebm - 0.5 mg/ml Vitamin E treated reproductive *C. elegans*
ecm - 1.0 mg/ml Vitamin E treated reproductive *C. elegans*
ham - 0.1 mg/ml Vitamin E and Vitamin C treated reproductive *C. elegans*
hbm - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hcm - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hdm - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated reproductive *C. elegans*

APPENDIX 9F: Graph 18- Log paper plot of purine metabolites for Vitamin C, E, C and E treated and untreated reproductive *C. elegans*. The data is per μg soluble protein.



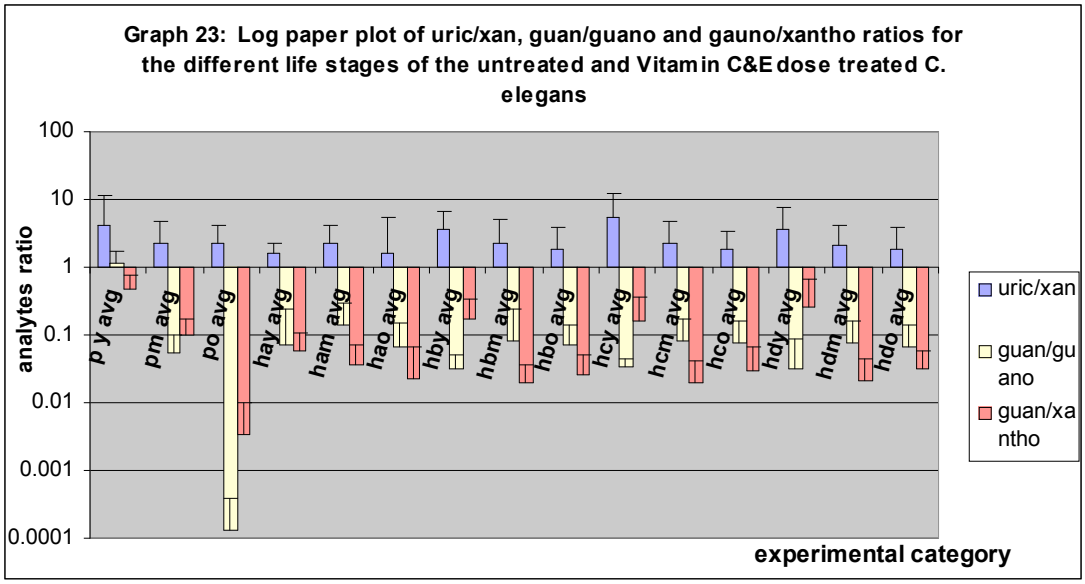
py - untreated pre-reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
eay - 0.1 mg/ml Vitamin E treated pre-reproductive *C. elegans*
eam - 0.1 mg/ml Vitamin E treated reproductive *C. elegans*
eao - 0.1 mg/ml Vitamin E treated post-reproductive *C. elegans*
eby - 0.5 mg/ml Vitamin E treated pre-reproductive *C. elegans*
ebm - 0.5 mg/ml Vitamin E treated reproductive *C. elegans*
ebo - 0.5 mg/ml Vitamin E treated post-reproductive *C. elegans*
ecy - 1.0 mg/ml Vitamin E treated pre-reproductive *C. elegans*
ecm - 1.0 mg/ml Vitamin E treated reproductive *C. elegans*
eco - 1.0 mg/ml Vitamin E treated post-reproductive *C. elegans*

Appendix 9G: Graph 20 – Log paper plot of uric acid/xanthine, guanine/guanosine, guanine/xanthosine ratios for the Vitamin E dose treatment and untreated *C. elegans* in the pre-reproductive, reproductive and post-reproductive life stages.



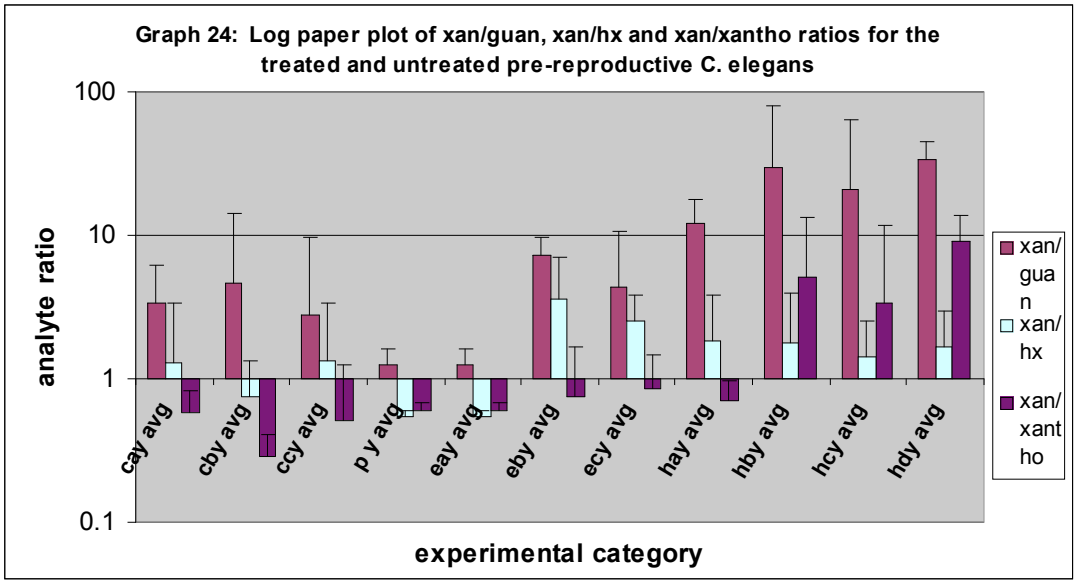
cao - 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*
cbo - 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
cco - 1.0 mg/ml Vitamin C treated post-reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
eao - 0.1 mg/ml Vitamin E treated post-reproductive *C. elegans*
ebo - 0.5 mg/ml Vitamin E treated post-reproductive *C. elegans*
eco - 1.0 mg/ml Vitamin E treated post-reproductive *C. elegans*
hao - 0.1 mg/ml Vitamin E and Vitamin C treated post-reproductive *C. elegans*
hbo - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hco - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hdo - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*

APPENDIX 9H: Graph 21- Log paper plot of purine metabolites for Vitamin C, E, C and E treated and untreated post-reproductive *C. elegans*. The data is per μg soluble protein.



