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Structure-Function Analysis, and Food Product Analysis

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A thesis submitted to the faculty of
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Master of Science

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ABSTRACT

Antioxidants in Cancer Research and Prevention: Assay Comparison, Structure-Function Analysis, and Food Product Analysis

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Recent epidemiological studies have suggested that the development and progression of several chronic diseases may be initiated or augmented by oxidative stress. Reactive oxygen species and reactive nitrogen species react readily with and can damage nucleic acids, proteins, and lipids. While biological systems are equipped antioxidant defenses to cope with oxidative stress, oxidative damage may still occur when oxidative stress overwhelms antioxidant defenses. This damage, if left unchecked, may lead to a variety of degenerative diseases, including heart disease, Alzheimer's Disease, Parkinson's Disease and cancer.

Several assays have been designed to describe the antioxidant activity of various phytochemicals, vitamins, and other compounds. The ORAC and TOSC assays have emerged as industry standards for measuring antioxidant activity due to their high reliability and sensitivity. Until recently, however, little has been done to assess the relative correlation between these two assays. Furthermore, no assay has been developed to measure changes in antioxidant activities of cells in response to oxidative stress. The current work investigates the correlation between measured antioxidant activities of samples in the both the ORAC and TOSC assays.

Recent antioxidant research also focuses on relating chemical structure to antioxidant activity. Previous research in this area has included a broad range of chemical groups, but no study has attempted to formulate a structure-function framework that has applicability to compounds of any group. The current work uses amino acids as a simplest-case model for studying the relationships between chemical structure and antioxidant activity.

One particular area of emerging research has centered around comparing organic and conventionally grown food products. The impetus of these investigations lies in claims made by organic supporting groups that these food products are generally more beneficial than their conventional counterparts. Despite the rapid rise in popularity of organic foods, there remains a dearth of research investigating these claims. The current work compares the antioxidant activities of organic and conventionally grown blueberries and apples.

Key Words: Antioxidants, cancer, prevention, ORAC, TOSC, amino acid, structure-function, blueberries, apples, organic.

ACKNOWLEDGEMENTS

"Verily, thus saith the Lord: It shall come to pass that every soul who forsaketh his sins

and cometh unto me, and calleth on my name, and obeyeth my voice, and

keepeth my commandments, shall see my face and know that I am;

And that I am the true light that lighteth every man that cometh into the world"

Doctrine and Covenants 93:1-2

This work is dedicated

to my father, whose example teaches me more about life than I could ever learn from biology;

to my children, present and future, who remind me why life is lived;

and to my wife Suzana, who makes life beautiful.

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INTRODUCTION

Chemical Stress and Cancer

Recent epidemiological studies have suggested that the development and progression of several chronic diseases may be initiated or augmented by oxidative stress. Much of this oxidative stress is generated as a by-product of fundamental metabolic processes that occur constantly in the body. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) react readily with biological substances and can destroy or damage nucleic acids, proteins, and lipids. While many of these biological systems are equipped to cope with oxidative stress, oxidative damage may still occur when oxidative stress overwhelms antioxidant defenses. This damage, if left unchecked, may lead to a variety of degenerative diseases, including heart disease, Alzheimer's Disease, Parkinson's Disease and cancer (1-5).

Cancer is a leading cause of death in the world. In 2004, over 7.4 million people died of cancer, accounting for more than 13% of all deaths worldwide (6). In 2010, it is estimated that there will be over 1.5 million new cancer cases in the United States alone. Estimates for 2010 also project that close to 570,000 Americans will die from cancer, which translates into greater than 1,500 cancer deaths per day (7). It is also projected that in 2020, 15 million new cancer cases will be reported and 12 million cancer patients will die worldwide (8). Despite numerous outreach, education, and research efforts, cancer mortality rates continue to increase.

Notwithstanding its high mortality rate, cancer is largely a manageable condition if detected and treated early in its development. The World Health Organization estimates that up to one-third of all cancers could be cured if detected early and treated adequately (6). Furthermore, cancer is often a preventable condition. Recent estimates are that between 30% to 95% of all

cancer cases could be prevented by modifying diet, lifestyle, and behavioral habits alone (6, 7, 9). Cancer then, as has been insightfully observed, is a "preventable disease that requires major lifestyle changes" (9). For example, tobacco use is the single largest preventable cause of cancer in the world, yet millions use tobacco regularly; among the total projected cancer deaths in 2010, 171,000 (34%) are expected to be caused by tobacco use.

Evidence suggests that close to one-third of all projected cancer deaths in 2010 will be caused by obesity, physical inactivity, or poor nutrition (6, 7). These conditions can be especially detrimental because prolonged inflammation in obese patients is thought to be critical for tumor initiation and progression (10). Such conditions can be prevented through limiting consumption of energy-dense foods, avoiding sugary drinks and alcohol, limiting the frequent intake of red meats, and increasing consumption of foods of plant origin (11). Many cancers caused by infectious agents can be prevented through the use of vaccines and antimicrobials, as well as lifestyle changes.

Many types of skin cancers can be prevented by avoiding excessive exposure to UV radiation. This can be accomplished by the implementation of sunscreen, the proper use of hats and clothing, and by the use of indoor tanning equipment. The American Cancer Society also recommends regular screenings for many different types of cancer, and reports that at least half of all new cancer cases could be prevented if these conditions were detected early while still in their pre-cancerous states (7). It is clear that for improvements in cancer prevention to occur, significant lifestyle and behavioral changes must be made both in the United States and in the world at large to reduce the oxidative stress that can lead to cancer.

Types of Oxidants

Radicals are unpaired valence electrons found in various types of biological and chemical molecules. These compounds can either have one extra electron (giving them a slight negative charge), or be one electron deficient (giving them a slight positive charge). Either way, most compounds that have unpaired radicals are highly reactive chemically.

There are two main classes of free-radical compounds: reactive oxygen species (ROS) and reactive nitrogen species (RNS; see Table 1). The more common of the two groups is ROS, which are derivatives of O_2, with superoxide radical ($O_2^{\bullet-}$) being the most common. Other examples of ROS include hydrogen peroxide (H_2O_2), alkoxyl/peroxyl radical ($RO^\bullet/ROO^\bullet$), and peroxynitrite ($ONOOH/ONOO^-$). Recently, ROS have been linked to a variety of chronic diseases, including Alzheimer's disease, Parkinson's disease, and cancer (2, 12). ROS have also been linked to p53 function (3), diabetes (13), neurodegenerative diseases (4) and cognitive decline (14).

Reactive nitrogen species are species of free radical compounds derived from nitric oxide ($NO^\bullet$), and include peroxynitrite ($ONOO^-$), nitrogen dioxide ($^\bullet NO_2$), and dinitrogen trioxide (N_2O_3). These and other RNS, similar to ROS, have been shown to cause damage to lipids, amino acids, nucleic acids, and other small molecules (15). In a recent review, the signaling, cytotoxic, and pathogenic characteristics of nitric oxide and peroxynitrite were discussed in detail (16). Nitric oxide is an important signaling molecule because of its unique chemical properties, which include its rapid cellular diffusion (its diffusion coefficient in water is slightly higher than oxygen and carbon dioxide), and its propensity to quickly produce oxygen radicals. Peroxynitrite is another well-known RNS formed by the reaction of hydrogen peroxide and nitric oxide.

Although peroxynitrite itself does not contain free radicals, it is a powerful oxidant, and its decomposition in the phagosome results in the formation of H_2O_2 and NO_2^- (16).

Table 1: Five Major Types of Free Radicals in Biological Systems	
Superoxide Ion	The superoxide anion is O_2^-. It is the product of the one electron reduction of molecular oxygen. Although used in the immune system to kill invading microorganisms, Superoxide ion can cause damage to mitochondria and other important cellular constituents.
Hydroxyl Radical	Hydroxyl Radical, OH, is the neutral form of the Hydroxide Ion. The Hydroxyl Radical is important in atmospheric reactions, and is also formed via the reduction of O_2 in the electron transport chain.
Singlet Oxygen	Singlet O_2 refers to the metastable states of molecular O_2 with higher energy than the ground state triplet oxygen. It is produced by light, as well as by the immune system. These are especially prone to cause damage to double bonds between carbon atoms in saturated fatty acids.
Hydrogen Peroxide	Hydrogen peroxide is an important reactive oxygen species because it is involved in the production of many other types of ROS. Cells contain catalases that decompose H_2O_2 to O_2 and H_2O.
Reactive Nitrogen Species	Nitric acid forms peroxynitrite by reacting with the superoxide ion. This in turn causes damage to many types of body tissues.

Similar to ROS, not all RNS are detrimental, as both are important components of the innate immune system. A study performed by Iovine et al. demonstrated that murine macrophages significantly upregulated RNS production following exposure to *Campylobacter jejuni,* and were much more effective at eliminating it when compared to mutant macrophages unable to produce RNS (17). An earlier study by Neu et al. also demonstrated that prolonged inhibition of nitric oxide synthesis in human umbilical vein epithelial cells supports neutrophil adhesion, although it also lead to an increase in intracellular oxidative stress in those cells (18).

Antioxidants in Cancer Research

While some antioxidants are generated by the body (Coenzyme Q10, Glutathione, and Uric Acid being the most prominent examples), many can only be obtained through food sources (19-21). Food sources which contain high vitamin and mineral content (including antioxidants) are called bioactive foods.

Bioactive foods may have the potential to decrease both the initiation and progression of cancer due to their rich antioxidant and nutrient contents, as previously discussed, and evidence is growing that may help define the role of bioactive foods in the prevention of cancer development and progression. Multiple studies have found correlations between plasma levels of bioactive food components (i.e. caroteniods, tocopherols, etc.) and the likelihood of survival of patients undergoing chemotherapy (22-24). Research has also shown a positive correlation between the amount of plant material consumed and a patient's chance of recovery. Two studies in particular followed the dietary intake of women with breast cancer as they went through treatment. These studies found an increased chance of survival in those women who had a high intake of plant-based foods and/or supplements containing bioactive food components when compared to those who consumed diets containing less of these components (22, 25).

Resveratrol

Resveratrol is a stilbene polyphenol that has recently garnered much attention for its anti-inflammatory, antitumorigenic and antioxidant properties (26, 27). Found in high concentrations in *Vitis* and *Vaccinium* fruits, studies showing that resveratrol extends lifespans in *S. cerevisiae, C. elegans, Drosophila*, and murine models (through SIR2 activation) has given support to reports that regular consumption of red wine may increase longevity in humans (26, 28, 29). The results from these studies may not be so easily applied to humans, however, due to resveratrol's

low potency and poor bioavailabilty after metabolism (30). Further research is needed to more fully elucidate the extent to which resveratrol may affect longevity and aging in humans.

In addition to its potential health-promoting effects, resveratrol may directly influence the development and progression of cancer. A recent report demonstrated that resveratrol-induced apoptosis in colorectal cancer cells is mediated by adaptive response gene ATF3 *in vitro*, supporting the idea that ATF3 may play an antitumorigenic role in colorectal tumorigenesis (31). Another recent report demonstrated the ability of resveratrol and resveratrol analogs to elicit blocks in the cell cycle of cultured human prostate cells, as well as the resulting increase in p53 and p21, providing further evidence of resveratrol's antitumorigenic abilities (30).

Carotenoids

Carotenoids, particularly lycopene, are known to be powerful antioxidants linked to oxidation-preventing mechanisms. In one case-control study, carotenoid plasma levels were measured to compare 118 non-Hispanic Caucasian men suffering from non-metastatic prostate cancer with 52 healthy men in southeast Texas. Results showed that the risk for men with high levels of α-carotene, trans-β-carotene, β-cryptoxanthin, and lutein and zeaxanthin in their plasma was less than half that of those with low levels of these compounds. No correlation was found between carotenoid plasma levels and the stage of aggressive disease in these patients. This study suggests that high plasma levels of carotenoids may help reduce prostate cancer development, but not its progression (32).

Vitamin D

Another well-known bioactive food component is vitamin D. Although vitamin D deficiency is mainly associated with bone-related diseases, interest has risen in vitamin D deficiency

as a risk factor for different cancer types, mainly colon, breast, ovarian and prostate cancer. A case-control study concluded that patients with plasma levels of 25(OH)D (the main form of circulating vitamin D and main marker for vitamin D deficiency) below 30 ng/ml had about twice the risk of developing colon cancer (33), while another revealed a doubling of colon cancer incidence for patients with less than 20ng/ml of vitamin D (34). The association of 25(OH)D levels in different stages of colon cancer was investigated, and results suggested that vitamin D metabolites may have protective effects in all the stages of colon carcinogenesis (35).

Low levels of vitamin D have also been associated with breast cancer risk. Studies suggested that women in the lowest quartile of serum 25(OH)D had a five times greater risk of breast cancer than those in the highest quartile (36), while other case studies suggested that low 25(OH)D plasma levels were associated with a more rapid progression of metastatic breast cancer (37).

In a study including 19,000 men with prostate cancer, those with 25(OH)D levels below 16 ng/ml had a 70% higher incidence rate of prostate cancer than those with higher levels of vitamin D, and the incidence of prostate cancer for younger men was 3.5 times higher if their levels were below 16 ng/ml (38). These studies suggest that vitamin D levels have significant effects on the initiation of colon and prostate cancer, and in the progression of metastatic breast cancer.

Vitamins A, E, and C

Additional studies on vitamins A, C, and E have shown inverse correlations between the presence of these vitamins and cancer incidence. In a study investigating the correlation of vitamin A, C and E in 5,454 colon cancer patients, results showed that the inverse correlation between vitamin intake and colon cancer incidence was statistically significant (39). Also, a case-

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control study that examined the association of antioxidant vitamins A, C, and E and beta-carotene for 144 cervical cancer patients in South Korea, showed that total intakes of vitamins A, C and E and beta-carotene were inversely correlated with cervical cancer risk (40).

Vitamin E is a fat-soluble antioxidant known to quench oxygen radical species formed during fat oxidation, while vitamin A (retinol) is known for its role in vision and the production of retinoic acid. Both of these vitamins are well-known components in bioactive foods. A study involving 26 patients with gastroesophageal cancer examined vitamin A and E plasma levels compared to the plasma levels of healthy individuals in Eastern Anatolia. Contrastingly, these results showed that the difference in the plasma levels of vitamin A and E between healthy and cancer patients was not statistically significant (41).

In contrast to vitamin E, vitamin C is a water-soluble vitamin that acts as a powerful anti-oxidant and is highly concentrated in citrus fruits. To better understand the role of citrus fruit in cancer risk, 955 patients with oral and pharyngeal cancer, 395 with esophageal, 999 with stomach, 3,634 with large bowel, 527 with laryngeal, 2,900 with breast, 454 with endometrial, 1,031 with ovarian, 1,294 with prostate, and 767 with renal cell cancer were studied in Switzerland and Italy. Results showed that there was a statistically significant inverse correlation between citrus fruit consumption and the risk of cancer for the digestive tract and the larynx (42).

Several additional studies have also demonstrated that the overall nutrient state of an individual is positively correlated with the probability of both surviving cancer treatment and experiencing remission (24, 43, 44). Obesity (which can be prevented through modifying diet and physical activity) has been linked to increased probability of recurrence and incidence of secondary cancers in cancer patients.

Assessing Antioxidant Activity

Several assays have been designed to describe the antioxidant activity of various phyto-chemicals, vitamins, and other compounds. Some of these measure the inhibition of a test reaction by various antioxidant compounds, including the TEAC (Trolox Equivalent Antioxidant Capacity), Radox TEAC, FRAP (Ferric Reducing/Antioxidant Power), HORAC (Hydroxyl Radical Averting Capacity), FOX (Ferrous Oxide-Xylenol Orange), and TRAP (Total Radical Antioxidant Potential) assays (45, 46). Others involve electron spin resonance (ESR) spectroscopy and use techniques such as time-resolved pulse radiolysis and spin trapping. Many others are also used (12, 45-48).

These existing assays are not without limitation, however (12, 47). First, not all of these assays give the same trends for antioxidant activity. For example, poor correlation has been observed between the FRAP and TEAC, the ORAC and the FRAP, and the ORAC and TEAC assays (47, 48). Second, some of the data from *in vitro* studies are difficult to extrapolate to an *in vivo* model because some of these assays are not run at physiological pH. Third, some of these assays do not measure the bioavailability of antioxidant metabolites *in vivo*. Thus, multiple assays should be investigated and validated continuously in order to resolve questions about the true antioxidant capacity of a given sample.

The ORAC and TOSC assays, however, have emerged as industry standards (with the ORAC being particularly recognized) for measuring antioxidant activity due to their high reliability and sensitivity. Until recently, however, little has been done to assess the relative correlation between these two assays. Furthermore, no assay has been developed to measure changes in antioxidant activities of cells in response to oxidative stress.

