Development of Assays for Biomarkers of Oxidative Damage to Assess the Efficacy of Fruit-derived Antioxidants

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Abstract

The diet is a very important part of maintaining a healthy lifestyle. Increased consumption of fruits and vegetables is one practice postulated to decrease the incidence of diseases such as cancer, cardiovascular disease and other disorders. Although there are a number of possible beneficial compounds in fruit, it is believed that the antioxidant components found in these foods may decrease the oxidative damage that could lead to such diseases. Oxidative damage to cellular proteins, lipids and DNA is considered to result from an increase in the production of free radicals, which overwhelm the body's defence system.

This research investigated fruit-derived antioxidants, and developed biomarker assays to measure the potential health benefits they may offer. To determine the *in vivo* antioxidant efficacy of berry fruit anthocyanins, oxidative damage to proteins, lipids and DNA was measured in rats fed several combinations of natural and synthetic diets. Mild oxidative damage was induced by the inclusion of fish oil in these diets.

DNA oxidation was determined by measuring urinary 8-hydroxy-2'-deoxyguanosine using reversed-phase high performance liquid chromatography with electrochemical detection. ELISA and colorimetric techniques were used to measure protein carbonyl content of plasma as a reflection of protein oxidation. Oxidation to lipids was assessed by measuring malondialdehyde, which results from lipid peroxidation.

Supplementation with fish oil induced a mild form of dietary oxidative damage, as shown by an increase in lipid and protein oxidation. In most cases the berry fruit extracts had little effect on the level of fish oil-induced oxidative damage, however, boysenberry anthocyanin extract significantly reduced protein oxidation when used in combination with the natural diet. Taken together the results suggest that oxidative damage to biomacromolecules may occur by different pathways of oxidative stress, which selectively target either DNA, protein or lipids at varying levels, and the antioxidant is effective only with selected mechanisms of oxidative damage.

Abbreviations

8OHdG	8-hydroxy-2'-deoxyguanosine
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid)
AUC	area under curve
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
$CHCl_3$	carbon tetrachloride
CoA	coenzyme A
DNA	deoxyribonucleic acid
DNPH	dinitrophenylhydrazine
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme-linked immunoabsorbent assay
FPG	formamidopyrimidine DNA N-glycosylase
FO	fish oil
FRAP	free radical antioxidant power
GC-MS	gas chromatography – mass spectrometry
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
IPA	isopropyl alcohol
LC-MS	liquid chromatography with mass spectrometry
LDL	low density lipoprotein
MDA	malondialdehyde
MeOH	methanol
NaOAc	sodium acetate
ORAC	oxygen radical absorbance capacity
$ORAC_{FL}$	oxygen radical absorbance capacity assay using fluorescein
PUFA	polyunsaturated fatty acid
RP-HPLC	reversed-phase high-performance liquid chromatography
SBO	soybean oil
SEM	standard error of the mean

SPE	solid phase extraction
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TE	trolox equivalent
TEAC	trolox equivalent antioxidant capacity
Trolox	6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid

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Chapter One - Introduction

1.1 Introduction

Numerous health benefits are claimed for dietary antioxidants because of their ability to reduce oxidative damage (Joseph, et al., 1999). Oxidative damage of cellular proteins, lipids and DNA is considered to result from an increase in the production of chemically reactive species known as free radicals, which overwhelm the body's defence system. Even normal bodily function's such as exercise and breathing, produce free radicals. These free radicals attack healthy cells, thus reducing the body's resistance to some types of cancers and can lead to other disorders such as cardiovascular (De Zwart, et al., 1999) and neurodegenerative diseases such as Alzheimer's disease (Halliwell and Gutteridge, 1995).

The activities of dietary antioxidants are often determined by *in vitro* measurements, such as the oxygen radical absorbance capacity (ORAC) assay (Cao and Prior, 1998). While this measure is accepted as a valid indicator of the antioxidant potential of a substance (Frankel and Meyer, 2000), assays of this type do not prove that a specific substance acts as a biological antioxidant when consumed. Other *in vivo* approaches are required to assess the biological efficacy of a particular antioxidant.

1.2 Oxidative Damage

Oxidative stress is defined as: 'an increase in free radical production, leading to oxidative damage of cellular proteins, lipids and DNA and altering the natural antioxidant defence mechanisms present in most tissues' (Cesarone, et al., 1999; Renner, et al., 2000). This oxidative stress leads to oxidative damage, which can be detected in healthy cells of any age due to incomplete antioxidant and internal repair activities. A balanced intracellular redox status is essential for the function of cellular systems and is

maintained by many antioxidant mechanisms. When this redox balance becomes impaired, cells are exposed to the deleterious effects of oxidative stress.

1.2.1 Free Radicals

Free radicals, including reactive oxygen and nitrogen species (Table 1.1), are highly reactive atoms or molecules with an unpaired electron. Free radicals occur naturally and are an important part of biological processes such as inflammation (Bancroft, et al., 2003), growth (Yen, et al., 2002), and repair (Sohal, et al., 2002). Other non-radical molecules (Table 1.1) are also known to be highly reactive and have similar biological activities to those of free radicals.

Radicals	*Non-radicals*
$O_2^{\bullet-}$ superoxide anion	H_2O_2
$HO^{\bullet}$ hydroxyl radical	$^1O_2$ singlet oxygen
$HO_2^{\bullet}$ hydroperoxyl radical	O_3 ozone
$L^{\bullet}$ lipid radical	LOOH lipid hydroperoxide
$LO_2^{\bullet}$ lipid peroxyl radical	Fe=O iron-oxygen complexes
$LO^{\bullet}$ lipid alkoxyl radical	HOCl hypochlorite
$NO_2^{\bullet}$ nitrogen dioxide	
$^{\bullet}NO$ nitric oxide	
$RS^{\bullet}$ thiyl radical	
$P^{\bullet}$ protein radical	

Table 1.1: *Active oxygen and related species (Taken from Pokorny, et al., 2001).*

When the *in vivo* concentration of free radicals exceeds the antioxidant capability of the cell, oxidative stress occurs and the free radicals become detrimental to cell function (Astley and Lindsay, 2002). Cells have developed an extensive set of antioxidant defence mechanisms, which include enzymes to inactivate peroxides, and compounds to scavenge free radicals. When oxidative stress is at a level where the cellular enzyme

activity is compromised, dietary antioxidants may be effective at scavenging free radicals (Mayne, 2003).

1.2.2 The Biological Consequences of Oxidative Damage

1.2.2.1 Cellular Consequences

Free radical damage to proteins, lipids and DNA results in not only direct damage, but also indirect damage, as both oxidised lipids and DNA can cross-link with proteins (Bevan, et al., 2003).

It has been suggested that oxidative damage to DNA, over the human lifespan is a significant contributor to the development of age-related diseases and cancer (Halliwell, 2002), diabetes (Coleman, et al., 2002), neurodegenerative diseases (Perry, et al., 2002) and ageing in general (Youdim and Deans, 2000). Oxidatively damaged nucleic acids include damaged base and sugar groups and single and double strand breaks in the DNA backbone (Beckham and Ames, 1998). Chemical modification of DNA nucleotides by oxidation may induce strand breaks or base release (Cadet, et al., 2002). *In vivo*, damaged DNA is repaired by endonucleases, which release the deoxynucleotides; and glycosylases, which release the bases for urinary excretion. Elevated oxidative damage to DNA may not always be associated with increased cancer development (Halliwell, 2002). Additional factors including unrelated genetic changes might also be required for cancer development. Furthermore, oxidative damage may be effective only over a certain range, where excessive damage may have an anti-cancer effect by promoting apoptosis. Oxidative attack may also cause DNA-protein cross-linking and damage to the deoxyribose-phosphate backbone (Halliwell, 2002).

Lipids are known targets for free radical damage (Esterbauer, et al., 1992). Peroxidation of fatty acyl groups, mostly in membrane phospholipids, has three well-characterised phases: initiation, propagation and termination. The propagation phase continues until the substrate is consumed or termination occurs (Montine, et al., 2002). Oxidation of

membrane lipids, especially unsaturated carbohydrate side-chains of phospholipids, may lead to membrane dysfunction and cell lysis (Behl and Moosman, 2002).

Along with the other types of oxidative damage, protein oxidation is believed to be mechanistically involved in ageing, the development of cancer, Parkinson's disease, and other pathological effects (Kasprzak, 2002). The free radical oxidation of proteins produces carbonyl groups, which can deactivate the protein by changing their tertiary structure, which may prevent either binding to cofactors or to other proteins (Levine, 2002).

1.2.2.2 Disease Pathogenesis

An imbalance between antioxidant and oxidant-generating systems leading to oxidative stress has been proposed to contribute to the pathogenesis of many diseases. With respect to ageing and cancer, DNA is considered a major target of free radical damage (Ames, et al., 1993). Apart from neurological disorders such as Alzheimer's disease and Parkinson's disease, other clinical disorders attributed to free radical damage include cardiovascular disease and ischemic dementia (De Zwart, et al., 1999). In Alzheimer patients, oxidative damage is thought to increase the levels of cerebral glucose-6-phosphate dehydrogenase and reduce cell glutathione peroxidase activities. This increases cellular susceptibility to membrane lipid peroxidation and reduces levels of antioxidant macronutrients such as vitamins A and E and carotenoids (De Zwart, et al., 1999). In Parkinson's disease a high concentration of iron and low glutathione and glutathione peroxidase levels in the brain, infer higher levels of oxidative stress (Giasson, et al., 2002).

1.3 Antioxidants

An elaborate network of detoxifying enzymes and low molecular weight antioxidant molecules exists in the body to maintain redox homeostasis (Drummen, et al., 2002).

Antioxidant mechanisms include metal sequestering by proteins, use of compounds such as vitamins C and E to scavenge reactive oxygen species and other free radicals, and specialised antioxidant enzymes, such as superoxide dismutase (Fattman, et al., 2003). Studies have revealed that the levels of endogenous enzymes and antioxidants *in vivo* are variable and in same cases are significantly reduced (Faure, et al., 1996). Because antioxidants act by disrupting pathways that result in free radical damage (Frei, et al., 1988) they can only be useful if they target reactive oxygen species and other free radical formation pathways.

1.3.1 Non-Enzymatic Antioxidant Systems

1.3.1.1 Endogenous Antioxidants

Glutathione

Glutathione is a substrate for the hydrogen peroxide-removing enzyme, glutathione peroxidase, and is also a scavenger of hydroxyl radicals and singlet oxygen. Glutathione is known to protect cells from toxic injury, presumably through conjugation with the toxin, resulting in lower amounts of toxic intermediates (Chen, et al., 2002).

Uric Acid

Uric acid is present in human blood plasma as an end product of purine metabolism. It is a powerful scavenger of singlet oxygen, peroxyl and hydroxyl radicals. Half the plasma antioxidant status comes from urate and albumin, with the other half of the antioxidant activity derived from small molecules, such as vitamin E, enzymes and flavonoids (Rice-Evans, et al., 1997).

1.3.1.2 Dietary Antioxidants

Vitamin C (Ascorbate or Ascorbic Acid)

Vitamin C is an antioxidant required for tissue growth and repair, adrenal gland function, and healthy gums. Vitamin C is not synthesised endogenously in humans,

although it is in rats. An example of the antioxidant activity of ascorbate is its reducing action, where it acts as an electron donor, reducing ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) (Halliwell and Gutteridge, 1995).

Vitamin E (α-tocopherol)

Vitamin E is a lipid soluble antioxidant. There are 6 molecules of α-tocopherol per low-density lipoprotein (LDL) particle, a ratio higher than all other antioxidants (Esterbauer, et al., 1992). Tocopherol exists in four homologues: α, β, γ and δ of which α-tocopherol has the highest biological activity (Astley and Lindsay, 2002). MacDonald-Wicks and Garg (2003) reported that vitamin E had no affect on the basal level of oxidative stress, but may be of greater significance when animals were challenged with a pro-oxidant. Ibrahim, et al. (1999) investigated the affects of dietary lipid, vitamin E and iron on generation of lipid and protein oxidation products in mouse liver. Dietary vitamin E had a significant effect on decreasing the levels of hepatic oxidation products and prevented fish oil and iron having a pro-oxidant effect on cells, which acts on both lipid- and water-soluble cell constituents.

Vitamins C and E in Synergy?

There is evidence to suggest a synergy between vitamins C and E. For example, vitamin C may affect redox cycling and be an efficient co-antioxidant *in vitro* for the regeneration of α-tocopherol (Hamilton, et al., 2000). Increased vitamin C could therefore lead to improved vitamin E status (Hamilton, et al., 2003). Consequently, any alteration in the status of a single vitamin could affect the status of other antioxidants although as yet, there is no evidence that applies the potential benefits of vitamin E and or C supplementation beyond the simple antioxidant effects. However, a study by Huang, et al. (2002) showed that dietary supplementation with vitamin C or E alone, reduced lipid peroxidation to a similar extent. In the presence of both vitamins however, there was no effect beyond that of either vitamin alone.

Fruit-Derived Antioxidants

In addition to the antioxidant vitamins and minerals known to be present in fruits and vegetables (Wada and Ou, 2002), phytochemicals such as carotenoids, flavonoids and other phenolics are very potent antioxidants because of their high radical scavenging

activity and their capacity to complex iron (Areias, et al., 2001). These phytochemicals have also been shown to possess antibacterial, antiviral, antithrombotic, anti-inflammatory and anticancer activities (Hou, 2003; Astley and Lindsay, 2002). It has been suggested that the majority of the antioxidant capacity of fruits and vegetables comes from total phenolics and flavonoids, rather than vitamin C (Liu, et al., 2002). Dietary phenolic compounds have generally been considered as non-nutrients and their possible benefit to human health has only recently received increasing attention.

Berry fruit contain the highest reported antioxidant activity of any fruit (Prior, et al., 1998). Recent scientific research has shown that incorporation of berry fruit constituents in the diet may: 1) protect from loss of cognitive and motor function during ageing (Joseph, et al., 1999); 2) maintain vision and relieve eye stress (Nakaishi, et al., 2000); and 3) provide protection against some forms of cancer (Stoner, et al., 1999). Thus, there is evidence to support the strong antioxidant activity of berry fruit, although in most cases there is a lack of understanding of the active components and mechanism(s) of the health effect. In a study by Walle, et al. (2003) cell-based assays demonstrated covalent binding of quercetin, the most commonly present of the dietary flavonoids, to cellular DNA and protein, which implied a protective effect. The hypothesised active antioxidants in berry fruit are the flavonoids.

The flavonoids are the largest group of phenolic compounds. (Approximately 4000 flavonoids are known to exist.) They are 15-carbon molecules biosynthetically derived from phenylalanine and malonyl CoA and are composed of two phenolic rings connected via a pyranoid ring. These rings make up the basic flavonoid structure, of which many other compounds are derived. The combined rings are known as the anthocyanidin and an attached sugar makes up the anthocyanin (Figure. 1.1). Other flavonoid derivatives include tannins, flavonols and flavanols. Anthocyanins "are secondary plant metabolites responsible for the blue, purple and red colour of many plant tissues" (Ramirez-Tortosa, et al., 2001). Each anthocyanin differs with respect to: 1) the number and type of chemical groups, either hydrogen (H), hydroxyl groups (OH) or methoxy (OCH_3) groups attached to the rings of the anthocyanidin moiety and; 2) to the sugar that forms the glycoside linkage. Anthocyanins in berry fruit have a high oxygen radical absorbance capacity, thus exhibiting antioxidant activity (Wang, et al., 1997).

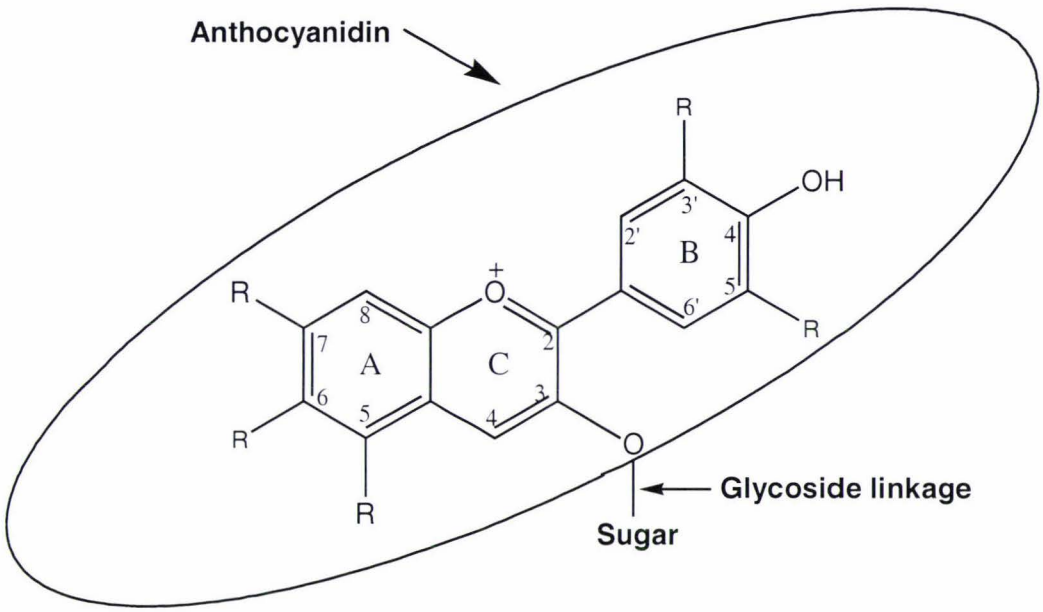


Figure 1.1: *Generalised structure of an anthocyanin molecule. The extended conjugation and possible charge enables the absorption of visible light and therefore anthocyanins are coloured. R group substituents include H, OH, and OCH_3 groups.*

They have also been shown to inhibit oxidation of LDLs (Staue-Gracia, et al., 1997), yet their mechanism of action has not been defined. There exist two theories as to how anthocyanins act as antioxidants. The first is that anthocyanins scavenge free radicals thus breaking the free radical chain reaction. This infers that the anthocyanins act directly to reduce the level of oxidative damage. The other explanation is that anthocyanins indirectly decrease oxidative damage by inducing enzymes "to disarm and facilitate the disposal of reactive electrophiles and oxygen species" (Talalay, et al., 1995).

A study by Sarma and Sharma (1999) showed the association of anthocyanins and DNA to form a complex that protects both DNA and the anthocyanin from damage caused by hydroxyl radicals. Oxidative stress in retinal cells has been shown to be induced by the presence of ascorbate and ferric ions and is highly reduced in the presence of flavonoids (Areias, et al., 2001). Khokhar and Owusu Apenten (2003) showed that flavonoids can chelate metals, specifically iron, which suppresses the accessibility of the iron to oxygen molecules and decreases oxygen toxicity to cells. These experiments however, provide only *in vitro* evidence of the association of flavonoids to metal ions, and use flavonoid

concentrations far greater than those in the diet. Further studies are required to investigate the iron binding efficiency of physiologically active forms of flavonoids.

1.3.2 Enzymatic Antioxidant Systems

1.3.2.1 Redox Cycling Enzymes

Redox cycling enzymes, or phase 2 enzymes are transcriptionally regulated by low concentrations of a variety of chemical agents, such as antioxidants, in the diet (Ramirez-Tortosa, et al., 2001). Experiments by Talalay (2000) showed phase two enzymes promoted conjugation between endogenous ligands such as glutathione and synthetic oxidative stress products, which lead to more water-soluble and thus more easily excretable products. Redox cycling enzymes include glutathione peroxidase, glutathione transferase, quinone reductase and superoxide dismutase. Superoxide dismutase removes free radicals from the cell by catalysing the dismutation of two superoxide radicals to form oxygen and hydrogen peroxide, which undergoes further reactions for removal from the cell.

1.3.2.2 Repair Enzymes

Various enzymes can act to repair the damage caused by free radicals. These enzymes therefore act indirectly to decrease oxidative damage. Methionine sulphoxide reductase is one such enzyme and its function is to repair methionine sulphoxide, which is formed by free radical oxidation of the amino acid methionine. Proteins containing this altered amino acid, together with the presence of low methionine sulphoxide reductase levels have been implicated in cataract formation. However the importance of methionine sulphoxide reductase in repairing radical-induced damage has yet to be elucidated (Halliwell and Gutteridge, 1995). Oxidatively damaged DNA is repaired by specific repair enzymes involved in pathways such as base and nucleotide excision repair pathways (McCann and Berti, 2003).

1.4 Methods for Measuring Oxidative Damage

1.4.1 The Concept of Biomarkers

A biomarker is: " 1) A pharmacological or physiological measurement which is used to predict a toxic event in an animal. 2) A specific biochemical in the body, which has a particular molecular feature that makes it useful for measuring the progress of disease or the effects of treatment" (as defined in hyperdictionary.com).

By using the primary and secondary products of free radical damage as biomarkers, the status of defence mechanisms against free radical damage can be inferred. An ideal biomarker of effect must be highly specific for the effect of interest, have an inexpensive and simple analysis of non-invasively obtained samples such as urine, and have low background levels (De Zwart, et al., 1999). As outlined by Halliwell (2002), in order to validate a particular biomarker, two different steps are required: 1) analytical validation, including development of procedures, analysis of reference materials, and quality controls and 2) validation of the fact that changes in the level of biomarker do reflect the later development of disease.

Current challenges in human biomarker research include: sensitivity, selectivity, simplification and explanation of the molecular mechanisms underlying biomarker formation, namely, is there a causal relationship between biomarker excretion and the disease or toxin-induced free radical damage. The current methodology for biomarker analysis involves measuring products of free radical damage that result from disease, or exposure to certain toxins *in vivo* that can best demonstrate if free radical damage is occurring. The current biomarker analyses for the products of free radical damage do not determine whether free radicals are the cause or consequence of the damage that occurred. Ultimately, it is necessary to develop accurate methods to measure the biomarkers of oxidative stress. These measurements can provide insights into the efficacy of dietary antioxidants and environmental stress induced by specific professions involved in, for example, handling radioactive compounds, or give an indication of predisposition to certain diseases.

1.4.2 Oxidative Damage to DNA

All evidence pointing to oxidative damage to DNA as an indicator of cancer is, thus far, circumstantial. Therefore, it is important to determine if elevated levels of oxidised DNA bases in the urine or tissues correlate with the onset of such diseases. Oxidised bases are used as biomarkers for oxidative stress, whether in cellular DNA or as an elimination product in the urine (Renner, et al., 2000). About 70 oxidised nucleosides including diastereomers have been identified in model studies. However, chemical and biochemical assays have revealed that only eight of these are generated within cellular DNA (Cadet, et al., 2002).

This section will focus specifically on 8-hydroxy-2'-deoxyguanosine (8OHdG), (also known as 2'-deoxy-7,8-dihydro-8-oxoguanosine) because of its significant role in the induction of mutations (Cheng, et al., 1992), its quantitative abundance, and the availability of sensitive methods for its determination in different biological matrices. 8OHdG is formed by the attack of hydroxyl radicals or singlet oxygen at the C-8 position of the guanine base (Park and Floyd, 1992). The presence of 8OHdG causes guanine to thymine transversions during DNA replication due to an altered hydrogen bonding capacity. 8OHdG analysis, however, can underestimate oxidative damage to DNA, as other oxidatively damaged bases (such as 8-hydroxy-2'-deoxyadenine) are ignored. Dietary 8OHdG does not appear to contribute to excreted 8OHdG, as shown in radiolabelled 8OHdG studies (De Zwart, et al., 1999; Gackowski, et al., 2001).

1.4.2.1 8OHdG Keto-enol Tautomerism

Tautomerism refers to a naturally occurring equilibrium between different molecular structures of the same molecular formula. Tautomers usually differ in the point of attachment of a hydrogen atom. 8% of the molecules exist in the enol form at physiological pH (pH 7.4), where the other tautomer predominates (Culp, et al., 1989). Of the many analytical techniques used to measure 8OHdG, there is a large variation in the pH used for sample preparation, although no reason for the pH of choice is given and the tautomerism is never alluded to. When analysing 8OHdG it is important to consider the possibility of 8OHdG keto-enol tautomerism (Figure 1.2) and adjust the pH of the

system appropriately so as to convert the racemic mixture to one tautomeric form for quantification.

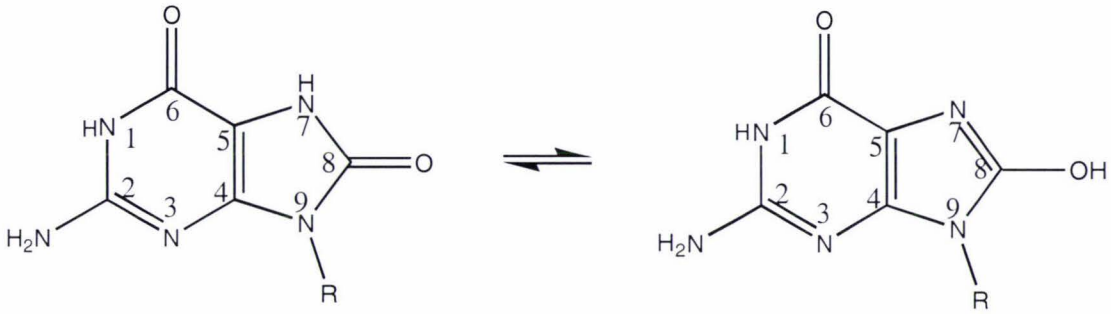


***Figure 1.2**: 8-hydroxy-2'-deoxyguanosine keto-enol tautomerism. The keto form (left) and enol form (right). The enol form is more stable for aromatic compounds. R = ribose.*

1.4.2.2 Non-invasive Urinary Analysis

The main repair mechanism to ameliorate 8OHdG in DNA is base excision repair (Mitra, et al., 2002), which results in 8OHdG excretion in the urine. Urinary excretion is interpreted as a reflection of the integrated rate of oxidative damage within the body. Although analysis of 8OHdG in urine is a non-invasive method for biomarker analysis, when dealing with the urinary excretion no information is given on the distribution of oxidatively damaged DNA in the genome; this is important, as some mutations occur in non-coding DNA. 8OHdG has been chosen for analysis by many groups because it is more closely linked with mutagenesis than oxidation products of adenine or thymine for example (Moller and Loft, 2002). The amount of 8OHdG in urine seems to be unaffected by diet and 8OHdG is thought not to be metabolised in humans (Halliwell, 2002). It is more difficult to isolate 8OHdG by reversed-phase high-performance liquid chromatography (RP-HPLC) in rat urine than human urine (Ames, et al., 1993; Loft, et al., 1998; Lengger, et al., 2000) although the advantage of studying rats is that their diets can be more easily manipulated and controlled. The urinary 8OHdG concentration has been expressed as nmol 8OHdG per mole of creatinine (creatinine is routinely used to correct for anomalies in urine concentrations), or nmol 8OHdG kg^{-1} body weight 24 $hours^{-1}$ (Cooke et al., 2000).

Many analytical techniques used to measure urinary 8OHdG lack specificity, have insufficient sensitivity and have extremely difficult and extensive sample preparation (Wilson, et al., 2002). Electrochemical detection following RP-HPLC eliminates the problem of separating the signal of 8OHdG from normal deoxynucleosides because 8OHdG is the only electrochemically active oxidised DNA base (Jenner, et al., 1998). An electrochemical detector has increased sensitivity compared to ultraviolet spectrophotometric techniques for electrochemically active compounds and can be used to detect and quantify trace levels of electroactive compounds in complex matrices. A type of electrochemical detector, the CoulArray® detector builds on this with the use of 8-16 electrochemical cells, which can be set at different potentials to oxidise or reduce the compounds that elute from the HPLC column. This results in different oxidation profiles for each eluting compound. Peak identification in the sample can be predicted by comparing the retention time of the desired component to an 8OHdG standard. Other compounds present in the urine can have the same retention time, but should have different oxidation profiles with CoulArray® detection, thus enabling accurate identification of 8OHdG. Measurement of 8OHdG alone may give misleading information, as an increasing level of 8OHdG could be due to either an increase in oxidative damage or a decrease in repair, therefore, caution must be taken when interpreting urinary excretion results.

Gas chromatography followed by mass spectrometry (GC-MS) is another method that can be used to measure many oxidised DNA species in the urine. However, extensive sample preparation, including solid phase extraction and/or RP-HPLC pre-purification means the method is tedious (Jenner, et al., 1998). Although many oxidised DNA bases can be measured at the same time in one sample, which could be an advantage over analysis of 8OHdG alone.

1.4.2.3 Analysis of Cells for Oxidative Damage to DNA

Oxidative damage in cells can be studied both directly and indirectly. The cellular 8OHdG concentration is expressed as the number of oxidised bases per non-oxidised residues. Until the repair rates and capacity for the repair of specific tissues can be adequately assessed, the rate of oxidative damage can only be estimated from the

urinary excretion of repair products as a mean of the oxidative damage to the entire body.

Direct Analysis of Oxidative DNA Damage to Cells

The direct approach involves extraction of the DNA followed by either enzymatic or chemical hydrolysis to release the bases followed by analysis by, for example, RP-HPLC with electrochemical detection or GC-MS. Isolation of DNA from tissues to measure the products of oxidative damage has an advantage over urine, as there are fewer co-eluting peaks. The disadvantages of using tissue, however, are the levels of oxidative damage differs between each tissue (Fraga, et al., 1990), and some of the DNA can artifactually oxidise during sample preparation and handling where a high derivatisation temperature is required, for example, in the GC-MS technique.

A monoclonal antibody, which recognises 8OHdG has been isolated (Park, et al., 1992). While this antibody is useful for quantitative work, it gives an overestimate of the level of oxidative damage, due to cross-reactivity to the normal deoxynucleoside, deoxyguanosine. RP-HPLC with electrochemical detection or GC-MS detects monomeric 8OHdG and may be used for tissue DNA, or urine. Intra-individual variation of human urinary 8OHdG appears to be largely underestimated, and therefore single values of 8OHdG should be considered with caution, particularly in small study groups (Knasmuller and Verhagen, 2002). This means in order to validate the methodology, the study group must cover a large sample from the population.

When studying oxidised DNA in a specific tissue, care should be taken to obtain the urinary data as well, as it is difficult to define which tissue is the most appropriate for analysis. The wide range of 8OHdG concentrations measured by HPLC with electrochemical detection in DNA from various sources is thought to result from different DNA isolation techniques.