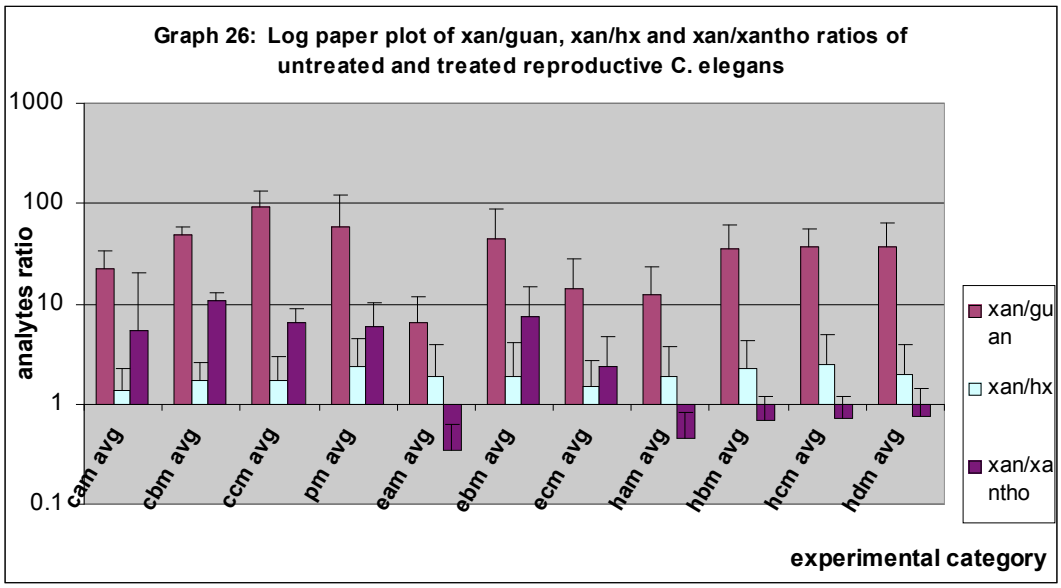
py - untreated pre-reproductive *C. elegans*
pm - untreated reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
hay - 0.1 mg/ml Vitamin E and Vitamin C treated pre-reproductive *C. elegans*
ham - 0.1 mg/ml Vitamin E and Vitamin C treated reproductive *C. elegans*
hao - 0.1 mg/ml Vitamin E and Vitamin C treated post-reproductive *C. elegans*
hby - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hbm - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hbo - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hcy - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hcm - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hco - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hdy - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hdm - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated reproductive *C. elegans*
hdo - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*

Appendix 9I: Graph 23 – Log paper plot of uric acid/xanthine, guanine/guanosine, guanine/xanthosine ratios for the Vitamin C & E combination dose treatment and untreated *C. elegans* in the pre-reproductive, reproductive and post-reproductive life stages.



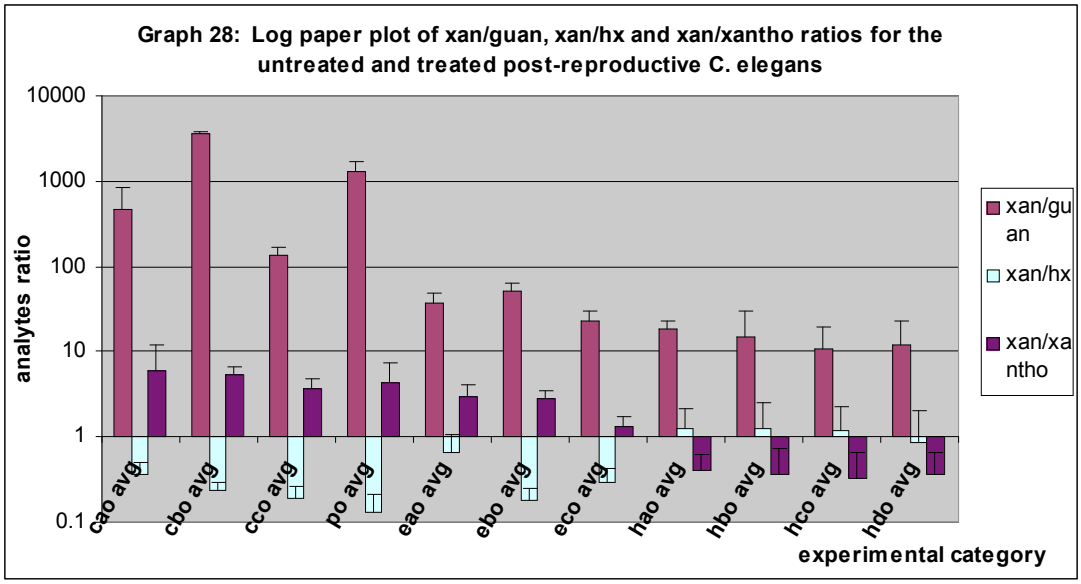
cay - 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*
cby - 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
ccy - 1.0 mg/ml Vitamin C treated pre-reproductive *C. elegans*
py - untreated pre-reproductive *C. elegans*
eay - 0.1 mg/ml Vitamin E treated pre-reproductive *C. elegans*
eby - 0.5 mg/ml Vitamin E treated pre-reproductive *C. elegans*
ecy - 1.0 mg/ml Vitamin E treated pre-reproductive *C. elegans*
hay - 0.1 mg/ml Vitamin E and Vitamin C treated pre-reproductive *C. elegans*
hby - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hcy - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated pre-reproductive *C. elegans*
hdy - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated pre-reproductive *C. elegans*

Appendix 9J: Graph 24 – Log paper plot of xanthine/guanine, xanthine/hypoxanthine, xanthine/xanthosine ratios for the Vitamin C, Vitamin E, Vitamins C & E treated and untreated pre-reproductive *C. elegans*.



cam - 0.1 mg/ml Vitamin C treated reproductive *C. elegans*
cbm - 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
ccm - 1.0 mg/ml Vitamin C treated reproductive *C. elegans*
Pm - untreated reproductive *C. elegans*
eam - 0.1 mg/ml Vitamin E treated reproductive *C. elegans*
ebm - 0.5 mg/ml Vitamin E treated reproductive *C. elegans*
ecm - 1.0 mg/ml Vitamin E treated reproductive *C. elegans*
ham - 0.1 mg/ml Vitamin E and Vitamin C treated reproductive *C. elegans*
hbm - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hcm - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated reproductive *C. elegans*
hdm - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated reproductive *C. elegans*

Appendix 9K: Graph 26 – Log paper plot of xanthine/guanine, xanthine/hypoxanthine, xanthine/xanthosine ratios for the Vitamin C, Vitamin E, Vitamins C & E treated and untreated reproductive *C. elegans*.



cao - 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*
cbo - 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
cco - 1.0 mg/ml Vitamin C treated post-reproductive *C. elegans*
po - untreated post-reproductive *C. elegans*
eao - 0.1 mg/ml Vitamin E treated post-reproductive *C. elegans*
ebo - 0.5 mg/ml Vitamin E treated post-reproductive *C. elegans*
eco - 1.0 mg/ml Vitamin E treated post-reproductive *C. elegans*
hao - 0.1 mg/ml Vitamin E and Vitamin C treated post-reproductive *C. elegans*
hbo - 0.1 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hco - 0.5 mg/ml Vitamin E and 0.5 mg/ml Vitamin C treated post-reproductive *C. elegans*
hdo - 0.5 mg/ml Vitamin E and 0.1 mg/ml Vitamin C treated post-reproductive *C. elegans*

Appendix 9L: Graph 28 – Log paper plot of xanthine/guanine, xanthine/hypoxanthine, xanthine/xanthosine ratios for the Vitamin C, Vitamin E, Vitamins C & E treated and untreated post-reproductive *C. elegans*.