Structure-Function Analysis

Another area of research relating to food extracts correlates different chemical structures to biochemical functions. Previous research in this area, using the ORAC assay, has included a broad range of chemical groups, including flavonoids (49-51), chalcones (52-54), nitrones (55), and anthocyanadins (56). While these and other studies have been effective in their analyses of these specific chemical groups, no study has attempted to formulate a structure-function frame-work that has applicability to compounds of other groups. A system such as this would be bene-ficial in pharmaceutical and academic settings alike, and may possibly allow for rapid quantifica-tion of antioxidant activity of a compound based on its chemical structure alone.

By using a simplest-case model for isolating chemical functional groups, tests could be run to measure the overall effect of a functional group or set of functional groups on the overall antioxidant activity of a molecule. Compounds used in a simplest-case model would have to be largely similar, but varying enough in one piece or portion of the molecule so as to isolate the specific effects of one particular portion of that molecule. They would also have to be easy to use and run in the ORAC assay for accurate and widely applicable results. Amino acids as a group meet these criteria well, and will be used as the basis for my structure-function analyses. With amino acids, I will be able to specifically isolate certain functional groups to observe over-all trends in structure and antioxidant function.

Fruit and Vegetable Products

There is an abundance of recent studies demonstrating the importance of consuming a diet rich in antioxidants, and the health benefits of such a diet. These studies have ranged from broad, epidemiological studies to basic scientific investigations. The results from these studies appear to be clear in their message, however: that consuming foods rich in antioxidants (with

fruits and vegetables being paramount), helps prevent a variety of chronic and degenerative diseases (9, 57). While several studies have investigated the antioxidant activities of individual food and food components, more work remains in this area.

One particular area of emerging research has centered around comparing organic and conventionally grown food products. The impetus of these investigations lies in claims made by organic supporting groups that these food products are generally more beneficial than their conventional counterparts. Despite the rapid rise in popularity of organic foods, there remains a dearth of research investigating these claims. Moreover, because of the potential implications for business and consumer perception of these products, research in this area is needed. Bioavailability studies are also lacking, assessing the degree to which antioxidants are able to change the overall antioxidant activity in tissues or cells.

CHAPTER I: ANTIOXIDANT ASSAY COMPARISON

1. Gas Chromatograph: A gas chromatograph was used for the TOSC assay, as well as the corresponding software for data collection (for example, the HP5890A gas chromatograph along with the Hp ChemStation GC 5890 online program).

2. Microplate Reader: A fluorescence microplate reader is needed to measure fluorescence intensity for the ORAC assay. Some have used the COBAS FARA II spectroflourometric analyzer (Roche Diagnostic Systems) (47), others the BMG FLUOstar Optima fluorescence microplate reader (58). The BMG FLUOstar Optima was used for all fluorescence readings reported in this work.

3. Clear-bottom well plates: The assays may be run with 96-well plates, 48-well plates, 24-well plates, or six-well plates. The plate size should be selected based on the instrument capabilities, and the number of desired samples per run. All assays for these projects were run in clear-bottom, 96-well plates.

REAGENTS

ORAC Assay

1. AAPH (Figure 1.1): For the ORAC assay AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) is used as the free-radical initiator because it forms free radicals at a constant rate. Caution must be used when handling AAPH, as it can cause apoptosis, damage to the liver and kidney, and damage to capillaries and lymphocytes. Antioxidants quench the oxygen radicals generated by AAPH, thereby inhibiting fluorescence decay (59, 60).
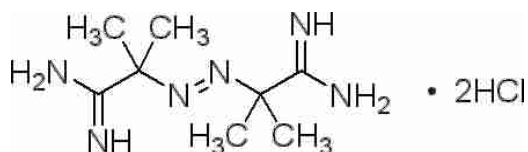
Figure 1.1: Structure of AAPH ((2,2'-Azobis(2-methylpropionamidine), dihydrochloride).
AAPH is used in both the ORAC and TOSC assays, and causes the release of ethylene gas
when added to KMBA.

2. Flourescein Sodium Salt (Figure 1.2): A modified ORAC that used fluorescein instead of β-

phycoerythrin was developed after the original ORAC assay was validated. Fluorescein was

preferred because of its several advantages: it is less expensive than β-phycoerythrin, it does

not interact with other compounds, and does not photobleach (61).
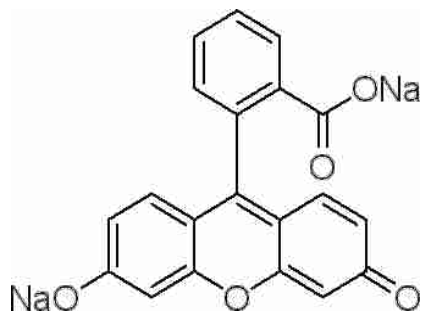
Figure 1.2: Structure of Fluorescein Sodium Salt ($C_{20}H_{10}Na_2O_5$).
Fluorescein is used as the fluorescent molecule in the ORAC assay.

3. Trolox (Figure 1.3):   Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid)

is a widely recognized water soluble Vitamin E derivative used as a baseline against which to

measure relative antioxidant activity. Thus, results for test samples (including chemical com-

pounds and foods) are reported and published as μmol Trolox equivalents/mg or TE.
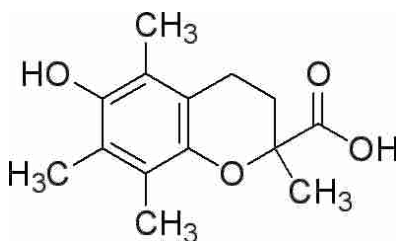
Figure 1.3: Structure of Trolox (((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid). Trolox is used as a standard for both the TOSC and ORAC assays for antioxidant capacity.

4. Sample: Samples that have been run in the ORAC assay include a variety of antioxidant compunds, such as retinols, phenols, carotenoids, tocopherols, phytochemicals, and whole fruits, vegetables, seeds, spices, grains and legumes (57).

TOSC Assay

1. AAPH: In the TOSC assay, the thermal homolysis of AAPH (2,2'-Azobis(2-methyl-propionamidine), dihydrochloride) generates peroxyl radicals.

2. KMBA (α-keto-γ-(methylthio)butyric acid sodium salt, figure 1.4): KMBA is oxidized by AAPH to produce ethylene gas, which is then measured by gas chromatography. When antioxidants are present, however, they quench the peroxyl radicals formed by AAPH, and consequently ethylene gas production is inhibited (58, 62).
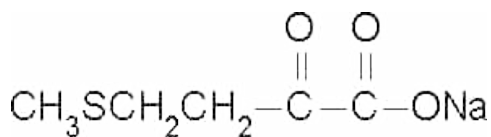
Figure 1.4: Structure of KMBA (α-keto-γ-(methylthio)butyric acid sodium salt). KMBA is oxidized in the TOSC assay to produce ethylene gas.

3. Trolox: Similar to the ORAC, Trolox is used as a standard for determining antioxidant activity in the TOSC assay. As with the ORAC assay, results from the TOSC are also reported as μmol Trolox equivalents/mg. KMBA, AAPH, and Trolox can all be obtained from Sigma-Aldrich, Wako Chemicals, and other commercial chemical manufacturers and distributers.

4. Sample: Many types of samples can be run in the TOSC assay, including vitamins, cellular enzymes, organic and inorganic compounds, fruit and vegetable juices, and other antioxidant compounds (58, 63). Recent studies have included TOSC analyses of Acai fruits, Hydrothermal Vent Mussels, and pyrene from marine invertebrates (58, 62, 63). TOSC samples must be water soluble or DMSO (dimethyl sulfoxide) soluble.

<center>M E T H O D S</center>

ORAC Methods

The ORAC assay is an important assay because of its practicality and ease of use. It can be confusing to some researchers, however, because many investigators have incorporated their own variations on the assay (12, 47, 61, 64). Steps below are adapted from Tomer, et al. (48). Fluorescein is then prepared by dissolving 22.5 mg into 50 mL PBS to make a first stock solution. A second stock solution is prepared by adding 50 μL of the first fluorescein stock solution to 10 mL of phosphate buffer. Finally, 320 μL of the second fluorescein stock solution is added to 20 mL of phosphate buffer.

1. AAPH is prepared at a concentration of 79.65 mmol/L by adding 216 mg to 10 mL phosphate buffer (75 mM, pH 7.4).

2. Experimental samples and control samples (including Trolox) are also prepared at this point. Special care should be taken to ensure that each sample is prepared and stored properly. Samples may be either water or DMSO soluble.

3. 400 μL of fluorescein and 40 μL of sample are first pipetted into each well. Phosphate buffer is used as a blank, and a Trolox dilution series (50, 25, 12.5, and 6.25 μM) is used as a standard.

4. When these aliquots are added to each well, fluorescence readings are measured for gain adjustment, and are subsequently measured as "time zero". During cycle four, the oxygen radical reaction is initiated by injecting 150 μL of AAPH into the respective wells. Sixty and 90 second time intervals may also be used (58, 64).

5. Fluoresence readings are taken every 3.5 minutes, up to 35 cycles. Flourescence readings for each cycle are saved, as they will all be used to calculate areas under the curves and Trolox Equivalents.

TOSC Assay

The time required for a complete run is between 2½ - 3 hours (1½ hours actual run time in gas chromatograph). Additional time should be expected for comprehensive data analysis.

1. Reagent Preparation: A 200 mM concentration of AAPH is prepared by adding 434 mg AAPH and ddH_2O up to 8 mL total volume. A 20 mM concentration of KMBA is prepared by adding 2.7 mg KMBA and ddH_2O up to 8 mL total volume. Following their preparation, the respective vials of AAPH and KMBA should be maintained on ice. A 100

mM potassium phosphate buffer at pH 7.4 is also prepared (58). Experimental and control samples to be tested should also be prepared at the desired concentrations at this point.

2. Vial Preparation: In 10 mL glass vials, 100 μL PBS (Phosphate Buffered Saline) is added to the bottom of each. Next, 690 μL ddH$_2$O is added to the sample vials. Vials are then vortexed. After vortexing, 100 μL KMBA are added to all vials. Next, vials are capped and vortexed a second time. After each sample is vortexed the second time, a 10 μL sample is then added to each sample vial, and the vials are again capped.

3. Incubation & AAPH Injection: The septa-sealed vials should next be incubated for 5 minutes in a 37°C water bath to equilibrate. After the 5 minute incubation period, 100 μL AAPH is added into the vials to initiate the reaction (AAPH should be vortexed before each injection).

4. Gas Chromatograph Preparation: GC temperatures are set as follows: 160°C for the injector port, 60°C for the oven, and 220°C for the flame ionization detector (FID). It is recommended that the injection port septa be changed before each run. The hydrogen/air balance at the detector exhaust should be set to 8-12%. Mobile phase helium can be used to push the other gases though the gas chromatograph at a flow rate of 30mL/min. Once lit, the baseline signal value is normally around 10. Slight variations may be due to the amount of air/hydrogen intake. Once the GC injection syringe barrel is cleaned and the machine prepped, injections and measurements may begin.

5. Gas Chromatography: Twelve minutes after the first AAPH injection, a 1 mL aliquot of gas from each vial should be injected into the GC at staggered intervals. At timepoint 10 min., data should be saved, as area under the curve for these data will later be calculated.

At time 12 min., the 8 injections are repeated at the same time intervals 7 times, for a total

of 8 passes which make up a 96 minute run. Data saved from every cycle will be used to

calculate area under the curve.

<center>D A T A   A N A L Y S I S</center>

Trolox Equivalents

The most important aspect of the ORAC and TOSC assays is calculating Trolox equiva-

lents, or TE. As mentioned, Trolox equivalents represent µmol Trolox equivalents/mg sample.

The area under the fluorescence kinetic curve (AUC) for each sample is calculated as:

$$AUC = (0.5 + f_1/ f_1 + f_2/ f_1 + f_3/ f_1 + ... + f_n/ f_1) \times CT$$

Where f_1 = initial fluorescence reading at cycle 1, f_n = fluorescence reading at cycle n, and CT =

cycle time in min (12).

In order to calculate Trolox equivalents, a Trolox standard curve must first be obtained.

This is done by performing the ORAC or TOSC assay first on varying concentrations of Trolox

(most preferably a serial dilution, which will make calculating a standard curve simpler and more

accurate), and calculating the individual AUC values for each concentration using the method

described above. Once the AUC values for the Trolox concentrations have been calculated, the

AUC for the blank well must also be calculated and then subtracted from the total AUC for each

Trolox concentration. Subtracting the blank AUC from the Trolox (and later from each sample)

AUC gives the net AUC, which accounts for the background readings given by the fluorescence

reader. This concept is shown in the graph below. The area *between* the two curves represents

the net AUC (figure 1.5):

Figure 1.5: Sample ORAC Decay Curves
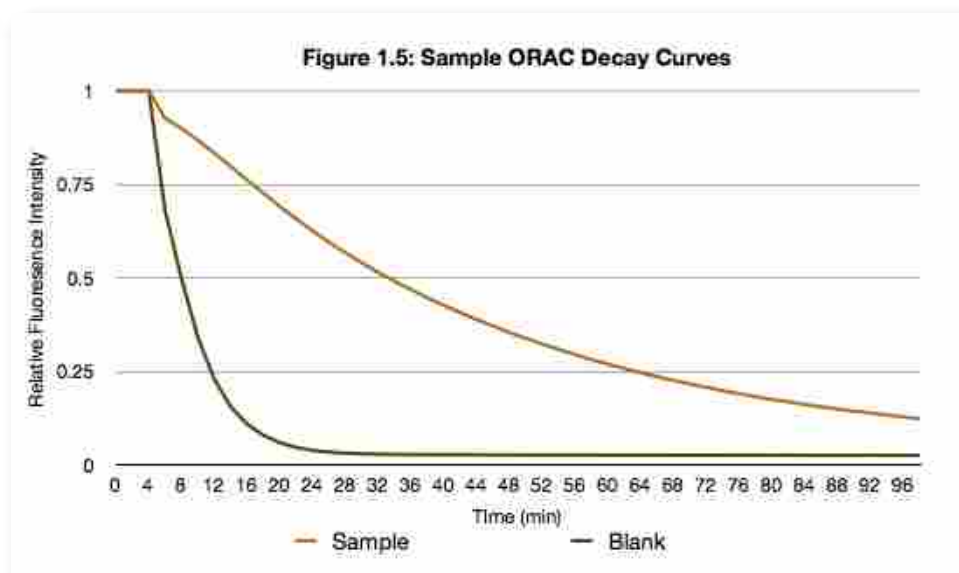
Net AUC = Sample AUC - Blank AUC

A Trolox standard curve is then created from the Net AUC values for each concentration of Trolox used. The slope of the standard curve is then calculated and used for conversions of raw data to μmol Trolox Equivalents per liter (TE/L), which are then converted to the preferred units (data here are reported as μmol Trolox Equivalents per milligram sample).

R E S U L T S

To date, only a few studies have been performed that assess the correlations among the differing antioxidant assays (47, 48, 58). Of these, Cao and Prior have found weak but significant correlation between the ORAC assay and the FRAP assay, and no correlation between the ORAC and the TEAC assays (47).

One study has also been performed to assess the correlations between the ORAC and TOSC assays (48). While precise correlation was not observed, the trends observed in each case

were the same: Green Tea Polyphenols and MegaNatural Gold Grape seed extract yielded high TOSC values and high ORAC values; Lemon Fruit 12:1 and cirus bioflavonoids yielded low TOSC values and low ORAC values; and Grape skin extract, GSKE-40 grape seed extract, quercitin, pycogenol, and pine bark theraplant all yielded moderate TOSC values and moderate ORAC values.

The figures below contain some of the data from the study. The first figure contains the relative activity of the 11 phytochemicals measured by the TOSC assay (light bars) and by the ORAC assay (dark bars). Minimum and maximum values for each assay were scaled to 0% and 100%, respectively, to facilitate inter-assay comparisons.   The numbers represent the phytochemicals as follows: (1) lemon fruit, (2) citrus bioflavonoids, (3) pomegranate, (4) grape seed extract, (5) pine bark theraplant, (6) quercitin, (7) pycnogenol, (8) grape seed extract, (9) rutin, (10) α-lipoic acid, and (11) green tea polyphenols. Values reflect the mean for each sample (Figure 1.6). Table 2 below presents the numerical values calculated for each sample.