Indirect Analysis of Oxidative DNA Damage to Cells

Indirect analysis involves the isolation of cells, and the combined use of a specific DNA repair enzyme and a technique to measure DNA strand breaks. An example of this method for analysis is using a DNA repair enzyme, formamidopyrimidine DNA N-glycosylase (FPG), which creates single strand breaks in DNA at the sites of altered

purines, including 8OHdG (Minguez-Mosquera, et al., 1994). These breaks can then be measured by the comet assay (single cell alkaline gel electrophoresis).

The comet assay detects breaks by their ability to relax the DNA thereby altering the migration pattern in gel electrophoresis, producing a comet-like image in which the percentage of the total comet which is the tail reflects break frequency. The DNA is stained with a fluorescent dye and the relative tail intensity is assessed by sorting comets into classes from 0 (no detectable tail) to 4 (large tail, minimal head), and related to a calibration curve to determine actual DNA break frequency. This type of analysis is simple, economical and relatively sensitive, however, underestimates can occur in clustered regions of lesions where the lesions may behave like a single strand break and some damage sites may be inaccessible to the enzyme.

1.4.3 Oxidative Damage to Proteins

Oxidative damage to proteins increases their carbonyl content and consequently carbonyl groups on amino acids are thought to be a hallmark of oxidative modification (Yan and Sohal, 1998). However, the appearance of carbonyl groups on proteins does not result exclusively from oxidative modification, for example, glycation of proteins may add carbonyl groups onto amino acid residues (Levine, et al., 1994). Thiol groups, which form on cysteine residues and oxidised tryptophan and tyrosine residues (Astley and Lindsay, 2002) may also serve as biomarkers of oxidative modification to proteins. Studies by Grune, et al. (1995) concluded that mild oxidative stress modified cellular proteins thereby increasing intracellular proteolysis. In contrast, severe oxidative stress diminishes intracellular proteolysis, probably by generating severely damaged cell proteins such as cross-linked or aggregated proteins that cannot be easily degraded.

1.4.3.1 Methodology

In order to detect carbonyl groups, the proteins must first be derivatized. Protein carbonyls can be reduced to alcohols with tritiated sodium borohydride, and identified

using polyacrylamide gel electrophoresis. Protein bands are visualised by Coomassie blue staining then excised and incubated in hydrogen peroxide to solubilise the gel. The tritium level is then quantified by liquid scintillation counting (Yan and Sohal, 1998).

Another method of derivatization is the use of 2,4-dinitrophenylhydrazine (DNPH), which was pioneered by Levine, et al. (1994). The DNPH undergoes reaction with carbonyl groups to form the 2,4-dinitrophenylhydrazone derivative, which is then colorimetrically detected. In addition to colorimetric detection, HPLC (Levine, et al., 1994) or an ELISA (Buss, et al., 1997) can be used to analyse the 2,4-dinitrophenylhydrazone derivative. HPLC can be problematic in that protein precipitation can occur and cause the column to block. An additional problem is with the use of 2 M hydrochloric acid used in derivatization, which is too concentrated for use in the HPLC system, as it can destroy the chromatography columns and tubing.

A colorimetric method is a simple, effective way of determining the level of oxidised protein by analysing the appearance of 2,4-dinitrophenylhydrazone in a plasma protein matrix. Using this method does not require any handling of radioactively labelled reagents, and uses inexpensive equipment. The ELISA method, although more expensive, may be considered more cost-effective as the assay is not as time-consuming as the colorimetric method and both methods exhibit the same overall trends (Buss, et al., 1997).

1.4.4 Oxidative Damage to Lipids

The overall result of lipid peroxidation is to decrease membrane fluidity, increase the 'leakiness' of the membrane to substances that do not normally cross it (such as calcium ions), and inactivate membrane-bound enzymes. Continued fragmentation of fatty acid side-chains to produce aldehydes and hydrocarbons such as pentane will eventually lead to complete loss of membrane integrity. Oxidative damage to lipids may be measured by analysis of the products of lipid peroxidation, or direct analysis of LDL lipid peroxidation using copper-mediated oxidizability studies *ex vivo* (Wiseman, et al., 2002).

1.4.4.1 Malondialdehyde Analysis

Malondialdehyde (MDA; 1,1,3,3-tetramethoxypropane) results from peroxidation of arachidonic acid and is formed in small amounts in most tissues. According to Largilliere and Melancon (1988) the amount of circulating free MDA is very low and in healthy subjects may not be detected. In contrast to free radicals, aldehydes are relatively stable and therefore are able to diffuse within or out of cells. Aldehydes such as MDA, and their metabolites are therefore potentially good indicators of lipid peroxidation (De Zwart, et al., 1999).

The most common assay used to analyse MDA in plasma is an acid-catalysed reaction with thiobarbituric acid (TBA) to generate a pinkish-red TBA-MDA adduct (Fan, 2002). This test, however, lacks specificity, and the high derivatisation temperature means that artefacts can form. Furthermore, other compounds present in the sample are also reactive towards TBA. The TBA assay has also been used to determine MDA in urine (Draper, et al., 1984), bronchoalveolae of rats (Petruska, et al., 1991) and heart tissue (Lazzarino, et al., 1995). These results however did not agree with work done by other groups, probably due to MDA formation during sample preparation.

A recently developed GC-MS based assay allows the specific analysis of MDA in plasma, cell cultures, solid organs, and sperm (Fenaille, et al., 2001). The assay requires initial derivatisation of the MDA in plasma samples with phenylhydrazine followed by separation of the derivatised MDA by GC-MS.

In a study by Draper, et al. (1984) urinary MDA content was found to be responsive to conditions associated with increased lipid peroxidation *in vivo* such as vitamin E deficiency, iron administration and high tissue concentrations of polyunsaturated fatty acids. This study showed that the diet did not contribute to the formation of urinary MDA. Although MDA is extensively metabolised to carbon dioxide, small amounts are excreted in an acid-hydrolysable form in rat urine (Draper, et al., 1984). Further studies on MDA in urine will evaluate MDA analysis as a non-invasive method for the evaluation of MDA as a biomarker of oxidative damage.

Some cells are more resistant to lipid peroxidation than others (Drummen, et al., 2002) and an absence of free MDA in normal human plasma is not unexpected. MDA has a tendency of to form complexes with proteins and amino acids (Largilliere and Melancon, 1988); therefore, ideal analysis would be to use two or more methods. It is, therefore important to consider the compound being measured in relation to how it relates to the overall process of lipid peroxidation (Halliwell and Gutteridge, 1995). Thus, it is advisable to perform an overall cellular measurement in conjunction with other biomarker analyses to get a more accurate measure of oxidative damage.

1.4.4.2 Isoprostane Analysis

Isoprostanes (also referred to as F_2-isoprostanes) are prostaglandin-like compounds, produced in humans by a non-cyclo-oxygenase free radical-catalysed mechanism involving peroxidation of arachidonic acid (Wilson, et al., 2002). When measured by GC-MS isoprostanes are considered a useful marker of lipid peroxidation *in vivo* (Pratico, et al., 1996; Roberts and Morrow, 2000). In addition to being determined in the tissue, F_2-isoprostanes have been determined in the urine (Cracowski, et al., 2001). The primary biological source of urinary F_2-isoprostanes, however, is unknown. Analytical methods for isoprostane measurement include GC-MS and ELISA. Both methods require two solid phase extraction steps and two preparative thin layer chromatography steps, and therefore are extremely time-consuming (Morrow, et al., 1992). An advantage of isoprostane analysis is that there is no background in air or solvents, thus reducing the possibility of contaminants, however the difficult sample preparation and invasively obtained samples means that isoprostanes are unlikely to be an acceptable biomarker at this stage.

1.4.4.3 Volatile Hydrocarbon Analysis

Measurement of volatile hydrocarbons, mainly ethane and pentane has been used and validated as a measure of lipid peroxidation in both *in vitro* and *in vivo* studies pioneered by Riely and Cohen (1974) who investigated a possible relationship between carbon tetrachloride and ethane exhalation in mice. Subsequent studies measured propane, 2-

methyl propane, butane, and 2-methyl butane. Studies by Van Gossum, et al. (1988) showed vitamin E supplementation increased the plasma vitamin E levels and decreased the pentane exhalation. Increased pentane was found in subjects with various disorders such as multiple sclerosis (Drury, et al., 1997). The major limiting factor in ethane exhalation analysis is the high probability of contamination of inhaled air, which can lead to artifactually high alkane levels and the inability to determine which tissue has high levels of lipid peroxidation.

1.4.5 Overall Antioxidant Capacity

There are many methods for analysing the overall antioxidant capacity, which may give an indication of the total oxidative damage to a system, tissue or organism. Each method has its own advantages and disadvantages and the optimum analysis would be in conjunction with analysis of at least one of the biomarkers discussed above. The increase in total antioxidant capacity in plasma after consumption of antioxidants should indicate absorption of the antioxidant and an increase in the *in vivo* antioxidant defence status. Little change in the antioxidant status may be observed with this type of analysis, as the total antioxidant capacity in plasma appears to be tightly regulated (Prior and Cao, 1999).

The most common methods are free radical antioxidant power (FRAP), the ORAC assay and the trolox equivalent antioxidant capacity (TEAC) assay which will be discussed in more detail below. Other methods that measure the *in vitro* antioxidant capacity include:

- 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay, a colorimetric method to study the scavenging effects of antioxidants on the stable radical DPPH. (Gil, et al., 2002)
- Superoxide anion scavenging assay, which measures superoxide anion depletion by superoxide dismutase (Bagchi, et al., 1997)
- Dichlorofluorescein (DCF) assay, which uses non-fluorescent fluorescein derivatives of DCF, which, after being oxidised by various oxidants, will become fluorescent.

The level of oxidative stress can be ascertained by quantifying the fluorescence (Wang and Joseph, 1999).

- The ferric-xylenol orange peroxidase (FOX) assay, which involves reduction of the hydroperoxide by the ferrous ion and quantification at 560 nm (Nouroozi-Zadeh, et al., 1994). The assay lacks stoichiometry between amount of iron and hydrogen peroxide produced and is sensitive to small changes in pH. Also the fact that biological solutions contain natural buffering compounds such as proteins should be recognised (Gay and Gebicki, 2002).

1.4.5.1 Oxygen Radical Absorbance Capacity Analysis

The ORAC assay, developed by Cao, et al. (1993) depends on the free radical activity to reduce the signal from a fluorescent probe. The inhibition of free radical damage by an antioxidant is directly correlated with fluorescence intensity, as measured by antioxidant protection against the change of fluorescence upon the introduction of a free radical inducer (2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)). The final result is expressed relative to the standard, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (trolox), which is a water-soluble analogue of vitamin E. The standards undergo the same treatment, producing a fluorescence decay curve from which a linear standard curve of area under the curve against concentration is plotted.

The "total antioxidant capacity of samples is estimated by taking the oxidation reaction to completion" (Cao, et al., 1993). Ou, et al. (2001) has recently developed an improved method ($ORAC_{FL}$) which used fluorescein instead of B-phycoerythrin. The $ORAC_{FL}$ was found to be more accurate in providing a direct measure of hydrophilic chain-breaking antioxidant capacity against the peroxyl radical. The $ORAC_{FL}$ assay however, cannot be considered as 'total antioxidant activity' since the assay is performed in aqueous solution, therefore the $ORAC_{FL}$ assay primarily measures hydrophilic antioxidant activity against peroxyl radicals. Using $ORAC_{FL}$ analysis, Cao, et al. (1997) demonstrated that some flavonoids could behave as both pro-oxidants and antioxidants depending on the concentration and the free radical source.

1.4.5.2 Free Radical Antioxidant Power Analysis

The FRAP assay measures the ability of an antioxidant in the sample, to inhibit the oxidative effects of reactive oxygen species generated in the reaction mixture. At low pH, antioxidants in the sample non-specifically reduce ferric to ferrous iron, which has an intense blue colour that can be monitored by measuring the change in absorption at 593 nm. The increase in absorbance is related to total reducing power of the electron-donating antioxidant present in the sample (Benzie and Strain, 1999).

1.4.5.3 Trolox Equivalent Antioxidant Capacity Analysis

The TEAC assay (also known as the ABTS or decolourisation assay) is based on inhibition of the radical cation 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) formation by antioxidants in the sample. The sample can be analysed in the lipophilic and hydrophilic phases separately. The antioxidant activity is influenced by the concentration of antioxidant present and the duration of the reaction (Re, et al., 1999).

1.4.5.4 Comparison of FRAP, ORAC and TEAC

Studies by Ou, et al. (2002) showed a good correlation between FRAP and ORAC although the ORAC assay reflects peroxyl radical scavenging activity or hydrogen atom transfer and therefore is not a total antioxidant activity assay, and FRAP estimates only the iron (III) activity. Both FRAP and TEAC each use single electron-transfer mechanisms to assess antioxidant activity therefore neither TEAC or FRAP measures chain-breaking antioxidant activity or preventative antioxidant activity. No oxygen radical is involved in FRAP or TEAC so the results do not necessarily reflect antioxidant activities, and FRAP does not measure thiols which can act as antioxidants, such as glutathione. Also the FRAP and TEAC assays rely on the hypothesis that the redox reactions proceed so fast that all reactions are complete within a short time, which may not always be true.

Prior and Cao (1999) showed that the ORAC assay was more sensitive than the FRAP and TEAC. It appears impossible to measure total antioxidant capacity using only a single assay. Thus, methods specific for each radical need to be used to get a more precise indication of the total antioxidant capacity.

1.4.6 Oxidative Damage in Mammalian Systems

1.4.6.1 Inducing Oxidative Damage

As discussed above, results from an experiment by MacDonald-Wicks and Garg (2003) showed vitamin E had no effect on the basal level of oxidative stress, but may be of greater significance when animals are challenged with a pro-oxidant. In order to attain a measurable difference in oxidative damage, the basal level of oxidative damage may need to be increased.

Many different chemicals induce free radical damage, for example: ethanol exposure increases superoxide anion in liver mitochondria; carbon tetrachloride ($CHCl_3$) induces oxidative stress through formation of $CCl_3^{\bullet}$ radicals; iron nitrilotriacetate induces oxidative damage to DNA and lipids in the kidney; and paraquat can produce superoxide anions due to redox cycling via P450 reductase.

When increasing basal oxidative damage, a subtle increase is desired to provide a measurable increase in oxidative damage without shocking the organism. A method for achieving this is the addition of fish oil to the diet (Cho, et al., 1995; Ibrahim, et al., 1997). Due to its high level of unsaturation, fish oil can be rapidly oxidised to produce lipid peroxide, although it is not well established how the fish oil induces oxidative damage. The lipid peroxidation process involves the formation of free radicals, which can attack other tissue components such as nucleic acids.

1.4.6.2 Cell-Based Studies

Oxidative damage can be measured by biomarker analysis of cells. Szeto, et al. (2002) studied DNA damage in lysed cells using the comet assay and demonstrated that antioxidants in a subcellular environment did not necessarily exert the same effect as for the whole cell, due to the cell membrane, which acted as a barrier. An additional problem with cell-based analyses is that media can catalyse the oxidation of compounds added to them, resulting in cellular effects that may be due to free radical production (Halliwell, 2003). Such artefacts have affected many studies on the effects of ascorbate, thiols and flavonoids. The cells used in such studies are cancer-derived, and therefore may not contain pathways that are pertinent to normal cells.

The comet assay involves the removal of synergistic intracellular components, which may reduce antioxidant action, or even promote pro-oxidant effects of some dietary antioxidants. Thus when analysing antioxidant action by the comet assay, parallel *in vivo* testing is advisable.

Noroozi, et al. (1998) showed by cell studies using the comet assay that the protective effect of vitamin C against DNA damage was significantly less than that of many flavonoids tested. However, the vitamin C and flavonoids appeared to work in synergy to achieve a greater combined level of protection against oxidative damage. Cellular studies may be used to provide a basis for mammalian studies, although the cellular results alone cannot be related directly to mammalian systems.

1.4.6.3 Mammalian Studies

Recently many mammalian studies have emerged ((Boyle, et al., 2000; Collins and Horvathova, 2001; Panayiotidis and Collins, 1997; Ramirez-Tortosa, et al., 2001; Richelle, et al., 1999)) which look at the affects of dietary antioxidants on oxidative stress. It is important to implement mammalian studies, using rats, for example, to first test the hypothesis, then study the human population to measure the biological efficacy of dietary antioxidants.

1.5 Hypothesis and Aims

1.5.1 Hypothesis

Fruit-derived antioxidants decrease oxidative damage in mammals, as measured by biomarkers of oxidised DNA, lipids and proteins.

1.5.2 Aims

- Development and implementation of an assay for a biomarker of oxidised DNA (8OHdG)
- Implementation of an assay to measure protein carbonyl groups in plasma
- Application of the developed assays to a number of rat trials
- Analysis of urine and plasma from the rat trials for changes in the concentration of biomarkers.

In order to gauge the *in vivo* antioxidant potential of specific compounds, an experimental procedure to assess the efficacy of dietary antioxidants was developed. The antioxidant efficacy was determined by measuring oxidative damage to DNA (analysis of 8OHdG in urine), proteins (as plasma protein carbonyls) and lipids (as plasma MDA) in experimental rats when fed several combinations of natural and synthetic diets.

Analysis of 8OHdG involved solid phase extraction of urine samples, followed by reversed-phase high-performance liquid chromatography with electrochemical detection. Protein carbonyl analysis used both colorimetric and ELISA methodology. MDA was assessed using GC-MS methodology.

Chapter Two – Method Development

Validation of biomarkers requires 2 different steps: 1) analytical validation, including development of procedures, analysis of reference materials, and quality controls; and 2) validation of the fact that changes in their level do reflect the later development of disease (Wada and Ou, 2002). Since oxidative damage is associated with the development of many diseases (Joseph, et al., 1998) it has been assumed that, since biomarkers are formed due to free radical damage, they will reflect the possibility of the development of free radical-related diseases.

Method development is a key component of this project. This chapter describes the methodology and its development, implementation and alterations in order to refine the assays. For 8-hydroxy-2'-deoxyguanosine (8OHdG) analysis, this includes optimisation of the sample recovery by pH control studies and implementation of quality controls to assess both the intra- and inter-assay variation. The implementation of the rat feeding experiments attempts to validate the developed biomarker methods. This validation was aimed at providing a link between the biomarker concentration and the level of oxidative stress and to test the efficacy of the berry fruit extracts, which are known to be effective antioxidants (Ramirez-Tortosa, et al., 2001), in the context of rat feeding experiments.

2.1 8OHdG Assay to Measure Oxidative Damage to DNA

The concentration of 8OHdG in urine provides a measure of the integrated amount of DNA damage within the body. 8OHdG analysis may be interpreted as a measure of DNA turnover, as a response to oxidative stress, and provides a measurement of DNA flux *in vivo*.

Many complex methods are available for analysis of 8OHdG, including: two-step solid phase extraction followed by high-performance liquid chromatography (HPLC) with electrochemical detection (Germadnik, et al., 1997); the multiple chromatographic

technique using three HPLC pumps, three switching valves and four HPLC columns following solid phase extraction (Bogdanov, et al., 1999) and the use of immunoaffinity columns (Helbock, et al., 1998). The methodology that has been developed in this study aimed to provide a simpler, faster method of analysis of 8OHdG in urine.

2.1.1 Standard Preparation

500 $\mu g ml^{-1}$ of 8OHdG stock standard (cat # H5653; Sydney, Australia) was made up in 0.1 M phosphoric acid and stored at 4°C. Working standards were initially diluted in 0.1 M phosphoric acid, however, this resulted in the peak for 8OHdG being skewed to the front edge in the HPLC analysis. Preparing the working standards in 100 mM sodium acetate (NaOAc), pH 3.5, gave more symmetrical peaks on the HPLC.

2.1.2 Sample Preparation by Solid Phase Extraction

2.1.2.1 Preliminary Method Development: Is Solid Phase Extraction Necessary?

A urine sample was injected directly into the one pump dual column HPLC system to determine whether solid phase extraction (SPE) was necessary and compared with a 1 $\mu g ml^{-1}$ standard and urine that had been through the SPE clean up procedure (described in subsequent sections) (Figure 2.1).

The presence of 8OHdG was determined by the retention time and compared to the channel ratios of the standard (A) from electrochemical detection. This shows SPE is necessary to visualise the 8OHdG peak in the electrochemical detector (B) and (C). The SPE is therefore important in terms of concentrating the 8OHdG in the urine and cleaning up the urine sample matrix.

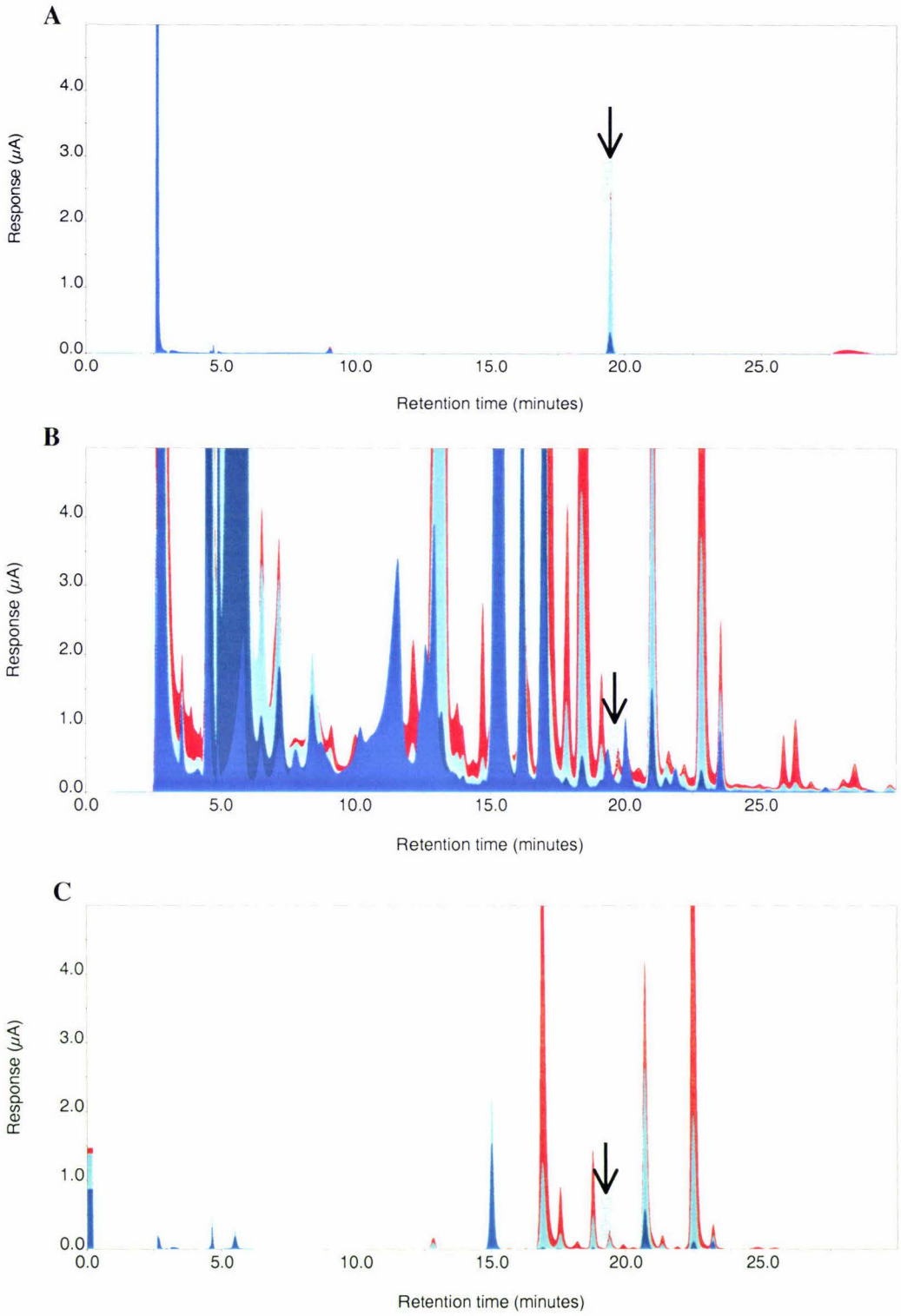


Figure 2.1: *Chromatograms for 100 μl injections of: 8OHdG 1 μgml^{-1} standard (A), urine that has not been through SPE (B) and urine that has been through the SPE purification procedure (C). Samples were analysed on one pump, dual column RP-HPLC with CoulArray® detection. Channels shown had the applied oxidation potential of: 200 mV (bright blue); 300 mV (dark blue); 400 mV (cyan) and 500 mV (red). 8OHdG was quantified at 400 mV. Arrow shows elution of 8OHdG.*

2.1.2.2 *Solid Phase Extraction Protocol*

The solid phase extraction protocol was based on Shigenaga, et al. (1989). 500 mg Bond Elut™ C18-OH SPE (part # 12113045, Phenomenex Inc, California, USA) cartridges were used to clean up the urine samples. The cartridges were preconditioned sequentially with 10 ml of methanol (MeOH), 10 ml of water and 10 ml of 50 mM potassium dihydrogen phosphate, pH 7.5 (buffer A). A 1 ml aliquot of rat urine was mixed with 1 ml of 1 M sodium chloride solution using a vortex, before application to the SPE cartridge. The cartridge was washed with 4 ml of buffer A followed by 5 ml of buffer B (5% MeOH in buffer A) to elute water-soluble compounds present in the urine. Following this, lipophilic compounds were washed out with 3 ml of ethyl acetate, then the 8OHdG eluted in 2 ml of buffer C (15% MeOH in buffer A). Each fraction was collected and analysed by HPLC to ensure the 8OHdG had not eluted in the wash buffer or solvents (see section 2.1.4 for HPLC analysis). These three washing steps were needed to reduce interference by other compounds present in the complex urine matrix.

An alternative SPE cartridge, Strata X™ (part # 8B-S100-HCH-S, Phenomenex, California, USA), was investigated in order to optimise recovery and reduce interference from other compounds present in the urine. The Strata X™ was described as being versatile, as it can be used for many applications. Two different resin weights of Strata™ resin: 200 and 500 mg Strata X™ were compared to the 500 mg Bond Elut™ SPE cartridge. Four of each of these three SPE cartridges were compared by addition of 1 ml of 1 $\mu g ml^{-1}$ 8OHdG standard to the cartridge following preconditioning as normal.

The results (Figure 2.2) showed that the recoveries were very similar for all cartridges although the standard error was substantially more for the 200 mg Strata X™ cartridge. Due to price and availability, the 500 mg Strata X™ cartridges were used for SPE of urine from the final two rat feeding trials.

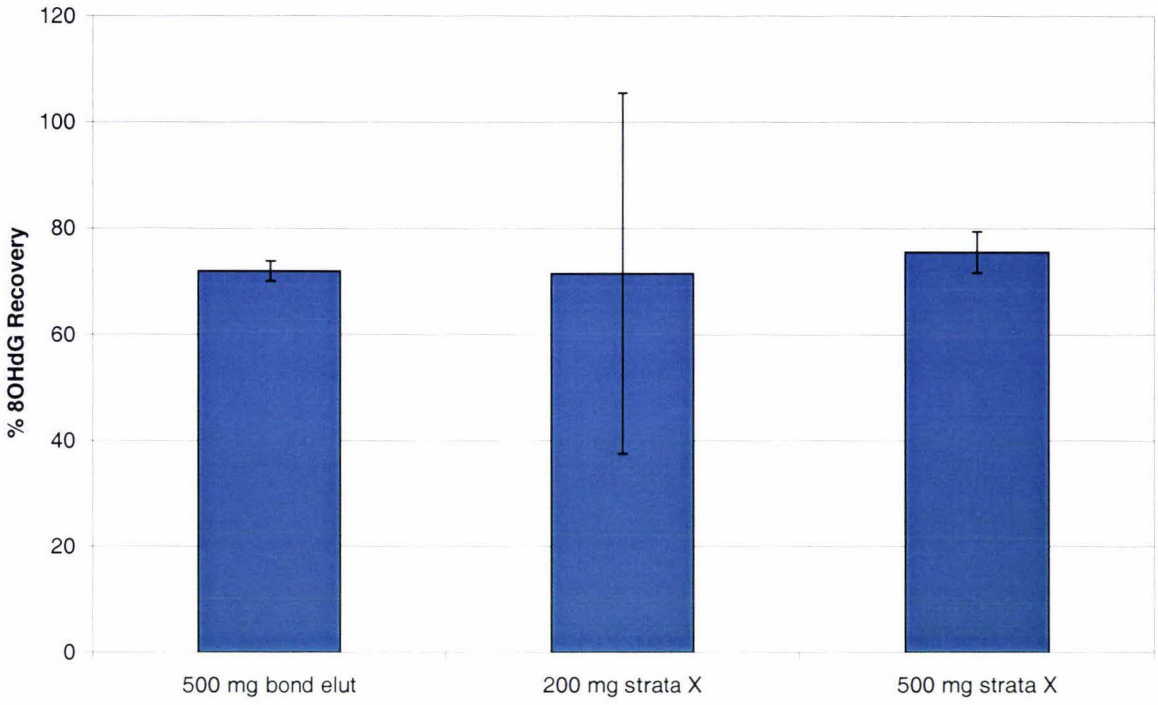


Figure 2.2: *Percentage recoveries for 1 μgml^{-1} 8OHdG standard through different SPE cartridges to determine which cartridge produced the optimum recovery of 8OHdG. Error bars represent SEM (n=4).*

2.1.2.3 pH Control of 8OHdG to Optimise Recovery

As 8OHdG may exist in different tautomeric forms (see section 1.4.2.1 introduction), the effect of pH on the sample was investigated. Alternatively, the pH of the system may induce acid hydrolysis of 8OHdG, cleaving off the sugar, forming 8-oxo-guanine.

8OHdG standards (1 μgml^{-1}) were prepared in 100 mM NaOAc at both pH 3.5 and pH 7.5. These standards were prepared in triplicate through the SPE cartridges using both the standard protocol, and a similar protocol but where the final elution was with 100% MeOH. The concentration of 8OHdG was calculated by referencing the peak height of the SPE standard to the peak height of a 1 μgml^{-1} standard prepared at both pH 3.5 and pH 7.5.

The elution of 8OHdG off the SPE cartridge was optimised by using 100% MeOH (Figure 2.3) therefore this version of the method was used for analysis for the last two

rat trials. The standard prepared at pH 3.5 showed a slightly higher recovery. In addition, when the 8OHdG concentration was quantified against standards prepared at pH 3.5 and pH 7.5, the standard prepared at pH 3.5 was preferred for quantification (data not shown), as the results gave higher 8OHdG recoveries. This may be because a different form of 8OHdG was present at pH 7.5, with a different retention time, therefore it did not get captured in the loop when switching occurred, and then loaded onto column B.