APPENDIX 10

Python program used to perform data substraction.

```
# jwork6a.py
# JM Kinser & Gita Sudama
# Date:  2006

import string
from Numeric import *
from MLab import max, min
import jsignal
import Numeric

# read in the data files
def Reader( fname ):
    fp = open( fname )
    a = fp.read( )
    fp.close()
    # parse
    b = a.split( '\n' )
    N = len( b )    # number of rows
    print N
    if len(b[-1]) ==0:
        N -=1
    data = zeros( (N,16), Float )
    for i in range( N ):
        c = b[i].split()
        for j in range(16):
            data[i,j] = string.atoi( c[j+1] )
```

```
    return data

def Shifter( sig1, sig2, windowsize=900 ):
    # collapse to 1D
    t1 = sum( sig1, 1 )
    t2 = sum( sig2, 1 )
    # find the relative shifts for short windows
    N = len( t1 )

    shifts = []
    for i in range( 0, N-windowsize, windowsize ):
        print i
        aa = jsignal.Norm( t1[i:i+windowsize] )
        bb = jsignal.Norm( t2[i:i+windowsize] )
        corr = jsignal.Correlate( aa,bb )
        corr = corr.real
##        if i==2048:
##          gnu.Save ( 'dud.txt', aa)

        mx = max( corr )
        loc = nonzero( equal( corr, mx ))[0]
        shift = windowsize/2 - loc
        shifts.append( shift )
    return shifts

def Adjust( sig2, shifts, windowsize=900 ):
    N,D = sig2.shape
    nsig = zeros( (N,D), Float )
    tsig = zeros( (N+windowsize*2,D), Float )
```

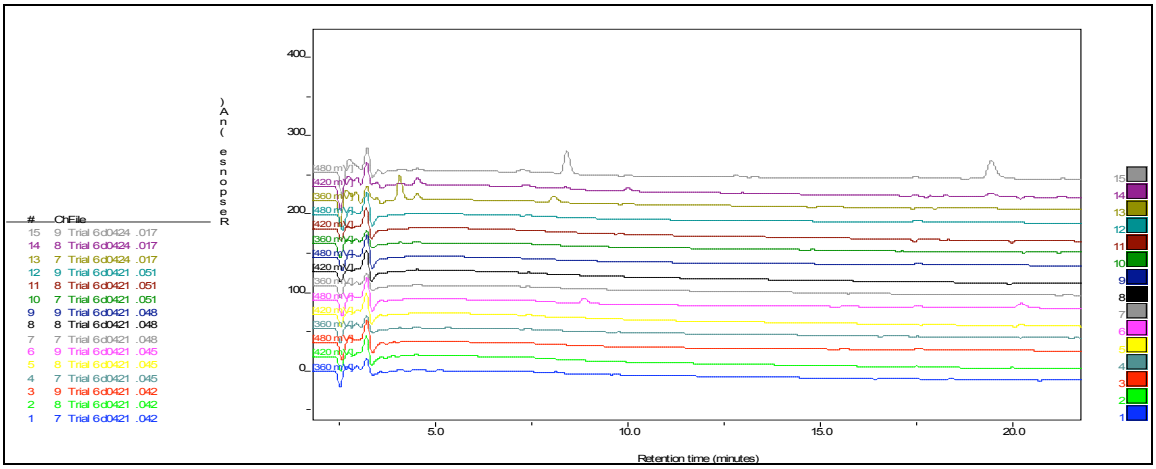
```
    tsig[windowsize:windowsize+N] = sig2+0
    M = len( shifts )
    print "working"
    for i in range( M ):
        k = i *windowsize
        nsig[k:k+windowsize] = tsig[k+shifts[i]+windowsize:k+shifts[i]+2*windowsize]+0
        print "looping"
    return nsig

data = Reader("ea1y.txt")
sig1 = data
print "done reading sig1"
data = Reader("ca1y.txt")
sig2 = data
print "done reading sig2"
shifts = Shifter( sig1, sig2 )
print "finished shifter"
nsig = Adjust( sig2, shifts )
print "THE END- A part"

filename = raw_input('Enter file name to write results to: ')
results = open(filename, 'w')  ##** creates file to store results

factorstr1 = raw_input('Enter factor value for file1: ')
factor1=float (factorstr1)        ##** the normalization
                                  ##** factor for dataset1

factorstr2 = raw_input('Enter factor value for file2: ')
factor2=float (factorstr2)        ##** the normalization, ##** factor for dataset2
```

```
FileA = Numeric.array(sig1)
print "sig1 read"
FileB = Numeric.array(nsig)
print "nsig read"
File1 = FileA*factor1
File2 = FileB*factor2
FileDiff = File2 - File1

print len(FileDiff), " = N"
for i in range (0,7200):
    results.write(str(i))
    results.write (",")

    for j in range (10,11):
        ##print FileDiff[i, j]

        results.write(str(FileDiff[i,j]))
        results.write (",")
        results.write(str(nsig[i,j]))
        results.write (",")
        results.write(str(File1[i,j]))
        results.write (",")

    results.write('\n')

results.close()
```

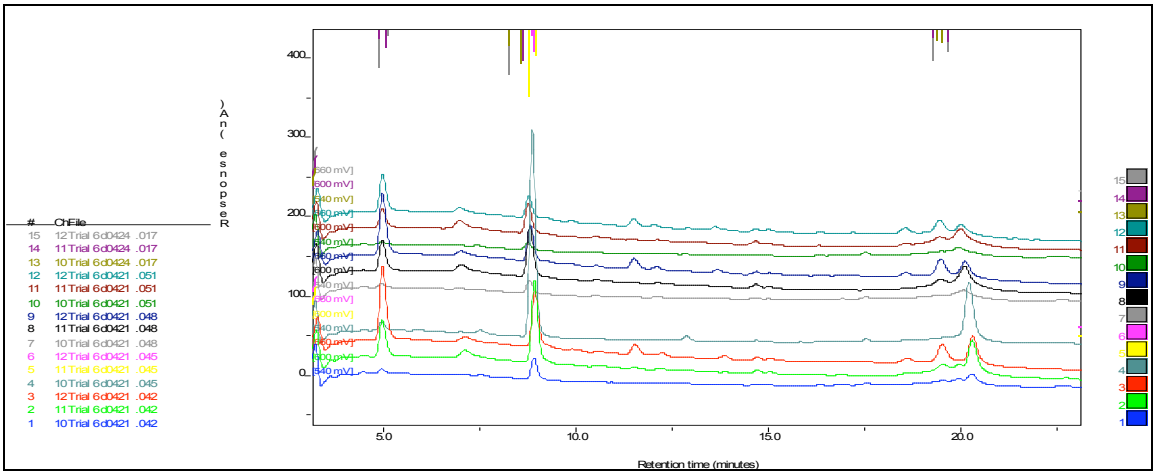
APPENDIX 11

Appendix 11: Portion of a matrix generated from a chromatogram.