Figure 1.6: ORAC and TOSC Values (as percentage of relative maximum value)
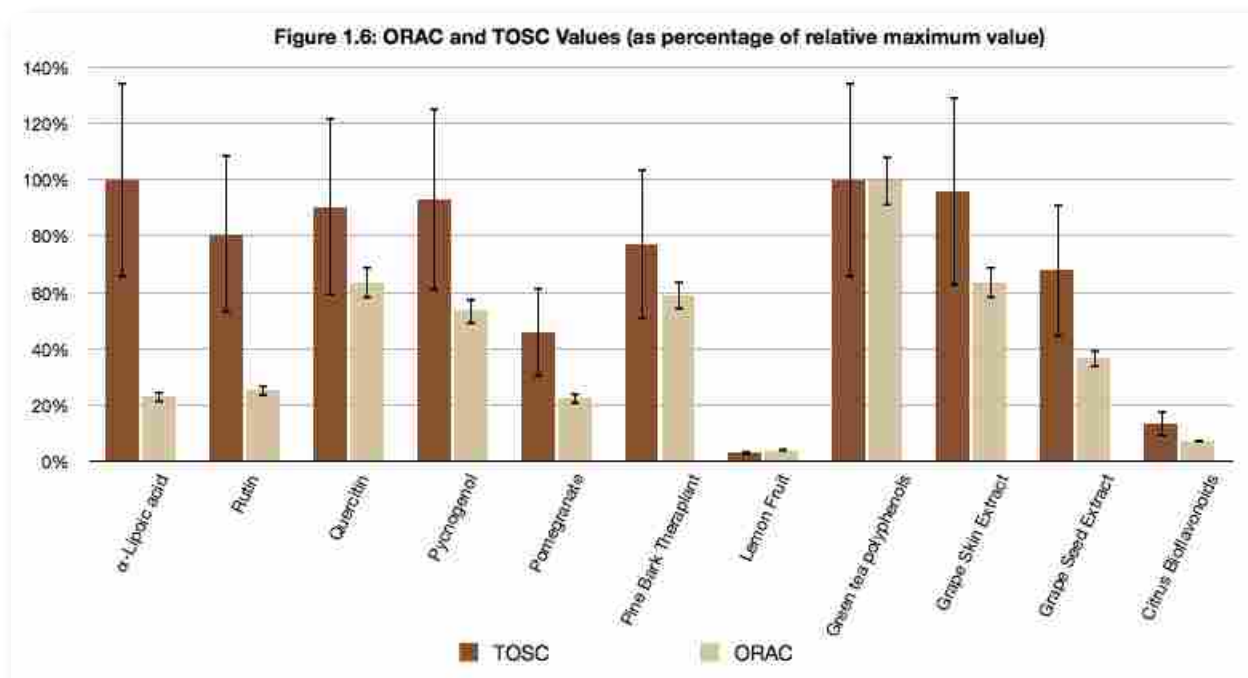
Table 2: Raw ORAC and TOSC Results

Sample	TOSC	ORAC
Lemon Fruit	103	3885
Citrus Bioflavonoids	446	7410
Pomegranate	1543	22530
Grape Seed Extract	2294	37125
Pine Bark Theraplant	2608	59483
Quercitin	3059	63750
Pycnogenol	3154	53970
Grape Skin Extract	3246	63668
Rutin	2727	25440
α-Lipoic acid	3381	23235
Green tea polyphenols	3377	100658

In order to assess correlation between the results of the two assays, Pearson Product Moment Correlation (PMCC) coefficient was calculated from the regression line calculated from the data sets. The PMCC is a measure of linear dependence (correlation) between two sets of data. The PMCC ranges from -1 to 1, with each extreme signifying a perfect correlation between the two data sets. The overall PMCC between each sample run in the ORAC and TOSC assays was 0.738, signifying moderate correlation.

A coefficient of determination was also calculated for the dataset, which reflects the proportion of variability in the dataset, and is a reflection of how well future data can be predicted using the current model. The coefficient of determination for all 11 samples was 0.54. Figure 1.7 shows a scatterplot representation of the data. TOSC and ORAC values are both represented as μmol Trolox equivalents/mg; the average of several replicates is shown.

Figure 1.7: Assay Correlation - All Samples Included

$R^2 = 0.5446$
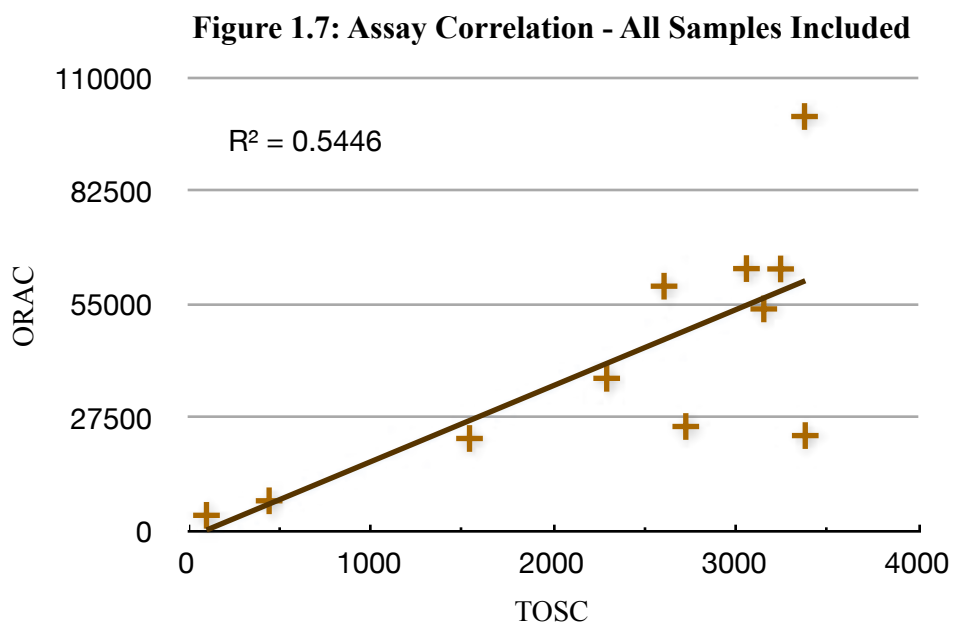
A *post hoc* analysis of the data sets was performed to look for and exclude any outliers in the data, to see if greater correlation between the data may be masked by extreme values. Indeed, when the outliers rutin, α-lipoic acid, and green tea polyphenols were removed from the dataset, high correlation between the remaining TOSC and ORAC assay values was observed (PMCC=0.97; coefficient of determination=0.94). These samples may have revealed outlying data due to slight procedural errors, but because several replicates were run, these departures were most likely due to the individual chemical properties of the samples and their unique behaviors when run in each assay. Figure 1.8 shows a revised scatterplot graph with the three outliers excluded, and table 3 below presents a summary of the statistical data.

Figure 1.8: Assay Correlation - Three Outliers Excluded

$R^2 = 0.941$
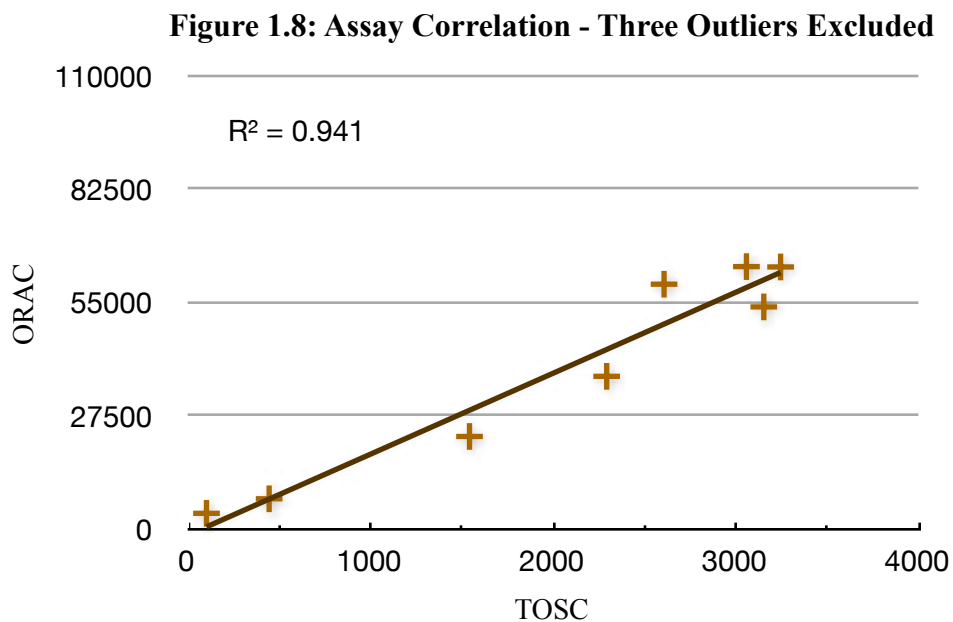
Table 3: Statistical Summary of ORAC-TOSC Correlation Data

	ALL SAMPLES	OUTLIERS EXCLUDED
PMCC	0.74	0.97
Coefficient of Determination	0.54	0.94

CONCLUSION

Comparing the two assays revealed that more variability in antioxidant activity could be seen for the TOSC assay than for the ORAC assay. Such variability may have been due to slight procedural errors, including error in the injection process into the gas chromatograph. Establishing specific numbers for the antioxidant activity of certain phytochemical compounds also proved difficult because the conditions under which the fruit or vegetable samples were cultivated and stored varied greatly from sample to sample. Despite small variations in sampling, the antioxidant activity ranking of these phytochemical compounds was similar among replicates.

Absolute values of the antioxidant counts for the ORAC and TOSC assays vary because of the different means of assessing antioxidant activity (ie, measuring ethylene gas production vs measuring degradation of fluorescein). Because these two methods are very different, analysis of the same compound using different antioxidant assays is recommended for elucidating the true antioxidative potential of a compound or extract.

While the measurement of any antioxidant compound *in vitro* is a valuable first step for identifying good antioxidants for further use in *in vivo* testing, it should not be assumed that the antioxidant activity of a compound *in vitro* will necessarily reflect the antioxidant activity *in vivo*. As mentioned, studies have shown that consumption of fruits and vegetables results in ele-

vated plasma ORAC values in humans (65), and synergy is often observed between multiple an-

tioxidant compounds *in vivo* (65, 66).

The discrepancies seen between the ORAC and TOSC assays suggest that in some cases the results from both assays may be useful in determining an antioxidant's true antioxidant capacity. As other antioxidant assays are also used and developed, discrepancies between these assays pertaining to the relative antioxidant activity of various samples will likely be greatly diminished. As more research is performed, the realm of predictive power will increase, and the ORAC and TOSC assays will continue to be useful tools for assessing antioxidant activities.

CHAPTER II: STRUCTURE-FUNCTION ANALYSIS

A novel method for predicting antioxidant activity based on amino acids as a simplest-case model

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Introduction

For years, antioxidants have been the focus of many epidemiological studies aimed at elucidating the relationship between oxidative stress and degenerative diseases. Among these, correlations have been observed between oxidative stress and the development and progression of Alzheimer's disease, cognitive decline, type-2 diabetes, hyperthyroidism, and cancer (1, 2, 5, 10, 67). Increased understanding of the pathogenic mechanisms of these diseases will come through further study of the oxidative/antioxidative balance occurring in the tissues involved.

Antioxidants are chemical compounds that are capable of preventing oxidation of other molecules by quenching free-radicals. During normal cellular metabolic processes, basal levels of oxidative stress are produced which, if left unchecked, have the potential to cause oxidative damage to DNA, lipids, and other cellular components (15, 68). While the body is capable of protecting itself against oxidative damage through activation of innate antioxidant mechanisms, a diet rich in antioxidant-containing foods remains the most important way to supplement antioxidative defenses in the body.

Fruits and vegetables have specifically been included in a wide variety of antioxidant studies because of their high concentrations of antioxidant compounds, such as tocopherols, carotenoids, retinoids, polyphenols, and flavonoids (57, 64, 69). These and other antioxidant com-

pounds can also be found in grains, nuts, legumes, oils, berries, perennials, and herbs (70). A recently-published long-term study investigating the effects of vitamin supplement use during breast cancer treatment concluded that vitamin supplement use during the first six months after breast cancer diagnosis may be associated with reduced risk of mortality (71). Multiple studies have found correlations between plasma levels of bioactive food components (i.e. carotenoids, tocopherols, etc.) and the likelihood of survival of patients undergoing chemotherapy (22-24). Several additional studies have also demonstrated that the overall nutrient state of an individual is positively correlated with the probability of both surviving cancer treatment and experiencing extended remission (24, 43, 44).

Antioxidant molecules have also been used in numerous structure-antioxidant function analyses (52-55). Although these analyses have been effective in studying structure-function relationships within each compound class, to our knowledge, no attempts have been made to elucidate structural trends that have general applicability to any class of compounds. Thus, while results obtained from previous studies are beneficial in the investigation of the specific compounds investigated, little if any conclusions may be applied to other models.

To address this, we propose using simplest-case models to make observations regarding general structure-antioxidant function trends. In the case of antioxidant activity, a simplest-case model is a model that attempts to directly observe the effects of a single functional group or group of functional groups by analyzing molecules that are generally very similar, but only vary at one locus. Using a simplest-case model will allow for optimal observation of the effects of single functional groups on antioxidant function, and will allow for the greatest amount of applicability to other compound classes.

In the current paper, amino acids were selected as the optimal compounds for our simplest-case model for two reasons. First, amino acids can function as antioxidants both as free compounds (i.e., glutathione, which is a cysteine, glutamic acid, glycine tripeptide), and in the active sites of antioxidant enzymes (some peroxidases, for example, contain redox-active cysteine or selenocysteine residues in their active sites). Second, amino acids all contain the same amine-carboxylic acid-side chain parent structure, while each side chain is unique. This unique property allows for maximal isolation of different functional groups for antioxidant analysis. Such a compound class is ideal for antioxidant structure-function study because the effects of different functional groups in each side chain may be directly observed.

The current paper reports data from an investigative chemical structure-antioxidant function analysis using amino acids as a simplest-case model. Initial trends were examined in the context of non-amino acid compounds, as well as previously-published data to assess the robustness of the results when compared to other types of compounds. Results suggest that there are certain structure-function trends that may have broad applicability to a variety of compound classes.

MATERIALS AND METHODS

ORAC ASSAY

For all antioxidant activity measurements, we used a modified oxygen radical absorbance capacity (ORAC) assay based on previously published methods (12, 48, 64). The ORAC measures the fluorescence decay of fluorescein sodium salt after exposure to AAPH (2,2'-azobis(2-amidino-propane) dihydrochloride), which generates oxygen radicals at a constant rate (72). Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as a standard, run

at 50, 25, 12.5, and 6.25 μM concentrations during every run. Costar 96-well clear bottom plates were loaded with 200μL fluorescein (0.1μM), 20μL AAPH (4μM), and 20μL sample (ie, Trolox standards, antioxidant samples, or PBS blank). Measurements were taken using the BMG FLU-Ostar Microplate reader at 37°C, utilizing a 485nm excitation filter and a 520nm emission filter. Readings were taken every two minutes for 120 minutes. Fluorescence readings were measured and analyzed according to Trolox standards, and final data are reported as μmol Trolox Equivalents per milligram sample (TE), consistent with previously published analytical methods (12). Data were analyzed using Apple Numbers.

AMINO ACIDS

As previously stated, amino acids were selected as a simplest-case model for antioxidant analysis because the effects of various functional groups are most readily observed. Twenty essential and non-essential amino acids were purchased from Sigma-Aldrich (St. Louis, MO) or Wako Chemicals (Richmond, VA), and are as follows: Alanine, Arginine, Asparagine, Aspartic Acid, Cysteine, Glutamic Acid, Glutamine, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Hydroxyproline, Serine, Threonine, Tryptophan, and Tyrosine (L-isomers were used in all cases). Samples were stored at 4°C. For analysis in the ORAC assay, samples were freshly prepared at a concentration of 200μM, and diluted 1:25 before being aliquoted into the 96-well plate to ensure optimal concentration for ORAC analysis.

RATIONALE FOR CORRELATION STATISTICS

The rationale for structure-function analysis is based on the ideas that: (1) certain chemical groups may be either beneficial or detrimental to the overall antioxidant activity of a molecule, (2) the presence of beneficial antioxidant chemical groups will result in increased antioxi-

dant activity of the molecule, and (3) functional groups that add to a molecule's antioxidant activity will add to the antioxidant activity of other molecules in almost all cases. Together, these ideas suggest that correlation may be observed between the presence of certain chemical structures and antioxidant activity, and that the correlation is not a result of probabilistic independence, which is the idea that the occurrence of one event does not influence the occurrence of another event.

If such correlations are linear, they can be assessed using Pearson Product-Moment Correlation Coefficient analysis (PMCC, or r) and coefficient of determination (R^2), which together suggest both linear dependence and the ability to make future predictions based on the given statistical model. Polynomial regression, which is a special case of linear regression, can be used to describe relationships that are non-linear, and is expressed as an *nth*-order polynomial. This type of non-linear regression analysis could suggest possible synergistic or antagonistic effects of structure on antioxidant function.

STRUCTURAL GROUP CATEGORIES

Following antioxidant analysis of each individual amino acid, TE data were entered into a structure database containing chemical structure information for each amino acid. The chemical features of each amino acid were quantified and analyzed according to 15 structural criteria: number of carbons (or C), number of valence electrons (or E), valence electron/carbon ratio (E/C), carbon/valence electron ratio (C/E), number of sp^2-hybridized carbons, sp^2-hybridized carbon/total carbon ratio, number of conjugated double bonds (defined here as the number of contiguous double bonds, with a minimum of two; definition includes C=O groups when adjacent to at least one other double bond, but does not include C=O groups on their own because

there cannot be a conjugated system with only one double bond), longest conjugated double bond chain in molecule, number of phenol-OH groups, number of benzene rings, number of R-OH groups, number of carboxylic acids, valence electron/carboxylic acid ratio (E/CA), total carbon/carboxylic acid ratio (C/CA), and number of nitrogens (see Figure 2.1).