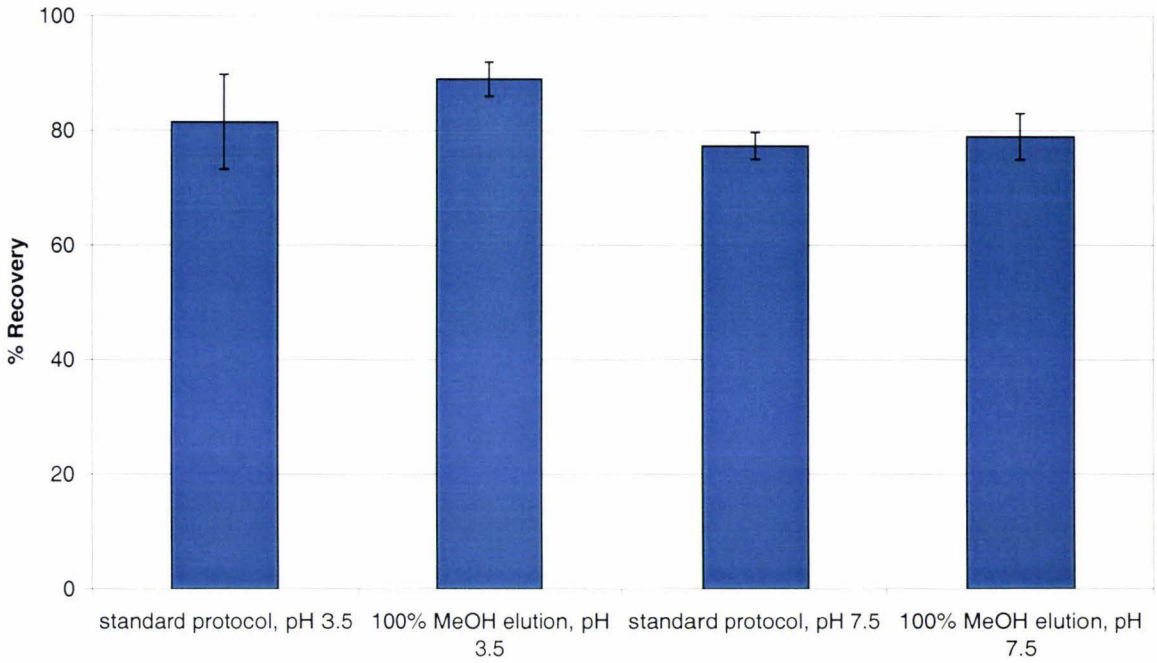


Figure 2.3: *Relative percentage recoveries after SPE and HPLC for 1 μgml^{-1} 8OHdG standards prepared in buffer at pH 3.5 or pH 7.5. Quantification against pH 3.5 standard is shown. Error bars represent SEM (n=3).*

Another alteration to the method postulated to optimise 8OHdG recovery was to change the pH of the urine to pH 3-4 before storage at –20°C (Germadnik, et al. 1997). Urine was frozen for one month before analysis and was spiked with 8OHdG standards (1 and 2 μgml^{-1}) and analysed before and after addition of 70 µl of 2 M hydrochloric acid (HCl) per 1 ml of urine prior to freezing and storage.

The results showed that reducing the pH of the urine before storage decreased the percentage recovery and increased the error of the concentration of 8OHdG in the urine (Figure 2.4). This may be due to the conversion of 8OHdG into another tautomeric form due to the low pH, rather than 8OHdG degradation. The percentage recoveries were greater than 100% due to the 8OHdG already present in the urine.

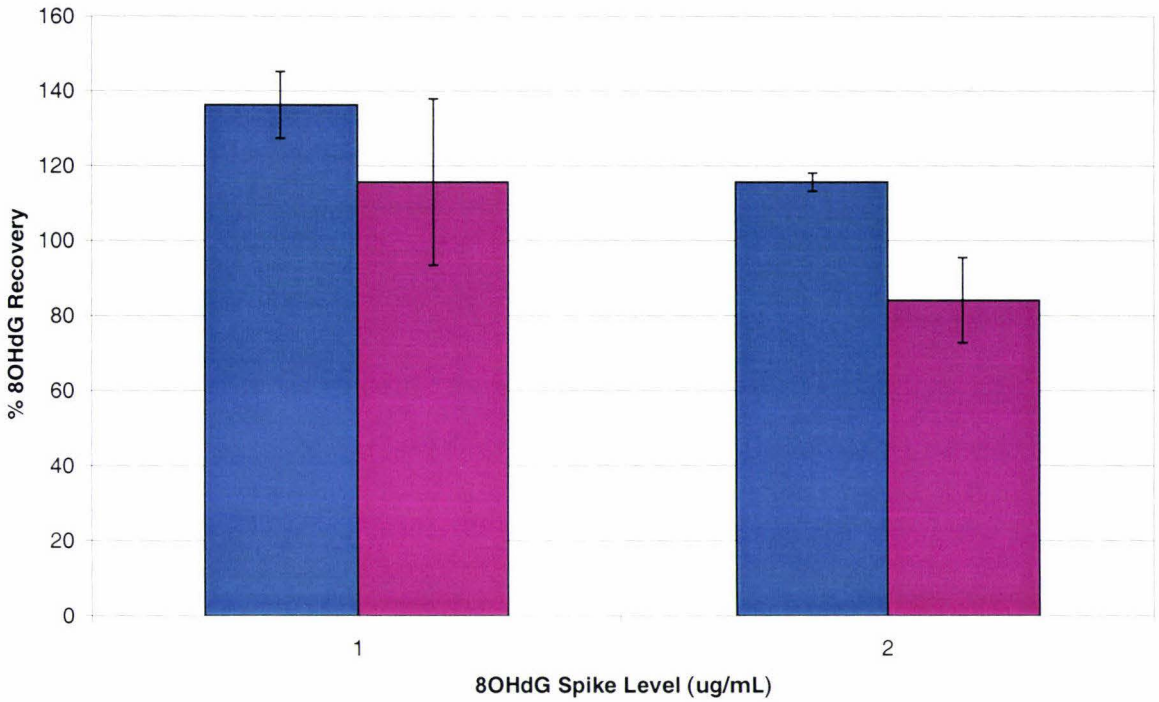


Figure 2.4: *Percentage recovery for 1 and 2 $\mu g ml^{-1}$ spiked and non-spiked urine where the pH had not (indigo) and had (maroon) been changed to 3-4 with 2M hydrochloric acid prior to storage. Error bars represent SEM (n=3).*

Therefore, although storage life may be extended by reducing the pH of the urine (Germadnik, et al., 1997), the recovery of 8OHdG is compromised. The samples from the rat trials were prepared by SPE within one week of collection therefore extending the storage life was not an issue. Samples were therefore frozen without any alteration to the pH.

2.1.2.4 Intra-sample Variation

The intra-sample variation was assessed by calculating the concentration of 8OHdG in urine pooled from the test rats prior to diet supplementation, which was spiked with 0.05 μgml^{-1} of 8OHdG. Four replicates underwent SPE and the mean percent recovery was 108.6% with a standard error of 0.04%. The intra-sample coefficient of variation for the SPE section of the protocol was 10.2%.

2.1.2.5 Implementation of Quality Controls for Sample Recovery Analysis – Inter-batch Variation

For convenience, SPE of urine samples was performed in batches of 11 samples or less. In order to control for inter-batch variation in the method, quality control analysis was implemented. The quality control sample consisted of 990 µl of rat urine containing 0.25 μgml^{-1} of 8OHdG. The analysis of the quality control from each SPE batch of samples provided the inter-sample variation for the SPE part of the method. Analysis of these samples was by the same method used to calculate the 8OHdG concentration in standard urine samples. The coefficient of variation over ten SPE batches was 9.6%, which was judged acceptable.

2.1.3 High Performance Liquid Chromatography Analysis

2.1.3.1 HPLC Columns and Solvent Programmes

HPLC analysis was based on the solvents used in Shigenaga, et al. (1989). Initially a 5 µm ODS2 Waters Spherisorb, 4.6 x 250 mm column (part # PSS839540, Waters Inc, Massachusetts, USA) was used with 100 mM NaOAc buffer, pH 5.5 (solvent A). Solvent B consisted of 20% MeOH in solvent A. The 8OHdG was detected at 260 nm using a Jasco UV/Vis detector. 8OHdG standards (5, 4, 3, 2, 1 and 0.5 μgml^{-1}) were analysed to prepare a standard curve. A 100 µl injection was used for all samples. To

increase the selectivity of the method, two columns were connected in series, where column A was a Phenomenex Jupiter, C5 (part # 00F-4167-EO, Phenomenex, California, USA) and column B was the Waters Spherisorb, C18 used above. The two columns have different separation properties and selectivities, and therefore act to further clean up the urine to separate the 8OHdG from interferences.

It was noted that in the standard, a large hump was present at approximately five minutes downstream from the 8OHdG peak (Figure 2.5). This could be a tautomer of 8OHdG, formed due to keto-enol tautomerism (see Introduction, 1.4.2.1), or 8-oxo-guanine, formed by acid hydrolysis of 8OHdG. The pH of the mobile phase was changed from 5.5 to 3.5 to push the equilibrium to only one tautomeric form. If the latter occurred, it may be that all analysis in this study referred to as 8OHdG is actually 8-oxo-guanine. This could be checked using standards of both 8-oxo-guanine and 8OHdG on an HPLC with detection by mass spectrometry (LC-MS). The 8-oxo-guanine cannot be purchased, so must be synthesised following the method described in Lee, et al. (2002).

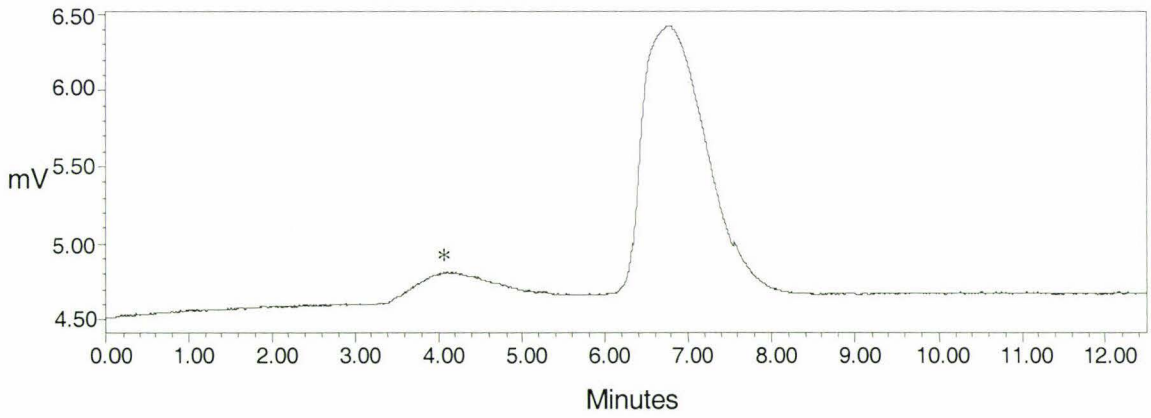


Figure 2.5: *Example chromatogram at 260 nm of a 1 μgml^{-1} 8OHdG standard where mobile phase conditions were pH 5.5. The large hump (*) at approximately 4 minutes may represent the other tautomeric form of 8OHdG, or the acid hydrolysed form, 8-oxo-guanine. When the pH was changed to pH 3.5, this hump disappeared.*

Dilution of stock standard prepared in 0.1 M phosphoric acid with a pH 3.5 buffer eliminated the large hump in the chromatograms. Additionally, because the peak was

broad and skewed, the 100 µl injection loop was replaced with a 10 µl loop, to reduce the volume of sample being loaded onto the column. This in turn reduced the peak width, allowing for more effective capture of the compound when the switching valve, described in subsequent paragraphs, was installed.

To further increase the sensitivity and selectivity, a Rheodyne 2-way switching valve was installed (Rheodyne, California, USA). The aim of this was to capture the 8OHdG in sample from the first column and load this onto the second column. This reduced interference by increasing the separation and selectivity for the 8OHdG in the sample.

To ensure the 8OHdG was captured in the 1 ml loop on the switching valve, at the start of each day, a 5 µg/ml standard was run through the first column to determine the exact time of 8OHdG elution, and thus the switching time. The main problem with this method was the large increase in pressure when column B was brought in line, from 2190 psi to 3995 psi, with a flow rate 1 $mlmin^{-1}$. The peak was very skewed from the first column, possibly indicating the pressure could have damaged the column. The flow rate was decreased to 0.8 $mlmin^{-1}$ to decrease the pressure, however the pressure of the system still remained relatively high (3270 psi). The valve was switched back after the 8OHdG was loaded onto column B, then following 15 minutes of rinsing column A, the flow was switched back to column B, to elute the 8OHdG with a binary gradient.

Due to the damage the high pressure caused to column A, the Jupiter C5 column was replaced with an Alltech Adsorbosphere C8, 5 µm, 150 x 4.6 mm (part # 287123, Alltech, Inc, Illinois, USA). Since the column was C8, it had different separation properties than the C5 column which altered the retention time, and in turn, the switching time. A new calibration curve was plotted, using the mean of five sets of 5, 4, 3, 2 and 0.1 μgml^{-1} standards (Appendix one). Spiked urine samples were analysed in conjunction with urine only to ensure the correct peak was quantified. The standard curve was found to be linear and therefore one-point standards were used during sample analysis. In calculating the concentration of 8OHdG in urine, the height of the 8OHdG peak in the urine was directly related to the mean height of the standard run both before and after the sample. This compensated for any 8OHdG lost in the switching (Materials and Methods, 3.2.1.3, for a sample calculation).

To resolve the problems associated with large pressure fluctuations, a second HPLC pump was installed, thus isolating each column and resolving the pressure problem (Materials and Methods, Figure 3.1, for column configuration). In addition to the second pump, a solvent programme was used as follows: Column A had an isocratic (single solvent) programme, using an LKB pump, and solvent A was replaced with 95% 100 mM sodium acetate, pH 3.5 and 5% MeOH. Column B was changed to Phenomenex Luna C18, 250 x 4.6 mm (part # 00G-4252-EO, Phenomenex, California, USA), which used a binary solvent gradient, controlled by a Jasco pump (Materials and Methods, Table 3.3). All samples were concentrated to dryness and redissolved in 200 µL of mobile phase A without MeOH, pH 3.5. The total run time to analyse 8OHdG in the urine was 40 minutes.

A 20 µl loop was installed to increase the amount of 8OHdG loaded onto the column, without creating a broad peak due to overloading, as with the 100 µl loop. Solvent flow for both columns was maintained at 0.05 $mlmin^{-1}$ when not in use. Each column was flushed with 15% MeOH/water every 5-6 days for at least 30 hours.

To reduce the variation in retention time of 8OHdG due to temperature variability, the column temperature was increased from 35°C to 40°C and all lines were run through the column heater (ESA Inc, Massachusetts, USA).

2.1.3.2 Detection of 8OHdG

To maximise sensitivity a CoulArray® electrochemical detector with a broad range of oxidation potentials from 100 mV to 800 mV in 100 mV increments was used (as for Figure 2.1). The 8OHdG was quantified at 400 mV oxidation potential. This channel was influenced by interference from other compounds. Therefore, a narrower range of oxidation potentials was used where the initial potential was 200 mV, with 45 mV increments, and the 8OHdG quantified at 290 mV. At 290 mV, the peak height was lower, but the interference by other co-eluting compounds was also reduced. This enhanced the selectivity for detection of 8OHdG.

2.1.3.3 Inter- and Intra-assay Variation Associated with HPLC of 8OHdG

The intra-assay variation contributed by HPLC analysis was calculated as the coefficient of variance of the same standard ($1\mu gml^{-1}$), which was injected at least six times during a single day. For any given day, the coefficient of variance was between 1.5 and 8.8%. Due to the relatively low variation, which was considered to be acceptable, the urine samples from the last trial were analysed with a standard either side of two samples.

The inter-assay variation was calculated by the 8OHdG concentration for the same standard analysed every day for 12 days. The coefficient of variation was 8.8%, where the standard was injected 63 times in 12 days. As a coefficient of variation of less than 10% is considered to be acceptable, HPLC analysis of the samples had acceptable performance.

2.1.4 Summary

The optimised HPLC method for the analysis of 8OHdG in urine benefits from simple sample preparation, although HPLC analysis is somewhat lengthy, as a standard must be run before and after every 2-3 samples to ensure correct column switching. In comparison with other methods of analysis described at the start of this chapter (Bogdanov, et al., 1999; Germadnik, et al., 1997; Helbock, et al., 1998) this method is much less time-consuming. The stability of 8OHdG under analysis conditions needs to be determined as to whether it is 8OHdG or its hydrolysis product, 8-oxo-guanine that is being detected. While this does not directly affect the interpretation of the results, for completeness, appropriate standards should be synthesised (Lee, et al., 2002) and the identity of the HPLC peak confirmed.

2.2 *Protein Carbonyl Assay to Measure Oxidative Damage to Proteins*

Oxidative modification of proteins has been confirmed to increase their carbonyl content, thus carbonyl groups on amino acids are a hallmark for oxidative modification (Yan and Sohal, 1998). Two methods of carbonyl analysis were compared to identify the most efficient method of carbonyl analysis.

2.2.1 Colorimetric Determination of Carbonyl Content

2.2.1.1 Initial Methodology Used

The oxidised protein method requires measurement of the amount of protein in the samples to be analysed therefore, a protein assay was performed using the Bradford method (Bradford, 1976), with an appropriate dilution (100-fold) of plasma in water + 5 ml BioRad reagent (BioRad, California, USA). The calibration curve for analysis of protein content is shown in appendix two. The absorbances at 595 nm were quantified against standards.

The optimum amount of plasma to be used was 100 µl, which contains approximately 40-50 mg of protein. Following the determination of protein content, two 100 µl aliquots of each sample were analysed according to the colorimetric method for carbonyl assay, described in Reznick and Packer (1994). In this assay, one aliquot of plasma was reacted with 4 ml of dinitrophenylhydrazine (DNPH), while the other aliquot served as a blank and was treated with 4 ml of 2.5 M HCl. Each sample was treated as described in Chapter Three, Materials and Methods. The absorbance of each sample was determined at 355 and 390 nm using a Beckman spectrophotometer. The carbonyl content was calculated from the absorbance at A_{355nm}-A_{390nm}, using an absorption coefficient, ε, of 22,000 $M^{-1}cm^{-1}$ for both wavelengths. This version of the method was used to determine the carbonyl concentration of the plasma samples from the fish oil rat feeding trial and the first fruit-derived antioxidants feeding trial.

2.2.1.2 *Final Methodology Used*

Upon comparison with the enzyme-linked immunosorbent assay (ELISA) method, the initial version of the colorimetric method produced very low values, due to the partial loss of pellets from the numerous washing steps involved. Further investigation into this methodology revealed a method by (Witko-Sarsat, et al., 1998) where the absorbance of each sample was read at 370 nm, rather than taking the difference between the absorbance at 355 and 390 nm. A wavelength scan was performed of a DNPH-containing plasma sample, and 370 nm was found to be the wavelength of maximum absorbance in the region between 355 and 390 nm. This method of measurement is simpler, since it only requires an absorbance measurement at one wavelength. Therefore for simplicity the carbonyl content was assessed at 370 nm, with the same extinction co-efficient used for the previous version of the method, as in Witko-Sarsat, et al. (1998).

2.2.2 Determination of Carbonyl Content using an Enzyme-linked Immuno-adsorbent Assay (ELISA)

In the colorimetric method described above, the results rely directly on the blank values due to protein loss in the washing steps. It is assumed, therefore, that protein loss in the treated sample is the same as the loss for the sample blank. In the ELISA method for determination of the carbonyl content the plasma samples were reacted with DNPH, then the protein was non-specifically adsorbed onto the ELISA plate. Unconjugated DNPH and non-protein constituents were washed away and the adsorbed protein probed with a biotinylated anti-DNP antibody followed by a streptavidin-linked horseradish peroxidase.

Each sample was analysed in triplicate and samples were quantified by comparison with bovine serum albumin (BSA) standards. Absorbances were read using a Dynatech™ MR5000 plate reader (Molecular Devices Corporation, California, USA), and were related to a standard curve prepared for serum albumin standards. Because this method includes adsorption of the protein to an ELISA plate, unconjugated DNPH and non-

protein constituents were easily washed away and gave minimal interference, therefore producing greater sensitivity and accuracy. The absolute carbonyl values, however, will depend on the oxidised protein standard used.

2.2.3 Comparison of ELISA and Colorimetric Methods for Determination of Protein Carbonyl Content of Plasma

A comparison of the results from the first two fruit-derived antioxidant trials from the ELISA and colorimetric is shown in Figure 2.6. The correlation co-efficient shows there is a very poor relationship between the values obtained using the two methods, thus the methods must not be measuring the same parameter. These results disagree with those of Buss, et al. (1997) where there was a "good correlation between ELISA and colorimetric assays for plasma samples" and R^2=0.7 (n=26).

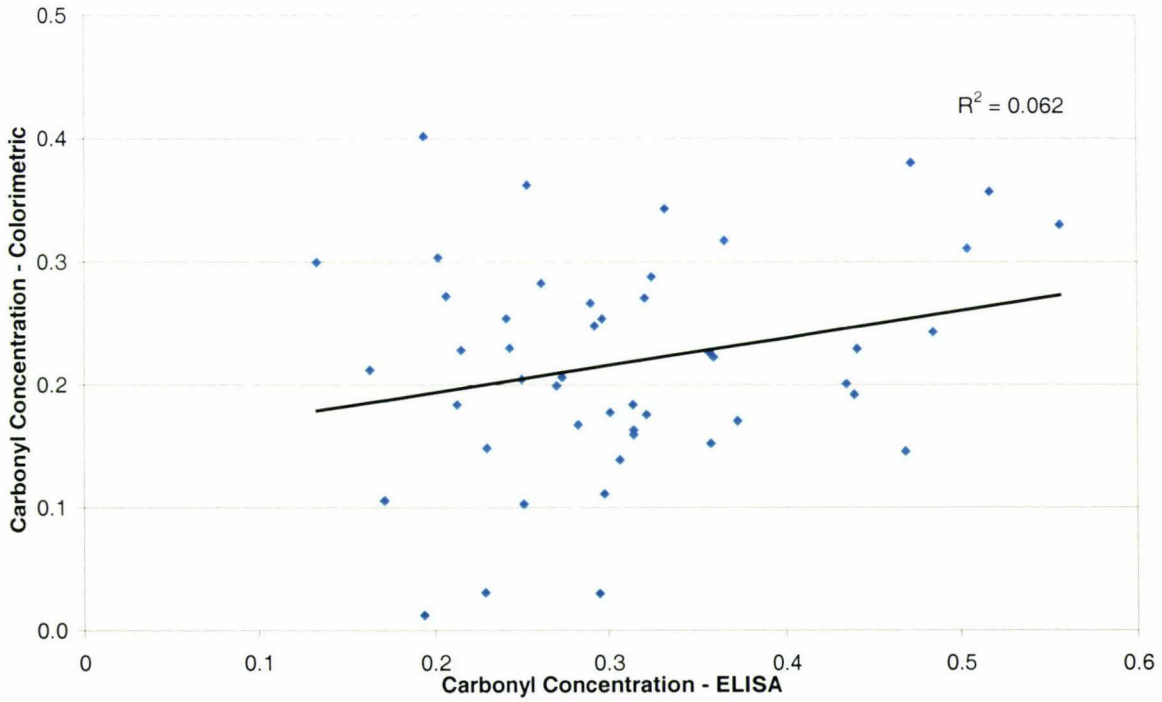


Figure 2.6: *Comparison of the ELISA method and colorimetric method (at 370 nm) for determination of protein carbonyl content for fruit-derived antioxidant feeding trials #1 and #2. (n=50).*

The study in Buss, et al. (1997), had many negative values for the carbonyl content determined by the colorimetric assay. Treating the negative samples as zero, may have revealed less of a correlation.

The fact the colorimetric values were lower may be due to the loss of some protein in the wash steps. It was assumed, however, that the amount of protein lost in the sample is equivalent to that lost in the sample blank. The ELISA method may also be over- or under-estimating the protein concentration due to the fact that the amount of protein was assumed to be equal in all samples for the ELISA method, which a protein concentration assay revealed was not the case. Alternatively, with plasma, it is likely that some proteins may adhere better to the ELISA plate than others, thus giving artefactually high carbonyl values (Buss, et al., 1997). The plasma protein content of samples from the first three rat trials gave a range of 35.8-68.4 $mgml^{-1}$ with a mean of 49.92 $mgml^{-1}$ and a standard deviation of 5.97. When the protein concentration was taken into account for the carbonyl concentration for samples determined by the ELISA method, the correlation between the two methods was virtually unchanged ($R^2 = 0.068$).

Replicate samples (n = 5) one with a high, and one with a low carbonyl concentration, as determined by the ELISA method from the second fruit-derived antioxidant feeding trial, were analysed by the colorimetric method to measure the variability of the assay. The results gave a mean coefficient of variation of 30.4%, with a range of 27.6% - 33.1%. The variation was similar in the plasma samples with low and high carbonyl content. Therefore, this method has high error with both low and high carbonyl concentrations.

To determine the variability of the ELISA assay, the mean coefficient of variation was calculated for the triplicate measures from each fruit-derived antioxidant feeding trial. The mean coefficient of variation was 11.7% (n = 104). This was considered to be acceptable. Thus, due to the accuracy and speed of the ELISA method, only this method was used for carbonyl content analysis for the final rat trial.

Chapter Three - Materials and Methods

3.1 Rat Feeding Experiments

3.1.1 General Protocol

Rat trials were performed to evaluate the accuracy of the developed biomarker methods described in Chapter Two, Method Development. The first trial incorporated fish oil into the natural diet to increase oxidative stress, so a decrease could be measured in subsequent trials when assessing the efficacy of berry fruit-derived antioxidants.

3.1.1.1 Animals

Feeding trials involved approximately 8-week-old male Sprague-Dawley rats, individually housed in a controlled environment in the Small Animal Production Unit at Massey University. Air temperature and relative humidity in the room were 22-24°C and 50-60% respectively with a diurnal 12-hour light cycle. Housing, handling and sample collection procedures were approved by the Massey University Ethics Committee (Approval numbers 01/72; 01/73; 01/98 and 03/60). Rats were randomised into groups according to their body weight, so the mean weight of each group was approximately the same.

3.1.1.2 Diets

The rats in the fish oil feeding trial (Chapter Four) were raised on a natural diet (Materials and Methods, Table 3.1), and the rats in each subsequent trial (Chapters Five and Six) were raised on synthetic diet (Table 3.1), unless otherwise specified. Each trial was based on a similar format, with differences discussed in the respective results' chapters. Both the natural and synthetic diets contained lowered levels of vitamin E so the main antioxidant present was the anthocyanin from the berry fruit extracts.

For each feeding trial, the rats were raised on a natural diet, then two weeks prior to the start of the trial they were fed the synthetic diet, to allow the rats to acclimatise to the synthetic diet. The diet weight was evaluated daily to determine the diet intake of each rat. All rats were allowed water *ad libitum*.

Natural Diet	*Synthetic Diet*
40.35% wheat	67% wheaten cornflour
30% barley	12% lactic casein
7% fishmeal	10% fish or soybean oil
6% meat and bone	*5% vitamin/mineral premix
5% broll	5% salt mix
5% skim milk powder	1% cellulose
5% Lucerne	
1% soybean oil	
*5% vitamin/mineral premix	
0.1% methionine	
0.05% sodium chloride	

Table 3.1: *Components of the natural and synthetic diets used in the rat feeding trials. *Contains 27 IU/kg vitamin E.*

Berry fruit Anthocyanin Extraction for Diet Supplementation

Boysenberry anthocyanin powders used in each trial were prepared by mixing 10 kg of previously frozen fruit with 10 l of isopropyl alcohol (IPA) and leaving overnight, at room temperature. Following this, the pulp was removed by filtration then a further 10 l of IPA was added to the pulp. The two IPA fractions were pooled and the solvent was removed by rotary evaporation at 30°C and the remaining liquid freeze-dried to give a powder.

The boysenberry and blackcurrant (Currantex-20™, provided by Just the Berries Corporation, Palmerston North, New Zealand) powders used had the compositions shown in Table 3.2. Activin™ is a grape seed extract that is a concentrated source of biologically active flavonoids, specifically proanthocyanidins and was supplied by Healtheries Limited, (Auckland, New Zealand) to use as a positive control.

*Component**	*Boysenberry Anthocyanin Powder*	*Blackcurrant Powder (Currantex-20™)*
Energy (kJ)	1580.0	155
Protein (g)	4.0	1.4
Available Carbohydrate (g)	88.8	7.4
Total Available Sugars (g)	66.3	7.3
Total fat (g)	<0.1	0.4

***Table 3.2**: Composition of boysenberry anthocyanin powder used in trial #3, and blackcurrant powder used in first feeding trial. * composition per 100 g of boysenberry powder or per 100 g blackcurrant fruit, fresh weight.*

3.1.1.3 Sample Collection

Following each trial, terminal exsanguination was performed by intracardiac puncture with an 18 G needle after administration of isofluorane to anaesthetise the rats. Approximately 10 ml of blood was obtained from the heart, and collected in lavender vacutainer tubes containing ethylene diamine tetra-acetic acid (EDTA). The plasma was recovered by centrifugation at 2000 *g* for 15 minutes at 4°C. Aliquots of plasma were distributed into various tubes for each assay and placed in dry ice until storage at -20 °C. Overnight urine was collected from each rat by placing a vial under the specially-designed cages where each rat was housed, and the collected urine frozen immediately. All samples were analysed within six months of sample collection, for oxidised lipid, DNA, protein and plasma antioxidant status (ORAC and vitamin E analysis).

3.2 Sample Preparation and Analysis

All solvents used were HPLC grade. Chemicals and reagents were analytical grade and were obtained from Merck New Zealand Limited (Palmerston North, New Zealand).

3.2.1 8OHdG Assay to Measure Oxidative Damage to DNA

A stock standard (500 μgml^{-1}) was prepared from 5 mg 8OHdG + 10 ml of 0.1 M phosphoric acid in a volumetric flask. Working standards were diluted in 100 mM sodium acetate (NaOAc) (pH 3.5).