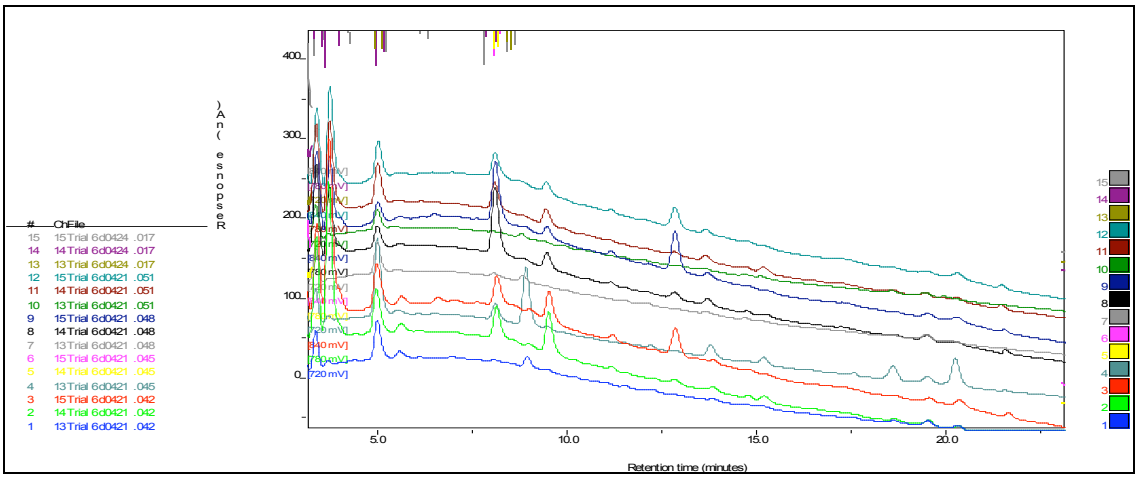
0	-219	317	402	531	1294	3516	1774	2296	1772	3819	2233
	3460	2526	1904	-2971	-12833						
1	-1122	-63	480	0	1006	3331	1623	2136	1747	3757	2239
	3438	2426	1802	-2994	-12499						
2	-785	36	588	99	973	3262	1572	1921	1646	3627	2067
	3305	2352	1710	-3054	-12366						
3	-522	181	635	228	1034	3279	1606	1894	1630	3529	1987
	3187	2317	1662	-3066	-12170						
4	-379	294	660	294	1078	3280	1621	1899	1633	3470	1986
	3112	2273	1639	-3034	-11975						
5	-242	377	646	353	1119	3238	1628	1903	1636	3409	1955
	3025	2250	1599	-2999	-11785						
6	-205	414	626	392	1162	3224	1643	1945	1636	3324	1961
	2955	2227	1604	-2962	-11577						
7	-245	395	589	363	1164	3192	1639	1943	1613	3284	1954
	2900	2185	1550	-2936	-11399						
8	-297	377	579	289	1143	3118	1618	1919	1583	3267	1937
	2825	2138	1495	-2921	-11231						
9	-314	368	590	287	1133	3058	1618	1883	1569	3267	1887
	2752	2097	1418	-2903	-11067						
10	-306	383	608	262	1127	2970	1586	1783	1530	3162	1819
	2696	2057	1367	-2899	-10826						
11	-232	398	615	298	1122	2901	1557	1760	1513	3077	1784
	2629	2003	1367	-2871	-10583						
12	-111	377	579	309	1141	2878	1568	1774	1578	2967	1775
	2583	1984	1343	-2818	-10371						



Appendix 12a: Metafile of ca3y, cb3y, cc3y, p3y, and ha3y for channels 7 to 9. Trial6d0421.042 represents ca3y, pre-reproductive 0.1 mg/ml treated with Vitamin C population. Trial 6d0421.045 represents cb3y, pre-reproductive 0.5 mg/ml treated with Vitamin C population. Trial6d0421.048 represents cc3y, pre-reproductive 1.0 mg/ml treated with Vitamin C population. Trial6d0421.051 represents p3y, pre-reproductive untreated population. Trial6d0424.017 represents ha3y, pre-reproductive 0.1 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E population.

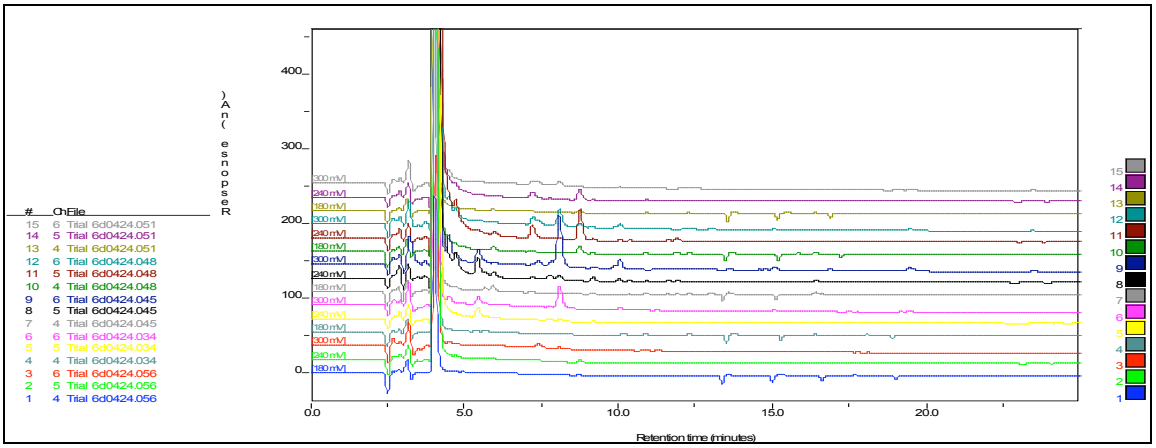


Appendix 12b: Metafile of ca3y, cb3y, cc3y, p3y, and ha3y for channels 10 to 12. Trial6d0421.042 represents ca3y, pre-reproductive 0.1 mg/ml treated with Vitamin C population. Trial 6d0421.045 represents cb3y, pre-reproductive 0.5 mg/ml treated with Vitamin C population. Trial6d0421.048 represents cc3y, pre-reproductive 1.0 mg/ml treated with Vitamin C population. Trial6d0421.051 represents p3y, pre-reproductive untreated population. Trial6d0424.017 represents ha3y, pre-reproductive 0.1 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E population.