	# of C	# of E	E/C Ratio	C/E Ratio	sp^2-Hybridized Carbons	sp^2-Hybridized Carbon/Total Carbon Ratio	Number of Conjugated Double Bonds	Longest Conj. DB Chain	Phenol-OH	# of Benzene Rings	R-OH	Carboxylic Acids	E/CA Ratio	C/CA Ratio	Nitrogens
Alanine	3	10	3.33	0.30	1	0.33	0	0	0	0	0	1	10	3	2.0
Arginine	6	14	2.33	0.43	2	0.33	0	0	0	0	0	1	14	6	4.0
Asparagine	4	16	4.00	0.25	2	0.50	0	0	0	0	0	1	16	4	2.0
Aspartic Acid	4	18	4.50	0.22	2	0.50	0	0	0	0	0	2	9	2	1.0
Cysteine	3	14	4.67	0.21	1	0.33	0	0	0	0	0	1	14	3	1.0
Glutamic Acid	5	18	3.60	0.28	2	0.40	0	0	0	0	0	2	9	2.5	1.0
Glutamine	5	14	2.80	0.36	2	0.40	0	0	0	0	0	1	14	5	2.0
Glycine	2	10	5.00	0.20	1	0.50	0	0	0	0	0	1	10	2	1.0
Histidine	6	16	2.67	0.38	4	0.67	2	2	0	0	0	1	16	6	3.0
Hydroxyproline	5	14	2.80	0.36	1	0.20	0	0	0	0	1	1	14	5	1.0
Isoleucine	6	10	1.67	0.60	1	0.17	0	0	0	0	0	1	10	6	1.0
Leucine	6	10	1.67	0.60	1	0.17	0	0	0	0	0	1	10	6	1.0
Lysine	6	12	2.00	0.50	1	0.17	0	0	0	0	0	1	12	6	2.0
Methionine	5	14	2.80	0.36	1	0.20	0	0	0	0	0	1	14	5	1.0
Phenylalanine	9	16	1.78	0.56	7	0.78	3	3	0	1	0	1	16	9	1.0
Serine	3	14	4.67	0.21	1	0.33	0	0	0	0	1	1	14	3	1.0
Threonine	4	14	3.50	0.29	1	0.25	0	0	0	0	1	1	14	4	1.0
Tryptophan	11	20	1.82	0.55	9	0.82	4	4	0	1	0	1	20	11	2.0
Tyrosine	9	20	2.22	0.45	7	0.78	3	3	1	1	0	1	20	9	1.0
Valine	5	10	2.00	0.50	1	0.20	0	0	0	0	0	1	10	5	1.0

Figure 2.1: Structure database used for amino acid analysis. PMCC and coefficient of determination were calculated between ORAC data and each of the 15 categories below to assess relative correlation.

For all initial analyses, Pearson's *r* and coefficients of determination were calculated to assess the relative correlation between ORAC data and each structural category separately. For example, *r* and R^2 values were calculated between ORAC values and total number of carbons, total number of valence electrons, electron-to-carbon ratio, etc. These initial analyses formed the basis of all correlative investigations, and are reported below.

RESULTS AND DISCUSSION

AMINO ACID RESULTS AND LINEAR CORRELATION ANALYSIS

The twenty amino acids comprising our simplest-case model were first analyzed using the ORAC assay, and rank-ordered from highest to lowest according to antioxidant activity (see Figure 2.2). Of these, Tryptophan revealed the highest overall antioxidant activity at 735.25 TE/mg, while Glycine revealed the lowest at -347.43 TE/mg.

	Antioxidant Activity (TE/mg)	Sample Size (n)
Tryptophan	735.24	48
Tyrosine	477.43	78
Methionine	361.75	16
Asparagine	-0.18	12
Cysteine	-0.19	12
Histidine	-7.50	30
Arginine	-76.00	30
Phenylalanine	-123.07	66
Threonine	-128.65	36
Glutamic Acid	-140.62	30
Aspartic Acid	-140.87	30
Isoleucine	-150.02	30
Leucine	-155.43	30
Glutamine	-159.78	12
Serine	-179.86	18
Hydroxyproline	-183.89	24
Lysine	-183.92	12
Valine	-187.66	24
Alanine	-210.72	30
Glycine	-347.42	12

Figure 2.2: Antioxidant activities of the 20 amino acids used for simplest-case model as measured by the ORAC assay. Data represent means of several replicates (n). All samples were prepared at 200 μM concentrations, and subsequently diluted 1:25 prior to analysis in the ORAC assay. Results are presented as μmol Trolox Equivalents per milligram sample or TE/mg.

PMCC regression analysis between ORAC data and the structural categories mentioned initially yielded mixed results among each category (see Figure 2.3). The overall mean of the PMCC values was $r=0.41$. The highest overall PMCC was observed for the ORAC-valence elec-

tron to carboxylic acid ratio category (r=0.75), and the second highest was for the ORAC-number of sp^2-hybridized carbons category (r=0.71). The lowest correlations were observed in the ORAC-number of nitrogens category (r=0.08), and the ORAC-number of carboxylic acids category (r=-0.13). Of the groups, only three categories returned negative PMCC values, suggesting an inverse relationship between the category and antioxidant activity; but these were very weak correlations.

	# of C	# of E	E/C Ratio	C/E Ratio	sp^2-Hybridized Carbons	sp^2-Hybridized Carbon/ Total Carbon Ratio	Number of Conjugated Double Bonds	Longest Conj. DB Chain	Phenol -OH	# of Benzene Rings	R-OH	Carboxylic Acids	E/CA Ratio	C/CA Ratio	Nitrogens
PMCC (r)	0.68	0.66	-0.31	0.26	0.71	0.52	0.70	0.70	0.46	0.66	-0.20	-0.13	0.75	0.67	0.08
R^2	0.47	0.43	0.09	0.07	0.51	0.27	0.49	0.49	0.21	0.44	0.04	0.02	0.56	0.45	0.01

Figure 2.3: Complete PMCC and coefficient of determination data from initial statistical analysis of each structural category.

A *post hoc* analysis was then performed to assess the effects of single outliers on overall correlation data, as well as to investigate the possibility that specific compounds tended to be outliers in multiple categories. To do this, outliers in each group were systematically removed until the PMCC of each group was greater than 0.90. The data from the most significant groups are summarized in figure 2.4. The groups which appeared to be most affected by outliers were the ORAC-number of sp^2-hybridized carbons group, the ORAC-number of conjugated double bonds group, and the ORAC-longest conjugated double bond chain group, as the PMCC values rose to over 0.90 after removing only two or three outliers. Thus, these groups revealed the highest PMCC values with the most compounds being considered, suggesting that these categories may be most helpful in accurately predicting antioxidant activity.

	Initial r	Modified r	Number of Outliers Excluded	Outliers Excluded
ORAC-number of carbons	0.68	0.90	4	Phenylalanine, Methionine, Asparagine, Threonine
ORAC-number of electrons	0.66	0.90	5	Glutamic Acid, Aspartic Acid, Methionine, Isoleucine, Leucine
ORAC-number of sp^2-hybridized carbons	0.71	0.95	2	Methionine, Phenylalanine
ORAC-number of conjugated double bonds	0.70	0.96	3	Methionine, Phenylalanine, Histidine
ORAC-longest conjugated double bond chain	0.70	0.96	3	Methionine, Phenylalanine, Histidine

Figure 2.4: Summary of post hoc analysis in which the effect of outliers on PMCC linear regression results were investigated. Only the categories which showed a modified r of greater than 0.90 after 5 or less outliers were excluded are shown in this table.

Interestingly, during investigation of outlier data, we observed that certain compounds tended to be outliers in several groups, while other compounds were rarely outliers. Methionine and phenylalanine, for example, were outliers in 9 out of the 10 groups, where tryptophan, tyrosine, and serine were never outliers. This suggests that methionine and phenylalanine possess unique antioxidant characteristics not accounted for by our analysis; they may also have uniquely high or low antioxidant activity given their structure.

To examine the effects of phenylalanine and methionine on the overall PMCC data, secondary PMCC data were again calculated for every category, with methionine and phenylalanine excluded. The overall mean of the PMCC values rose from 0.41 to 0.53, which was statistically significant ($p<0.0002$). With these outliers excluded, the ORAC-number of sp^2-hybridized carbons group PMCC rose to 0.95, suggesting that, in most cases, the antioxidant activity of a molecule may correlate with the number of sp^2-hybridized carbons it contains.

Non-Linear Correlation Analysis

In many cases, correlation between antioxidant activity and chemical structure is not likely to be a purely linear relationship, as many interactions are involved. Thus, there may be non-linear structure-function relationships that can be elucidated with the help of polynomial regression. These relationships may help to discover both theoretically ideal amounts of a certain structural characteristic, as well as possible synergistic or antagonistic effects of combinations of structural characteristics.

Polynomial regression analysis was performed on the following structural categories: number of carbons, number of valence electrons, valence electron to carbon ratio (E/C), carbon to valence electron ratio (C/E), number of sp^2-hybridized carbons, number of sp^2-hybridized carbon to total carbon ratio, and valence electron to carboxylic acid ratio (E/CA). These were selected because they were the most relevant, as not all compounds contain certain structural groups like benzene rings or R-OH groups.

Initial polynomial regression analyses revealed low to moderate R^2 values (reflect variance accounted for by the regression line) in all categories, suggesting that there were no discernible non-linear correlative effects between antioxidant activity and chemical structure among all compounds tested. When outliers methionine and phenylalanine were excluded, however, mean R^2 values increased significantly in all categories ($p < 0.00001$). Figure 2.5 summarizes polynomial regression analysis data when methionine and phenylalanine were excluded.

	2nd Order	3rd Order	4th Order	5th Order	6th Order
Number of Carbons	0.70	0.71	0.78	0.81	0.81
Number of Electrons	0.58	0.58	0.58	0.63	0.64
E/C ratio	0.12	0.16	0.36	0.36	0.38
C/E ratio	0.12	0.16	0.30	0.31	0.31
Number of sp^2-hybridized carbons	0.92	0.92	0.92	0.93	0.93
sp^2-hybridized carbon/total carbon ratio	0.56	0.64	0.73	0.73	0.73
E/CA ratio	0.70	0.71	0.73	0.74	0.74

Figure 2.5: R^2 data from polynomial regression analysis. These data represent analysis of amino acid groups that did not contain methionine or phenylalanine.

The group which revealed the highest coefficient of determination data in the polynomial regression analysis was the ORAC-number of sp^2-hybridized carbon group. Interestingly, graphical analysis of the polynomial regression line revealed a sigmoid-like shape, suggesting that there may be a threshold number of required sp^2-hybridized carbons to cause a rapid increase in antioxidant activity (see figure 2.6). Moreover, upon removal of the third outlier, histidine, the sigmoidal shape becomes increasingly apparent (figure 2.7). This suggests that sp^2-hybridized carbons may have a compounding effect on antioxidant activity within a certain range. The exact details of this range, however, can only be further described with the analysis of additional compounds for antioxidant activity.
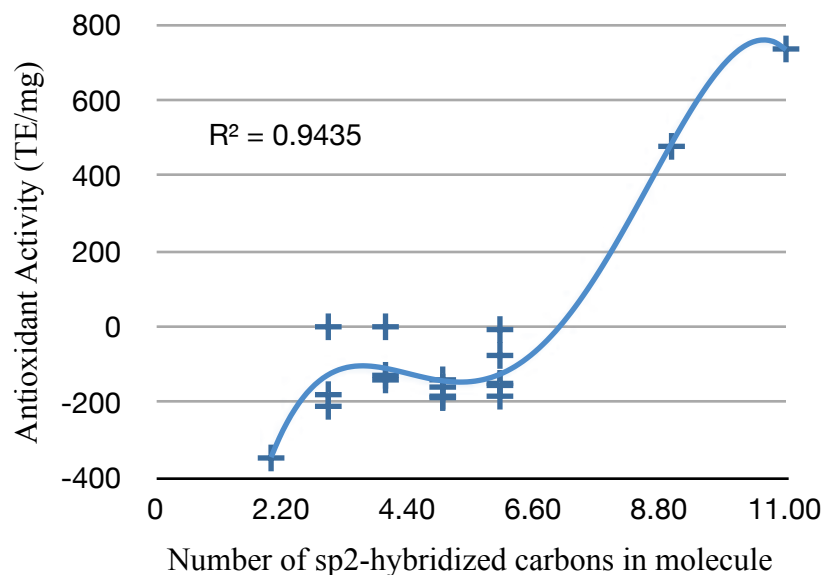
Figure 2.6: Polynomial regression data and curve relating the number of sp²-hybridized carbons and antioxidant activity in the amino acid model. Only methionine and phenylalanine are excluded. Partial sigmoidal shape was observed here, and is more apparent with the exclusion of histidine (see figure 2.7).
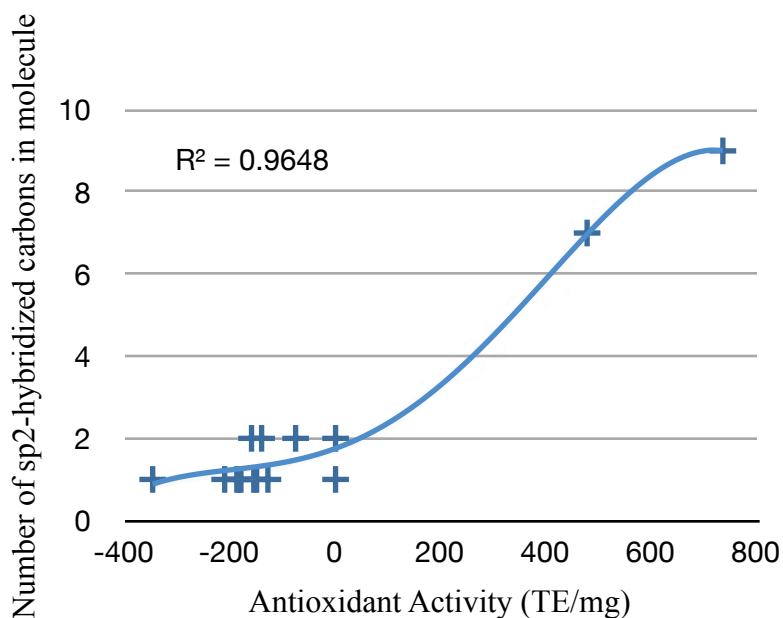
Figure 2.7: Polynomial regression data and curve relating the number of sp²-hybridized carbons and antioxidant activity in the amino acid model. Methionine, phenylalanine, and histidine are excluded. Coefficient of determination data suggest that the relationship between antioxidant activity and the overall number of sp²-hybridized carbons in an antioxidant molecule may be non-linear.

We next wanted to extend these results to other compounds, to test the predictive power of these new data. To do this, seven additional compounds were analyzed for their antioxidant activities using the ORAC assay. The compounds and their resulting ORAC data are summarized in figure 2.8. In order to assess the predictive power of the amino acid results, regression equations would need to be used to generate predicted ORAC values. From amino acid analysis, data regarding sp^2-hybridized carbons suggested highest predictability, so sp^2-hybridized data of the new compounds was entered into the amino acid polynomial regression equations in order to generate predicted ORAC values.

	Antioxidant Activity (TE/mg)
Acetylsalicylic Acid	831.00
Ascorbic Acid	50.31
Citric Acid	-112.78
Glutathione	146.27
α-Lipoic Acid	-251.24
Resveratrol	1279.00
Retinoic Acid	672.00

Figure 2.8: Additional compounds analyzed using the ORAC assay. These compounds were prepared and analyzed at the same concentrations as the amino acids to ensure greatest continuity. Data represent means of several replicates.

First, regarding overall number of sp^2-hybridized carbons, regression equations yielded results that correlated well with actual values (r=0.88). Moreover, when outlier acetylsalicylic acid was removed, PMCC data and coefficient of determination data improved greatly (r=0.97; R^2=0.98 for 2nd order polynomial; figure 2.9).