3.2.1.1 Sample Preparation: Solid Phase Extraction to Clean-up Samples and Concentrate 8OHdG:

The solid phase extraction (SPE) method was based on that used in Shigenaga, et al. (1989). 500 mg/6 ml Strata-X™ were sequentially preconditioned with 10 ml of each of the following: methanol (MeOH), water, and buffer A (50 mM potassium dihydrogen phosphate, pH 7.5 with 2 M potassium hydroxide). 1 ml of urine sample was mixed with 1 ml of 1 M sodium chloride solution and applied to the preconditioned column. The cartridges were washed sequentially with 4 ml of buffer A, 5 ml of buffer B (5% MeOH in buffer A) and 3 ml of ethyl acetate. Following this, the 8OHdG was eluted with 2 ml of MeOH. The eluant was concentrated to dryness under nitrogen and redissolved in 200 µL of 100 mM NaOAc, pH 3.5.

For quality control, a spiked urine sample (0.25 μgml^{-1} 8OHdG) was analysed with every 11 samples run through the SPE protocol. The quality controls consisted of 990 µl of rat urine spiked with 10 µl of 5 μgml^{-1} of 8OHdG to give a final concentration of 0.25 μgml^{-1} in the 200 µl sample to be analysed.

3.2.1.2 Reversed-Phase High-performance Liquid Chromatography

Due to the complexity of the urine matrix, two HPLC columns were used, in conjunction with a switching valve to isolate the 8OHdG, providing more selective separation. A schematic of the HPLC configuration is shown in Figure 3.1.

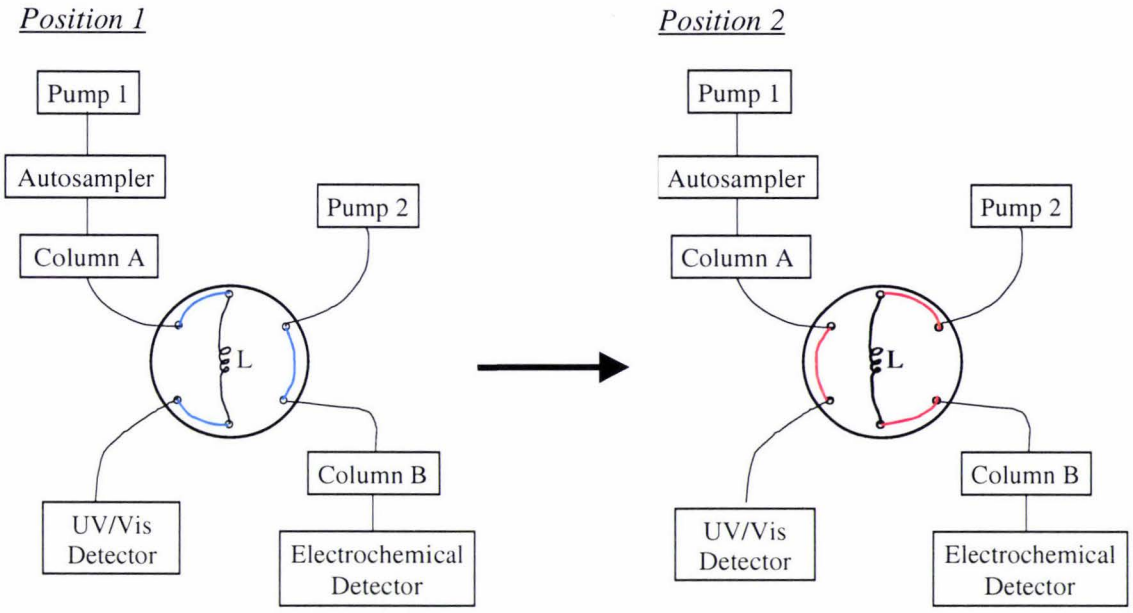


***Figure 3.1**: Schematic diagram of switching valve positions used to isolate 8OHdG in the 1 ml loop (**L**) and apply onto column B for detection with a CoulArray® electrochemical detector.*

Column A, an Alltech Adsorbosphere, C8 5 µm, 150 x 4.6 mm had an isocratic solvent programme with mobile phase A (5% MeOH in 100 mM NaOAc pH 3.5). An LKB 2249 gradient pump (pump 1) was used to pump the solvent through this column at 0.8 ml/min. Column B, a Phenomenex Luna, C18 5 µm, 250 mm x 4.6, used a Jasco pump (pump 2), (PU-980 intelligent HPLC pump, DG-980-50 3-line degasser and LG-980-02 ternary gradient pump) with a binary gradient (Table 3.3) at a flow rate of 0.8 ml/min. Mobile phase A was the same as for column A; mobile phase B consisted of 20% MeOH in mobile phase A. The temperature of both columns was maintained at 40°C by an ESA column heater. A 20 µl injection loop was fitted.

All standards were stored at 4°C. Working standards were diluted from the stock with 100 mM NaOAc prior to use. Before each batch of samples was analysed, a 5 $\mu g ml^{-1}$ 8OHdG standard was run four times through the first column only (Position 1), with detection at 260 nm by a Jasco UV-975 intelligent UV/VIS detector (see Figure 3.2 for an example chromatogram).

Time	*% A*	*% B*
0	100	0
13	100	0
30	5	95
35	5	95
40	100	0

Table 3.3: *Solvent gradient used by the Jasco pump for column B in the assay for urinary 8OHdG using a dual column, switching valve method with HPLC and CoulArray® detection. A = 5% MeOH in 100 mM NaOAc and B = 20% MeOH in A.*

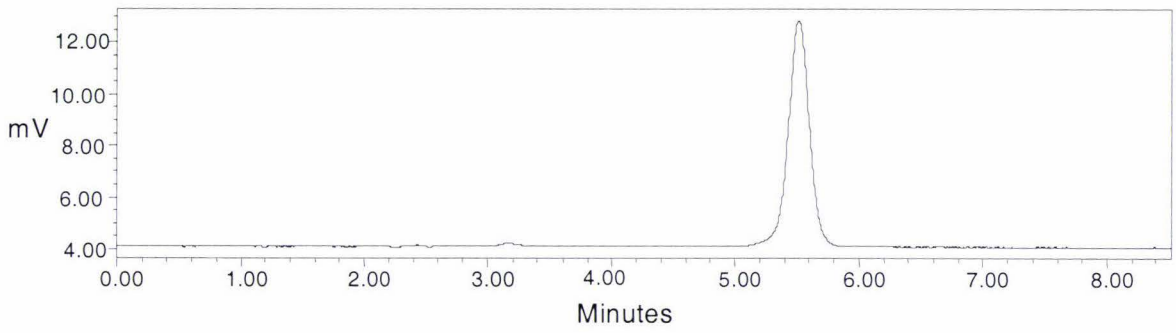


Figure 3.2: *Example of chromatogram from UV detection (at 260 nm) of a 5 μgml^{-1} 8OHdG standard run five times prior to a sample run to deduce the time of elution of 8OHdG off the first column. The time for the switching was programmed into the CoulArray® software. Switching time for this chromatogram was programmed as 5-6 minutes.*

The chromatograms of each standard were overlaid and the time of 8OHdG elution from column A was determined, which was then programmed into the CoulArray® software (CoulArray® for windows, version 1.00. ESA Inc, Massachusetts, USA) to control the switching valve. This ensured that 8OHdG was contained in the 1 ml loop at the exact time of the switch in order to be loaded on to column B for further separation. The valve switched at approximately 5.0 minutes to load the 8OHdG present in the sample onto column B, and switched back at approximately 6.0 minutes, where pump 2 began a gradient to separate the 8OHdG from the 1 ml of sample applied to the second column.

3.2.1.3 Quantification of 8OHdG in urine

The amount of 8OHdG was detected using an eight-channel ESA CoulArray® detector, with an applied oxidation potential of 200 mV for the first channel with each successive channel incremented by 45 mV. In order to quantify the level of 8OHdG in the samples, the peak height for the 290 mV channel was calculated using the CoulArray® software. The height of the 8OHdG peak for the 290 mV channel was related to the mean of the 1 μgml^{-1} standards run before and after the sample, giving the concentration of 8OHdG in the urine.

Example Calculation:

Peak height for 1 μgml^{-1} 8OHdG standard run prior to sample = 163 nA.

Peak height for 1 μgml^{-1} standard run after sample = 158 nA.

Mean peak height of the standards = 160.5 nA.

Peak height for an arbitrary sample = 2.86 nA. Thus, 1 μgml^{-1} 8OHdG = 160.5 nA, therefore, the concentration of 8OHdG in sample = 2.86 nA x (1 μgml^{-1}/160.5 nA) = 0.0178 µg/ml in the 200 µL concentrated sample.

To express the result per 1 ml of urine: (0.0178 μgml^{-1} x 0.2 ml)/1 ml = 0.0036 μgml^{-1} (or 33.6 $ngml^{-1}$) of 8OHdG in the rat urine.

3.2.2 Protein Carbonyl Assay to Measure Oxidative Damage to Proteins

When determining the protein carbonyl concentration, 2,4-dinitrophenylhydrazine (DNPH) was used to derivatize the carbonyl groups for quantification either by colorimetric or ELISA methodology.

3.2.2.1 Colorimetric Method

Initially, a protein assay was performed using the Bradford method (Bradford, 1976) in reference to bovine serum albumin (BSA), with 100 µL of an appropriate dilution of

plasma and 5 ml BioRad reagent (BioRad, California, USA). The absorbances at 595 nm were quantified against 0, 0.28, 0.56, 0.84 and 1.09 $mgml^{-1}$ BSA standards. (see Appendix two for protein concentration standard curve).

Following determination of the protein content, two 100 µl aliquots of each sample were analysed based on the method in Reznick and Packer (1994). One aliquot was the sample blank (sample + 4 ml of 2.5 M hydrochloric acid (HCl)), while the other was derivatised (sample + 4 ml of 10 mM DNPH in 2.5 M HCl) for measurement of the carbonyl concentration. The samples were kept in the dark for one hour and vortexed every 15 minutes. 5 ml of 20% (w/v) trichloroacetic acid (TCA) was added to all tubes to precipitate the protein. The samples were kept on ice for 10 minutes to maximise protein precipitation. Each tube was centrifuged for 10 minutes at 800 *g*, the supernatant was discarded and the pellet washed with 4 ml of 10% (w/v) TCA. Each tube was again centrifuged, and the pellets were washed three times with absolute ethanol/ethyl acetate 1:1 to remove excess free DNPH and lipid contaminants. The supernatants were carefully removed and discarded. After the final wash the pellets were dried in a vacuum desiccator for 10 minutes.

The pellets were dissolved in 2 ml of 6 M guanidine hydrochloride solution containing 20 mM of potassium dihydrogen phosphate (pH 2.3), and the tubes were placed in a 37°C water bath for 10 minutes. The absorbance of each sample was determined at 370 nm using a Beckman spectrophotometer (Beckman Coulter, Inc, California, USA).

Determination of Carbonyl Content

The carbonyl content was calculated from the absorbance at 370 nm, using an extinction coefficient, ε, of 22,000 $M^{-1}cm^{-1}$ (Witko-Sarsat, et al., 1998). The pellets from the hydrochloric acid treated samples were used as blanks and the absorbance subtracted from the absorbance of the DNPH-containing sample prior to calculations.

Sample Calculation:

$\varepsilon(DNPH)_{370} = 22{,}000/M = 22{,}000/10^6\ nMml^{-1}$

Carbonyl concentration (C) = Abs/ε; therefore C = Abs (at 370 nm)/$2.2x10^4/10^6$

Therefore, the carbonyl content (C) is: C = Abs (370) x 45.45 $nMml^{-1}$ (Reznick and Packer, 1994). The concentration in nmol/ml was divided by the protein concentration calculated from the protein concentration assay to give nmoles per mg of protein.

3.2.2.2 ELISA Method

A 'Zentech protein carbonyl test' ELISA kit was obtained from Zenith Technology, (Dunedin, New Zealand). In this method for the determination of the carbonyl content, the plasma samples were reacted with dinitrophenylhydrazine (DNPH) then the protein was non-specifically adsorbed onto the ELISA plate. Unconjugated DNPH and non-protein constituents were washed away, then a biotinylated anti-DNPH antibody was used to measure the adsorbed protein-DNPH conjugate. This was then probed using streptavidin-linked horseradish peroxidase. The samples were prepared and analysed as described in the instructions accompanying the ELISA kit. Absorbances were read using a Dynatech MR5000 plate reader.

Determination of the Carbonyl Content

The carbonyl content was calculated by relating the absorbance at 450nm to a standard curve produced in the same assay, with the oxidised protein standards provided in the kit. This assay assumes the protein concentration in all samples is constant, whereas the colorimetric method described above relates the carbonyl concentration to the protein concentration of each sample.

3.2.3 Malondialdehyde Assay to Measure Oxidative Damage to Lipids

(Performed by M. Hunt, HortResearch, Palmerston North)

3.2.3.1 Standard Preparation

The malondialdehyde (MDA) standard was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane (Sigma Inc, California, USA) at 40^0C to form MDA.

Standards were diluted to concentrations of 0, 18, 45, 90, 180, 450 and 900 $ngml^{-1}$.

3.2.3.2 Sample Preparation

This method was based on that of Fenaille, et al. (2001), with the following protocol: 500 µl of plasma (or standard) was treated with 350 µl of 10% TCA to precipitate the proteins. 300 µl of 0.8% butylated hydroxytoluene (BHT) in hexane was added, the sample/standard was mixed using a vortex, then centrifuged for 10 minutes at 10,000 *g*. The upper hexane layer was discarded. The aqueous layer was derivatised by the addition of 100 µl of 9 mM phenylhydrazine (Sigma Inc, USA) for one hour at room temperature.

Derivatised MDA was extracted by adding 300 µl of hexane, mixing well, and allowing the layers to separate for five minutes at room temperature. Internal standard (300 µl of benzophenone, 100 $ngml^{-1}$) was added, mixed and the sample left for a further 15 minutes at room temperature. The hexane layer was removed and the phenylhydrazine-MDA derivative analysed by gas chromatography with detection by mass spectrometry using single ion monitoring (Shimadzu GCMS-QP5050A gas chromatograph mass spectrometer, Shimadzu Corporation, Kyoto, Japan).

The parameters for analysis were: J & W Scientific DB™ – 5MS 30 m, 0.25 mm ID, 0.25 µm film thickness capillary GC column (part # 122-5532; J & W Scientific, California, USA). Temperature gradient: 40°C for 1 min, $10°Cmin^{-1}$ to 300 °C and maintained at 300 °C for 10 min. The injection temperature was 250°C with a 2 µl splitless injection, and a sampling time of 1 minute. The temperature of the GC-MS interface was 250°C, the detector voltage 1.8 kV. MDA and the internal standard were recorded at mass to charge ratios (m/z) of 76.90, 104.85, 143.90 and 182.05.

3.2.3.3 Determination of MDA Concentration

The peak area of the DNP-MDA derivative and benzophenone internal standard in the

samples was calculated using Shimadzu GC-MS Solutions GC-MS software, quantified using a standard curve prepared with benzophenone. The MDA concentration was calculated as $ngml^{-1}$ of plasma.

3.2.4 $ORAC_{FL}$ Assay to Measure the Overall Antioxidant Capacity

3.2.4.1 Sample Preparation

Plasma samples were diluted 125-fold in 75 mM potassium phosphate buffer (pH 7.4). Standard curves were prepared using 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (trolox), a water-soluble analogue of vitamin E, at 50-200 μM.

3.2.4.2 Procedure

The method was based on that of Ou, et al. (2001). Each sample or standard was analysed in triplicate in a 96-well format, with one blank per sample and a total of 16 samples per plate. Plates were prepared by adding 10 μl of sample (or phosphate buffer for the blanks) and 160 μl of fluorescein ($4.8x10^{-8}$ M) to each well. Plates were pre-incubated in the fluorescence plate reader, Spectramax Gemini (Molecular Devices Corporation, California, USA), to stabilise the temperature to 40°C.

The fluorescence plate reader, was set to zero, then 25 μl of 0.1 M 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was added to each well and the fluorescence measured every minute for 90 minutes. The excitation and emission wavelengths were 493 nm and 515 nm respectively. The plate was shaken between each reading.

3.2.4.3 Determination of Trolox Equivalent ORAC Value

The relative fluorescence for each standard was calculated, to give the area under the

curve (AUC), and a standard curve was plotted. The antioxidant capacity (in μM trolox equivalent) of the sample was calculated by $AUC_{sample} - AUC_{blank}$ and related to the results gained for the standards.

3.2.5 α-tocopherol Analysis

Plasma from each rat trial was also analysed for α-tocopherol to provide further assessment of the antioxidant status.

3.2.5.1 Sample Preparation

The method used was based on that in Gimeno, et al. (2001). 500 μl of plasma was mixed using a vortex with 100 μl of 50 μgml^{-1} of tocopherol acetate internal standard for 30 seconds. 500 μl of absolute ethanol was added and the sample mixed for a further 10 seconds. 500 μl of hexane was added, the sample was mixed and centrifuged for 15 minutes at 10,000 *g*. The hexane layer was removed and put into a 10 ml glass tube. Another 500 μl aliquot of hexane was added to the sample, vortexed, the pellet broken up, and the tube centrifuged for 15 minutes. The hexane layer was again removed and combined with the previous hexane aliquot and concentrated to dryness under nitrogen at 30°C. The dried sample was redissolved in 200 μl of MeOH and analysed by RP-HPLC.

3.2.5.2 RP-HPLC Analysis

A 40 μl sample was injected into a Jasco HPLC system (DG-980-50 3-line degasser; LG-980-02 ternary gradient unit; PU-980 Intelligent HPLC pump; AS-950 Intelligent autosampler and UV-975 Intelligent UV/Vis detector) fitted with a Merck Lichrocart C18 column. The isocratic solvent programme consisted of MeOH at 1 $mlmin^{-1}$ with detection at 290 nm.

3.2.5.3 Determination of α-tocopherol Concentration

A five point standard curve was plotted using the peak area of α-tocopherol related to the peak area of the tocopherol acetate internal standard. Peak areas from the samples were related to this curve and the concentration determined in μgml^{-1} of plasma.

3.2.6 Statistical Analysis

Statistical Calculations used Microsoft Excel 97. P-values used 1-tailed distribution and two-sample unequal variance. Error was determined by standard error of the mean (SEM).

Chapter Four – Fish Oil Feeding Trial Results and Discussion

A feeding trial was carried out using fish oil added to the diet to elevate the oxidative stress beyond the basal endogenous level in order to assess the amelioration of oxidative damage by dietary berry fruit extracts in subsequent trials. To determine the appropriate level of fish oil supplementation, different percentages (w/w) of fish oil and soybean oil were added to the natural rat diet for two weeks following a two-week acclimatisation period on the soybean oil control (Figure 4.1).

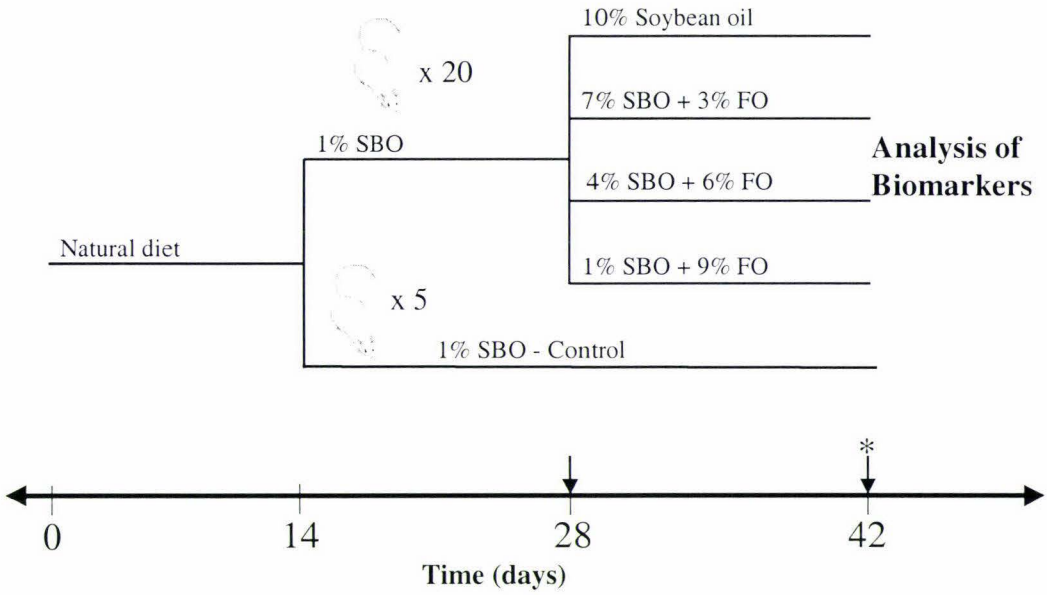


Figure 4.1: *Study design for the fish oil feeding trial to determine the level of fish oil (FO) most effective at inducing measurable levels of oxidative stress. SBO = soybean oil. * indicates collection of plasma samples. Solid arrows denote the collection of overnight urine samples.*

4.1 Overall Observations

The weight gain of each rat was assessed every two days for the final 12 days of the trial.

Biomarkers for oxidised DNA (urinary 8OHdG analysis throughout the trial), lipid peroxidation (plasma MDA), protein oxidation (plasma carbonyl concentration) and the overall plasma antioxidant status (measured by vitamin E concentration and ORAC analysis) were assessed in plasma samples collected at the end of the trial.

4.1.1 Rat Weight Gain

The mean rat weight increased by 8.8 $gday^{-1}$ during the final two weeks of the trial (Figure 4.2).

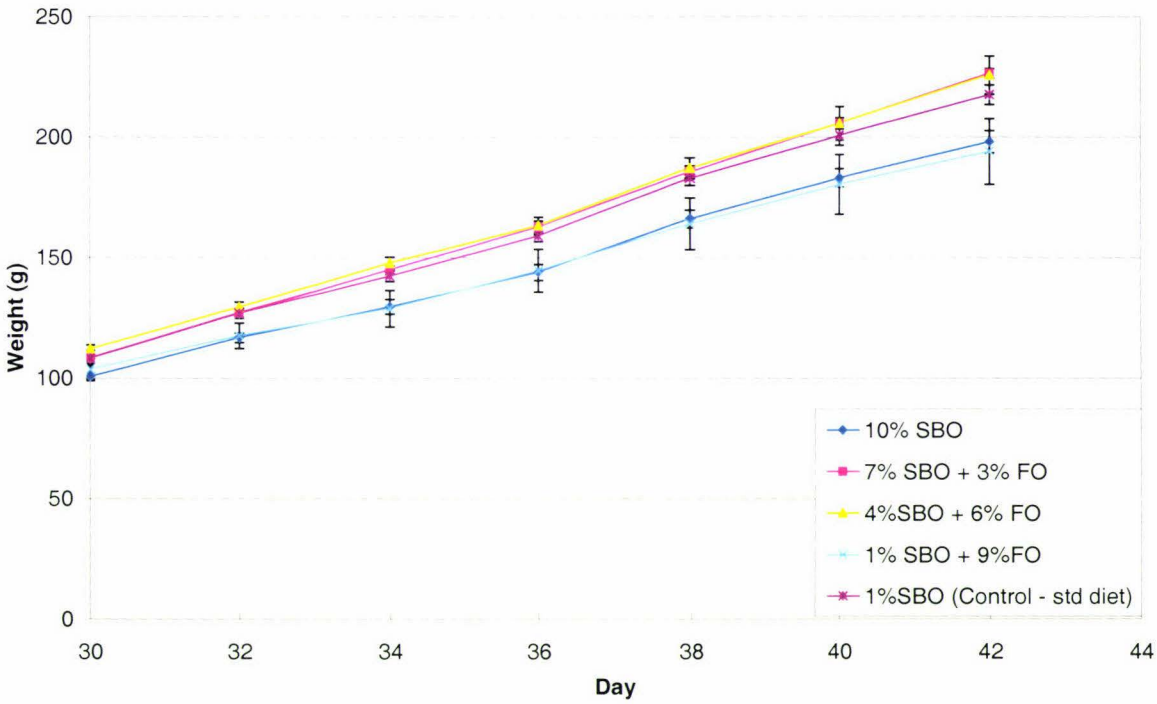


Figure 4.2: *Cumulative weight gain of rats fed diets supplemented with the given percentages of fish oil (FO) and soybean oil (SBO). Lines represent the mean rat weight in each diet group over the course of the trial. Error bars represent SEM (n=5).*

The total mean weight of rats fed the 10% soybean oil and 9% fish oil + 1% soybean oil were lower from the start of the trial and weight gain was constant in each diet group. There was no significant difference in weight gain between the different treatments ($p \leq$

0.05) throughout the trial therefore neither fish oil nor soybean oil present in the natural diet affected the growth of the rats.

4.2 *Biomarkers of Oxidative Damage*

The concentrations of biomarkers of oxidative damage are shown in Table 4.1. There was a trend towards increasing the amount of oxidative damage in rats fed increasing amounts of fish oil.

Dietary Supplement	*8OHdG Assay* $ngml^{-1}$	*Protein Carbonyl* $nmolmg^{-1}$ *protein*	*MDA Assay* $ngml^{-1}$	*$ORAC_{FL}$ Assay mM trolox equivalent*	*Vitamin E Assay* μgml^{-1}
10% SBO	48.3 ± 10.8	0.042 ± 0.009	22.3* ± 3.58	7.62 ± 0.58	2.34 ± 0.61
7% SBO + 3% FO	33.6 ± 4.23	0.038 ± 0.004	22.1* ± 4.80	8.50 ± 0.45	3.30# ± 0.49
4% SBO + 6% FO	54.0 ± 18.0	0.039 ± 0.008	37.1# ± 6.14	8.33 ± 0.43	3.08 ± 0.37
1% SBO + 9%FO	53.2 ± 13.9	0.049 ± 0.006	73.6 ± 14.9	8.46 ± 0.23	2.34 ± 0.45
1% SBO (Control)	45.2 ± 11.7	0.038 ± 0.007	49.2# ± 8.82	8.21 ± 0.52	2.10 ± 0.62

Table 4.1: *Mean concentrations of 8OHdG ($ngml^{-1}$), oxidised protein from colorimetric analysis ($nmolmg^{-1}$ protein), MDA ($ngml^{-1}$), $ORAC_{FL}$ value (mM trolox equivalent) and vitamin E (α-tocopherol, μgml^{-1}) for the fish oil rat trial. Values are ± SEM. SBO = soybean oil, FO = fish oil. Values were significantly different from the *1% SBO control and #10% soybean oil ($p \leq 0.05$). ($n=5$).*

4.2.1 Oxidised DNA

Due to the large variability in the 8OHdG assay, the concentrations were considered to be unreliable, as the results were not significantly different ($p \leq 0.05$) between each group. Excretion of 8OHdG in the urine, however, increased with increasing percentages of dietary fish oil. Varying the levels of soybean oil appeared to have little effect on the excretion of 8OHdG with the exception of the 7% soybean oil + 3% fish

oil, which had a lower excretion that was not significantly ($p \leq 0.05$) different from the control diet.

4.2.2 Oxidised Protein

The plasma protein carbonyl concentration determined by colorimetric analysis revealed no group was significantly different from another ($p \leq 0.05$). The diet containing 1% soybean oil + 9% fish oil, however, slightly increased the protein carbonyl concentration. The 10% soybean oil treatment also increased protein oxidation relative to the control to approximately the same level as the 9% fish oil. Diets with 7% soybean oil + 3% fish oil, and 4% soybean oil + 6% fish oil resulted in the same carbonyl concentration as the control (1% soybean oil).

4.2.3 Oxidised Lipid

The concentration of the lipid peroxidation product, MDA in the plasma increased with the inclusion of 9% fish oil + 1% soybean oil in the natural diet (Figure 4.3). This increase was significantly different from the 10% soybean oil group included as a control for the dietary lipid concentration ($p \leq 0.05$). Higher percentages of soybean oil (10% and 7%), when compared to the 1% soybean oil control diet, significantly lowered the concentration of MDA.

4.2.4 Plasma Antioxidant Status

There was no change in the total antioxidant capacity between animals fed either of the diets as measured by the ORAC assay.

Vitamin E concentration in plasma was decreased with both the 10% soybean oil and 9% fish oil + 1% soybean oil treatments, although the results were not significantly different ($p \leq 0.05$).

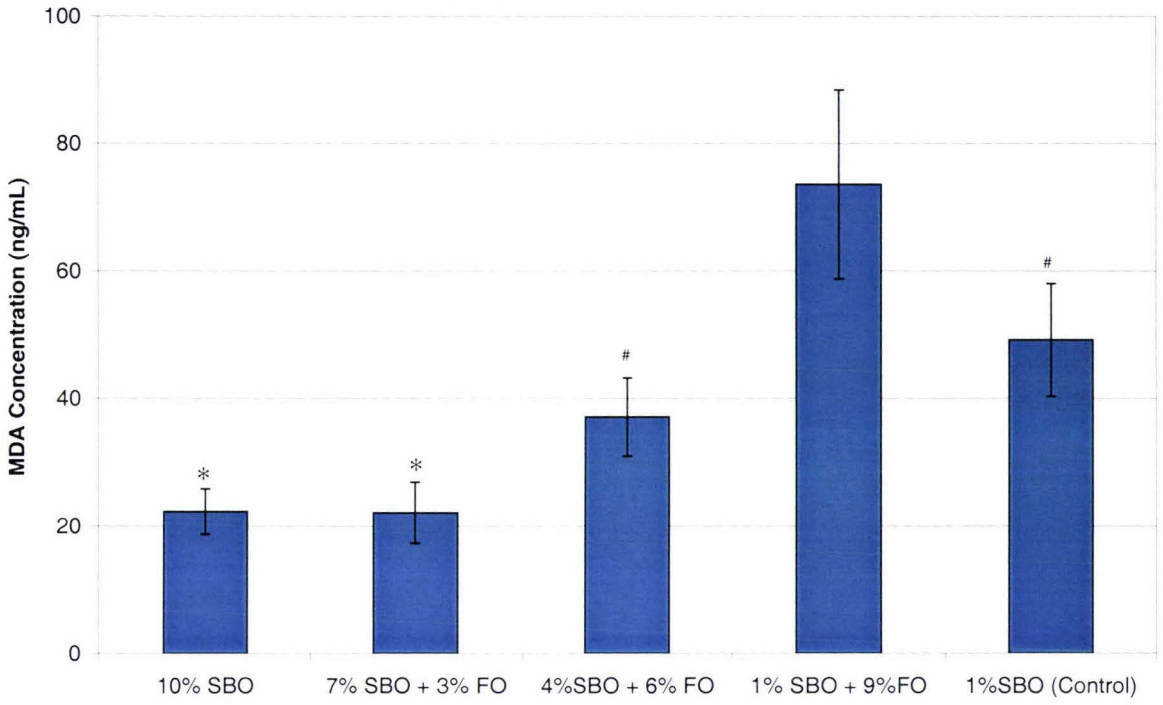


Figure 4.3: *Mean concentration of MDA ($ngml^{-1}$) in plasma samples where the rat diets were supplemented with the given percentages of fish oil (FO) in order to increase oxidative damage (SBO = soybean oil). Values were significantly different than *1% soybean oil control and #10% soybean oil treatment that controlled for the dietary oil content ($p \leq 0.05$). Error bars represent SEM ($n = 5$).*

4.3 Discussion

Fish oil can rapidly oxidise to produce lipid peroxides, due to the high level of polyunsaturated fatty acids (PUFA) (Cho, et al., 1995). The major PUFA in fish oil is docosahexaenoic acid [22:6 (n-3)], which can be incorporated into cell membranes, changing their composition and making the membrane more susceptible to oxidative

damage (Ibrahim, et al., 1997). The process of lipid peroxidation involves free radical formation, which can attack DNA and protein.