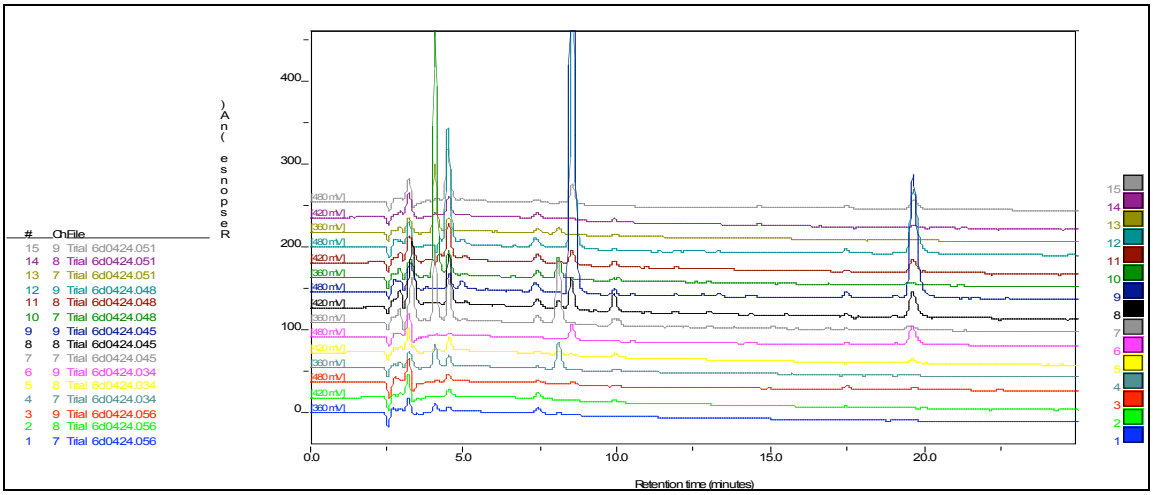


Appendix 12c: Metafile of ca3y, cb3y, cc3y, p3y, and ha3y for channels 13 to 15. Trial6d0421.042 represents ca3y, pre-reproductive 0.1 mg/ml treated with Vitamin C population. Trial 6d0421.045 represents cb3y, pre-reproductive 0.5 mg/ml treated with Vitamin C population. Trial6d0421.048 represents cc3y, pre-reproductive 1.0 mg/ml treated with Vitamin C population. Trial6d0421.051 represents p3y, pre-reproductive untreated population. Trial6d0424.017 represents ha3y, pre-reproductive 0.1 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E population.

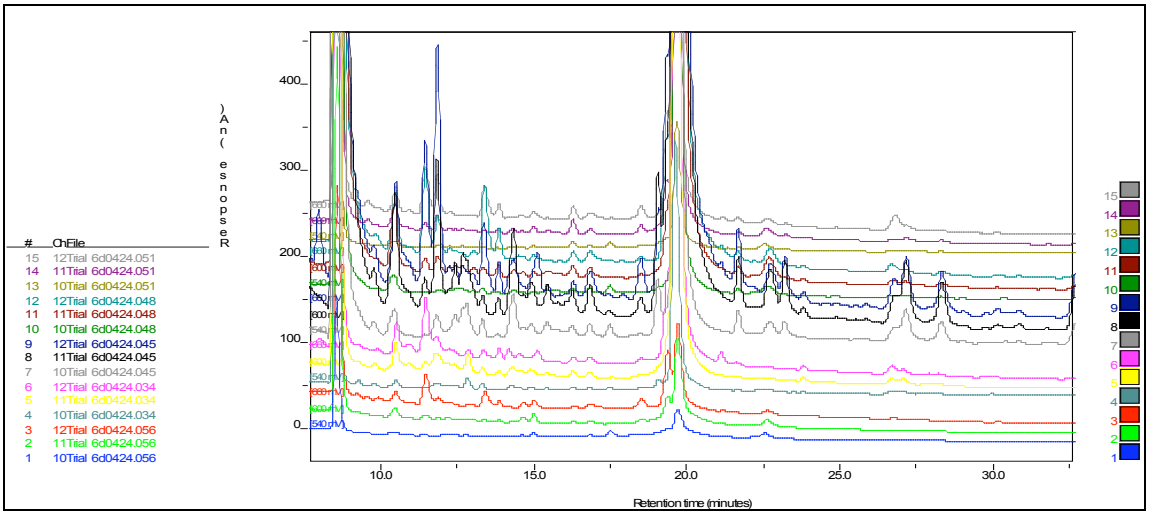
APPENDIX 13



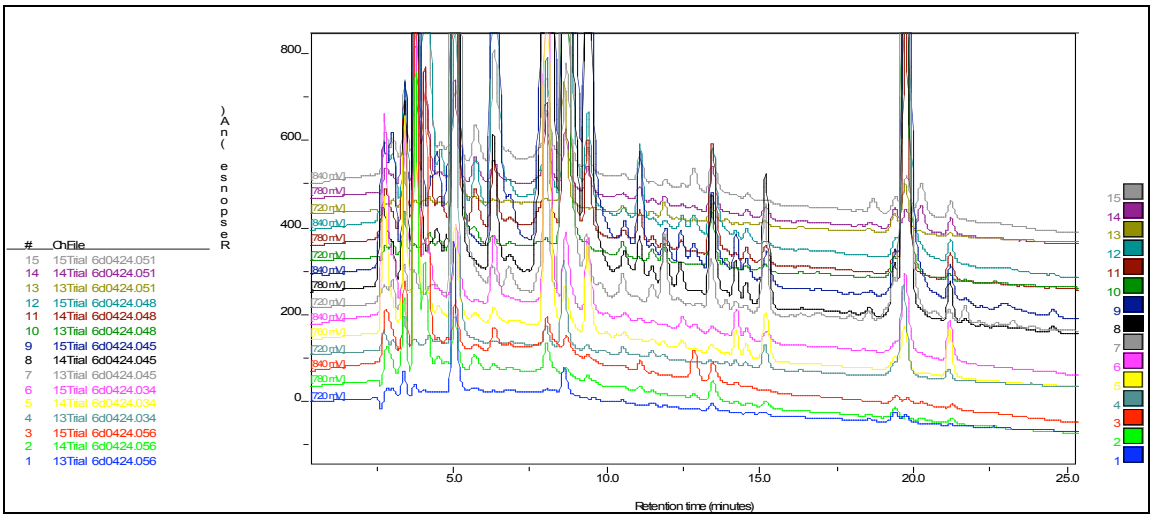
Appendix 13a: Metafile of p2m, hb2m, ea2m, eb2m and ec2m for channels 4 to 6. Trial6d0424.056 represents p2m, reproductive untreated *C.* elegans poplutaion. Trial 6d0424.034 represents hb2m, reproductive 0.5 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E population. Trial6d0424.045 represents ea2m, reproductive 0.1 mg/ml treated with Vitamin E population. Trial6d0424.048 represents eb2m, reproductive 0.5 mg/ml treated with Vitamin E population. Trial6d0424.051 represents ec3m, reproductive 1.0 mg/ml treated with Vitamin E population.