Regression equation prediction also yielded data that correlated well with observed sp^2-hybridized carbon to total carbon ratio data among the new compounds (r=0.85). Likewise, re-

moval of outlier retinoic acid greatly improved PMCC and coefficient of determination data

(R=0.95; R^2=0.999 for 2nd order polynomial; figure 2.10).
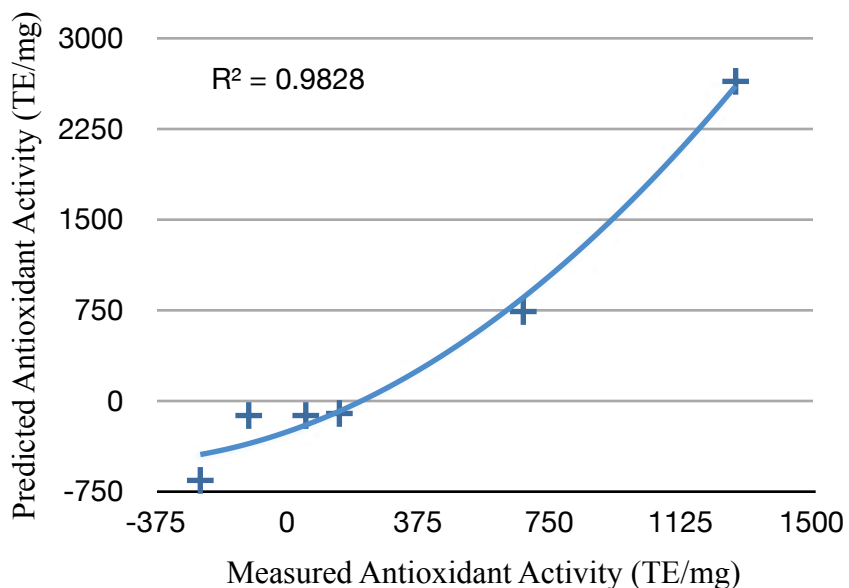
Figure 2.9: Regression analysis between actual measured ORAC values and predicted ORAC values that were generated using polynomial regression data from the amino acid model and total number of sp^2-hybridized carbons in the new compounds. This graph includes data for ascorbic acid, citric acid, glutathione, α-lipoic acid, resveratrol, and retinoic acid.

Together, these data suggest that the two categories (number of sp^2-hybridized carbons

and sp^2-hybridized carbon to total carbon ratio) can be used to predict antioxidant activity from

the polynomial regression equation generated from our amino acid data. Furthermore, the data

from the new compounds tested support the observations from the amino acid model, which sug-

gest that actual trends in antioxidant activity may resemble a more non-linear relationship than

linear.
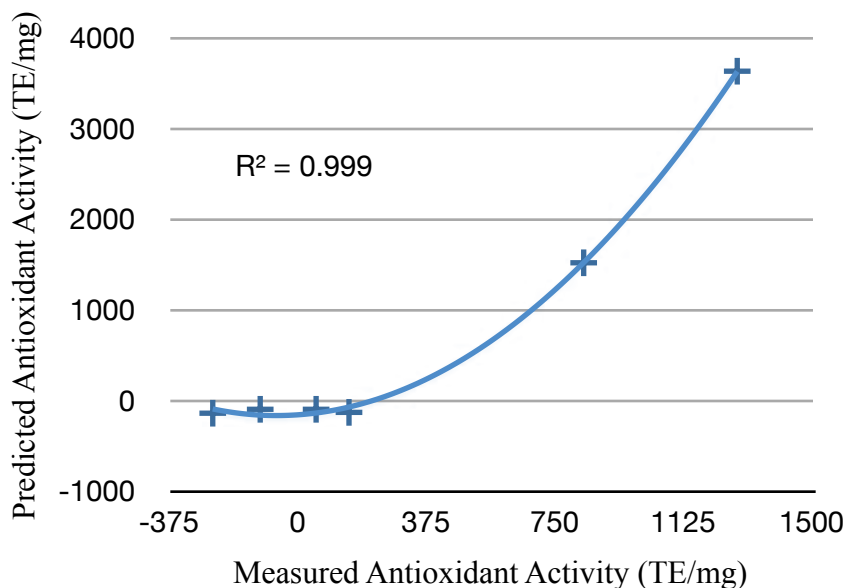
Figure 2.10: Regression analysis between actual measured ORAC values and predicted ORAC values that were generated using polynomial regression data from the amino acid model and sp²-hybridized carbon to total carbon ratios from the new compounds. This graph includes data for acetylsalicylic acid, ascorbic acid, citric acid, glutathione, α-lipoic acid, and resveratrol.

APPLICATION OF RESULTS TO PREVIOUSLY-PUBLISHED DATA

With this information, we then wanted to explore the applicability of these trends in other compound classes. In order to do this, we found published papers that have included similar antioxidant structure-function data in their work on chalcones (52), and nitrones (55). Figure 2.11 contains a table displaying each compound used, the work the data were taken from, and each compounds' antioxidant activity. We examined the antioxidant activity data from these papers in the context of our findings here to see if the trends we had observed in our amino acid model had applicability to other models.

First, linear regression analysis was performed between the antioxidant activities reported and each of the structural categories investigated in our study, with the addition of six new categories: number of allylic carbons, % of total conjugated double bonds in longest double bond chain, carbon to benzene ring ratio, valence electron to benzene ring ratio, valence electron to

phenol-OH ratio, and carbon to phenol-OH ratio. We felt these additional categories may be more helpful as they are more reflective of the structural characteristics of these compounds.

	Reported Antioxidant Activity
Chalcones (Vogel, Barbic et al. 2010)	
3'-Coumaroyl-2',4,4'-trihydroxy-6'-methoxychalcone (3)	7.70
3-Methoxyhelichrysetin (6)	6.00
2',3,4,4' -Tetrahydroxy-3'-(3''-hydroxy-methylbutyl)-6'- methoxychalcone, 3-hydroxyxanthohumol H (14)	3.90
2',4,4'-Tetrahydroxy-3'-(3''-hydroxy-methylbutyl)-3,6'- dimethoxychalcone, 3-methoxyxanthohumol H (15)	3.00
3-Methoxyxanthohumol (9)	2.60
Xanthohumol	2.30
2',3,4-Trihydroxy-6' -methoxy-2'',2' -dimethyl- pyrano[2'',3' :3',4']-chalcone, 3-hydroxyxanthohumol C (11)	2.10
2',4-Dihydroxy-3,6'-dimethoxy-2'',2' -dimethyl- pyrano[2'',3' :3',4']-chalcone, 3-methoxyxanthohumol C (12)	2.00
2',4' -Dihydroxy-4,6' -dimethoxy-3' -prenylchalcone, 4-O- methylxanthohumol (1)	1.60
4-Acetoxy-2',4'-dihydroxy-6'-methoxy-3'-prenylchalcone, 4-O-acetylxanthohumol (2)	0.60
Nitrones (Samadi, Soriano et al. 2011)	
(Z)-a-N-(5-Methoxy-1-methyl-1H-2-indolyl)-N-tert- butylnitrone (21)	798
(Z)-a-(20-Bromo-50-hydroxy-40-methoxyphenyl)-N- benzylnitrone (10)	736
(Z)-a-(20-Bromo-40-hydroxy-5-methoxyphenyl)-N- benzylnitrone (15)	640
(Z)-a-(20-Bromo-50-hydroxy-40-methoxyphenyl)-N-tert- butylnitrone (9)	558
(Z)-a-(1H-3-Indolyl)-N-tert-butylnitrone (23)	369
(Z)-a-(40-Hydroxy-30-methoxyphenyl)-N-methylnitrone (13)	335
(Z)-a-(40-Hydroxy-30-methoxyphenyl)-N-benzylnitrone (14)	293
(Z)-a-(2-Bromo-4,5-dimethoxyphenyl)-N-tert-butylnitrone (11)	3.0
(Z)-a-(20-Bromo-40,50-dimethoxyphenyl)-N-benzylnitrone (12)	3.0
(Z)-a-Phenyl-N-benzylnitrone (8)	1.0
(Z)-a-(20-Bromo-3-pyridyl)-N-tert-butylnitrone (18)	1.0
(Z)-a-(20-Chloro-3-pyridyl)-N-t-butylnitrone (19)	1.0

Figure 2.11: Table displaying the compounds used for additional analysis and their sources. The table is arranged in order of descending antioxidant activity within each group. Chalcone data were reported as "Trolox Equivalents", while Nitrone data were reported as micromol trolox/micromol compound.

Interestingly, similar trends were observed between our simplest-case amino acid model and the chalcone compounds, especially with respect to sp^2-hybridized carbons. While initial

PMCC linear regression analysis revealed a weak-to-moderate correlation between the sp^2-hybridized carbon to total carbon ratio and antioxidant activity in the compounds tested (r=0.59), removing the three largest outliers in this category (compound #s 1, 15, and 14) greatly improved the linear correlation data in this category (r=0.90). Furthermore, polynomial regression analysis of this category revealed high R^2 data (R^2=0.95), suggesting that this structural characteristic may also be beneficial in estimating antioxidant activity in chalcones as well as in amino acids (figure 2.12).

Figure 2.12: Graphical representation of fourth-order polynomial regression analysis of the correlation between antioxidant activity and the sp2-hybridized carbon to total carbon ratio in seven chalcones. Outlier compounds 1, 14, and 15 were excluded.

In the chalcone group, the highest PMCC value was found in the ORAC-valence electron to carbon ratio category (r=0.70). Removing the three largest outliers in this category (compounds 1, 2, and 3), however, greatly improved the linear correlation data (r=0.92). This sug-

gests that using valence electron to carbon ratio may be another helpful tool in predicting anti-

oxidant activity.

While the highest PMCC in the nitrone group was much lower (r=0.57, observed in the

ORAC-number of carbons category), removal of the five largest outliers (compounds 8, 11, 12,

18, and 19) resulted in very precise polynomial regression data (R^2=1.00). Polynomial regres-

sion data of the same compounds in the ORAC-number of sp^2-hybridized carbon category also

revealed high coefficient of determination data (R^2=0.77 (5th order), R^2=1.00 (6th order); see fig-

ure 2.13). Together, these data support the previous observations that the number and hybridiza-

tion of carbons in an antioxidant molecule may be powerful tools in assessing the relative anti-
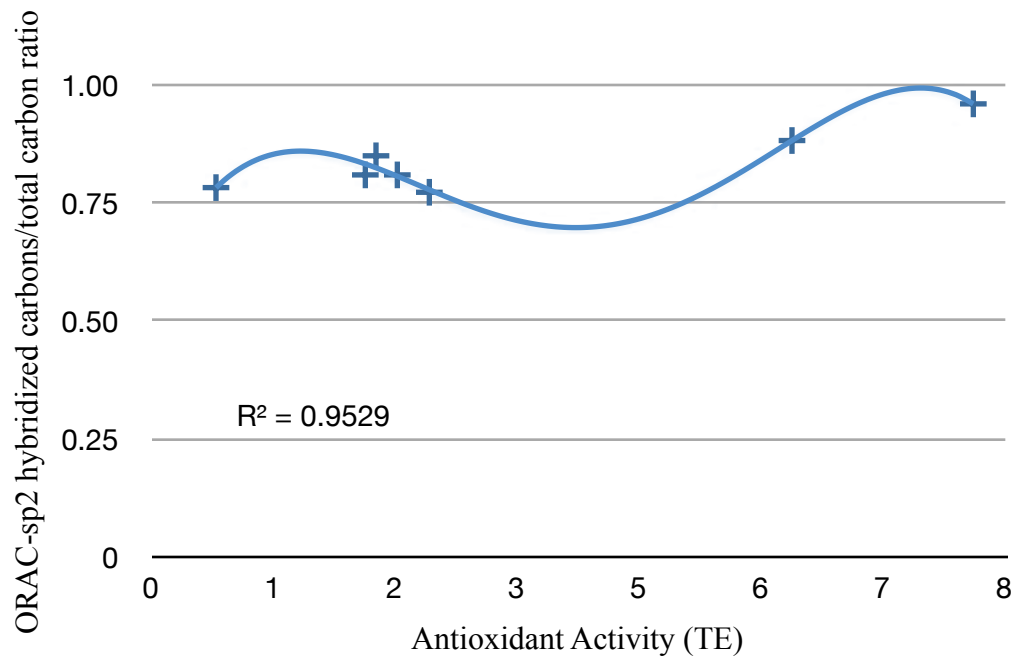
oxidant activity of that molecule.
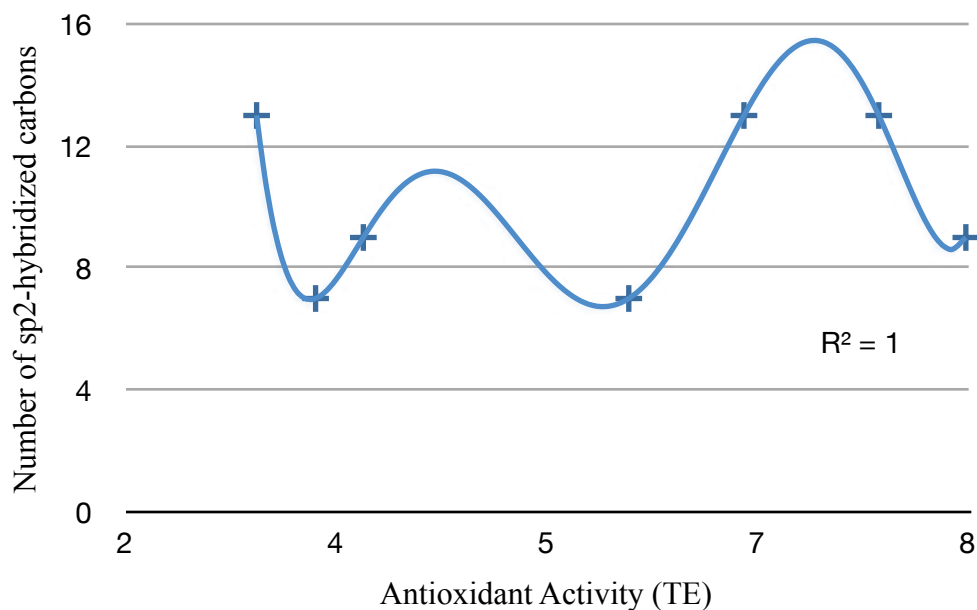
Figure 2.13: Graphical representation of sixth-order polynomial regression analysis of the correlation between an-tioxidant activity and the number of sp^2-hybridized carbons in seven nitrones. Outlier compounds 8, 11, 12, 18, and 19 are excluded here, as their individual antioxidant activities were much lower than the rest of the nitrones.

DISCUSSION AND CONCLUSION

Current studies into structure-function relationships in antioxidant molecules have been limited to comparisons between compounds of the same compound classes (52-54). While these studies have been helpful in further understanding the compound classes studied, there remains a dearth of investigations into structural characteristics that could be used to estimate antioxidant activity for any compound. The present study endeavored to elucidate which structural features of chemical compounds, if any, would be most effective for estimating antioxidant activity of chemical compounds. We used a simplest-case amino acid model to gather data from which predictions could be made regarding the antioxidant activity of non-amino acid compounds; we also tested our findings on chalcone and nitrone data from the current literature.

Overall, PMCC regression analysis suggested that there is be a linear relationship between antioxidant activity and the following structural characteristics: the overall number of sp^2-hybridized carbons the molecule contains (r=0.95), the number of conjugated double bonds a molecule contains (r=0.96), and the length of conjugated double bond chains (r=0.96). Through a *post hoc* analysis of outlying data, we observed that phenylalanine and methionine were consistently atypical compared to the trends of the rest of the group. Indeed, removal of these two compounds from analysis greatly improved coefficient of determination data in all groups ($p<0.00001$). This is undoubtedly due to the unique chemical features of these two groups (ie, benzene ring in phenylalanine and thiol group in methionine). Further study is needed to more extensively understand the antioxidative mechanisms of these two unique compounds.

Polynomial regression analysis revealed high coefficient of determination data in the ORAC-number of sp^2-hybridized carbon group ($R^2=0.94$). The sigmoid shape of this curve was even more clearly observed when the outlier histidine was removed from the group ($R^2=0.96$). The shape of this curve, supported by coefficient of determination data, suggested that number of sp^2-hybridized carbons in a molecule may be a valuable tool for predicting antioxidant activity. Using these data, we predicted the antioxidant activity of 6 new compounds from the number sp^2-hybridized carbons and sp^2-hybridized carbon ratios in the molecules. We observed that the model predicted antioxidant activity well in both categories ($r=0.97$ and $r=0.95$, respectively). Polynomial regression analysis further supported these claims.

To further explore the applicability of these models on other compounds, data were analyzed from two current works on chalcones (52) and nitrones (55) to evaluate the robustness of our findings. Again, we found that sp^2-hybridized carbon to total carbon ratio correlated well with antioxidant activity in polynomial regression analysis ($R^2=0.95$) as well as the valence electron-to-carbon ratio ($r=0.92$) in the chalcone data. Nitrone polynomial regression data also yielded high coefficient of determination data in the number of carbons group ($R^2=1.00$) and the number of sp^2-hybridized carbon group ($R^2=1.00$).

Overall, we observed that the sp^2-hybridized carbons the molecule contained was the most consistent predictor of antioxidant activity in all groups, which includes both the number of sp^2-hybridized carbons and the ratio of sp^2-hybridized carbons to total carbons. Valence electron to carbon ratio also emerged as an important structural characteristic, as well as the length of conjugated double bond groups a molecule contains. These structural characteristics undoubt-

edly act directly in quenching oxygen radicals by donating free electrons, but further study is needed to completely understand these unique structural components.