4.3.1 Increasing Oxidative Damage with Increasing Fish Oil

The values determined by 8OHdG analysis were not comparable to other feeding trials, where an increase in 8OHdG was interpreted as an increase in DNA repair.

Although a diet with a high percentage of fish oil induced carbonyl concentration, so did high soybean oil, although not to the same level. The calculated statistical error showed no group was significantly different from another ($p \leq 0.05$). Therefore, supplementation with neither fish nor soybean oil induced an increase in oxidative damage to plasma proteins.

MDA analysis showed a high percentage of fish oil increased lipid peroxidation, and that a higher level of soybean oil (10%) decreased lipid peroxidation relative to the 1% soybean oil diet which was a control for the lipid concentration in the diet. The effect was thought to be due to the presence of high vitamin E concentrations in the oil, which is a powerful antioxidant targeting lipids and reducing peroxidation. In the case of the 9% fish oil + 1% soybean oil, lipid peroxidation may have increased to higher levels if the soybean oil had not been present.

No change in the ORAC values with any treatment indicated the fish oil had no effect on the total antioxidant status of the plasma samples, this was probably due to the highly maintained redox control of the plasma (Rice-Evans, et al., 1997).

Varying percentages of fish oil in the diet was thought to cause a change in the plasma vitamin E concentration. This was because an increase in lipid peroxidation induced by the fish oil may have been countered by vitamin E, therefore lowering the concentration of vitamin E in the plasma. This was shown with analysis of the plasma of rats fed 9% fish oil + 1% soybean oil, where vitamin E values were lower relative to the control.

The plasma vitamin E should, therefore, have been higher in the 10% soybean oil treatment, but this was not the case. It has been demonstrated that fish oil or n-3 PUFA-rich diets reduce the α-tocopherol content of plasma and membranes and increase oxidative damage to lipids compared with diets containing soybean oil (Cho, et al., 1995; McGuire, et al., 1997; Quiles, et al., 2002; Rodriguez, et al., 1996). A significant reduction in plasma α-tocopherol was not observed in the current study. A diet containing fish oil only, without soybean oil should also have been tested, as Quiles, et al. (2002) reported that a diet consisting of fish oil alone increased oxidative stress. The *in vivo* targets of vitamin E, and the composition of both oils require further investigation.

The use of soybean oil as a control for total lipid in the diet may not have provided a suitable comparison, as there is evidence that soybean oil lowers oxidative stress due to the phytic acid and β-sitosterol present (Kennedy, 1995). Thus the oxidative stress may have decreased relative to the percentage of fish oil, rather than increased relative to the percentage of soybean oil. With the benefit of hindsight, a control consisting of no oil may have been more appropriate. Therefore, in subsequent trials 10% fish oil was fed to rats to increase their oxidative stress prior to supplementation with berry fruit extracts. No soybean oil was added, as it may have had an antioxidant effect on the oxidative stress in the rats, which could have been mistakenly attributed to anthocyanins in the berry fruit extracts.

Taken together, the results of biomarker analysis in this initial trial infer that supplementation of the rat diet with fish oil may induce a mild form of dietary oxidative stress.

Chapter Five – Fruit-derived Antioxidants Feeding Trials #1 and #2 Results and Discussion

To assess the ability of the selected berry fruit anthocyanin extracts (fruit-derived antioxidants) to decrease oxidative damage anthocyanin extracts were fed to rats concurrently with induction of oxidative damage by the addition of fish oil to the diet. The diet used in trials #1 and #2 was changed to a synthetic base diet (Table 3.1, Materials and Methods), compared to the natural diet used in the fish oil feeding trial, so future studies could be repeated with a diet of the same composition.

Rats were housed in square metal metabolic cages and both trial #1 and #2 were carried out in the same way (Figure 5.1). The second trial included a 10% soybean oil supplemented diet as an additional control to compare the oxidative damage induced by fish oil and soybean oil in the synthetic diet (Chapter Four).

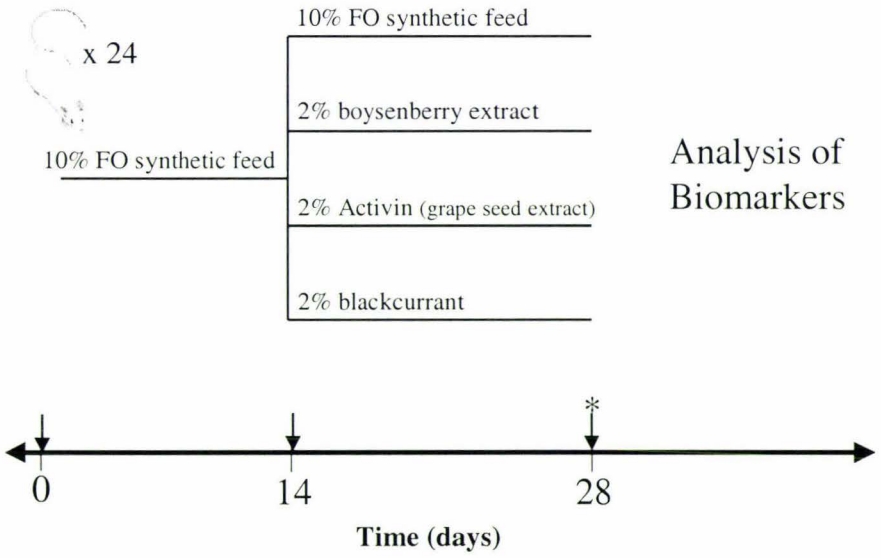


Figure 5.1: *Study design for fruit-derived antioxidants feeding trial #1. Six rats per treatment. FO = fish oil. * indicates collection of plasma samples. Solid arrows denote the collection of overnight urine samples.*

5.1 *Overall Observations*

In trial #1 the weight of each rat was assessed every two days throughout the trial. In trial #2 the rat weights were assessed every five days throughout the trial. Biomarkers for oxidative damage to proteins, DNA and the overall plasma antioxidant status were assessed in plasma samples collected at the end of the trial.

5.1.1 Rat Weight Gain

Trial #1

The rats in trial #1 consistently lost weight at a mean rate of 1.56 $gday^{-1}$, throughout the trial from the start of the trial when their diet was changed from the natural diet to the synthetic diet (Figure 5.2).

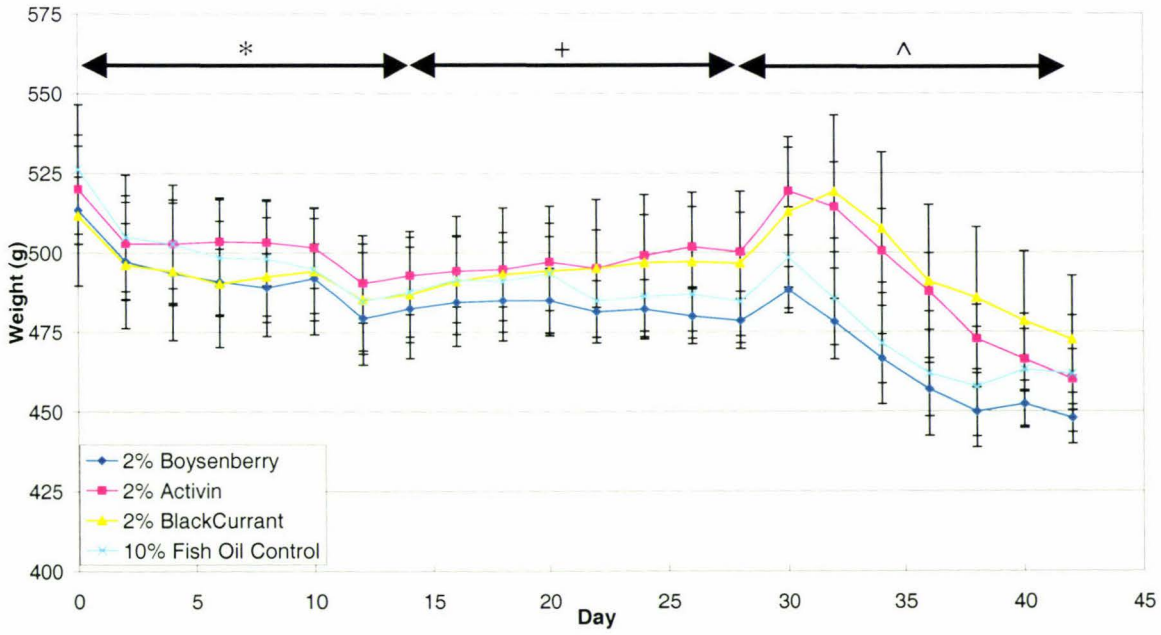


Figure 5.2: *Cumulated rat weight for trial #1. Lines represent the mean rat weight in each diet group over the course of the trial. *Days 1-14 = acclimatisation period; +days 15-28 = fish oil (FO) synthetic diet; ^days 29-42 = berry fruit extracts added to the base FO diet. Error bars represent SEM (n = 6).*

When the diet was further supplemented with fruit extracts, the rats gained weight over two days, then their body weight continued to decline. The lower weights of rats fed the fish oil control and higher weights of rats fed 2% boysenberry diet were not significantly different ($p \leq 0.05$). Rats fed the 2% Activin™ and 2% blackcurrant supplemented diets had higher weights than the rats on the control diet during the trial, although at the end of the trial (day 42) the mean rat weights were not significantly different.

Trial #2

The rats in trial #2 consistently gained weight during the acclimatisation period, however, upon diet supplementation with fish oil, the weight declined in all groups (Figure 5.3). The overall mean weight gain in each group of rats fed the fish oil diet was 1.44 $gday^{-1}$. In contrast, the animals fed soybean oil continued to gain weight at a steady rate of 3.43 $gday^{-1}$.

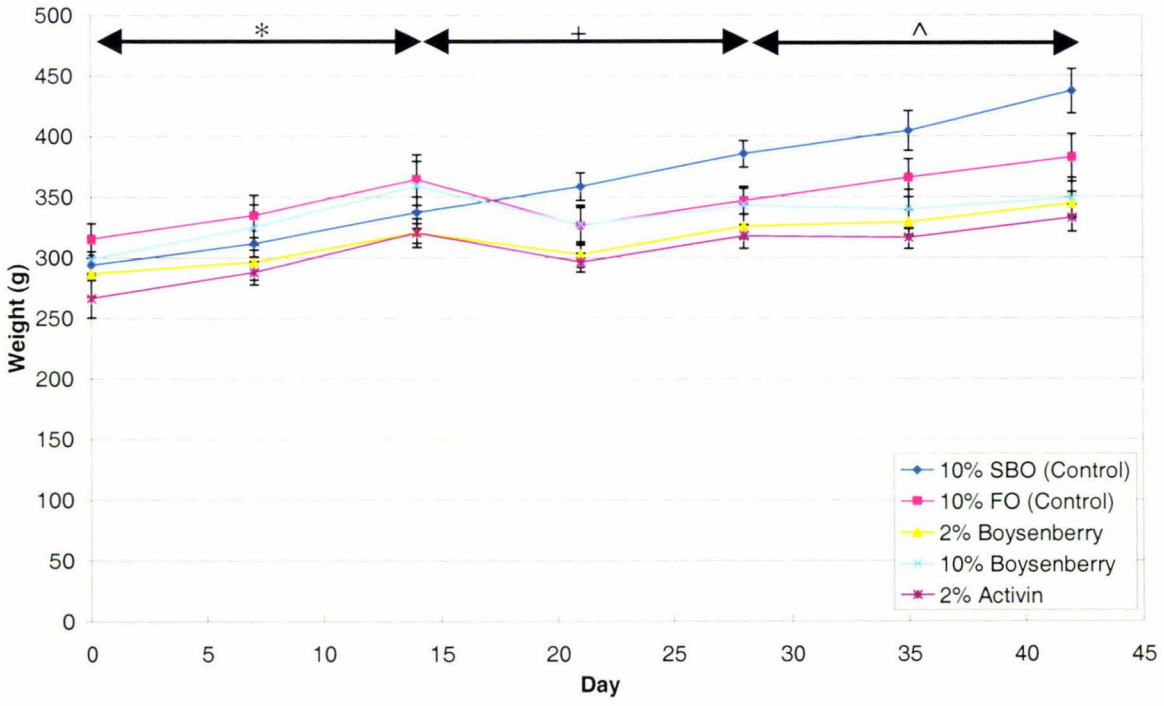


Figure 5.3: *Cumulative rat weight in trial #2. Lines represent the mean rat weight in each diet group over the course of the trial. *Days 1-14 = acclimatisation period; +days 15-28 = fish oil (FO) or soybean oil (SBO) synthetic diets; ^days 29-42 = berry fruit extracts added to the base diets. Error bars represent SEM (n = 5).*

When berry fruit extracts were added to the diets, generally the rat weights began to increase, although still remained significantly lower than both control groups ($p \leq 0.05$). The weights for the rats on the soybean oil diet were significantly ($p \leq 0.05$) higher than the other diet groups at the end of the trial.

It was apparent that supplementation with fish oil in the synthetic diet significantly affected the rats. This did not occur when the natural diet was supplemented with fish oil (Chapter Four). The weight loss in trial #1 indicates poor adaptation and tolerance to the synthetic diet. The weight increase in trial #2 was likely to be due to acclimatisation to the fish oil in the diet, as the weight of the fish oil control rats also increased. The adaptation for trial #2, and not trial #1 may have been due to the age of the rats at the start of the trial. Rats in trial #1 were older, therefore their ability to adapt to the fish oil synthetic diet may not have been as great as that of the younger rats used in trial #2. The rats on the soybean oil diet had steady growth, but those on the fish oil diets had variable growth throughout the trial.

5.1.3 Dietary Consumption

The composition of the fruit extracts added to the diets is shown in Table 5.1.

Compound	*Blackcurrant* mgg^{-1} *extract*	*Boysenberry* mgg^{-1} *extract*	*Activin*™ mgg^{-1} *extract*
Cyanidin 3-*O*-sophoroside	1.40	0.12	0.00
Cyanidin 3-*O*-2^{G} glucosylrutinoside	0.00	0.04	0.00
Cyanidin 3-*O*-galactoside	4.13	0.00	0.00
Cyanidin 3-*O*-glucoside	8.01	0.58	0.00
Cyanidin 3-*O*-rutinoside	4.22	0.12	0.00
Total Anthocyanin	10.91	0.88	0.00
Total Phenolics	61.25	5.76	100.86

Table 5.1: *Concentration* (mgg^{-1} *extract) of anthocyanin at* A_{530} *and total phenolics (determined by HPLC) at* A_{280} *of the extracts used in trials #1 and #2. Total values represent summation of all peaks at the designated wavelength and include known peaks.*

Each extract was dissolved in HPLC solvent and analysed by HPLC with photodiode array detection, using procedure described in McGhie et al. (2003). Anthocyanin concentrations were calculated relative to a cyanidin galactoside standard. Total phenolic concentrations were related to an epicatechin standard. Total anthocyanin and phenolic concentrations, and the antioxidant capacity (ORAC) of each diet is shown in Table 5.2.

Diet	*Total Anthocyanin* mgg^{-1} *diet*	*Total Phenolics* mgg^{-1} *diet*	*ORAC* *mmol Trolox equiv.* g^{-1} *diet*
2% Blackcurrant/FO	0.17	2.07	2.03
2% Boysenberry/FO	0.03	0.32	1.26
10% Boysenberry/FO	0.16	2.42	3.84
2% Activin™/FO	0.00	3.08	6.26
10% FO control	0.00	0.04	0.69
10% Soybean oil control	0.00	0.02	0.43

Table 5.2: *Total concentration* (mgg^{-1} *diet) of anthocyanin at* A_{530}, *phenolics at* A_{280} *and ORAC value (trolox equivalent mmol* g^{-1} *diet) for rat feed used in trials #1 and #2. Total values represent summation of all peaks at the designated wavelength and include known peaks. (FO = fish oil)*

The diet containing 10% boysenberry had five times the anthocyanin concentration of the diet containing 2% boysenberry, which was expected. The phenolic content was higher than expected in the 10% boysenberry diet. The anthocyanin concentrations in the diets were slightly higher than expected from the concentration determined for each extract. The total phenolic concentration, however was over ten times higher than expected in the diet. This may be explained by the components of the fish oil which also absorb at the wavelength used for quantification of total phenolic concentration, shown by the 10% fish oil control. For the same reason, the antioxidant capacity of the 10% fish oil control appeared to be higher than that of the soybean oil control.

Antioxidant capacity (ORAC) analysis showed the 10% boysenberry diet had well over five times the antioxidant capacity than the 2% boysenberry diet. Activin™, the positive control, had the highest antioxidant capacity, which was expected. The

antioxidant capacity of Activin™, comes from proanthocyanidins, which were analysed using the phenolic analysis. The phenolic concentration corresponded to the ORAC analysis of each extract, where the anthocyanin concentration did not. This inferred that the antioxidant capacity comes from the phenolic compounds (which include the anthocyanins). Possible synergistic interactions may occur between anthocyanins and other phenolics present in the diets.

5.2 *Biomarkers of Oxidative Damage*

The concentrations of biomarkers of oxidative damage for trials #1 and #2 are shown in Table 5.3.

Diet Supplement	*8OHdG Assay* $ngml^{-1}$	*Protein Carbonyl* $nmolmg^{-1}$ *protein*	*MDA Assay* $ngml^{-1}$	$ORAC_{FL}$ *Assay mM trolox equivalent*	*Vitamin E Assay* μgml^{-1}
Trial #1					
2% Boysenberry	2.44 ± 0.58	0.28 ± 0.04	177.6 ± 17.4	10.3 ± 0.34	1.45 ± 0.52
2% Activin™	4.47^{+} ± 0.58	0.23 ± 0.02	102.2^{+} ± 9.42	10.4 ± 0.34	0.94 ± 0.28
2% Blackcurrant	3.15 ± 0.78	0.10^{+} ± 0.04	169.4 ± 27.3	10.3 ± 0.54	1.38 ± 0.32
10% FO (Control)	3.17 ± 0.34	0.26 ± 0.03	205.4 ± 31.7	10.5 ± 0.77	1.33 ± 0.27
Trial #2					
2% Boysenberry	11.0^{*+} ± 3.11	0.40 ± 0.03	130.9* ± 10.9	15.4 ± 0.71	1.01* ± 0.30
10% Boysenberry	71.2^{*+} ± 17.80	0.33 ± 0.04	114.9* ± 13.3	14.1^{*+} ± 0.53	0.75 ± 0.29
2% Activin™	11.3 ± 5.30	0.43 ± 0.04	140.1* ± 25.1	14.5* ± 0.69	1.29^{*+} ± 0.18
10% SBO (Control)	3.90 ± 0.72	0.30 ± 0.02	25.0^{+} ± 7.75	16.3 ± 0.79	2.90^{+} ± 0.42
10% FO (Control)	4.72 ± 0.61	0.37 ± 0.06	117.6* ± 24.4	15.7 ± 0.56	1.16* ± 0.31

Table 5.3: *Mean concentrations of 8OHdG* ($ngml^{-1}$), *oxidised protein from ELISA analysis* ($nmolmg^{-1}$ *protein), MDA* ($ngml^{-1}$), $ORAC_{FL}$ *value (mM trolox equivalent) and vitamin E (α-tocopherol,* μgml^{-1}) *for samples from rat trials #1 and #2. Values are ± SEM. Values were statistically different from the *soybean oil (SBO) control; +fish oil (FO) control (p≤0.05). (Trial #1, n = 6 and trial #2, n = 5).*

5.2.1 Oxidised DNA

The mean concentration of 8OHdG in samples taken at the start of trial #1 was 3.65 ± 0.26 $ngml^{-1}$. After two weeks on the fish oil diet, the 8OHdG concentration dropped to 1.85 ± 0.40 $ngml^{-1}$, the results were, however more variable than at the start of the trial. At the end of the trial, following supplementation with berry fruit extracts, the 8OHdG had increased to the concentrations shown in Table 5.3. A typical chromatogram, representing 8OHdG analysis in urine from rats on the 2% boysenberry diet is shown in Figure 5.4.

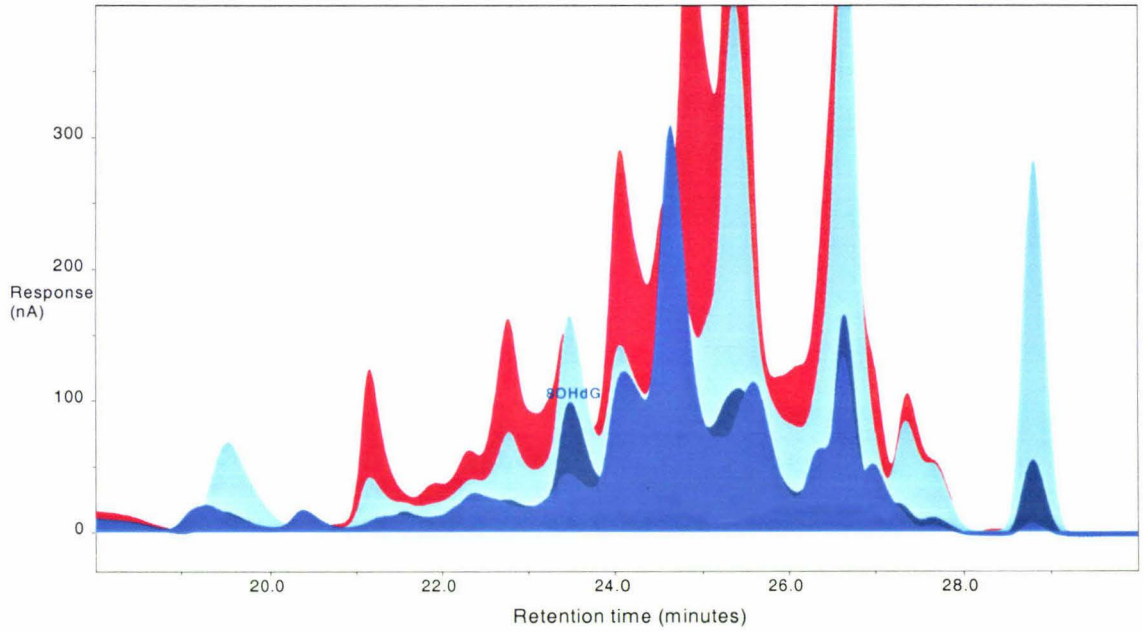


Figure 5.4: *A chromatogram for a typical urine sample, after solid phase extraction and HPLC with electrochemical detection. The 8OHdG peak (labelled 8OHdG) was determined by the relative ratios of each channel in comparison to a 1 ug/ml standard. Peak height was determined for 290 mV channel. Channels shown are: 245 mV (bright blue), 290 mV (dark blue), 335 mV (light blue) and 380 mV (red). The other four channels of the system (200, 415, 470 and 515 mV) have been omitted for clarity.*

In the trial #2, excretion of 8OHdG prior to addition of berry fruit extracts to the diet was significantly ($p \leq 0.05$) lower in rats fed the soybean oil containing diets, compared to those on the fish oil diets. The 8OHdG concentration in rats fed fish oil was 10.96 ±

2.69 $ngml^{-1}$, and 4.05 ± 0.58 $ngml^{-1}$ in the soybean oil control. In the samples taken at the end of the trial (Table 5.3), 8OHdG was slightly elevated with the addition of fish oil. The addition of antioxidant further increased the concentration of 8OHdG in the urine, with a larger increase for a higher percentage of boysenberry (Figure 5.5). This increase was consistent with an increase in DNA repair. (Raw data for the 8OHdG assay is shown in Appendix Four as an example of raw data for this assay.)

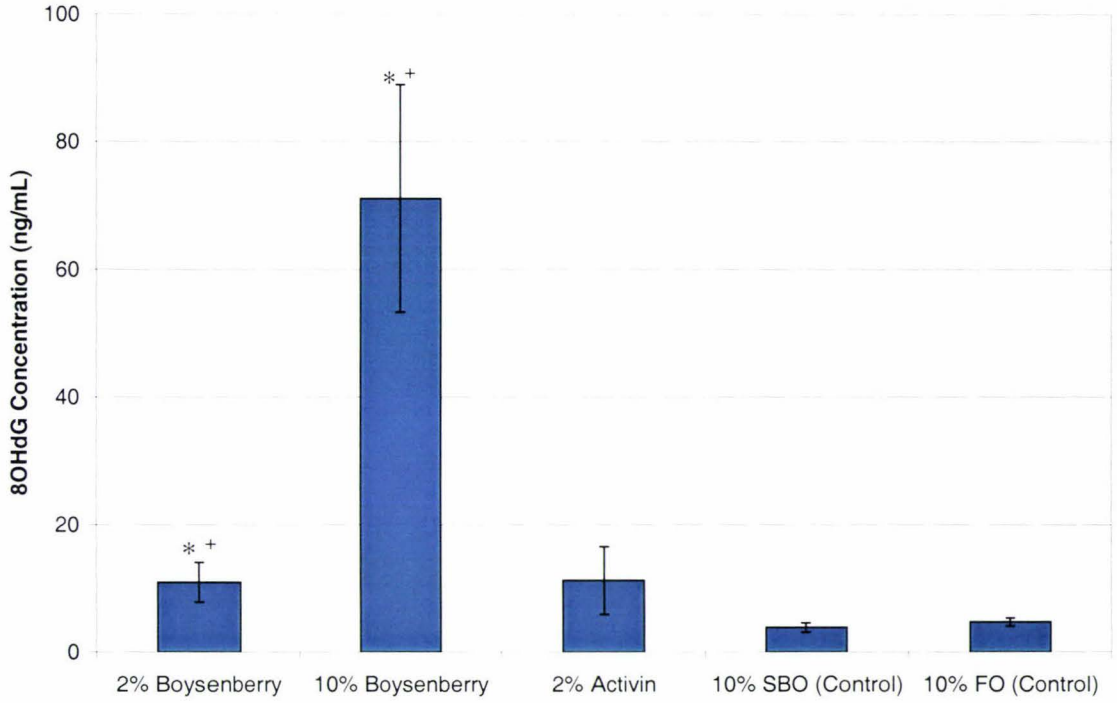


Figure 5.5: *8OHdG concentration ($ngml^{-1}$) for urine at the end of the fruit-derived antioxidants feeding trial #2. SBO = soybean oil, FO = fish oil. Error bars represent SEM (n = 5). Values were statistically different from the *soybean oil (SBO) control; +fish oil (FO) control (p≤0.05)*

5.2.2 Oxidised Protein

The carbonyl assay to assess the level of oxidised protein in plasma samples from trial #1 showed the carbonyl concentration in rats fed with Activin™ and boysenberry extracts were not significantly different from the control. The rats fed the diet

supplemented with 2% blackcurrant extract significantly lowered the plasma protein carbonyl concentration relative to the 10% fish oil control ($p \leq 0.05$).

The protein carbonyl concentration for samples from trial #2 showed neither extract had an effect on protein oxidation. Although not significant, the Activin™ and 10% boysenberry appeared to increase protein oxidation relative to the fish oil control. Upon comparison of both control groups, it was apparent that the fish oil slightly increased oxidative stress relative to the soybean oil. (Appendix five and six show raw data for the colorimetric and ELISA methods respectively as examples of data produced from these assays).

5.2.3 Oxidised Lipid

Oxidised lipid, as measured by the MDA concentration, was significantly different between Activin™ and the 10% fish oil control at the 95% confidence level ($P \leq 0.05$) in trial #1. Generally the fruit extracts appeared to decrease the lipid peroxidation induced by the fish oil.

In trial #2, addition of either extract failed to reduce the lipid peroxidation induced by the presence of fish oil in the synthetic diet. In fact, it was apparent that in rats fed 2% Activin™ and 2% boysenberry, the lipid peroxidation increased beyond that of the fish oil alone. All treatments had increased lipid peroxidation relative to the soybean oil control. (Appendix seven shows raw data from trial #2 as an example of data produced from this assay).

5.2.4 Plasma Antioxidant Status

As for the oxidised fish oil feeding trial, the results for the $ORAC_{FL}$ assay showed the trolox equivalents for each treatment group were approximately the same in trial #1.

Vitamin E analysis of plasma reflected the $ORAC_{FL}$ results in the trial #1 and showed no significant difference in the α-tocopherol concentration between each of the groups, although the plasma vitamin E concentration was lower in rats fed Activin™.

Plasma $ORAC_{FL}$ concentrations in trial #2 were significantly lower in the 2% Activin™ treatments compared to the soybean oil control, and in the 10% boysenberry, compared to both the soybean and fish oil control groups. (Appendix eight shows raw data from trial #2 as an example of data produced from this assay)

Plasma vitamin E status in trial #2 decreased upon addition of fish oil, inferring the dietary vitamin E, albeit at low levels, was acting as an antioxidant in preference to the fruit-derived antioxidants. (Raw data shown for this trial in Appendix nine as an example of the data produced from this assay). 2% Activin™ significantly increased the plasma vitamin E concentration relative to both controls.

5.3 Discussion

5.3.1 Fish Oil-Induced Oxidative Damage

The differences in biomarker concentrations between trials #1 and #2 may be attributed to different fish oils used, which could have had different compositions and peroxidation values, therefore may induce different types of oxidative damage. Further analysis on the composition and peroxide values of each oil is required to determine if one oil may induce higher levels of oxidative damage than the other.