Appendix 13b: Metafile of p2m, hb2m, ea2m, eb2m and ec2m for channels 7 to 9. Trial6d0424.056 represents p2m, reproductive untreated *C.* elegans poplutaion. Trial 6d0424.034 represents hb2m, reproductive 0.5 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E population. Trial6d0424.045 represents ea2m, reproductive 0.1 mg/ml treated with Vitamin E population. Trial6d0424.048 represents eb2m, reproductive 0.5 mg/ml treated with Vitamin E population. Trial6d0424.051 represents ec3m, reproductive 1.0 mg/ml treated with Vitamin E population.

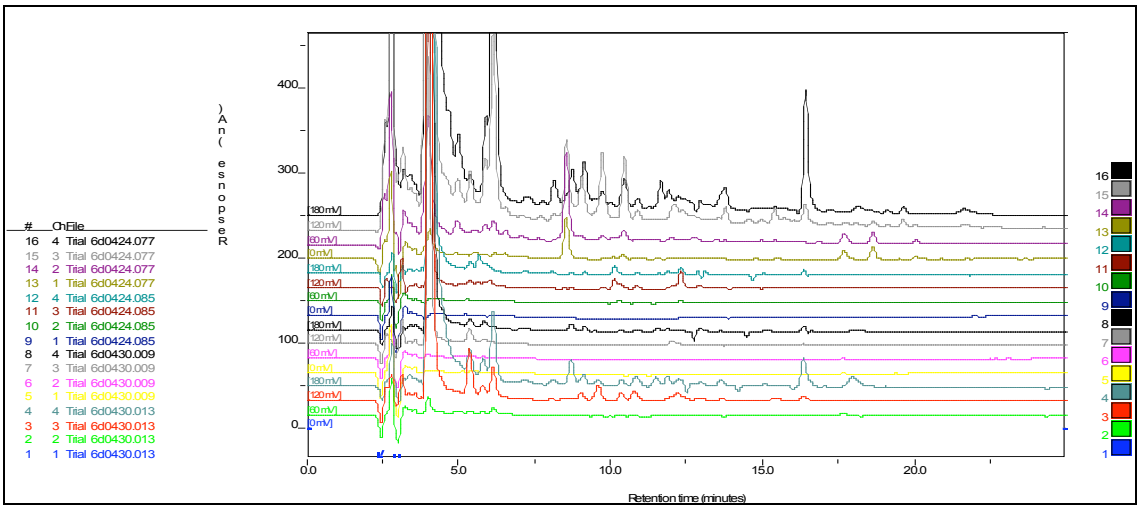


Appendix 13c: Metafile of p2m, hb2m, ea2m, eb2m and ec2m for channels 10 to 12. Trial6d0424.056 represents p2m, reproductive untreated *C.* elegans poplutaion. Trial 6d0424.034 represents hb2m, reproductive 0.5 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E population. Trial6d0424.045 represents ea2m, reproductive 0.1 mg/ml treated with Vitamin E population. Trial6d0424.048 represents eb2m, reproductive 0.5 mg/ml treated with Vitamin E population. Trial6d0424.051 represents ec3m, reproductive 1.0 mg/ml treated with Vitamin E population.

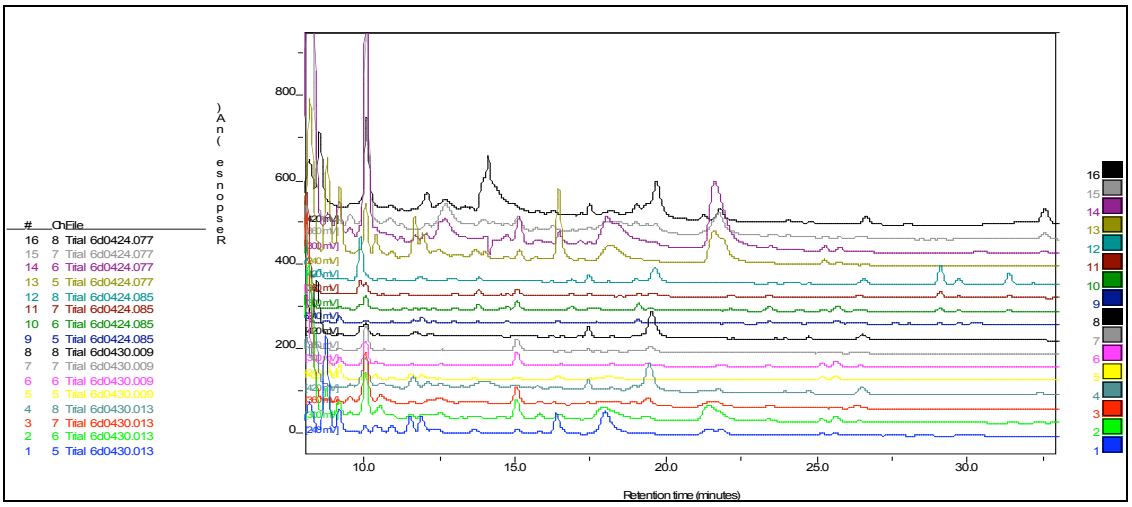


Appendix 13d: Metafile of p2m, hb2m, ea2m, eb2m and ec2m for channels 13 to 15. Trial6d0424.056 represents p2m, reproductive untreated *C.* elegans poplutaion. Trial 6d0424.034 represents hb2m, reproductive 0.5 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E population. Trial6d0424.045 represents ea2m, reproductive 0.1 mg/ml treated with Vitamin E population. Trial6d0424.048 represents eb2m, reproductive 0.5 mg/ml treated with Vitamin E population. Trial6d0424.051 represents ec3m, reproductive 1.0 mg/ml treated with Vitamin E population.