It is also interesting to note that while high coefficient of determination data were observed in both the chalcones and amino acids with regards to sp^2-hybridized carbon characteristics, the shape of the regression curve was not sigmoidal in the chalcone model, as observed in the amino acid model. Further testing of these compounds may help to elucidate more accurate trends, as well as nonlinear relationships in these data.

We recognize that there are limitations to this study. First, although the study contained a modest sample size (48 total compounds were analyzed), future studies should further extend the number of compounds included in correlation analysis. Only large-scale studies (as opposed to studies analyzing less than 10 compounds, for example), will be helpful in discovering trends that provide broad applicability. Second, while using amino acids as a simplest-case model proved helpful in this study, future studies should use combinations of water-soluble and lipid-soluble compounds for their simplest-case models. This will provide an enhanced degree of accuracy for observing antioxidant structure-function relationships in future work.

A limitation in the current literature is the variety of units in which researchers report data. Examination of external data to use in the validation of our model proved difficult, as inconsistencies among researchers were observed in the reporting of antioxidant data. We therefore recommend that, when reporting antioxidant data for raw chemicals (such as flavonoids, vitamins, amino acids, etc.), μmol Trolox Equivalents per milligram (TE/mg) is used (as opposed to TE/μmol); for bulk volume samples (such as liquid or juice samples), μmol Trolox Equivalents per liter (TE/L); and finally, for raw fruit or vegetable samples, we recommend that μmol Trolox

Equivalents per 100 grams (TE/100g) is used (57). Consistency in reporting data will undoubtedly result in more effective communication and comparative analyses among researchers.

Further research should also include more thorough analyses of the cellular roles of amino acids and other vitamins in quenching oxygen radicals. We believe that further use and application of the concepts outlined in this work will help in the increased understanding of antioxidant structure-function relationships, as well as provide useful tools for pharmaceuticals, industry, and academia alike.

CHAPTER III: FOOD PRODUCT ANALYSIS

MATERIALS AND METHODS

BLUEBERRIES
Background

In today's marketplace organic foods have become a very popular topic of discussion. In recent years, while popularity of organically-grown fruits and vegetables have grown, concerns have been raised over these fruits' problems with nutrient supply, as well as problems with soil-borne pests and diseases (73). While studies have begun to examine the differences between organic and conventional fruits in terms of nutrient content and antioxidant content, mixed results have been observed (74, 75).

Blueberries are known to possess antioxidant activity, contain high amounts of flavonoid antioxidants (57, 75, 76), and have also been shown to increase plasma antioxidant capacity in humans through increasing uric acid levels (77). In this study, antioxidant levels of organic blueberries and conventional blueberries were analyzed and compared in skin, seed, and juice components using the ORAC assay.

Oxygen Radical Absorbance Capacity (ORAC) Assay

All samples were analyzed using a modified ORAC assay based on published methods. Readings were taken every 2 minutes for 90 minutes. Measurements were taken using the PE Packard Fusion Universal Microplate Reader. All readings were taken at 37° C; 485 nm excitation and 590 nm emission filters were used. Data were analyzed using Apple Numbers. Skin and seed data are represented as Trolox Equivalents/100 mg sample (57). Juice is reported as Trolox Equivalents/L.

Blueberry Samples

Conventional and organic samples were randomly chosen for analysis from bulk samples obtained from local grocers. Bulk samples were purchased several times on different days, and from those bulk samples blueberries were selected randomly for analysis. Samples were analyzed according to their antioxidant capacity in skin, seed and juice after samples were separated, homogenized and diluted using DDH_2O. Seed and skin samples were prepared by homogenizing a 100mg sample and dissolving it in to 10mL DDH_2O. Juice samples were prepared by homogenizing 100mg of the entire fruit and dissolving it into 10mL DDH_2O. Samples were placed in aliquots of 2.5mL and stored at -20°C immediately following sample preparation.

Apples
Background

Apples have also been included in debates regarding the overall benefits of organic versus conventional fruits. Apples are known for their high concentration of vitamin C, as well as vitamin B_6, calcium, and flavonoids (57, 76, 78). Studies comparing organic and conventionally-grown apples are scarce, and have returned results finding no significant differences between the two groups (79, 80). In this study, antioxidant levels in six varieties of organic and conventional apples were analyzed and compared in skin and flesh components using the ORAC assay.

ORAC Assay

All samples were analyzed using the ORAC assay as previously described. Readings were taken every 2 minutes for 120 minutes. Measurements were taken using the PE Packard Fusion Universal Microplate Reader. All readings were taken at 37° C; 485 nm excitation and

590 nm emission filters were used. Data were analyzed using Apple Numbers. Skin and flesh data are represented as Trolox Equivalents/100 mg sample (57).

Apple Samples

Six common apple varieties were selected for inclusion in the study: Red Delicious, Golden Delicious, Fuji, Granny Smith, Gala, and Honey Crisp. Bulk samples consisting of organic and conventional apples from each group were purchased several times on different days from local grocers, and from those bulk samples apples were selected randomly for analysis. Table 4 below shows the weights that were homogenized and dissolved into 10mLs DDH$_2$O to prepare stock samples.

Table 4: Weights Used for Apple Sample Preparation.

Skin Weights	Grams	Flesh Weights	Grams
Conventional Honey Crisp	0.130	Conventional Honey Crisp	1.001
Organic Honey Crisp	0.159	Organic Honey Crisp	1.001
Conventional Granny Smith	0.522	Conventional Granny Smith	1.001
Organic Granny Smith	0.160	Organic Granny Smith	1.001
Conventional Gala	0.158	Conventional Gala	1.001
Organic Gala	0.154	Organic Gala	1.001
Conventional Fuji	0.143	Conventional Fuji	1.001
Organic Fuji	0.172	Organic Fuji	0.9571
Conventional Red Delicious	0.166	Conventional Red Delicious	1.005
Organic Red Delicious	0.165	Organic Red Delicious	1.044
Conventional Golden Delicious	0.152	Conventional Golden Delicious	1.019
Organic Golden Delicious	0.166	Organic Golden Delicious	1.040

Once prepared, flesh stock samples were diluted 1:25 and skin samples diluted 1:60 prior to analysis in the ORAC assay. Stock samples were sealed and stored at 4°C.

BLUEBERRIES

For solid blueberry components (ie, skin and seed), conventional skin was measured to have the highest overall antioxidant activity (7892 TE/100g), followed by conventional seed (1015 TE/100g). Organic skin (4623 TE/100g) and organic seed antioxidant activity (636 TE/100g) values were consistently lower. Student's t-test showed that the differences between the samples were statistically significant (p=0.0013 for skin samples; p=0.012 for seed samples; figure 3.1).

Figure 3.1: Solid Blueberry Components: Organic vs Conventional
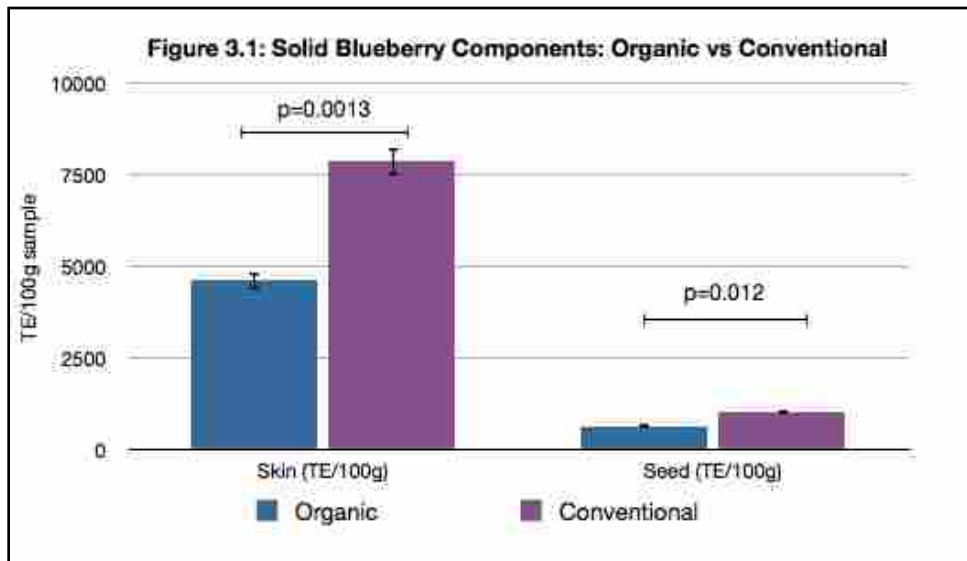
Together, the overall solid blueberry components resulted in a higher overall antioxidant activity in the conventional blueberries (8906 TE/100g) when compared to the organic blueberries (5258 TE/100g). This difference was statistically significant (p=0.0018). Figure 3.2 shows the total antioxidant activity of the conventional solid components compared to the total antioxidant activity of the organic solid components.

Figure 3.2: Total Antioxidant Activity
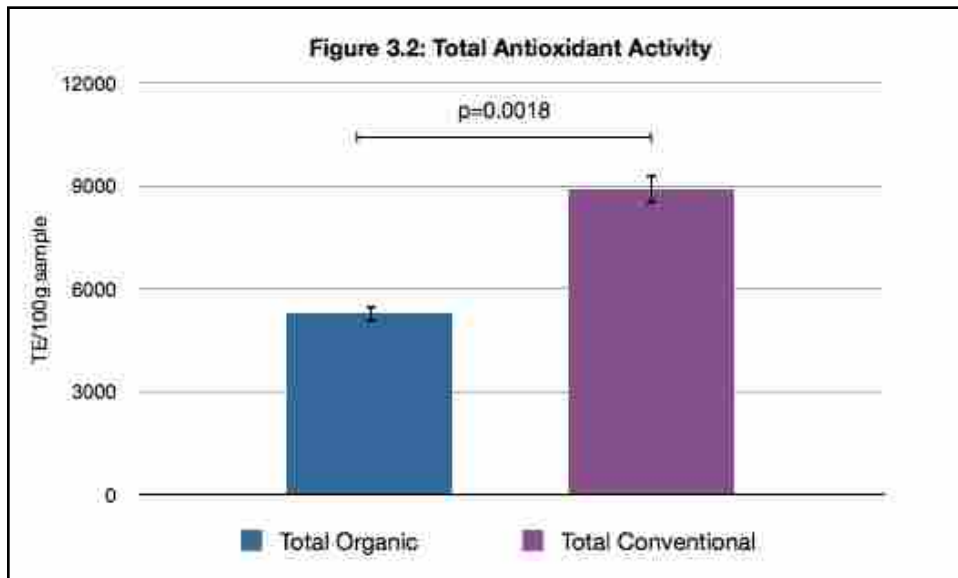
For liquid components, similar trends were observed: conventional juice (576166 TE/L) showed higher antioxidant capacity than organic juice (380452 TE/L). Student's t-test also revealed that the difference between the two groups was statistically significant (p=0.0012; figure 3.3).

Figure 3.3: Liquid Components: Organic v Conventional
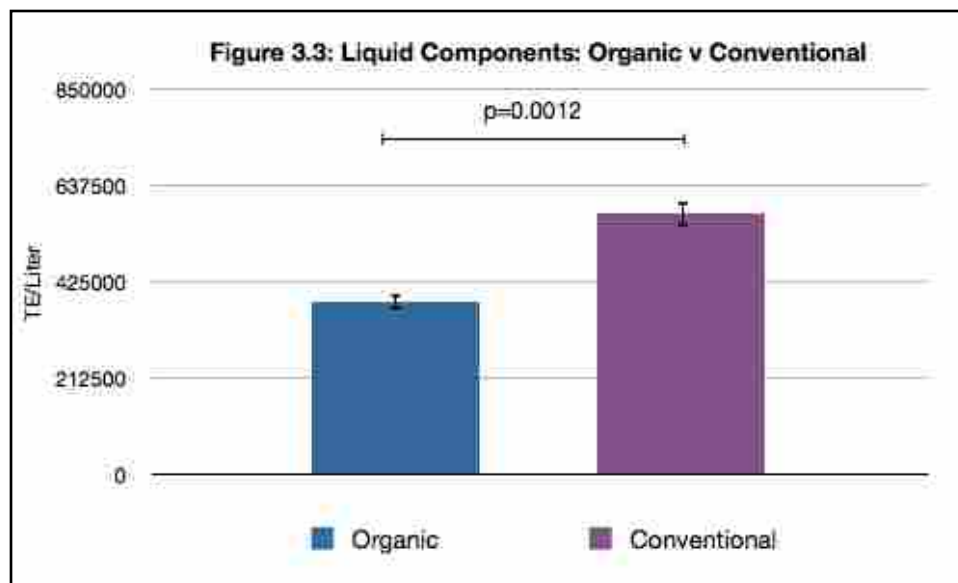
In all six apple varieties, skin components revealed higher antioxidant activities than flesh components, consistent with previous research (57), and were statistically significant, as measured by Student's t-test (p=0.0003). Overall, the highest measured antioxidant activity was found in the skin of the conventional Red Delicious variety (23653 TE/100g), and the lowest was found in the flesh of the organic Red Delicious variety (-127 TE/100g). The lowest of the skin group was the conventional Granny Smith samples (1345 TE/100g), which was equal to the highest of the flesh group, which was the organic Fuji group (1345 TE/100g).

Figure 3.4 shows a graph comparing organic and conventional skin samples, and figure 3.5 shows a graph comparing organic and conventional flesh samples.

Figure 3.4: Organic v Conventional Apple Varieties (Skin)

Figure 3.5: Organic v Conventional Apple Varieties (Flesh)
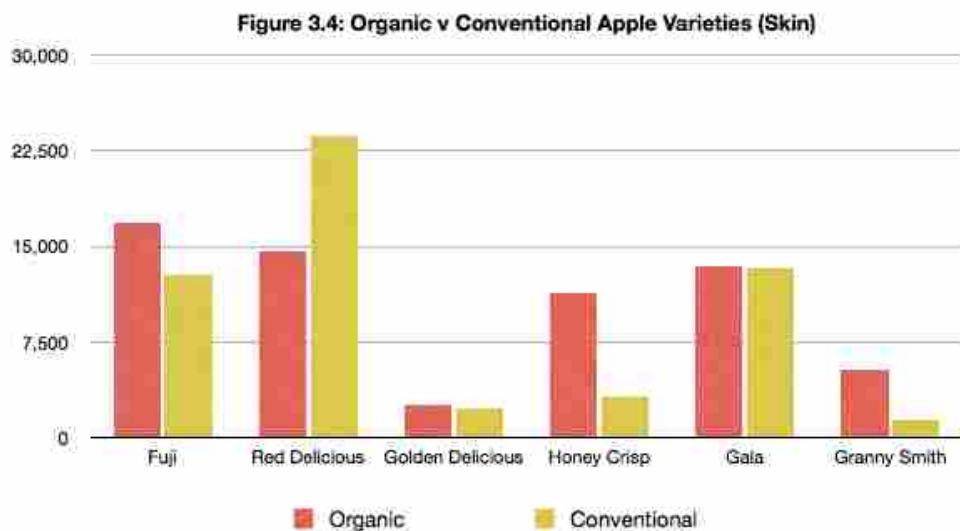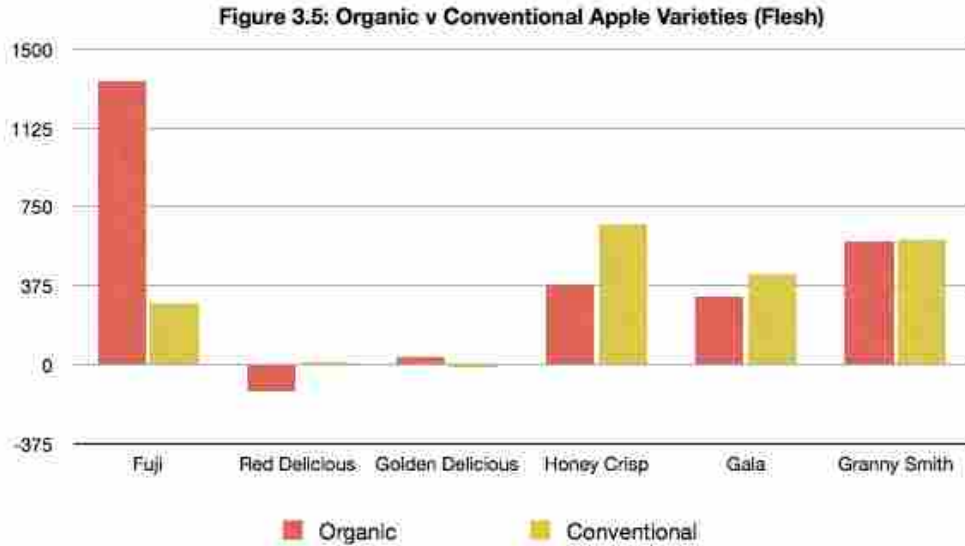
The data above represent averages of several replicates of each sample. The fewest number of replicates was done on the Organic Honey Crisp flesh group (n=33), and the greatest number of replicates was done on the Organic Fuji flesh group (n=105). On average, each group contained n=50 replicates.