It was apparent that in general, fish oil could not significantly induce protein oxidation, but induced high levels of lipid peroxidation. Analysis of lipid peroxidation and plasma vitamin E status, where the soybean oil control was included revealed that rather than fish oil inducing oxidative damage, the soybean oil may have decreased oxidative damage due to the presence of increased vitamin E. If this were the case, it would have been more appropriate to include a control group without oil added to the diet.

5.3.2 Reduction of Oxidative Damage by Berry Fruit Anthocyanins

It should be noted that as the 8OHdG concentration increased, so did the error (SEM). High errors in the trials #1 and #2 may have been due to the collection system. The diet and drinking water may have fallen into the urine and some evaporation may have occurred overnight. The 1 ml urine sample analysed for 8OHdG may not have been an accurate representation of urine due to the presence of some diet in the urine. The trial #3 (Chapter Six) used different cages to minimise evaporation and prevent food and water falling into the urine overnight, and correspondingly the errors for 8OHdG analysis in the final trial were lower.

An increase in the concentration of 8OHdG in the urine from the rats that were fed Activin™ was interpreted as an increase in DNA repair rather than an increase in oxidative damage to DNA. This therefore, could indicate that Activin™ promotes DNA repair, thus decreasing the level of oxidative DNA damage *in vivo*. The 8OHdG concentration of the 10% fish oil control rats was similar for trials #1 and #2, and the 2% Activin™ and boysenberry concentrations also gave comparable results in the two trials. A large increase in 8OHdG for 10% boysenberry/fish oil in the second trial may indicate a dose-dependent effect, where higher concentrations of antioxidants promoted a higher level of DNA repair.

In trials #1 and #2 the protein carbonyl concentration, determined by an ELISA, was ten times that determined in the fish oil feeding trial using the colorimetric method (Chapter Two, 2.2.4, for explanations as to why the values differ). When berry fruit extracts were added to the natural diet a reduction in the protein carbonyl formation was observed (McGhie, *et al.*, unpublished data). Generally, this reduction was not observed when similar antioxidant extracts were added to the synthetic diet containing fish oil. That is, generally there was no significant difference in carbonyl concentration in rats on the fish oil diet following supplementation with boysenberry extract.

The concentration of MDA in plasma varied slightly between the fruit-derived antioxidant feeding trials, and more significantly from the fish oil feeding trial. This may be attributed to the fact that the rats' dietary intake declined once put on the synthetic diet, thus increasing the MDA relative to the natural diet used in the fish oil

feeding trial (Chapter Four). The increase in MDA by the fish oil could not be countered by the berry fruit extracts, this may imply that the fish oil in the synthetic diet was increasing oxidative damage to lipids beyond a level where anthocyanins may be effective antioxidants. Differences in the MDA concentration between trials #1 and #2 may be due to a different fish oil being used in each trial, which if composed of different fatty acids, may induce different types of oxidative damage, one which could be countered by the Activin™, and one which could not.

There was no significant change in the vitamin E concentration between the fish oil and fruit-derived antioxidant supplemented diets in trials #1 and #2, thus the fruit-derived antioxidants were not being utilised, and may act in a different antioxidative mechanism to the vitamin E. An increase in vitamin E in trial #2, with rats fed Activin™ may infer Activin™ was the preferred antioxidant in rats fed this diet. A large change in the vitamin E concentration was observed between these treatments and the soybean oil control. This infers that the soybean oil contains vitamin E, which was increasing the plasma vitamin E status. Although dietary vitamin E is limited in the fish oil diets, vitamin E was thought to be utilised in preference to the berry fruit antioxidants. In trial #2 an increase in the serum $ORAC_{FL}$ value was expected, as it was hypothesised that the overall antioxidant capacity would have increased due to the presence of dietary antioxidants. A study by Richelle, et al. (1999) with strawberries, red wine, spinach and vitamin C showed that the ORAC values increased in the serum of elderly women. This may indicate the possible absorption of phenolic compounds, however, there were only eight subjects, thus further validation would be required in terms of epidemiological studies with biomarker analysis in addition to the ORAC and FRAP.

An increased antioxidant capacity in plasma or serum is not necessarily an acceptable outcome however, as it may be due to an adaptive response to an increase in oxidative stress at an early stage. In addition, a decrease in total antioxidant capacity may be desirable because an increase in the production of reactive oxygen species may result in a decrease in the total antioxidant capacity, due to the high level of activity of the antioxidants. An explanation for the decrease in trial #2 could be due to the caloric restriction the rats may have suffered when introduced to the synthetic diet. This result is in agreement with Cao, et al. (1999) where serum ORAC decreased in calorically restricted rats. Although the rats' dietary consumption did decrease, however, it may

not have decreased to a point where the rats may be considered to be calorically restricted.

The absolute values for ORAC analysis were different for trials #1 and #2. This may be explained by variations in the oil used. A different fish oil may have induced more oxidative stress in the plasma, and the 2% Activin™ and 10% boysenberry countered this by actively scavenging the free radicals, giving a decrease in antioxidant capacity.

The results indicate that in general, berry fruit anthocyanins had little effect on fish oil induced oxidative damage to lipids and proteins. This may be due to the occurrence of the anthocyanins scavenging the free radicals present in the *diet*, before ingestion, to a point where they are no longer effective as antioxidants *in vivo*. Oxidised DNA (8OHdG) analysis revealed that higher levels of extract may have a greater effect at increasing DNA turnover.

Chapter Six – Fruit-derived Antioxidant Feeding Trial #3 Results and Discussion

Trial #3 (Figure 6.1) was implemented to assess whether the synthetic diet and/or the fish oil, had a significant effect on the biomarker analyses by inducing different types of oxidative stress. In a previous feeding trial, (McGhie *et al*, unpublished data) natural 'chow' diets supplemented with 2% boysenberry, elderberry and blueberry significantly reduced the carbonyl content of the plasma relative to the control (the standard diet). In that trial, the rats were not pre-stressed with fish oil, so it was hypothesised that the addition of fruit-derived antioxidants (anthocyanins) in trials #1 and #2 were not able to counter the oxidative stress induced by the fish oil.

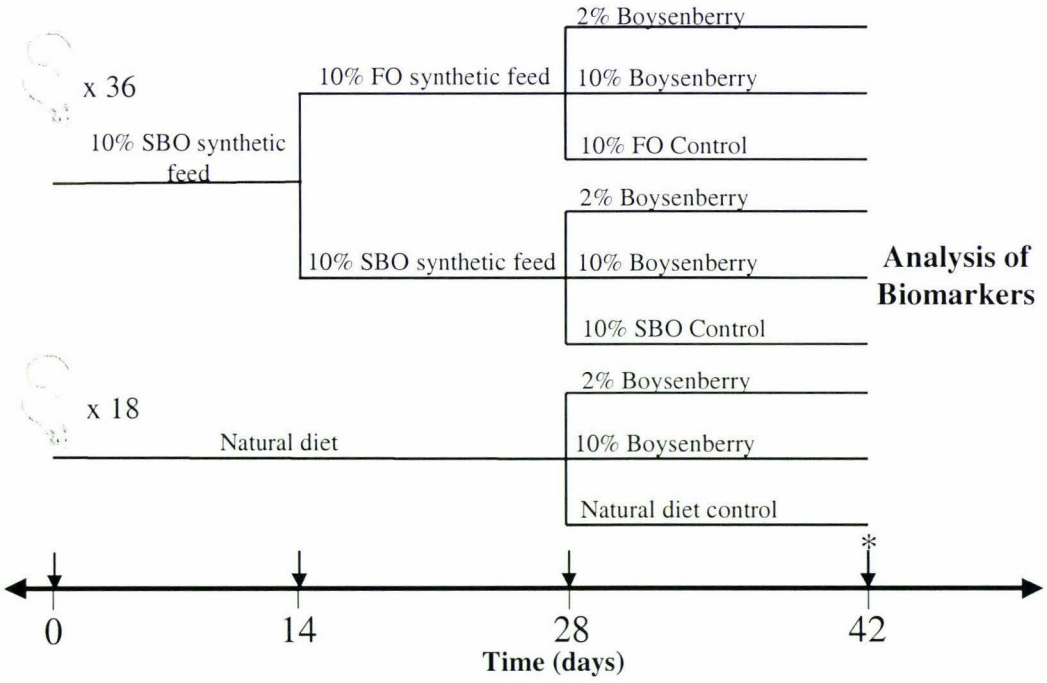


Figure 6.1: *Study design for fruit-derived antioxidant feeding trial #3 with six rats per diet. FO = fish oil and SBO = soybean oil. * indicates collection of plasma samples. Solid arrows denote the collection of overnight urine.*

6.1 Overall Observations

The rats were individually housed in polycarbonate 'shoebox' style cages with wire lids, different from those used in previous trials. On each night urine was collected each rat was housed in Nalgene™ metabolic cage systems for rodents. These cages had special diet and water containers to minimise the amount of feed or water in the urine collection vessel. The collection vessel had a specially-designed cap to allow urine to enter, but to minimise evaporation of the urine.

The weight of each rat was assessed every two days throughout the trial. Biomarkers for oxidative damage to proteins, DNA and the overall plasma antioxidant status were assessed in plasma samples collected at the end of the trial.

6.1.1 Rat Weight Gain

For the first 14 days of the trial there was no difference between rats fed the soybean oil synthetic diet and rats fed the natural diet. At the introduction of fish oil into the synthetic diet feeding group (day 14) there was a divergence in body weight between rats fed the fish oil synthetic diet and the natural and synthetic soybean oil diets (Figure 6.2). The rats on the natural diet gained significantly ($p \leq 0.05$) more weight than those fed either of the synthetic diets. In addition, the rats on the synthetic fish oil diet had significantly ($p \leq 0.05$) lower weights than rats in all other groups. When the berry fruit extract was added to the synthetic fish oil diet, the rats gained weight at a lower rate than their respective control groups.

As for trials #1 and #2 it was clear that the fish oil, when added to the synthetic diet affected the growth of the rats. When the growth of the rats is compromised, the rats may become stressed. Therefore, it is important to consider the growth of the rats when interpreting the biomarker assays, as the results may be attributed to the poor health of the rats, due to the diet rather than due to the fish oil in the diet.

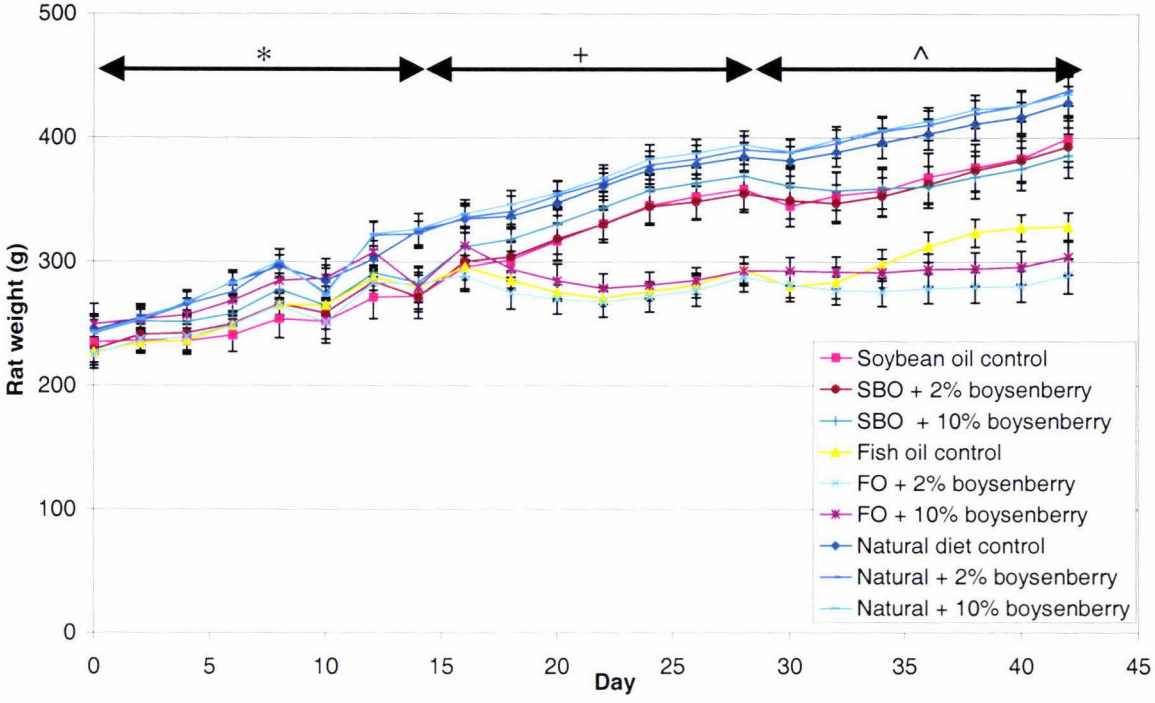


Figure 6.2: *Cumulative rat weight for the final feeding trial. Lines represent the mean rat weight in each feed group over the course of the trial. *Days 1-14 = acclimatisation period; +days 15-28 = fish oil (FO), soybean oil (SBO), or natural diet; ^days 29-42 = berry fruit extracts added to the base diets. Error bars represent SEM (n = 6).*

6.1.2 Dietary Consumption

The total anthocyanin concentration (mgg^{-1}) of the boysenberry extract in trial #3 was at least five times greater than that used in the previous trials (Table 6.1). The concentrations were determined as described in section 5.1.2, chapter 5. There was little reduction in the anthocyanin content in the feed over the course of the trial (Table 6.2), as determined by analysis of diet samples by HPLC, using the same method as for the extract analysis. During the acclimatisation period at the start of the trial, the mean consumption of the natural diet was 30 $gday^{-1}$, and the synthetic diet, 23 $gday^{-1}$, indicating the synthetic diet was less palatable than the natural diet.

When animals were fed diets containing fish oil, consumption of the synthetic fish oil diet was half that of the natural and soybean oil synthetic diets. Upon addition of the

berry fruit extracts, there was little difference in diet consumption of all rats fed the natural (mean of 33 $gday^{-1}$) and synthetic soybean oil (mean of 30 $gday^{-1}$) diets. Consumption of each fish oil diet (mean of 25 $gday^{-1}$) was lower than the natural and soybean diets. Growth of all rats was at an acceptable rate as defined by the Massey University Ethics Committee.

Compound	*Boysenberry*[1]	*Boysenberry*[2]
Cyanidin 3-*O*-sophoroside	0.12	4.52
Cyanidin 3-*O*-2^G glucosylrutinoside	0.04	1.54
Cyanidin 3-*O*-galactoside	0.00	0.00
Cyanidin 3-*O*-glucoside	0.58	5.86
Cyanidin 3-*O*-rutinoside	0.12	5.65
Total Anthocyanin	0.88	12.48
Total Phenolics	5.76	262.67

Table 6.1: *Concentration (mgg^{-1} extract) of anthocyanin at A_{530} and total phenolics (determined by HPLC) at A_{280} of the boysenberry extracts used in the feeding trials boysenberry[1] = trials #1 and #2 and boysenberry[2] = trial #3. Total values represent summation of all peaks at the designated wavelength and include known peaks.*

Diet	*Total Anthocyanin mgg^{-1} diet*	*Total Phenolics mgg^{-1} diet*
10% Soybean oil synthetic diet	0.00	0.00
2% Boysenberry in 10% soybean oil synthetic diet	0.16	1.24
10% Boysenberry in 10% soybean oil synthetic diet	0.82	6.61
10% Fish oil synthetic diet	0.00	0.00
2% Boysenberry in 10% fish oil synthetic diet	0.09	1.19
10% Boysenberry in 10% fish oil synthetic diet	0.48	5.23
2% Boysenberry in natural diet	0.10	1.70
10% Boysenberry in natural diet	0.63	6.12
Natural diet	0.00	0.00

Table 6.2: *Concentration (mgg^{-1} of diet) of anthocyanin at A_{530} and total phenolics at A_{280}. Total values represent summation of all peaks at the designated wavelength and include known peaks.*

It was apparent that the anthocyanins were being degraded in the diets containing fish oil. This implied the rats fed the boysenberry fish oil diets ingested fewer anthocyanins than rats on the other feeds, therefore less of a change in the biomarker analyses may be expected.

6.2 *Biomarkers of Oxidative Damage*

A summary of mean concentrations of each biomarker for the different treatments is shown in Table 6.3.

Diet Supplement	*8OHdG Assay* $ngml^{-1}$	*Protein Carbonyl* $nmolmg^{-1}$ *protein*	*MDA Assay* ng/ml^{-1}	*Vitamin E Assay* μgml^{-1}
10% SBO synthetic (control)	18.0 ± 3.89	0.37 ± 0.07	$182.8^{+\wedge}$ ± 26.6	2.54^{+} ± 0.26
2% Boysenberry/SBO synthetic	17.1 ± 3.15	0.33 ± 0.04	23.0*^ ± 4.31	2.90^{+} ± 0.17
10% Boysenberry/SBO synthetic	17.5 ± 2.45	0.18*^ ± 0.04	64.9* ±28.3	2.81^{+} ±0.16
10% FO synthetic (control)	14.6 ± 1.39	0.36 ± 0.05	44.8* ± 18.0	3.77* ± 0.37
2% Boysenberry/FO synthetic	16.7 ± 2.82	0.37 ± 0.06	189.2^{+} ± 156.8	2.58^{+} ± 0.28
10% Boysenberry/FO synthetic	18.7 ± 1.38	0.24 ± 0.05	114.8 ± 27.2	2.84^{+} ± 0.11
Natural diet (control)	21.8 ± 4.07	0.26 ± 0.03	83.9* ± 16.5	3.26 ± 0.51
2% Boysenberry/natural	25.7^{*+} ± 0.99	0.09*^ ± 0.02	47.3* ± 18.7	2.60^{+} ± 0.10
10% Boysenberry/natural	21.7^{+} ± 2.97	0.09*^ ± 0.03	50.6* ± 23.4	2.60^{+} ±0.12

Table 6.3: *Mean concentrations of 8OHdG* ($ngml^{-1}$)*, oxidised protein from ELISA analysis* ($nmolmg^{-1}$ *protein), MDA* ($ngml^{-1}$) *and vitamin E (α-tocopherol,* μgml^{-1}) *for samples from antioxidant rat trial #3. Values are ± SEM. SBO = soybean oil, FO = fish oil. Values were statistically different from the *soybean oil (SBO) control; +fish oil (FO) control; ^ natural diet control (p ≤ 0.05). (n = 6)*

The ORAC assay could not be performed for samples from this feeding trial due to instrument failure. Overall, biomarkers of oxidative damage revealed a difference in rats fed the natural diet, compared with rats fed the synthetic diets (both fish oil and soybean oil supplemented). Whether this difference was due to the diets, or the

presence of the oil is unknown and may be confirmed by including a synthetic diet containing no oil as a control in future studies.

6.2.1 Oxidised DNA

To measure 8OHdG excretion during the trial, urine samples were collected at four 2-week intervals (Figure 6.3).

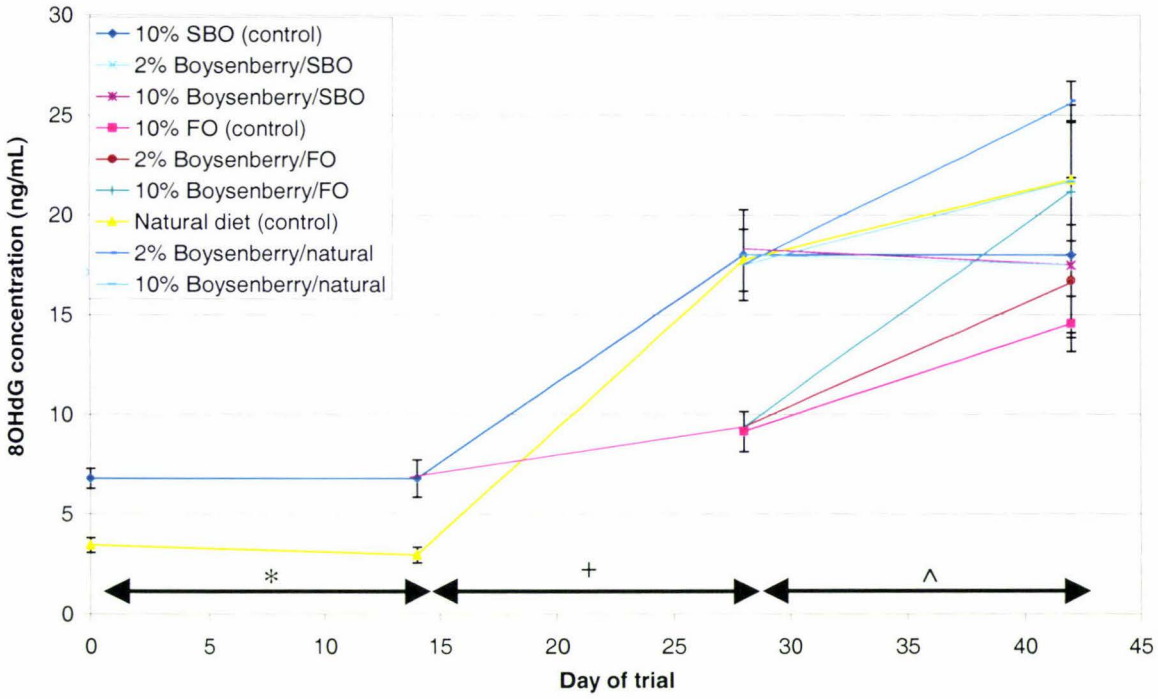


Figure 6.3: *8OHdG concentration (nml^{-1}) for each diet throughout trial #3. *days 0-14, acclimatisation period = synthetic soybean oil (SBO) or natural diets; +days 15-28 = fish oil (FO), SBO, or natural diets; ^days 29-42 = berry fruit extracts added to the base diets.*

After the two week acclimatisation period (days 0-14) the 8OHdG concentration was significantly ($p = 0.0004$) higher in the rats on the synthetic diet than those on the natural diet. At days 15-28 the 8OHdG concentration for rats on the synthetic soybean

oil and natural diets was the same, whereas rats on the fish oil diet had significantly ($p \leq 0.05$) lower concentrations of 8OHdG. The 8OHdG concentration, however, continued to increase throughout the trial in each feeding group. At the end of the trial the 8OHdG concentration was higher in the rats fed the natural diets than those on the synthetic diets. Neither percentage of boysenberry had a significant ($p \leq 0.05$) effect on the urinary excretion of 8OHdG for the rats fed the natural diet. Rats fed the fish oil diet had increasing 8OHdG excretion with increasing boysenberry concentration.

Representation of Urinary 8OHdG Excretion

Cooke et al. (2000) describes various representations of 8OHdG excretion. 8OHdG excretion may be expressed in terms of $nmolkg^{-1}24\ hr^{-1}$ or relative to urinary creatinine concentration. Creatinine is routinely used to correct for aberrations in urine concentrations, although it has been suggested that the creatinine concentration varies with the 8OHdG concentration (Cooke et al., 2000).

Figure 6.4 shows a comparison of the 8OHdG concentration for the final samples analysed from trial #3 expressed as ng/ml (A) nmol 8OHdG kg^{-1} body weight 24 $hours^{-1}$ (B) and nmol 8OHdG μmol^{-1} creatinine (C). Urinary creatinine concentrations were determined using the Jaffe method (Bartels, et al., 1972) in the nutrition laboratory, Massey University. This showed that correcting for the volume of urine excreted (either directly, or by reference to creatinine) gave a different relationship between each group.

When the urinary 8OHdG concentration was expressed as nmol 8OHdG kg^{-1} body weight 24 $hours^{-1}$, or nmol 8OHdG μmol^{-1} creatinine the results were very different from the $ngml^{-1}$ results as the concentration was higher in the rats fed the natural diet compared to the soybean oil synthetic diet. A significant decrease in nmol 8OHdG kg^{-1} body weight 24 $hours^{-1}$ was observed for 10% boysenberry relative to the control for the 10% soybean oil base diet ($p \leq 0.05$). The weight/time corrected values are in the same range as described in Shigenaga, et al. (1989), the molar values, however were over 1000-fold lower in this study than reported in Germadnik, et al. (1997). The values reported in Germadnik, et al. (1997), however, had very high errors associated with them.

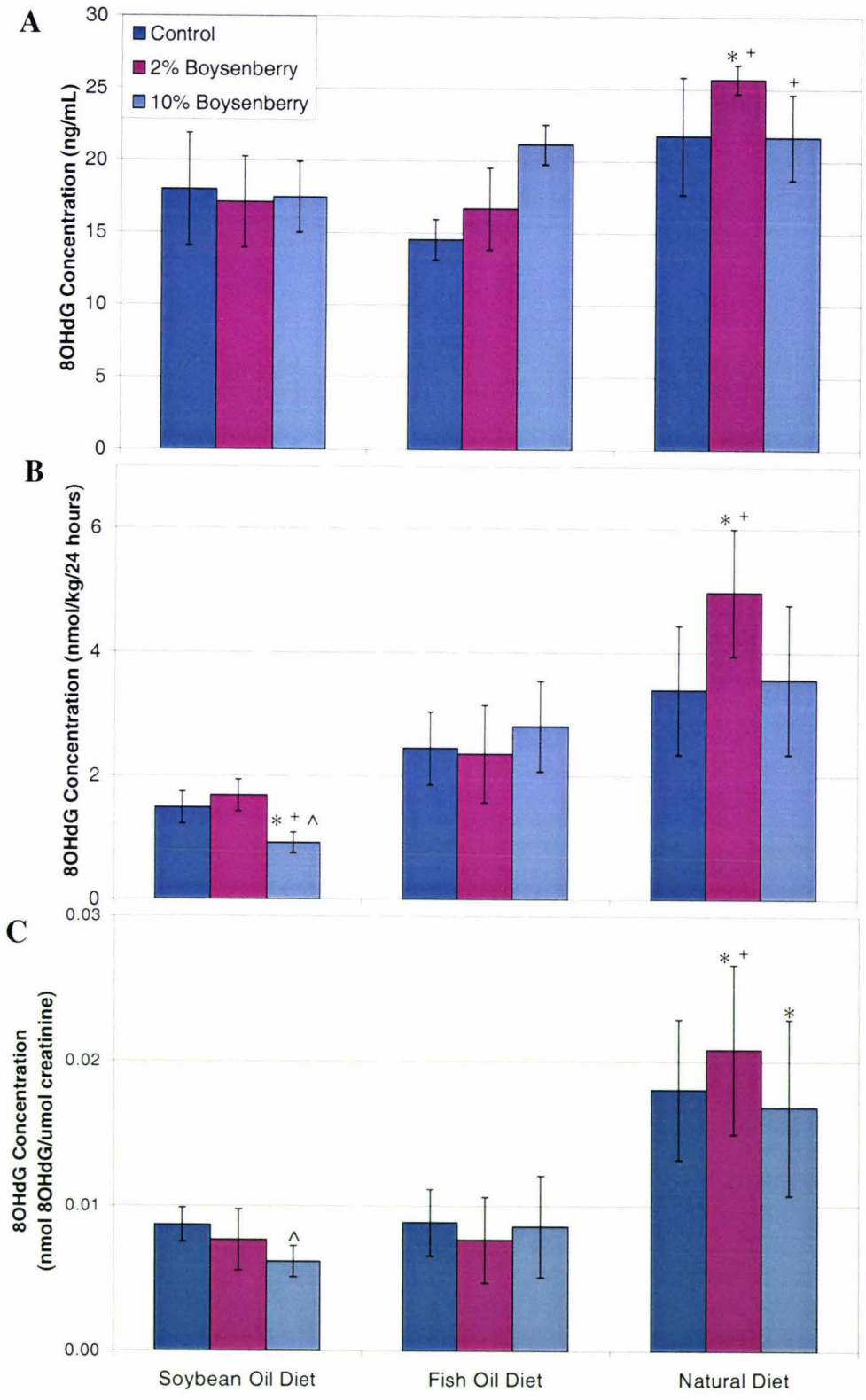


Figure 6.4: *Comparison of the 8OHdG concentration: ngml^{-1} (A); nmol 8OHdG kg^{-1} body weight 24 hours^{-1} (B); and nmol 8OHdG mmol^{-1} creatinine (C). Values were statistically different from the *soybean oil control; $^{+}$fish oil control; ^ natural diet control ($p \leq 0.05$). Error bars represent SEM ($n = 6$).*

The volume of urine excreted by each group of rats (Table 6.4) was higher for rats on the natural diet compared to rats on either synthetic diet. The addition of berry fruit extracts, however, did not significantly affect the volume of urine excreted in each group.

Diet	*Urine Excretion* *ml 24 hours^{-1}*
10% Soybean oil synthetic (control)	9.00^ ± 1.63
2% Boysenberry/soybean oil synthetic	11.38 ± 2.70
10% Boysenberry/soybean oil synthetic	5.70*+^ ± 0.62
10% fish oil synthetic (control)	13.00 ± 3.01
2% Boysenberry/fish oil synthetic	9.38^ ± 2.93
10% Boysenberry/fish oil synthetic	9.5^ ± 0.84
Natural diet (control)	17.25 ± 3.07
2% Boysenberry/natural diet	27.75*+^ ± 4.53
10% Boysenberry/natural diet	22.00* ± 5.84

Table 6.4: *Mean volume of urine excreted over 24 hours. Values were statistically different from the *soybean oil control; +fish oil control; ^ natural diet control ($p \leq 0.05$) ($n = 6$).*

6.2.2 Oxidised Protein

ELISA analysis of protein carbonyl concentration (Figure 6.5) showed the greatest change in rats fed the natural diet, where both levels of boysenberry reduced the carbonyl concentration markedly. Both levels of boysenberry also reduced protein carbonyl formation in the fish oil diet, although the results were not significantly different ($p \leq 0.05$). Supplementation with boysenberry showed a dose-dependent trend for the rats on both synthetic diets. Rats fed 10% boysenberry in the 10% soybean oil synthetic diet had a significant decrease in protein carbonyl content ($p \leq 0.05$).

The fact the boysenberry anthocyanins are actively reducing protein oxidation in rats fed the natural diet infers that the mechanism by which proteins are oxidatively damaged is different in rats fed the natural diet, than to those fed the synthetic diets.