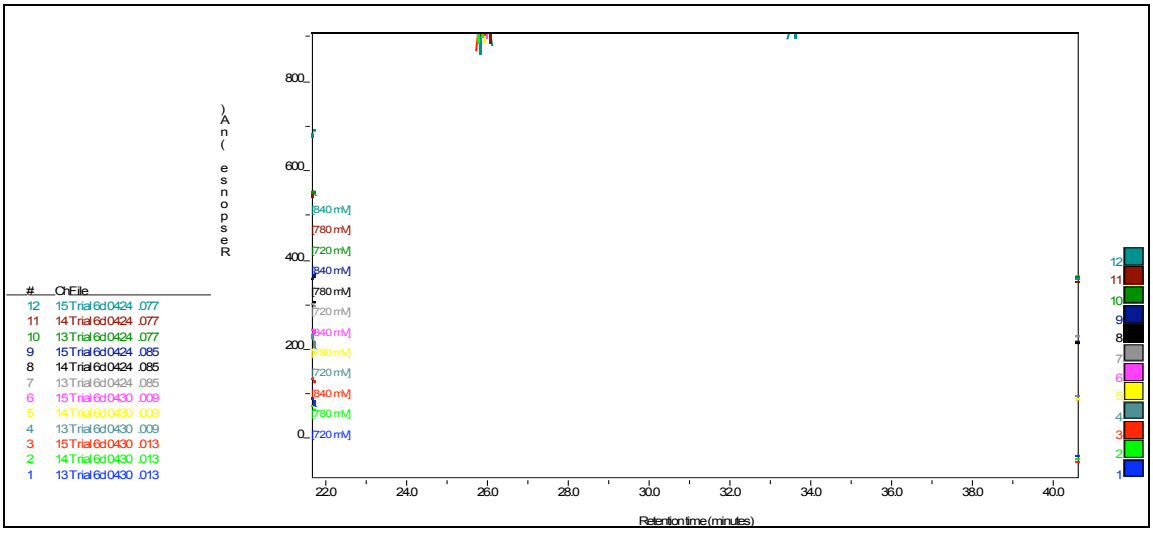
APPENDIX 14



Appendix 14a: Metafile of p1o, cb3o, hb2o and ec2o for channels 1 to 4. Trial6d0430.013 represents p1o, post-reproductive untreated *C.* elegans poplutaion. Trial 6d0430.009 represents cb3o, post-reproductive 0.5 mg/ml treated with Vitamin C population. Trial6d0424.085 represents hb2o, post-reproductive 0.5 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E population. Trial6d0424.077 represents ec2o, post-reproductive 1.0 mg/ml treated with Vitamin E population.



Appendix 14b: Metafile of p1o, cb3o, hb2o and ec2o for channels 5 to 8. Trial6d0430.013 represents p1o, post-reproductive untreated *C.* elegans poplutaion. Trial 6d0430.009 represents cb3o, post-reproductive 0.5 mg/ml treated with Vitamin C population. Trial6d0424.085 represents hb2o, post-reproductive 0.5 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E population. Trial6d0424.077 represents ec2o, post-reproductive 1.0 mg/ml treated with Vitamin E population.



Appendix 14c: Metafile of p1o, cb3o, hb2o and ec2o for channels 13 to 15. Trial6d0430.013 represents p1o, post-reproductive untreated *C.* elegans poplutaion. Trial 6d0430.009 represents cb3o, post-reproductive 0.5 mg/ml treated with Vitamin C population. Trial6d0424.085 represents hb2o, post-reproductive 0.5 mg/ml treated with Vitamin C and 0.1 mg/ml Vitamin E population. Trial6d0424.077 represents ec2o, post-reproductive 1.0 mg/ml treated with Vitamin E population.

APPENDIX 15

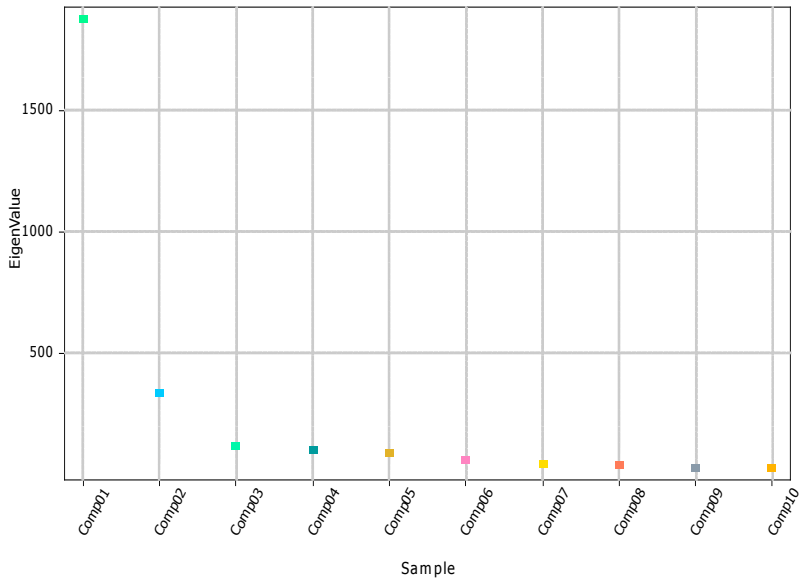
Appendix 15: Program written to convert the text chromatographic data into a matrix to be used in the Power Array software.

```
##GitaDataVector.py
##Written by Gita Sudama
#May 2006

import string
from Numeric import *
from MLab import max, min
import jsignal
import Numeric


# read in the data files
def Reader( fname ):
    fp = open( fname )
    a = fp.read( )
    fp.close()
    # parse
    b = a.split( '\n' )
    N = len( b )    # number of rows
    print N
    if len(b[-1]) ==0:
        N -=1
    data = zeros( (N,16), Float )
    for i in range( N ):
        c = b[i].split()
        for j in range(16):
            data[i,j] = string.atoi( c[j+1] )
    return data

data = Reader("P1Y.txt")

FileA = Numeric.array(data)
print "data read A"
##**print FileA

data = Reader("P2Y.txt")

FileB = Numeric.array(data)
```

```
print "data read B"

data = Reader("P3Y.txt")

FileC = Numeric.array(data)
print "data read C"

data = Reader("p1m.txt")

FileD = Numeric.array(data)
print "data read D"

data = Reader("p2m.txt")

FileE = Numeric.array(data)
print "data read E"

data = Reader("p3m.txt")

FileF = Numeric.array(data)
print "data read F"

data = Reader("p1o.txt")

FileG = Numeric.array(data)
print "data read G"

data = Reader("p2o.txt")

FileH = Numeric.array(data)
print "data read H"

data = Reader("p3o.txt")

FileI = Numeric.array(data)
print "data read I"


filename = raw_input('Enter file name to write results to: ')
results = open(filename, 'w')  ##** creates file to store

                              ##** results

#j=8
```