Figure 3.6 summarizes the total antioxidant activities of each apple variety (including skin and flesh), comparing organic and conventional samples. Overall, the data revealed that there were statistically significant differences among the different types of apples, but, interestingly, differences between organic and conventional samples were not statistically significant overall. The only statistically significant difference was observed between skin and flesh means (see table 5).

Figure 3.6: Total Antioxidant Activity of Six Apple Varieties (Organic v Conventional)
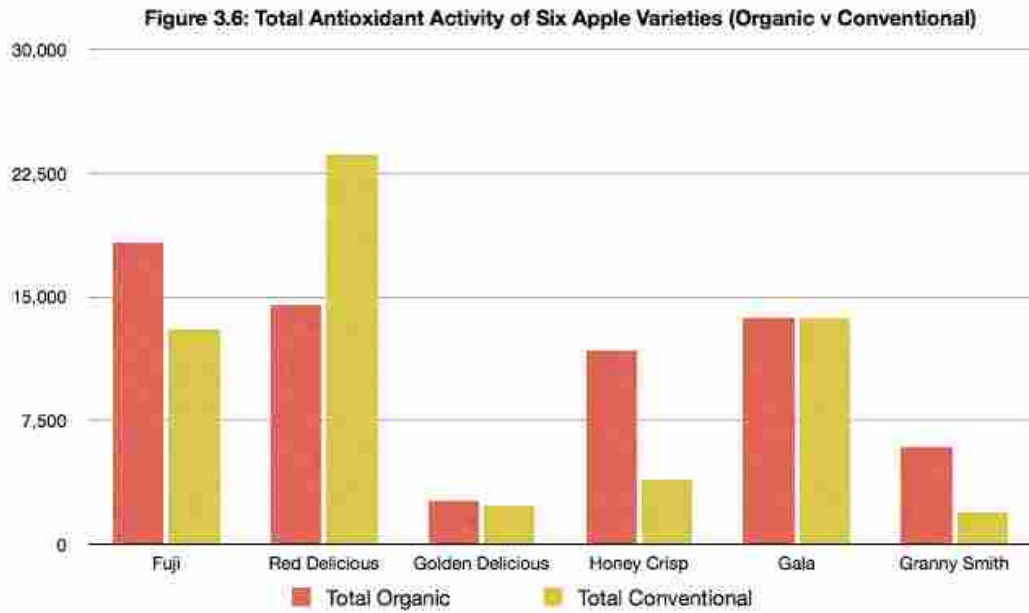
Table 5: t-Test Data Comparing Organic and Conventional Results for Each Category.

t-Test Data	**p**
Total Organic v Total Conventional	0.3018
Organic Skin v Conventional Skin	0.3115
Organic Flesh v Conventional Flesh	0.3240
Total Skin v Total Flesh	0.0003

Figure 3.7 below shows a matrix of t-test data comparing each apple group to the other apple groups (p values shown). As mentioned, there were statistically significant differences between the total antioxidant activities of each group.

Figure 3.7: t-Test Matrix Comparing Differences Among Apple Groups (p values)

	Fuji	Red Delicious	Golden Delicious	Honey Crisp	Gala	Granny Smith
Fuji	1.000					
Red Delicious	0.358	1.000				
Golden Delicious	0.059	0.088	1.000			
Honey Crisp	0.052	0.205	0.195	1.000		
Gala	0.299	0.226	0.004	0.185	1.000	
Granny Smith	0.017	0.130	0.285	0.146	0.064	1.000

CONCLUSION

Data in the current literature regarding differences in antioxidant activity between organic and conventionally grown fruit are both limited and inconclusive (73, 75, 79, 80). While papers have suggested that organically grown fruit may contain more antioxidant activity than their conventional counterparts (75), others have shown the opposite (74), and still others have observed non-significant differences (79, 80). Interestingly, data presented here both support and refute the current literature.

Blueberry data revealed highly significant differences between organic and conventional samples in skin (p=0.0013), seed (p=0.012), and juice (p=0.0012) components. Moreover, there were also statistically significant differences observed between overall antioxidant activities of organic and conventional blueberries (p=0.0018). These results suggest that there are significant differences between organic and conventional blueberries, with conventionally-grown samples possessing much higher antioxidant activity. Although the data are contrary to previously-published results, we are confident that the data are reliable because of the large sample sizes and the methods employed in producing fresh samples for each run. Future studies may further explore the flavonoid content of these samples to test for both their bioavailability and concentra-

tions in both organic and conventionally-grown blueberries. Studies on the effects of pesticide use on the antioxidant activities of organic and conventionally grown food products may also be beneficial.

In contrast, apple data revealed no statistically significant differences between organic and conventionally grown samples. While sample sizes were reliably large, no significant differences were observed between skin samples ($p=0.312$), flesh samples ($p=0.324$), or total antioxidant activity ($p=0.302$). The only significant differences that were observed were not between organic and conventional samples, but between skin and flesh samples in all apple varieties ($p=0.0003$); this trend was expected, as it has been observed in apples previously (57). Also, there were statistically significant differences between certain apple types (see figure 3.7); further analysis of these apple types may provide insights regarding these differences. Overall, these data support previously published data on apples (79), and add to the overall body of knowledge concluding that there are no significant differences between organic and conventional culture methods with respect to antioxidant activity.

Notwithstanding, these results are important contributions to the body of knowledge that constitutes this new and rapidly-growing area of research. As additional data are accumulated, true relationships between conventional and organically-grown blueberries may be elucidated. Further study is needed to more adequately examine the nutrient content in these products, to provide understanding regarding the observed differences among the groups. Further study will provide increased understanding of the effects of organic and conventional growth systems on antioxidant capacity, vitamin and mineral content, and overall food quality.

Chapter 17

Measuring Antioxidant Capacity Using the ORAC and TOSC Assays

Andrew R. Garrett, Byron K. Murray, Richard A. Robison, and Kim L. O'Neill

Abstract

Recent epidemiological studies have shown that there may be a link between oxidative stress and the development of several types of chronic diseases. Studies have also shown that diets rich in fruits and vegetables may decrease the incidence of cancer and other chronic diseases. The antioxidant activity of the phytochemicals these foods contain may be partially responsible for the decreased incidence of these diseases in people who regularly consume them. While there are several assays currently used to assess the antioxidant activity of phytochemicals and other antioxidant compounds, two are reviewed here in detail. The first is the oxygen radical absorbance capacity (ORAC) assay, which measures the decrease in fluorescence decay caused by antioxidants, and the second is the total oxyradical scavenging capacity (TOSC) assay, which measures the decrease in ethylene gas production caused by the inhibition of the thermal hydrolysis of ABAP (2,2′-Azobis(2-methyl-(propionamidine) dihydrochloride) by KMBA (α-keto-γ-(methylthio)butyric acid sodium salt) in the presence of antioxidant compounds. These two assays are discussed here, with an in depth review of their methodology and correlation.

Key words: ORAC, Oxygen radical absorbance capacity assay, TOSC, Total oxyradical scavenging capacity assay, Antioxidant, Phytochemical, Oxidative stress, Fluorescein, Trolox equivalents

1. Introduction

It has been theorized that the development and progression of several chronic degenerative diseases may be caused or augmented by oxidative stress (1-11). Much of this oxidative stress is generated as a by-product of fundamental metabolic processes that occur constantly. It is known that reactive oxygen species (ROS) react readily with biological substances, and can destroy or damage such molecules as nucleic acids, proteins, and lipids (12). This damage, in turn, can lead to a variety of degenerative diseases, including cancer, heart disease, Alzheimer's Disease,

and Parkinson's Disease (1). While many of these biological systems are somewhat equipped to cope with this oxidative stress, oxidative damage may still occur.

Several different assays have been specifically designed to accurately describe the antioxidant activity of various phytochemicals, vitamins, and other compounds. Some assays measure the inhibition of a test reaction by various antioxidant compounds (22, 23). These include the TEAC (Trolox Equivalent Antioxidant Capacity), Radox TEAC, FRAP (Ferric Reducing/Antioxidant Power), HORAC (Hydroxyl Radical Averting Capacity), FOX (Ferrous Oxide-Xylenol Orange), and TRAP (Total Radical Antioxidant Potential) assays. Other assays involve electron spin resonance (ESR) spectroscopy and use techniques such as time-resolved pulse radiolysis and spin trapping. Many others are also used (8, 14-16, 18, 19, 22-31).

However, these existing assays are not without limitation (1, 8, 24, 25). First, not all of these assays give the same trends for antioxidant activity. For example, poor correlation has been observed between the FRAP and TEAC, the ORAC and the FRAP, and the ORAC and TEAC assays (1, 8). Second, some of the data taken in vitro are difficult to extrapolate to an in vivo model because some of these assays are not run at physiological pH. Third, some of these assays do not measure the bioavailability of antioxidant metabolites in vivo. Thus, multiple assays should be investigated and validated continuously to resolve questions about the true antioxidant capacity of a given sample. The use of multiple assays will undoubtedly lead to the most accurate depiction of antioxidant capacity.

Two important assays, the TOSC and the ORAC, will be examined here, including an in depth look at methodology and correlation.

1.1. Oxygen Radical Absorbance Capacity Assay

The ORAC assay is a widely accepted tool for measuring the antioxidant activity of various vitamins, phytochemicals, and other organic and inorganic compounds (18). The ORAC assay measures the oxidative degradation of a fluorescent molecule (usually fluorescein sodium salt or beta-phycoerythrin) after being mixed with an oxygen radical initiator (14, 16). Fluorescence intensity decreases as oxidative degradation increases, with fluorescence typically recorded over 100 min from the addition of the oxygen radical initiator. When an antioxidant compound is mixed with the fluorescent molecule and the oxygen radical initiator, however, it can protect the fluorescent molecule from degradation, and the fluorescence intensity is thus maintained over time. Thus, the longer the fluorescent molecule maintains its intensity, the less it is being degraded by the oxygen radicals present, or the more it is being protected by the antioxidant compound that is present.

A phycoerythrin fluorescence-based assay for ROS was originally developed (20) and was later modified (21) to create the ORAC assay. Since then, the ORAC assay has become the current food industry standard for assessing antioxidant capacity of food additives, whole foods, juices, and raw vitamins (15, 16, 19).

The ORAC assay has several advantages, including reliability, high sensitivity, and the ability to measure the antioxidant activity of chain-breaking antioxidants (8, 18, 19). Also, the ORAC assay can accurately measure both the inhibition time and the inhibition degree of antioxidants to provide an accurate measurement of antioxidant activity (18).

Some problems with the early ORAC assays, however, were their sensitivity to temperature variations (a problem solved by preheating well plates) and their inability to measure a complete range of biologically relevant radicals, since they can only measure peroxyl radicals (18, 19, 27). In addition, it is important that the pH of the fluorescein be maintained above 7, as it is pH sensitive. Despite these small obstacles, the ORAC remains among the most preferred assays for measuring antioxidant capacity, and has even been suggested to be the most useful for ranking antioxidants. It is recommended that the ORAC be used to quantify peroxyl radical scavenging capacity (14).

1.2. Total Oxyradical Scavenging Capacity Assay

The Total Oxyradical Scavenging Capacity (TOSC) assay is another effective assay used to assess antioxidant activity. In contrast to the ORAC, the TOSC measures the decrease of ethylene production caused by antioxidants (32). Moreover, its advantages include its ability to measure the antioxidant capacity of biological tissues and to measure both lipid and water soluble antioxidants, similar to the ORAC. Unlike the ORAC, however, the TOSC has the capability of distinguishing between faster acting and slower acting antioxidants, as it is a kinetically based assay (28, 29).

2. Materials

2.1. Equipment

1. HP 5890A gas chromatograph along with the Hp ChemStation GC 5890 online program

2. Fluorescence Microplate Reader used with a COBAS FARA II spectroflourometric analyzer (Roche Diagnostic Systems), or BMG FLUOstar Optima fluorescence microplate reader (*see* Note 1).

3. Clear-bottom well plates: The plate size should be selected based on the instrument capabilities

2.2. Reagents

2.2.1. ORAC Assay

1. AAPH: For the ORAC assay, AAPH (2,2′-azobis(2-amidino-propane) dihydrochloride) is used as the free-radical initiator because it forms free radicals at a constant rate (26). Antioxidants quench the oxygen radicals generated by AAPH, thereby inhibiting fluorescence decay (14, 27).

2. Flourescein Sodium Salt: A modified ORAC that used fluorescein instead of β-phycoerythrin was developed after the original ORAC assay was validated (15). Fluorescein was preferred because of its several advantages: it is less expensive than β-phycoerythrin, does not interact with other compounds, and does not photobleach (15).

3. Trolox: Trolox (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97% is a widely recognized water soluble Vitamin E derivative used as a baseline against which relative antioxidant activity is measured. Thus, results for test samples (including chemical compounds and foods) are reported and published as μmol Trolox equivalents/mg or TE.

4. Sample: Samples that have been run in the ORAC assay include a variety of antioxidant compunds, such as retinols, phenols, carotenoids, tocopherols, vitamins, phytochemicals, and whole fruits, vegetables, seeds, spices, grains, and legumes (1, 18).

2.2.2. TOSC Assay

1. ABAP: In the TOSC assay, the thermal homolysis of ABAP (2,2′-Azobis(2-methyl-propionamidine), dihydrochloride) generates peroxyl radicals.

2. KMBA (α-keto-γ-(methylthio)butyric acid sodium salt): KMBA is oxidized by ABAP to produce ethylene gas, which is then measured by gas chromatography. When antioxidants are present, however, they quench the peroxyl radicals formed by ABAP, and, consequently, ethylene gas production is inhibited (31, 32).

3. Trolox: Similar to the ORAC, Trolox is used as a standard for determining antioxidant activity in the TOSC assay. As with the ORAC assay, results from the TOSC are also reported as μmol Trolox equivalents/mg. KMBA, ABAP, and Trolox can all be obtained from Sigma-Aldrich (St. Louis, MO) and other commercial chemical manufacturers and distributers.

4. Sample: Many types of samples can be run in the TOSC assay, including vitamins, cellular enzymes, organic and inorganic

compounds, fruit and vegetable juices, and other antioxidant compounds (32, 33). Recent studies have included TOSC analyses of Acai fruits, Hydrothermal Vent Mussels, and pyrene from marine invertebrates (34-36). TOSC samples must be water soluble or DMSO (dimethyl sulfoxide) soluble.

3. Methods

3.1. ORAC Methods

The ORAC assay is an important assay because of its practicality and ease of use. It can be confusing to some researchers, however, because many investigators have incorporated their own variations on the assay (15, 17, 18, 30, 32). Steps below are taken from Tomer, et al. (32) (*see* Note 2).

1. AAPH is prepared at a concentration of 79.65 mmol/L by adding 216 mg to 10 mL phosphate buffer (75 mM, pH 7.4). Caution must be used when handling AAPH, as it can cause apoptosis, and damage to the liver and kidney, as well as to capillaries and lymphocytes (26).

2. Fluorescein is then prepared by dissolving 22.5 mg into 50 mL PBS to make a first stock solution. A second stock solution is prepared by adding 50 µL of the first fluorescein stock solution to 10 mL of phosphate buffer. Finally, 320 µL of the second fluorescein stock solution is added to 20 mL of phosphate buffer.

3. Experimental samples and control samples (including Trolox) are also prepared at this point. Special care should be taken to ensure that each sample is prepared and stored properly. Samples may be either water soluble or DMSO soluble.

4. 400 µL of fluorescein and 40 µL of sample are first pipetted into each well. Phosphate buffer is used as a blank, and a Trolox dilution series (50, 25, 12.5, and 6.25 µM) is used as a standard.

5. When these aliquots are added to each well, fluorescence readings are taken for time 0. During cycle 4, the reaction is initiated by injecting 150 µL of AAPH into the respective wells (3.5 min and 90 s time intervals can also be used). Fluorescence readings may be taken until the flourescence counts have decreased to negative control amounts. Previous studies have been performed using various cycle times and varying time intervals (15, 17, 32).

6. Fluoresence readings are taken every 3.5 min, up to 35 cycles. Flourescence readings for each cycle are saved, as they will all be used to calculate areas under the curves and Trolox Equivalents.

3.2. TOSC Assay

The time required for a complete run is between 2½ and 3 h (1½ h actual run time in gas chromatograph). Additional time should be expected for comprehensive data analysis.

1. Reagent Preparation: A 200 mM concentration of ABAP is prepared by adding 434 mg ABAP and ddH$_2$O up to 8 mL total volume. A 20 mM concentration of KMBA is prepared by adding 2.7 mg KMBA and ddH$_2$O up to 8 mL total volume. Following their preparation, the respective vials of ABAP and KMBA should be maintained on ice. A 100 mM potassium phosphate buffer at pH 7.4 is also prepared (32). Experimental and control samples to be tested should also be prepared at the desired concentrations at this point.