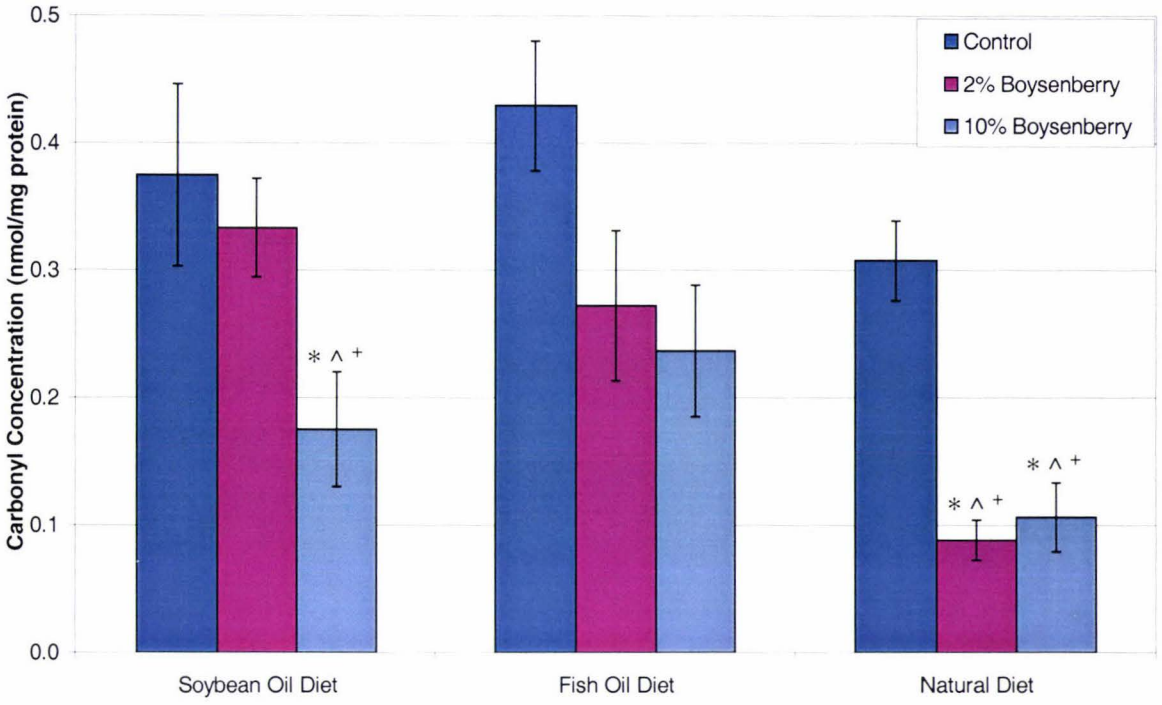


Figure 6.5: *Protein carbonyl concentration (nmolmg^{-1} protein) from ELISA analysis of plasma from trial #3. Values were significantly different from the *soybean oil control; $^{+}$fish oil control; ^ natural diet control ($p \leq 0.05$). Error bars represent SEM (n=6).*

6.2.3 Oxidised Lipid

Lipid peroxidation as measured by the MDA concentration in plasma (Figure 6.6), showed an increase in rats where their diet was supplemented with either 2% or 10% boysenberry in the 10% fish oil synthetic diet, which corresponded to the results from the previous trial. In the soybean oil base diets, both the 2% and 10% boysenberry significantly decreased the lipid peroxidation ($p \leq 0.05$), although the soybean control had over twice the concentration of MDA of the other control groups.

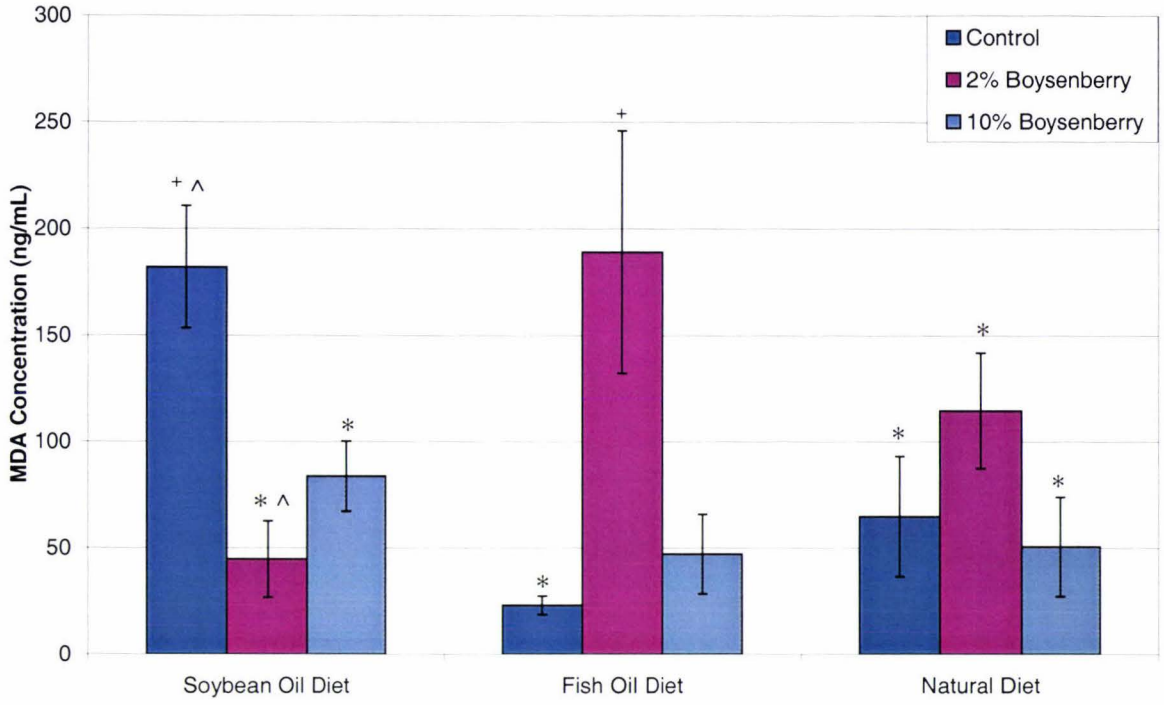


Figure 6.6: *MDA concentration ($ngml^{-1}$) in plasma samples from trial #3. Values were significantly different from the *soybean oil control; $^{+}$fish oil control; ^ natural diet control ($p \leq 0.05$). Error bars represent SEM ($n=6$).*

There was no change in the plasma MDA concentration between rats fed each natural diet treatment.

6.2.4 Plasma Antioxidant Status

The plasma α-tocopherol concentration was highest in rats fed the fish oil and soybean oil synthetic control diets and remained static in all groups fed the natural diet. Both boysenberry treatments decreased the concentration of α-tocopherol to that of the rats on the natural diet (Figure 6.7).

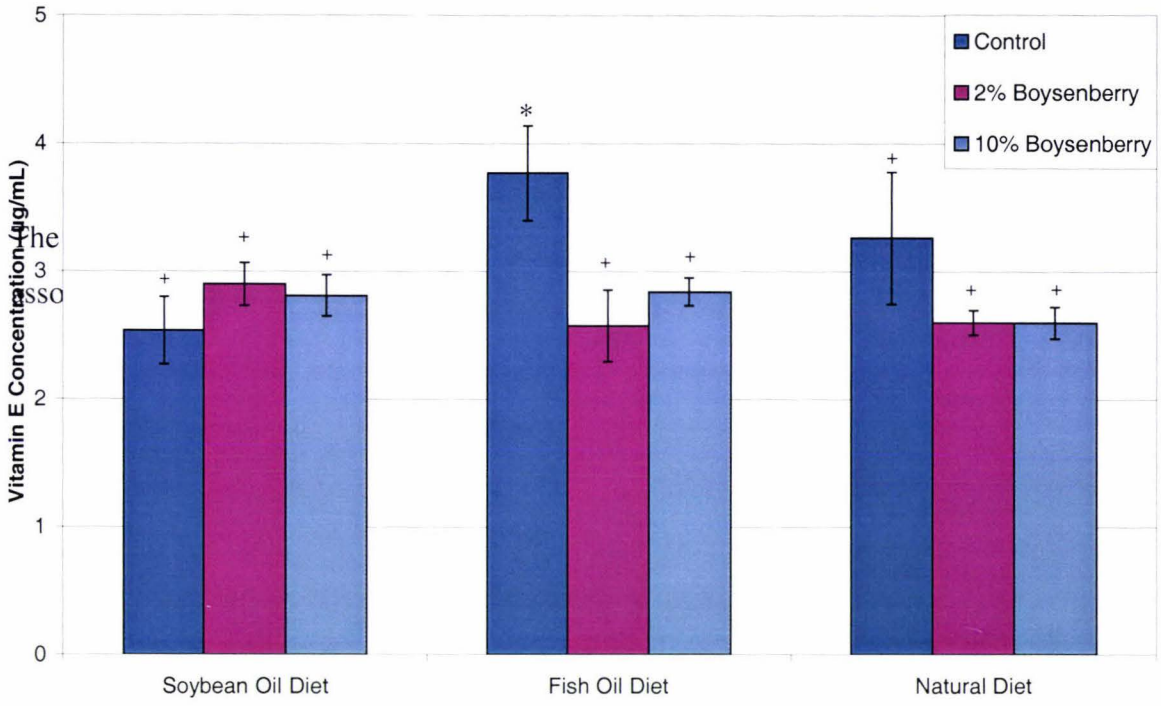


Figure 6.7: *Vitamin E content, as measured by plasma α-tocopherol concentration (μgml^{-1}) from samples from trial #3. Values are significantly different from the *soybean oil control; $^{+}$fish oil control; ^ natural diet control ($p \leq 0.05$). Error bars represent SEM ($n=6$).*

6.3 Discussion

6.3.1 Fish Oil-Induced Oxidative Damage

Failure to detect changes in biomarkers of oxidative damage in groups where oxidative damage should have been induced by dietary fish oil may reflect the ability of the rats to control the rate of free radical generation or prevent the free radicals from causing damage. This may be due to the vitamin C status of the rats. Rats synthesise vitamin C (Linster and Van Schaftingen, 2003) and this synthesis may have increased during the trial to compensate for the oxidative damage induced by the fish oil.

6.3.2 Reduction of Oxidative Damage by Berry Fruit Anthocyanins

The rats' dietary intake declined once put on the synthetic diet, which may explain why the biomarker values often differed between the synthetic and natural diets. The synthetic diet was used for consistency in diet preparation between trials and, like the natural diet, was vitamin E deficient. An alternative to the synthetic diet would be to prepare bulk natural diet and store over a period for use in all rat trials to avoid the source and/or quality of dietary ingredients changing.

Addition of the berry fruit extracts to the natural and fish oil synthetic diets induced an increase in the excretion of 8OHdG ($ngml^{-1}$) relative to the respective control. 8OHdG excretion was approximately equal for all groups on the soybean oil diets. A higher level of boysenberry caused an increase in 8OHdG excretion, and therefore DNA turnover, only for the fish oil base diet, as reflected by trial #2. There was little decrease in 8OHdG excretion when extracts were added to the soybean synthetic or natural base diets.

When interpreting the weight/time- or creatinine-corrected 8OHdG concentrations, a different relationship was observed than the $ngml^{-1}$ values. An increase in the 8OHdG concentration in these rat trials, interpreted as an increase in DNA repair, may be incorrect. Instead, the increase in 8OHdG excretion may be due to an increase in oxidative DNA damage. The results therefore may imply that the berry fruit extracts were not effective with only two weeks of dietary supplementation, and their efficacy at decreasing oxidative damage may be reflected after a longer period of time with berry fruit anthocyanin supplementation of the diet.

Overall it was apparent that modulating the levels of endogenous DNA oxidation through dietary intervention was possible, but determination of the effect was critically dependent on the number of variables such as the duration of trial, the type and dose of antioxidant, and the basal antioxidant level in plasma. Interpretation of results also requires careful consideration and a longer trial may provide more evidence of a beneficial effect of berry fruit supplementation, as the oxidative damage (8OHdG) may decrease following extended periods of time on the berry fruit supplemented diet. It may be that evidence of the antioxidant effect of berry fruit anthocyanins was not

determined by 8OHdG analysis in this short-term study, since 8OHdG excretion is a reflection of DNA repair. The repair thought to be induced by the berry fruit anthocyanins may be noted after a longer period of dietary supplementation.

A significant decrease was observed in the plasma protein carbonyl concentration in rats for both 2% and 10% boysenberry supplemented natural diets. Although a decrease in the carbonyl concentration in rats fed the fish oil diet was observed, the synthetic soybean oil diet supplemented with 10% boysenberry gave the only significant decrease in protein carbonyl concentration for the synthetic diets. This implied that the fish oil in the synthetic diet was increasing oxidative stress beyond a level where anthocyanins may be effective antioxidants. Alternatively, the mechanism by which fish oil induces oxidative stress did not target proteins, therefore they were not oxidised to a significant level. The synthetic diet with no fish oil or soybean oil added may be useful as additional controls to test this hypothesis.

Lipid peroxidation, as measured by the plasma MDA concentration decreased significantly ($p \leq 0.05$) in rats fed the soybean oil/boysenberry supplemented synthetic diets compared to the soybean oil control. The fact the soybean oil control was significantly higher than both the fish oil synthetic and natural diet controls indicates the soybean oil may have been inducing lipid peroxidation, although it was expected that the vitamin E in the soybean oil would have countered this damage. The decrease in damage with in the 10% soybean oil group supplemented berryfruit diets may indicate an interaction between the anthocyanins and vitamin E for the conditions associated with the soybean oil. An increase in MDA in rats fed the fish oil/2% boysenberry diet, relative to the control, requires further investigation. Low MDA or little difference between each treatment group for the natural diet may be due to the antioxidants in the plasma, such as vitamin E, terminating the lipid peroxidation reactions (Davison, et al., 2002). If this was the case, the plasma vitamin E concentration was expected to correlate with the MDA concentration. In this trial, the correlation co-efficient was 0.1133, indicating a relationship albeit at low levels. This inferred that the vitamin E was being utilised to decrease lipid peroxidation, therefore MDA was low, and accordingly the vitamin E was also low.

Plasma vitamin E concentrations were investigated because fish oil tends to reduce the vitamin E status and vitamin E is an important *in vivo* antioxidant (Cho, et al., 1995). Vitamin C was not considered in the current studies, as rats synthesise vitamin C and therefore are not a model for vitamin C investigations. Vitamin C could potentially impact on the vitamin E status, as inferred by Hamilton, et al. (2000) who provided evidence that vitamin C may exert a redox cycling effect and be an efficient co-antioxidant *in vitro* for the regeneration of α-tocopherol. The vitamin E content of plasma was tested to see if this hypothesis could apply for anthocyanins. Results from vitamin E analysis in this trial did not compare with the results from previous trials, as the α-tocopherol concentration decreased in the antioxidant treatments relative to the control group for the fish oil diets. This may indicate that, in the presence of fish oil, the anthocyanins induced the use of vitamin E from plasma.

It is possible that the overall plasma antioxidant status is under homeostatic control. Any alteration, therefore, in the status of a single antioxidant could affect the status of another antioxidant. The diet in these experiments had low vitamin E therefore anthocyanins may not be able to be as effective antioxidants as they could be, were vitamin E at natural levels in the diet. The potential benefits of supplementation with both antioxidants (vitamin E and anthocyanins) may therefore extend beyond the simple antioxidant effect. Results of a study by Hamilton, et al. (2000) supported an *in vivo* interaction between vitamin E and C, as indicated by similar changes in plasma vitamin E, antioxidant power and lipid concentration in response to supplementation with pharmacological doses of either vitamin C or E. Whether this can be extrapolated to include other antioxidants, such as anthocyanins remains to be seen from future experiments, where natural levels of vitamin E in the diet may be tested with elevated levels of anthocyanin extracts.

Vitamin E and 8OHdG analysis showed little change over time for groups fed soybean oil, therefore the anthocyanins may not have functioned as antioxidants because there were not high levels of oxidative stress in the rats associated with the soybean oil diets. This may be explained by tumour development analysis (Kennedy, 1995), which provided evidence that soybean oil lowers oxidative stress. This may infer that the *in vivo* antioxidant capacity was maintained by vitamin E in the soybean oil diet, and

therefore, the anthocyanin extracts had no further impact, as their antioxidant function was not required. Interpretation of the weight corrected 8OHdG data, however, inferred that the 10% boysenberry could decrease oxidative damage in the soybean oil synthetic diet. This may indicate a synergistic relationship between vitamin E and anthocyanins.

Monitoring plasma vitamin C status throughout the trial may have provided an indication of increased antioxidant capacity of the plasma due to increased synthesis of vitamin C. This may explain why generally the treatments containing fish oil showed little difference from the control for protein oxidation. Vitamin C may specifically target the mechanism of oxidative damage, which caused protein oxidation, so upon induction of oxidative damage by fish oil, vitamin C synthesis may rapidly increase to compensate for the large increase in oxidative damage by maintaining plasma redox control. The acclimatisation period may therefore be a disadvantage, as the rats may adapt to their growing conditions, so any oxidative damage that is induced at the start of the trial is countered by a change in the *in vivo* antioxidant capacity. If there were no acclimatisation period, however, such a change in oxidative damage may only be observed in the first few days of the trial, therefore, increased sampling would be required (as discussed below). An assessment of the total antioxidant capacity by the ORAC assay may have shown an increase in antioxidant capacity, reflecting an increase in vitamin C to actively oppose free radical damage induced by the fish oil. This assessment would be required early in the trial, however, as it is probable that the change in plasma vitamin C status occurred rapidly upon induction of oxidative damage. ORAC assessment at the end of the trial, to provide an indication of the level of vitamin C may, therefore have shown little change in the total plasma antioxidant status. To account for vitamin C synthesis when using rats as a model system, the enzymes involved with vitamin C synthesis should be investigated. If they are inducible, their function may be controlled, or prevented.

Any variations in the results between trials may be explained by the fact that a different line of rats was used for the final trial. For other trials where the rats were from the same breeding line, there were several generations between rats, and a strain may have been inadvertently selected that was more resistant to oxidative stress, which may explain different results for similar treatments in the different trials.

An experiment involving anthocyanins was carried out by Ramirez-Tortosa, et al. (2001) which aimed to assess the ability of 0.1% anthocyanin extract in the natural diet to modify the elevated indices of oxidative damage in rats induced by vitamin E deficiency. Liver 8OHdG concentration increased with low vitamin E, but was restored to the initial level significantly, but not fully, by anthocyanin. Whether this effect was due to the free radical scavenging ability of the anthocyanins, up-regulation of DNA repair mechanisms or antioxidant enzymes, or the formation of protective DNA-anthocyanin co-pigmentation complexes, was not determined. The same results were shown for antioxidant activity in the plasma. This suggests the anthocyanin-rich extract possessed antioxidant activity *in vivo*, however the anthocyanin extract was not pure, so it is possible that the *in vivo* effects of the extract may be due to the presence of other unidentified compounds, an explanation that applies for the trials in the current study. An HPLC profile showed no other compounds present other than anthocyanin, but there may have been compounds present that did not absorb light in the region used for quantification.

The results of this trial indicate the berry fruit antioxidants in this study had little effect on fish oil-induced oxidative stress, but was able to counter oxidative damage to proteins in the natural diet and diet supplemented with fish oil. Anthocyanin analysis of the diets implied that this may be due to the occurrence of the anthocyanins scavenging free radicals in the diet, prior to ingestion, to a point where they are no longer effective as antioxidants *in vivo*. Alternatively, the mechanisms by which anthocyanins act as antioxidants may be ineffective and target different mechanisms of fish oil induced oxidative damage.

Chapter Seven – Summary and Future Direction

7.1 Study Summary

While much research has been carried out on the antioxidant activities of food components *in vitro*, there is little evidence that these compounds display antioxidant activities *in vivo*. This is despite evidence that higher consumption of food high in antioxidant activity leads to a decrease in many chronic diseases (Polidori, et al., 2002). The rat trials in this study were undertaken firstly to develop an *in vivo* system to assess antioxidant activity by biomarker analysis, and secondly to investigate the mechanism of antioxidant activity of berry fruit anthocyanins. Combinations of the natural rat diet, and a synthetic diet with either fish oil or soybean oil were used in this study. Berry fruit anthocyanins were used in this study as they have reported antioxidant function as well as anti-inflammatory and anti-carcinogenesis activities (Hou, D.X., 2003).

In this study berry fruit antioxidants in the natural diet and at higher levels in a synthetic diet with added soybean oil, were effective in reducing the plasma protein carbonyl concentration, a biomarker of oxidative damage to proteins. Berry fruit anthocyanins were not effective at reducing the concentrations of lipid and DNA biomarkers (MDA and 8OHdG respectively) when consumed in a diet designed to induce mild oxidative damage (synthetic diet with added fish oil). This suggested that the extent of induced oxidative damage differed between the diets, and therefore the anthocyanins were not effective in reducing different types of oxidative damage. Taken together the results suggested that baseline oxidative damage may be caused by different mechanisms and may be fundamentally different depending on environmental conditions such as diet and that any given antioxidant will have differential efficacy in preventing oxidative damage.

The antioxidant capacity of the anthocyanins, and the structure in which they are absorbed may have relied on synergystic interactions with other antioxidants, such as vitamin E, which was depleted in the diets. Therefore with the various mechanisms

which oxidative damage is induced, and the varying concentrations of endogenous antioxidants, there are many possibilities as to why the anthocyanins were not effective at decreasing oxidative damage to lipids and DNA.

7.2 Future Plans

The free radical theory of ageing suggests that age-related changes occur as a result of the body's inability to cope with the oxidative damage that occurs throughout life (Van Hoorn, et al., 2003). If this is the case, it becomes very important to determine if these changes could be prevented or impeded by antioxidants, especially dietary antioxidants contained in fruits and vegetables (Joseph, et al., 1999).

The increasing average age of the population means that deterioration of mental and physical performance is increasingly seen as a major health issue. It is therefore important to develop methods to adequately assess oxidative damage for the purpose of determining the efficacy of antioxidants, and in the longer term, the efficacy of these antioxidants in reducing age-related degenerative diseases in the ageing population.

Future studies will continue to assess the impact of dietary antioxidants on the biomarkers of oxidative damage to predict an increased or reduced risk of disease, or an improved state of health, which may indirectly lead to a decreased risk of disease (Figure 7.1). To assess the effect of dietary antioxidants on oxidative damage, the concentration and the molecular structure of the absorbed antioxidant should be determined in conjunction with both the biomarkers of oxidative damage such as those used in this study. In addition, the markers of biological response may be assessed, such as changes in the activity of superoxide dismutase, as a marker of response to DNA damage.

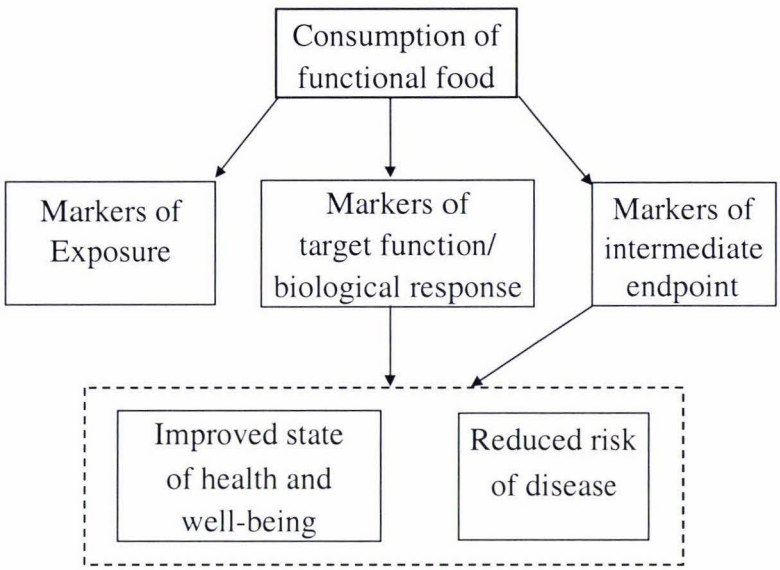


Figure 7.1: *Markers for development of methodology to assess the efficacy dietary antioxidants (based on Gibson and Williams (2000)).*

7.2.1 Biomarker Assays

In terms of biomarker analyses, the main questions which still need answering are: 1) is a decrease in oxidative damage due to antioxidants increasing enzyme activity (such as superoxide dismutase) **or** by the antioxidant directly scavenging up free radicals; and 2) how does this oxidative damage relate to alterations in gene expression and development of age-related diseases? Therefore the main requirement for future studies should be to identify targets for antioxidant protection to enable a better understanding of both the mechanisms of antioxidant action, and of oxidative stress using specific and selective measures for oxidative damage.

In addition to biomarkers of oxidised DNA, protein and lipid, measurement of endogenous antioxidant levels may provide a direct indication of oxidative stress. The application of biomarkers of oxidative damage and sensitive methods for analysing phenolic metabolites in plasma and urine are needed to fully evaluate their role in human health and diseases aswell. A recent study by Polidori, et al. (2002) used the direct approach of measuring vitamins and demonstrated that lipid-soluble antioxidants were lower in plasma from congestive heart failure patients. MDA in plasma was also

significantly higher in congestive heart failure patients compared to controls and positively correlated with severity in the disease patients.

7.2.1.1 Oxidised DNA

The measurement of oxidative damage to DNA is a fundamental method for determining oxidative damage *in vivo*, although the methodology is challenging. As discussed by Collins, et al. (1997), there is an urgent need for optimisation of assay conditions, validation of methods between laboratories and comparison of results gained from different methods. In addition to the alterations to the methodology used in this study, described below, it may be possible to use different methods to determine oxidative damage to DNA. This may include extracting the DNA from lymphocytes followed by analysis of 8OHdG, or analysis of the action of the enzyme whose action results in 8OHdG excretion.

Methodology

Further investigation into the pH-dependence of 8OHdG tautomeric forms may reveal the optimum pH for 8OHdG resolution and separation by HPLC. This may be achieved by preparing standards in various pH buffers and analysing by HPLC with mass spectrophotometric detection (LC-MS). The pH of the HPLC system currently used should mean that the 8OHdG is in only one tautomeric form. In addition, the synthesis of 8-oxo-guanine (Lee, et al., 2002) not available commercially, followed by analysis using LC-MS may reveal the 8OHdG in this study is in fact, 8-oxo-guanine and this may be due to the pH of the analytical system.

The method used in this study to measure 8OHdG had low levels of variation in the recovery of 8OHdG, which provides scope for additional refining of the method to increase the sensitivity and resolution further. The HPLC solvent programme could be shortened in order to decrease the run-time and increase the number of samples to be run per day. In the current solvent programme the 8OHdG is eluted from column B at a solvent composition of approximately 50% A and 50% B. The solvent programme may be modified so as to shorten the programme, but maintain the peak separation. Solvent B must still be maintained at 95% for five minutes to wash the column at the end of each

run. A possible change to the solvent programme is shown in table 5.1. This change will make the run time 30 minutes, as opposed to the current 40 minute run time. This was not implemented during the current studies, so the analysis of 8OHdG was the same for each rat feeding experiment.

Time	*% A*	*% B*
0	100	0
8 (13)	100	0
20 (30)	5	95
25 (35)	5	95
30 (40)	100	0

Table 7.1: *Suggested alterations to solvent gradient used by Jasco pump for column B in the assay for urinary 8OHdG using a dual column, switching valve method using RP-HPLC with CoulArray® detection. A = 5% MeOH in 100 mM sodium acetate and B = 20% MeOH in A. Current programme times are shown in brackets.*

To increase separation of 8OHdG off the first column, so a narrower peak is eluted, a binary solvent system may be implemented for the first column, instead of the single pump isocratic system currently used in this study. This may improve the resolution of 8OHdG from column A therefore fewer interfering compounds may be captured in the loop and transferred to column B. To further increase selectivity, a smaller loop may then be installed in the switching valve. This would also capture the 8OHdG in the urine samples eluted off column A, with less interfering compounds transferred to column B (Figure 7.2), but would require a more stable HPLC system with the main issue being more rigorous temperature control.

Inclusion of an internal standard would also improve the quantification of 8OHdG. There is little literature on internal standards for use in 8OHdG analysis in urine, however, in Ravanat, et al. (1998) 2,6-diamino-8-oxopurine was used as an internal standard for 8OHdG quantification in nuclear DNA isolated from rat organs, with HPLC and electrochemical detection. 2,6-diamino-8-oxopurine is electrochemically active and has similar properties to 8OHdG therefore it is likely this compound may be used as an

internal standard for 8OHdG quantification in urine. Investigation will be required to determine whether the elution time is the same as that of 8OHdG, so the internal standard is captured upon switching. An alternative to this internal standard may be the use of deuterium or ^{13}C-labelled 8OHdG and quantification by LC-MS. The isotopically different 8OHdG will elute at the same retention time as unlabelled 8OHdG and will provide a means to assess whether all the 8OHdG has been captured in the loop of the switching valve.

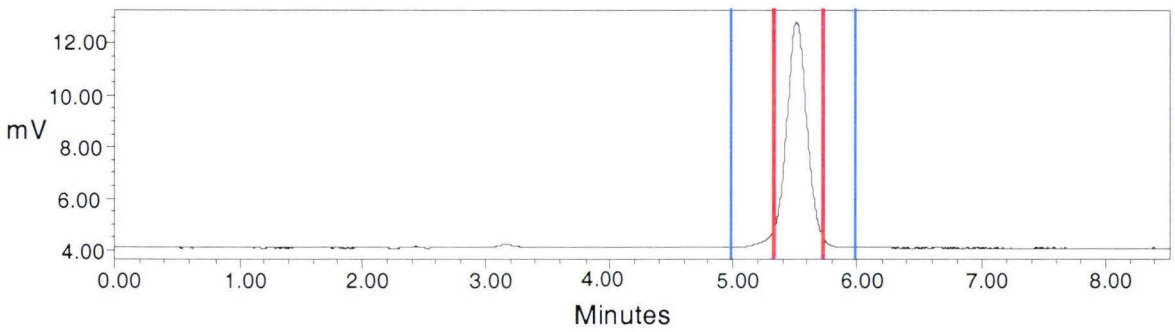


Figure 7.2: *Chromatogram for 5 μgml^{-1} 8OHdG standard from column A showing switching valve times currently used (blue), and proposed switching times (red) to increase selectivity of 8OHdG, resulting in less interfering compounds in the urine being loaded onto column B.*

7.2.1.2 Additional Biomarker Analyses

Investigation of other biomarkers for oxidatively damaged DNA, lipids and proteins may reveal different pathways of oxidative stress resulting from oxidative damage to these biomacromolecules. Other assays to support the results from the assays developed in this study may include those discussed below.