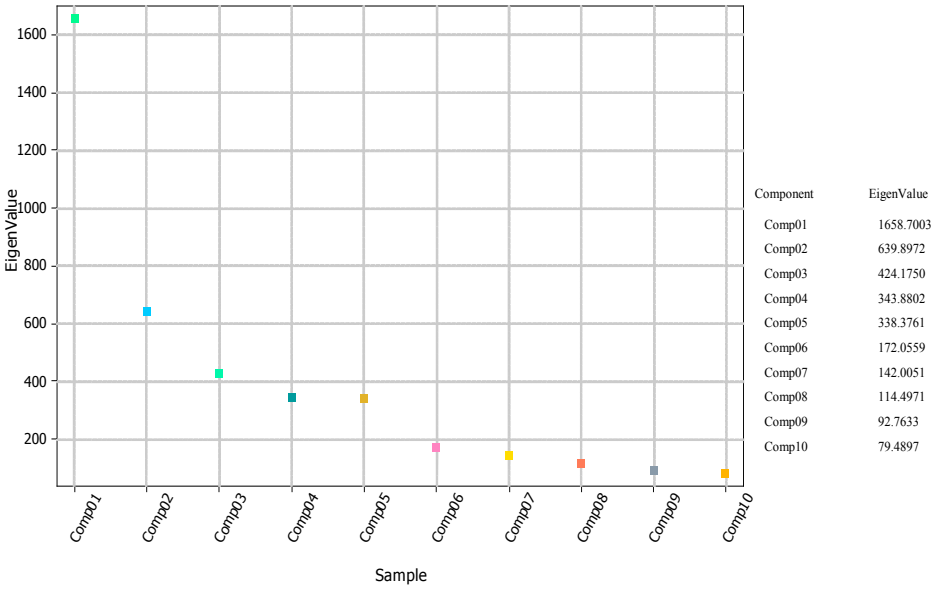
```
k=1

for j in range (16):

    for i in range (250,7200):

        results.write(str(i))
        results.write(",")
        results.write(str(FileA[i,j]))
        results.write(",")
        results.write(str(FileB[i,j]))
        results.write(",")
        results.write(str(FileC[i,j]))
        results.write(",")
        results.write(str(FileD[i,j]))
        results.write(",")
        results.write(str(FileE[i,j]))
        results.write(",")
        results.write(str(FileF[i,j]))
        results.write(",")
        results.write(str(FileG[i,j]))
        results.write(",")
        results.write(str(FileH[i,j]))
        results.write(",")
        results.write(str(FileI[i,j]))
        results.write(",")


        results.write ( '\n' )

        k + 1

##    results.write('\n')

results.close()
```

Appendix 16: PCA EigenValue summary for 10 components of treated and untreated pre-reproductive *C. elegans*.



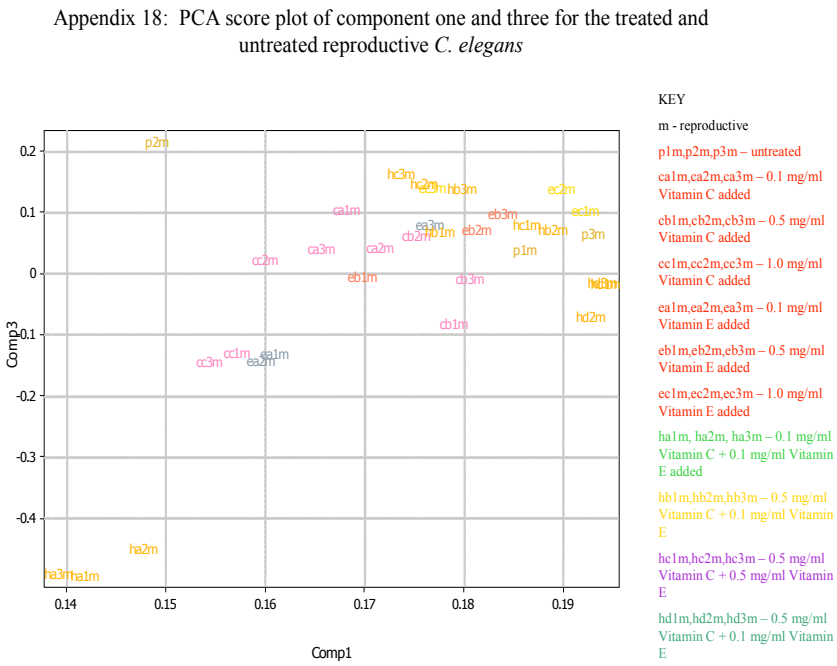
Appendix 16: PCA EigenValue summary plot for 10 components of treated and untreated pre-reproductive *C. elegans*

Appendix 17: EigenValues for each component in the PCA of reproductive *C. elegans*



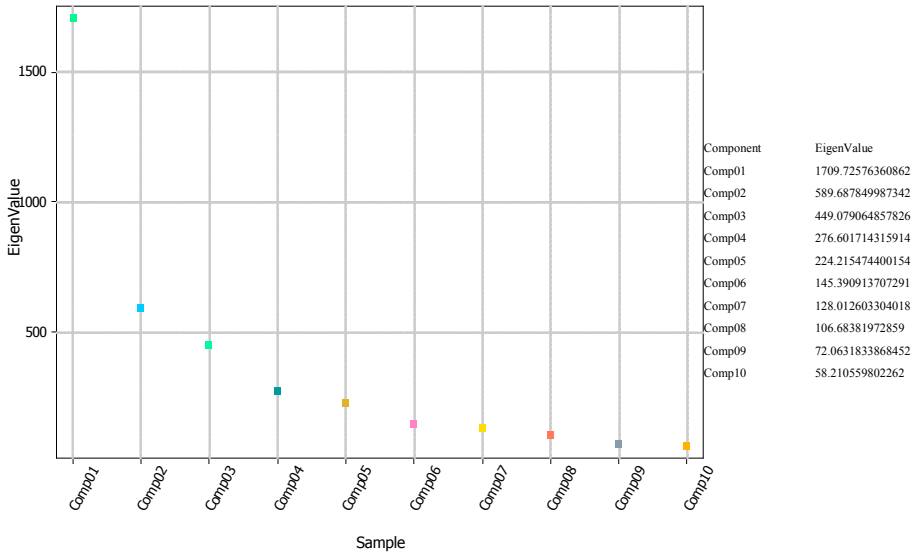
Component	EigenValue
Comp01	1658.7003
Comp02	639.8972
Comp03	424.1750
Comp04	343.8802
Comp05	338.3761
Comp06	172.0559
Comp07	142.0051
Comp08	114.4971
Comp09	92.7633
Comp10	79.4897

Appendix 17: EigenValues plot for the 10 components in the PCA of the reproductive (treated and untreated) *C. elegans.*



Appendix 18: PCA score plot of components 1 (one) and 3 (three) for the treated and untreated reproductive *C. elegans.*

Appendix 19: EigenValues for ten PCA components of the post-reproductive *C. elegans*



Component	EigenValue
Comp01	1709.72576360862
Comp02	589.687849987342
Comp03	449.079064857826
Comp04	276.601714315914
Comp05	224.215474400154
Comp06	145.390913707291
Comp07	128.012603304018
Comp08	106.68381972859
Comp09	72.0631833868452
Comp10	58.210559802262

Appendix 19: EigenValues plot for ten PCA components of the post-reproductive treated and untreated *C. elegans*.

LITERATURE CITED

LITERATURE CITED

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