1) Analysis of the DNA repair enzyme whose activity forms 8OHdG to assess how much 8OHdG is formed from free radical attack, and how much may be present in the nucleotide pool that is from dead cells (Cooke, et al., 2000). When analysing methods involving repair enzymes, the efficiency of the enzyme and pathway(s) of repair will need to be considered. This may allow the identification of more than one enzyme involved in 8OHdG formation. A more direct method may be to measure DNA repair

activity, for example, ^{3}H-thymine incorporation into newly synthesised cellular DNA, and comparison with cell division.

2) Low density lipoprotein (LDL) peroxidation analysis will provide a further indication of lipid peroxidation which will be different than the indication determined by the MDA assay. This measure however, involves copper ions and is an *ex vivo* study of protection of LDL peroxidation (Wiseman, et al., 2002). Following density gradient centrifugation of plasma to isolate LDL particles, LDL peroxidation analysis assesses the incorporation of a fluorescent probe, which sensitively detects oxidant reactions with LDL particles, into LDL particles and its inhibition by antioxidants (Serafini, et al., 2000). There is evidence that anthocyanins inhibit LDL oxidation (Sanchez-Moreno, et al., 2000), therefore this analysis may provide a better measure than MDA of anthocyanin-induced decreases in lipid peroxidation. Corresponding vitamin E analysis of the LDL particles may also provide some insight into the possible synergystic actions of vitamin E and the phenolics, as a previous study (Wiseman et al., 2002) showed that phenolics in olive oil spared vitamin E and protected LDLs from oxidation.

3) In terms of the oxidised protein assay, identifying the pathways that oxidise human proteins would greatly facilitate epidemiological studies and antioxidant trials. This could be done by western blot analysis of two-dimensional proteomic gels where the proteins have been reacted with 2'-4'-dinitrophenylhydrazine. Antibodies to the DNPH could then be used to identify which proteins have been oxidised. Without this knowledge clinical trials might focus on unaffected proteins, or irrelevant antioxidants, that is, antioxidants that do not target the pathway of oxidative damage to proteins. Mass spectrometry could also be utilised in the proteomic studies to identify the oxidised proteins.

7.2.2 Future Feeding Trials

7.2.2.1 Animal Trials

The experiments performed in this study have provided an initial basis for investigation

of biomarkers of oxidative stress and their relationship with fruit-derived antioxidants. The different diets seemed to have confused matters and to overcome this, more complete understanding to the antioxidant and oxidant compositions of the diets is needed to help explain the results. When this is established, future studies may extend the period in which the diets are supplemented, which may lead to further antioxidative effects produced from the fruit extracts.

The advantages of working with rat are that the rats grow quickly and their diets are easily manipulated. There are however disadvantages associated with using rats. These include the age of the rats, their environment, which has a controlled, unpolluted atmosphere, dissimilar to the human environment. In particular, the fact the rats synthesise endogenous vitamin C, where humans must utilise dietary vitamin C, may also be an important factor when interpreting and relating results to humans. The only rodents that do not synthesis vitamin C are gerbils. These could be used as a mammalian model in future studies, although they are not available in New Zealand.

Mixed results were shown by short-term intervention studies (Moller and Loft, 2002) possibly because the effects of the antioxidant may be too short-lived to be detected (by chance) in one blood sample taken during intervention. Thus, in future trials, as well as assessing 8OHdG at two-week intervals throughout the trial, plasma samples may need to be obtained from venipuncture of the rat tail vein to monitor lipid peroxidation and protein oxidation throughout an eight-week trial. The disadvantages of venipuncture are: it is time consuming; restraints used are expensive; and for repeated sampling, if the vein collapses early into the sampling, it makes future sampling more difficult.

7.2.2.2 Human Trials

When the efficacy of berry fruit antioxidants to decrease oxidative damage has been substantiated from rat experimental systems, human clinical studies may be initiated to validate anti-ageing effects in humans and provide evidence for product claims. These studies will assess the *in vivo* antioxidant activity of berry fruit extracts in a selected human population. Antioxidant efficacy will be assessed, as for the rat trials, by measuring biomarkers of oxidatively damaged protein, DNA and lipid. In addition to

biomarkers of oxidative damage, cognitive function tests may also be performed to determine whether the results may be related to the diet or may due to lowered age-related cognitive function.

7.2.3 Mechanistic Studies

7.2.3.1 Mechanisms of Oxidative Stress

Reversible and irreversible oxidative changes that occur *in vivo* during oxidative stress, such as antioxidant and free radical levels, fluctuate depending on the *in vivo* oxidative stress at that point in time. While the concentration of a specific antioxidant, such as vitamin E, may be relatively straightforward to measure, the correct antioxidant corresponding to the most active oxidative stress pathway must be measured. In measuring the concentration of free radical, the same argument applies, as the question arises whether the free radical being quantified is the most damaging one present in the organism being studied.

Investigating the mechanisms of oxidative stress, that cause oxidative damage will allow clarity as to why anthocyanins were ineffective at reducing oxidative damage to DNA and lipids when associated with fish oil induced oxidative stress. This will also allow prediction of which biomacromolecule(s) will be preferentially oxidatively damaged. To date, there have been no studies into the mechanisms of oxidative damage due to the many possible pathways involved and the highly reactive state of free radicals makes it difficult to monitor their presence to reflect the level of oxidative stress.

7.2.3.2 Mechanisms of Anthocyanin Antioxidant Action

With total berry fruit extracts it is possible that dynamic interrelationships exist between anthocyanins and other phenolic compounds contained in the extracts. Attributes related to the mechanisms of anthocyanin action are: the *in vivo* concentration and structure of the absorbed anthocyanin and its transport through the body; interactions with other

antioxidants; and the absorption, distribution, retention and metabolism characteristics of the anthocyanins. An important factor to consider is the fate of antioxidant-derived radical. When the anthocyanin reacts with a free radical, it becomes a radical which is relatively stable due to delocalisation of the charge through the ring structure. It is not known what happens to the anthocyanin radical, for example, whether it is recycled like α-tocopherol, or whether it is removed from the body by as yet undetermined mechanism.

The synergy between anthocyanins and endogenous antioxidants may be affected by the dose at which the anthocyanin is administered, and the presence of other antioxidants in the extract. Optimum synergistic relationships may be achieved by increasing the anthocyanin concentration relative to other phenolic compounds, for example. To test these synergistic and possible dose-dependent relationships, cell-based studies may be utilised with purified components of berry fruit. Although cell studies do not directly relate to *in vivo* interactions, they may provide a preliminary indication as to whether interactions occur between anthocyanins and other antioxidants. It is possible that the total phenolic content determines the antioxidant capacity due to synergistic relationships between anthocyanins and other phenolic compounds. Further investigation and functional studies are required to confirm this hypothesis.

Investigating the mechanism of bioabsorption and metabolism of anthocyanins is also important when determining the duration of trials. Feeding trials (Moller and Loft, 2002) showed a single dose of vitamin C vanished within a few hours, but the protective effects of carotenoids and vitamin E appeared later, possibly due to differences in bioavailability, which, in turn determines the antioxidative mechanism. Whether the results for carotenoids can be extrapolated to anthocyanins may be determined by increased sampling and extended duration of rat trials.

Anthocyanin Chemistry

Investigation of the structure of anthocyanins in terms of their antioxidant capacity and possible interactions may provide a link to the mechanism of action. The structure of anthocyanins, with a positive charge on an oxygen atom, may not be the form in which the anthocyanins are absorbed. Experiments performed at various pH's will ascertain in

what form the anthocyanin is absorbed and whether antioxidant activity is retained, since the pH of the stomach is different from that in the blood.

Another mechanism of anthocyanin antioxidant action is the chelating of various metal ions. Interactions of 4 structurally-related flavonoids with Cu^{2+} ions were investigated by Brown, et al. (1998). The extent to which they undergo complex formation through chelation or modification through oxidation, as well as their structural dependence was studied. The ortho-3',4'-dihydroxy substitution in the B ring is shown to be important for Cu^{2+} chelate formation, thereby influencing the antioxidant activity. Results of this experiment (Brown, et al., 1998) demonstrated that the structural properties of the flavonoids influenced their reactivities with respect to protecting LDL against Cu^{2+} ion-induced oxidation. These properties are dependent on whether chelation or oxidation by Cu^{2+} ions occurs, and their ability to partition between the aqueous compartment and the lipophilic environment within the LDL particle and their hydrogen-donating antioxidant properties.

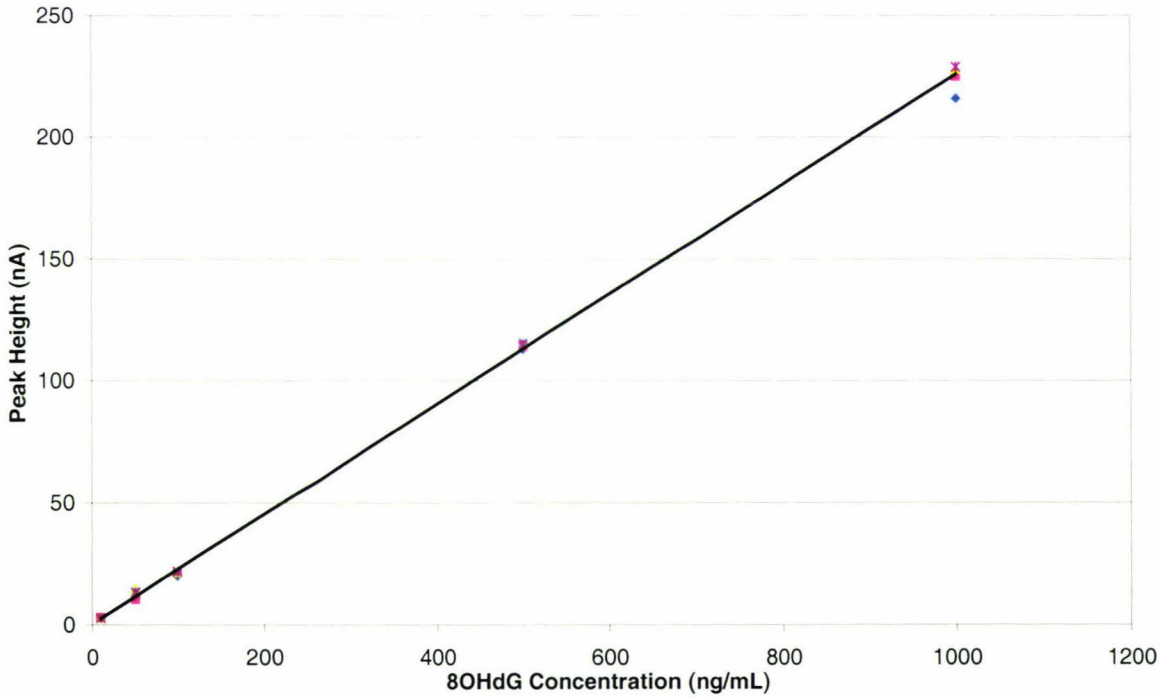
Experiments by Noroozi, et al. (1998) showed a protective effect of vitamin C and quercetin that was additive and also found evidence that free flavonoids are more protective than conjugated flavonoids (for example, quercetin compared with quercetin-3-glucoside). The antioxidant capacity of anthocyanins has been shown to be dependent on the degree of hydroxylation (Deighton, et al., 2002). In addition, glycosylation at the 3-position generally increases the antioxidant capacity (Deighton, et al., 2002). Therefore, ring substituents may have an effect on the antioxidant potency of anthocyanins and should be considered when looking at the mechanism of action. Various ring substituents may increase the reactivity toward the peroxyl radical, affect antioxidant stability, control dimerization and chemical reactivity of the anthocyanins as antioxidants.

Determination of both the actively absorbed structure of anthocyanins and the mechanisms of oxidative stress which anthocyanins target will determine which biomarker measurement is most relevant, that is, which biomarker measurement is likely to give the greatest change upon dietary supplementation with anthocyanins in future feeding trials.

Appendices

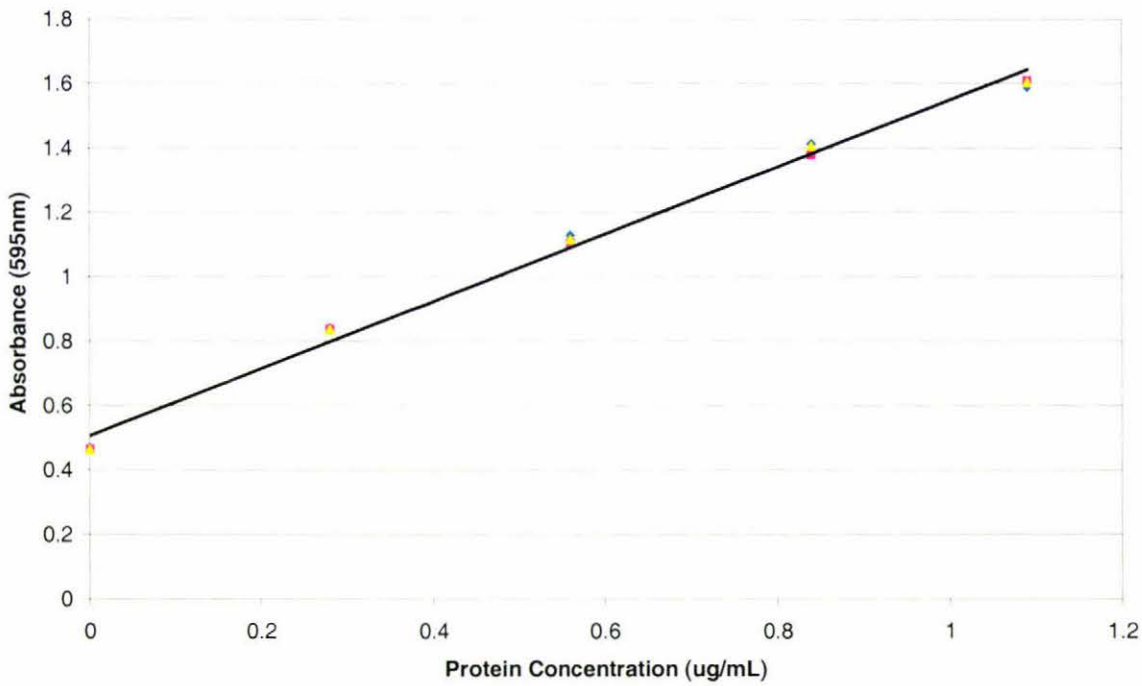
Appendix One: Calibration Curve for 8OHdG analysis

A set of standards (1000, 500, 100, 50 and 10 $ngml^{-1}$ 8OHdG) was run each day for five days and the peak height at an oxidation potential of 290 mV plotted with a correlation co-efficient of 0.9998. As the relationship between peak height at 290 mV and 8OHdG concentration was linear, the height of a one-point standard was used to quantify 8OHdG all samples. The variation on this plot represents the changes incurred by temperature fluctuations through one day and between days, which may cause the 8OHdG elution time to shift. Therefore the compound will not be fully captured upon switching of the 2-way valve, giving a lower than expected peak height.



Appendix Two: Calibration Curve for Determination of Protein Content (Bradford Method)

The calibration curve for the protein concentration assay (Bradford method) is shown. The absorbance of bovine serum albumin (BSA) at 595 nm was related to the concentration of the standards analysed in triplicate. A linear relationship (correlation co-efficient = 0.9867) was established between BSA concentration and absorbance, and used to calculate the protein concentration of plasma prior to the analysis of oxidised protein by the colorimetric carbonyl assay.



Appendix Three: Example of Raw Data and Data Manipulation for the 8OHdG Assay

Values used to calculate the mean concentration of 8OHdG and the associated statistics for trial #2 are shown. Values in red were excluded from calculations, as they were outliers because they were at least two times more or less than any other values.

The concentration (μgmL^{-1} in 200 µl) was derived from comparison of the peak height of the sample to that of a 1 μgml^{-1} 8OHdG standard. This result was used to calculate the amount of 8OHdG (µg) then the concentration per ml of urine.

Rat #	*Diet*	*Sample volume*	*μgml^{-1} in 200uL*	*µg*	*μgml^{-1}*	*Conc $ngml^{-1}$*
7	A	1	0.0350	0.0070	0.0070	6.992
10	A	1	0.0232	0.0046	0.0046	4.640
18	A	1	0.2131	0.0426	0.0426	42.628
23	A	1	0.0899	0.0180	0.0180	17.979
24	A	1	0.0716	0.0143	0.0143	14.326
6	B	0.8	0.3887	0.0777	0.0777	77.748
12	B	1	0.5479	0.1096	0.1096	109.571
13	B	1	0.1803	0.0361	0.0361	36.061
15	B	1	0.0013	0.0003	0.0003	0.254
21	B	1	0.3067	0.0613	0.0613	61.347
9	C	1	0.0354	0.0071	0.0071	7.070
14	C	1	0.6886	0.1377	0.1377	137.716
16	C	1	0.0171	0.0034	0.0034	3.425
22	C	1	0.1168	0.0234	0.0234	23.350
25	C	1	0.5382	0.1076	0.1076	107.636
8	D	1	0.0162	0.0032	0.0032	3.233
11	D	1	0.0214	0.0043	0.0043	4.270
17	D	1	0.0299	0.0060	0.0060	5.974
19	D	1	0.0269	0.0054	0.0054	5.383
20	D	1	0.6342	0.1268	0.1268	126.846
1	E	1	0.0168	0.0034	0.0034	3.359
2	E	1	0.0275	0.0055	0.0055	5.501
3	E	1	0.0211	0.0042	0.0042	4.213
4	E	1	0.0070	0.0014	0.0014	1.404
5	E	1	0.0250	0.0050	0.0050	5.000

Raw data for 8OHdG from the second berry fruit feeding trial. Diet types: A = 2% boysenberry; B = 2% Activin; C = 10% boysenberry; D = 10% fish oil control; E = 10 % soybean oil control.

Appendix Four: Example of Raw Data and Data Manipulation for the Colorimetric Protein Carbonyl Assay

Values used to calculate the mean protein carbonyl concentration and the associated statistics for feeding trial #2.

The net concentration of DNPH in 1 ml takes into account the blank value and the fact only 100 µl of plasma was used. The carbonyl concentration uses the extinction coefficient for the reacted DNPH. Values in red were excluded from calculations, as they were outliers because they were at least two times more or less than any other values.

Sample	*Diet*	*A370 DNPH*	*A370 HCl (Blank)*	*DNPH nmolml^{-1}*	*Net conc DNPH in 1 ml*	*Protein conc mgml^{-1}*	*Carbonyl conc nmolmg^{-1} protein*
7	A	0.0809	0.0202	2.7588	13.7941	51.8432	0.2661
10	A	0.0692	0.0223	2.1316	10.6580	47.1532	0.2260
18	A	0.0677	0.0186	2.2316	11.1580	48.7482	0.2289
23	A	0.0579	0.0159	1.9089	9.5445	47.6482	0.2003
24	A	0.0943	0.0122	3.7314	18.6572	49.0582	0.3803
6	B	0.0671	0.0220	2.0498	10.2490	53.4832	0.1916
12	B	0.0691	0.0632	0.2682	1.3408	45.0332	0.0298
13	B	0.0529	0.0131	1.8089	9.0446	49.2932	0.1835
15	B	0.0645	0.0293	1.5998	7.9992	52.6582	0.1519
21	B	0.0874	0.0167	3.2133	16.0666	50.6582	0.3172
9	C	0.0895	0.0374	2.3679	11.8397	48.8032	0.2426
14	C	0.0941	0.0162	3.5406	17.7028	51.6282	0.3429
16	C	0.0954	0.0143	3.6860	18.4300	51.6882	0.3566
22	C	0.0883	0.0193	3.1361	15.6803	50.5232	0.3104
25	C	0.0940	0.0284	2.9815	14.9076	51.8332	0.2876
8	D	0.0732	0.0323	1.8589	9.2945	52.4832	0.1771
11	D	0.0763	0.0158	2.7497	13.7486	50.5932	0.2717
17	D	0.0600	0.0215	1.7498	8.7491	60.1982	0.1453
19	D	0.0929	0.0451	2.1725	10.8626	59.2182	0.1834
20	D	0.0942	0.0214	3.3088	16.5438	50.1782	0.3297
1	E	0.0617	0.0231	1.7544	8.7719	51.5282	0.1702
2	E	0.0510	0.0189	1.4589	7.2947	52.6482	0.1386
3	E	0.0529	0.0143	1.7544	8.7719	50.0032	0.1754
4	E	0.0488	0.0131	1.6226	8.1128	48.5282	0.1672
5	E	0.0753	0.0445	1.3999	6.9993	47.2332	0.1482

Raw data for colorimetric oxidised protein assay from the second berry fruit feeding trial. Diet types: A = 2% boysenberry; B = 2% Activin; C = 10% boysenberry; D = 10% fish oil control; E = 10 % soybean oil control.

Appendix Five: Example of Raw Data and Data Manipulation for the ELISA Protein Carbonyl Assay

Absorbance values for triplicate analyses (abs 1, 2, 3) of plasma from berry fruit feeding trial #2. The carbonyl concentration of the samples was calculated by reference of the mean value of the triplicate to a five-point standard curve.

Sample	*Diet*	*Abs 1*	*Abs 2*	*Abs 3*	*Av Abs*	*Conc nmolmg^{-1}*
7	A	0.586	0.629	0.5	0.5717	0.2897
10	A	0.865	0.581	0.524	0.6567	0.3570
18	A	0.718	0.758	0.813	0.7630	0.4412
23	A	0.851	0.632	0.783	0.7553	0.4351
24	A	0.941	0.849	0.616	0.8020	0.4721
6	B	0.717	0.784	0.782	0.7610	0.4396
12	B	0.693	0.711	0.331	0.5783	0.2949
13	B	0.48	0.541	0.403	0.4747	0.2128
15	B	0.791	0.742	0.44	0.6577	0.3578
21	B	0.744	0.792	0.466	0.6673	0.3654
9	C	0.924	0.678	0.851	0.8177	0.4845
14	C	0.743	0.81	0.323	0.6253	0.3322
16	C	0.877	0.902	0.796	0.8583	0.5167
22	C	0.85	0.857	0.819	0.8420	0.5038
25	C	0.675	0.572	0.6	0.6157	0.3245
8	D	0.633	0.601	0.524	0.5860	0.3010
11	D	0.313	0.636	0.451	0.4667	0.2065
17	D	0.625	0.922	0.846	0.7977	0.4687
19	D	0.662	0.682	0.463	0.6023	0.3139
20	D	1.025	0.972	0.729	0.9087	0.5566
1	E	0.735	0.64	0.655	0.6767	0.3728
2	E	0.705	0.606	0.468	0.5930	0.3066
3	E	0.54	0.591	0.705	0.6120	0.3216
4	E	0.505	0.641	0.542	0.5627	0.2825
5	E	0.541	0.488	0.46	0.4963	0.2300

Raw data for ELISA oxidised protein assay for trial #2. Diet types: A = 2% boysenberry; B = 2% Activin; C = 10% boysenberry; D = 10% fish oil control; E = 10 % soybean oil control.

Appendix Six: Example of Raw Data and Data Manipulation for the MDA Assay

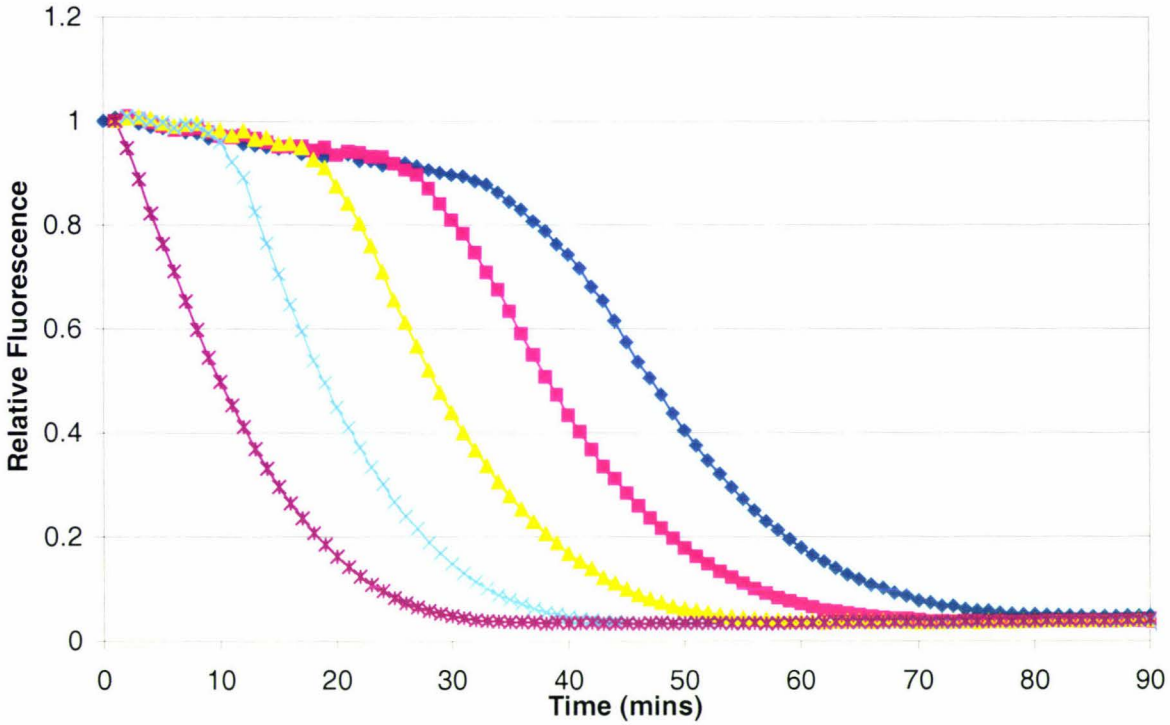
Raw data for the MDA assay from trail #2. The MDA concentration was determined using GC-MS software which created a seven point standard curve and related the peak area of the standard to the peak area of the phenylhydrazine-malondialdehyde derivative in the sample.

Rat #	*Diet*	*MDA ngml^{-1}*
7	A	169.94
10	A	106.77
18	A	135.07
23	A	115.18
24	A	127.70
6	B	135.04
12	B	148.62
13	B	72.18
15	B	102.30
21	B	116.30
9	C	78.83
14	C	99.41
16	C	156.80
22	C	223.22
25	C	142.24
8	D	75.33
11	D	74.67
17	D	117.41
19	D	208.25
20	D	112.31
1	E	21.52
2	E	11.45
3	E	16.00
4	E	20.90
5	E	55.12

Raw data for the MDA assay for feeding trial #2. Diet types: A = 2% boysenberry; B = 2% Activin; C = 10% boysenberry; D = 10% fish oil control; E = 10 % soybean oil control.

Appendix Seven: Raw Data and Data Manipulation for the ORAC Assay

A fluorescence decay curve for trolox curve, which is similar for the ORAC analysis for each of the feeding trials is shown. The area under the curve for each standard is plotted on a calibration curve in order to calculate the ORAC values, in trolox equivalents for each plasma sample.



Fluorescence decay curve for trolox. Relative fluorescence over time for trolox standard concentrations: 200 μM (dark blue), 150 μM (pink), 100 μM (yellow), 50 μM (cyan) and 0 μM (purple). The area under each curve from this chart relative to the standard concentration is used to form the standard curve for the determination of the ORAC value, in trolox equivalent, μM, for the samples.

ORAC values for samples for the second berry fruit feeding trial are shown. The trolox equivalent (TE) was calculated by relating the relative area under the curve of the sample to a standard curve. The actual TE took into account the dilution factor. The results were then expressed as mM.

Rat #	*Diet*	*Dilution Factor*	*TE μM*	*Actual TE μM*	*mM*
7	A	125	124.85	15605.73	15.61
10	A	125	131.71	16463.71	16.46
18	A	125	138.04	17254.75	17.25
23	A	125	114.64	14330.06	14.33
24	A	125	106.78	13347.26	13.35
6	B	125	119.03	14879.10	14.88
12	B	125	113.11	14139.15	14.14
13	B	125	99.58	12446.93	12.45
15	B	125	124.18	15522.55	15.52
21	B	125	108.45	13555.64	13.56
9	C	125	127.03	15878.48	15.88
14	C	125	111.75	13969.01	13.97
16	C	125	100.71	12588.85	12.59
22	C	125	114.42	14301.97	14.30
25	C	125	124.97	15621.87	15.62
8	D	125	113.81	14226.18	14.23
11	D	125	120.06	15007.16	15.01
17	D	125	122.67	15333.34	15.33
19	D	125	139.93	17490.89	17.49
20	D	125	129.64	16204.94	16.20
1	E	125	142.37	17796.69	17.80
2	E	125	147.00	18374.38	18.37
3	E	125	129.90	16237.72	16.24
4	E	125	115.13	14390.89	14.39
5	E	125	118.66	14832.78	14.83

Raw data for the ORAC assay from feeding trial #2. Diet types: A = 2% boysenberry; B = 2% Activin; C = 10% boysenberry; D = 10% fish oil control; E = 10 % soybean oil control.

Appendix Eight: α-tocopherol Analysis Raw Data and Data Manipulation

The actual α-tocopherol concentration, was derived from the concentration in 200 µl, which was the volume of MeOH the sample was redissolved in following concentration to dryness. This value was multiplied by 2.5 to give the concentration in the 500 µl plasma sample analysed. Values in red were excluded from calculations, as they were outliers because they were at least two times more or less than any other values.

Rat Number	*Diet*	*alpha-toc conc(ugml^{-1}) in 200 uL*	*Actual a-t conc ugml^{-1}*
7	A	1.185	0.474
10	A	0.648	0.2592
18	A	2.381	0.9524
23	A	3.838	1.5352
24	A	4.603	1.8412
6	B	1.644	0.6576
12	B	0.706	0.2824
13	B	0	0.0000
15	B	3.079	1.2316
21	B	3.918	1.5672
9	C	3.813	1.5252
14	C	1.816	0.7264
16	C	2.936	1.1744
22	C	4.413	1.7652
25	C	3.122	1.2488
8	D	3.646	1.4584
11	D	4.497	1.7988
17	D	1.492	0.5968
19	D	0.571	0.2284
20	D	4.264	1.7056
1	E	9.444	3.7776
2	E	7.676	3.0704
3	E	7.447	2.9788
4	E	8.43	3.372
5	E	3.237	1.2948

Raw data for the vitamin E assay from feeding trial #2. Diet types: A = 2% boysenberry; B = 2% Activin; C = 10% boysenberry; D = 10% fish oil control; E = 10 % soybean oil control.

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