2. Vial Preparation: In 10 mL glass vials, 100 μL PBS (Phosphate Buffered Saline) is added to the bottom of each. Next, 690 μL ddH$_2$O is added to the sample vials. Vials are then vortexed. After vortexing, 100 μL KMBA are added to all vials. Next, vials are capped and vortexed a second time. After each sample is vortexed the second time, a 10 μL sample is then added to each sample vial, and the vials are again capped.

3. Incubation & ABAP Injection: The septa-sealed vials should next be incubated for 5 min in a 37°C water bath to equilibrate. After the 5 min incubation period, 100 μL ABAP is added into the vials to initiate the reaction (ABAP should be vortexed before each injection).

4. Gas Chromatograph Preparation: GC temperatures are set as follows: 160°C for the injector port, 60°C for the oven, and 220°C for the flame ionization detector (FID). It is recommended that the injection port septa be changed before each run. The hydrogen/air balance at the detector exhaust should be set to 8–12%. Mobile phase helium can be used to push the other gases though the gas chromatograph at a flow rate of 30 mL/min. Once lit, the baseline signal value is normally around 10. Slight variations may be due to the amount of air/hydrogen intake. Once the GC injection syringe barrel is cleaned and the machine prepped, injections and measurements may begin.

5. Gas Chromatography: Twelve minutes after the first ABAP injection, a 1 mL aliquot of gas from each vial should be injected into the GC at staggered intervals. At time 10 min, data should be saved, as area under the curve for these data will later be calculated. At time 12 min, the 8 injections are repeated at the same time intervals 7 times, for a total of 8 passes that make up a 96 min run. Data saved from every cycle will be used to calculate area under the curve.

3.3. Results

3.3.1. Trolox Equivalents

The most important aspect of both of these assays is calculating Trolox equivalents, or TE. As mentioned, Trolox equivalents represent μmol Trolox equivalents/mg sample.

The area under the flourescence kinetic curve (AUC) for each sample is calculated as

$$AUC = (0.5 + f_1/f_1 + f_2/f_1 + f_3/f_1 + \dots + f_n/f_1) \times CT$$

where f_1 = initial fluorescence reading at cycle 1, f_n = flourescence reading at cycle n, and CT = cycle time in min (15, 32).

In order to calculate Trolox equivalents, a Trolox standard curve must first be obtained. This is done by performing the ORAC or TOSC assay first on varying concentrations of Trolox (most preferably a serial dilution, which will make calculating a standard curve simpler and more accurate), and calculating the individual AUC values for each concentration using the method described above. Once the AUC values for the Trolox concenrations have been calculated, the AUC for the blank well must also be calculated and then subtracted from the total AUC for each Trolox concentration. Subtracting the blank AUC from the Trolox (and later from each sample) AUC gives the net AUC, which accounts for the background readings given by the flouresence reader. This concept is shown in Fig. 17.1. The area *between* the two curves represents the net AUC:

Net AUC = Sample AUC – Control AUC

A Trolox standard curve is then created from the net AUC values for each concentration of Trolox used. The slope of the standard curve is then calculated and used to convert net area under the sample curve to Trolox Equivalents per liter (TE/L). TE/L are then converted to Trolox Equivalents per miligram sample for reporting.

3.3.2. ORAC/TOSC Correlation

To date, only a few studies have been performed that assess the correlations among the differing antioxidant assays (8, 32). Of these, Cao and Prior (8) have found weak but significant
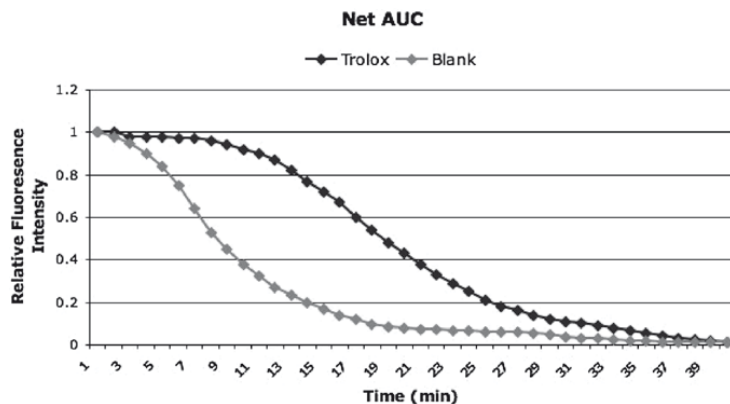
Fig. 17.1. Subtracting the blank AUC from the Trolox or sample AUC gives the net AUC.

correlation between the ORAC assay and the FRAP assay, but no correlation between the ORAC and the TEAC assays.

One study has also been performed to assess the correlations between the ORAC and TOSC assays (32). While precise correlation was not observed, the trends observed in each case were the same: Green Tea Polyphenols and MegaNatural Gold Grape seed extract yielded high TOSC values and high ORAC values; Lemon Fruit 12:1 and cirus bioflavonoids yielded low TOSC values and low ORAC values; and Grape skin extract, GSKE-40 grape seed extract, quercitin, pycogenol, and pine bark theraplant all yielded moderate TOSC values and moderate ORAC values.

Figure 17.2 contains some of the data from the study (32). This figure demonstrates the relative activity of the 11 phytochemicals measured by the TOSC assay (light bars) and by the ORAC assay (dark bars). Minimum and maximum values for each assay were scaled to 0% and 100%, respectively, to faclilitate inter-assay comparisons. The numbers represent the phytochemicals as follows: (1) lemon fruit, (2) citrus bioflavonoids, (3) pomegranate, (4) grape seed extract, (5) pine bark theraplant, (6) quercitin, (7) pycnogenol, (8) grape seed extract, (9) rutin, (10) alpha lipoic acid, and (11) green tea polyphenols. Values reflect the mean and standard deviation.

Moderate correlation between average TOSC and average ORAC values was observed among the 11 phytochemicals ($R^2 = 0.60$). TOSC and ORAC values are both in µmol Trolox equivalents/mg and are the average of several replicates using both the TOSC and ORAC assays (Fig. 17.3).
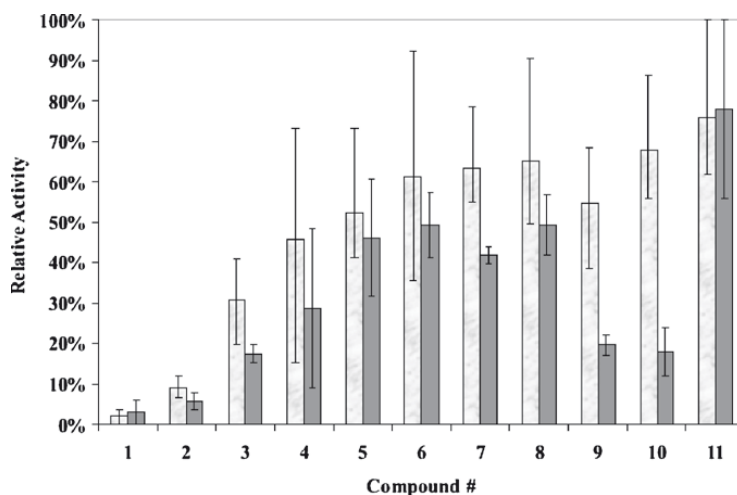
Fig. 17.2. Correlation between the ORAC and TOSC assays across 11 compounds.
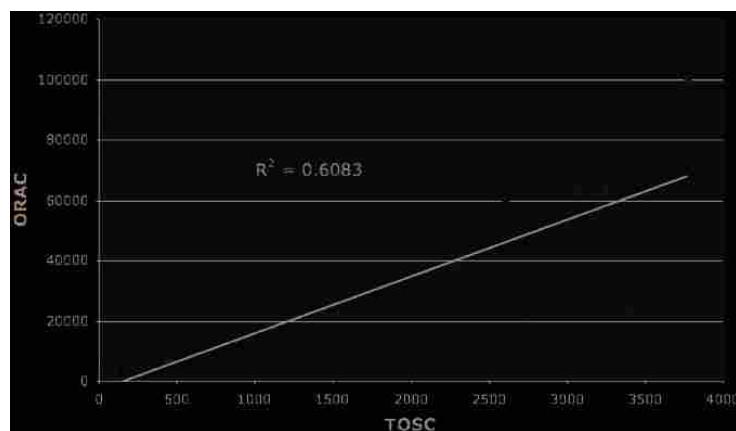
Fig. 17.3. Correlation between the ORAC and TOSC assays acorss 11 compounds.
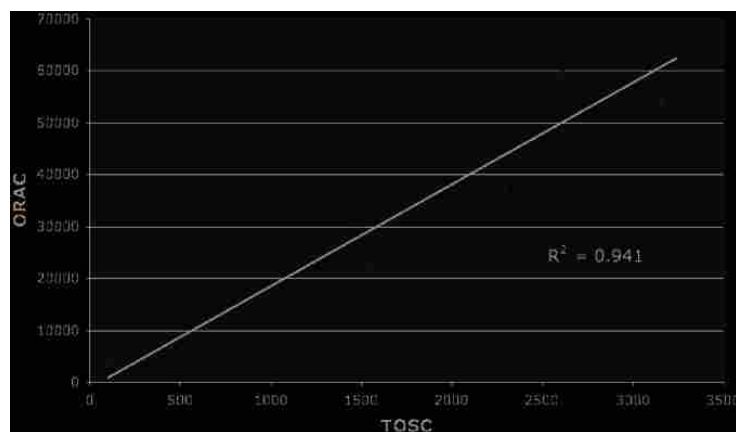
Fig. 17.4. Correlation between the ORAC and TOSC assays (7 outliers excluded).

When the outliers rutin, alpha lipoic acid, and green tea polyphenols were removed from the dataset, high correlation between the remaining TOSC and ORAC assay values was observed ($R^2 = 0.94$). Again, TOSC and ORAC values are represented in μmol Trolox equivalents/mg and are the average of several replicates using both the TOSC and ORAC assays (Fig. 17.4).

Included are tables that show the measured average TOSC and ORAC values obtained over several replicates (Fig. 17.5).

Comparing the two assays revealed that more variability in antioxidant activity could be seen for the TOSC assay than for

Phytochemical	TOSC[a]	ORAC
Lemon Fruit	103	3885
Citrus Bioflavonoids	446	7410
Pomegranate	1543	22530
Grape Seed Extract	2294	37125
Pine Bark Theraplant	2608	59483
Quercitin	3059	63750
Pycnogenol	3154	53970
Grape Skin Extract	3246	63668
Rutin	2727	25440
Alpha Lipoic acid	3381	23235
Green tea polyphenols	3377	100658

Fig. 17.5. The measured average TOSC and ORAC values for 11 compounds.

the ORAC assay. Such variability may have been due to slight procedural errors, including error in the injection process into the gas chromatograph. Establishing specific numbers for the antioxidant activity of certain phytochemical compounds also proved difficult because the conditions under which the fruit or vegetable samples were cultivated and stored varied greatly from sample to sample. Despite small variations in sampling, the antioxidant activity ranking of these phytochemical compounds was similar among replicates (32).

Absolute values of the antioxidant counts for the ORAC and TOSC assays varied greatly in this study because different radical sources are used for the two assays (AAPH is used for the ORAC assay, while ABAP is used for the TOSC assay), and also because samples measured in the ORAC and TOSC assays were prepared at different concentrations because of the enhanced sensitivity of the ORAC assay.

While the measurement of any antioxidant compound in vitro is a valuable first step for identifying good antioxidants for further use in in vivo testing, it should not be assumed that the antioxidant activity of a compound in vitro will necessarily reflect the antioxidant activity in vivo. As mentioned, studies have shown that consumption of fruits and vegetables results in elevated plasma ORAC values in humans (13), and synergy is often observed between multiple antioxidant compounds in vivo (7, 13).

The discrepancies (32) seen between the ORAC and TOSC assays suggest that in some cases, the results from both assays may be useful in determining an antioxidant's true activity. As other antioxidant assays are also used and developed, discrepancies between these assays pertaining to the relative antioxidant activity of various samples will likely be greatly diminished. As more research is performed, the realm of predictive power will increase, and the ORAC and TOSC assays will continue to be useful tools for assessing antioxidant activities.

4. Notes

1. Microplate readers may vary somewhat in their available excitation and emission filters. Cao and Prior used an excitation wavelength of 540 nm and an emission wavelength of 565 nm (8); Tomer used an excitation wavelength of 485 nm and an emission wavelength of 520 nm (32). Ou used an excitation wavelength of 493 nm and an emission wavelength of 515 nm (15). The wavelengths available on the instruments must be set to be able to read fluorescein emissions in order for ORAC data to be collected and analyzed properly.

2. There have been many different methods used in published research articles for the ORAC assay. Some alternative methods include, for example, the following: Ou used an AAPH concentration of 12.8 mM and a fluorescein concentration of 48 nM. When putting aliquots into each well, Ou used 365 µL Fluorescein, and 20 µL sample (15). Huang (18) used a final AAPH concentration of 153 mM by dissolving 0.414 g AAPH into 10 mL of 75 mM phosphate buffer (pH 7.4). A flourescein stock solution of 4×10^{-3} mM was prepared in 75 mM phosphate buffer (pH 7.4), which was stored and wrapped in foil at 5°C. This stock solution was diluted 1:1,000 with 75 mM phosphate buffer (ph 7.4) immediately prior to use.

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CHAPTER V: BIOACTIVE FOODS PAPER

Bioactive Foods in Aging: Role in Cancer Prevention and Treatment

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Bioactive Foods in Aging: Role in Cancer Prevention and Treatment

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1. The Burden of Cancer

Cancer is a leading cause of death worldwide. In 2004, over 7.4 million people died of cancer, accounting for more than 13% of all deaths worldwide (WHO 2010). In 2010, it is estimated that there will be over 1.5 million new cancer cases in the United States alone. Estimates for 2010 also project that close to 570,000 Americans will die from cancer, which translates into great than 1,500 cancer deaths per day (ACS 2010). It is also projected that in 2020, 15 million new cancer cases will be reported and 12 million cancer patients will die worldwide (Bray and Møller 2005). However, despite numerous outreach, education, and research efforts, cancer mortality rates continue to increase.

Notwithstanding its high mortality rate, cancer is largely a manageable condition if diagnosed and treated early. The World Health Organization estimates that up to one-third of all cancers could be cured if detected early and treated adequately (WHO 2010). Furthermore, cancer is often a preventable condition. Recent estimates state that between 30% to 95% of all cancer cases could be prevented by modifying diet, lifestyle, and behavioral habits alone (Anand, Kunnumakara et al. 2008; ACS 2010; WHO 2010). Cancer, as has been astutely observed, is a "preventable disease that requires major lifestyle changes" (Anand, Kunnumakara et al. 2008).

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Such changes include modifying dietary and lifestyle habits. Tobacco use is the single largest preventable cause of cancer in the world, yet millions use tobacco regularly; among the total projected cancer deaths in 2010, 171,000 (34%) are expected to be caused by tobacco use. Evidence also suggests that close to one-third of all projected cancer deaths in 2010 will be caused by overweight or obesity, physical inactivity, or poor nutrition (ACS 2010; WHO 2010). These conditions can be especially detrimental because prolonged inflammation in obese patients is thought to be critical for tumor initiation and progression (Schmid-Schonbein 2006). Such conditions can be prevented through limiting consumption of energy-dense foods, avoiding sugary drinks and alcohol, limiting the frequent intake of red meats, and increasing consumption of foods of plant origin (AICR 2007).

Many cancers caused by infectious agents can be prevented through the use of vaccines and antimicrobials, as well as lifestyle changes. Many types of skin cancers can also be prevented by avoiding excessive exposure to UV radiation. This can be accomplished by the implementation of sunscreen, the proper use of hats and clothing, and by the use of indoor tanning equipment. The American Cancer Society also recommends regular screenings for many different types of cancer, and reports that at least half of all new cancer cases could be prevented if these conditions were detected early while still in their pre-cancerous states (ACS 2010). It is clear that for serious improvements in cancer prevention to occur, significant lifestyle and behavioral changes must be made both in the United States and in the world at large.

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2. Bioactive Foods

2.1 What are Bioactive Foods?

As mentioned, one of the most important ways to decrease risk of cancer development and progression is by modifying diet, which is best done through limiting intake of energy dense foods, reducing excessive salt intake, limiting alcohol consumption, and replacing these with foods that contain high levels of critical nutrients; specifically foods of plant origin, including fruits, vegetables, legumes, whole grains, nuts, and oils. Such foods provide essential vitamin and minerals, while also providing healthy lipids, sugars and protein. "Bioactive" foods are those which contain high levels of bioactive compounds, such as antioxidants, antithrombotics, and anti-inflammatory substances. These compounds protect the body from inflammation, accumulation of low-density lipoprotein (LDL) cholesterol, and oxidative stress, thereby helping to prevent the development of cancer, as well as other conditions such as heart disease and other cardiovascular diseases (Kris-Etherton, Hecker et al. 2002).

2.2 Examples of Bioactive Foods

Among the most widely studied bioactive compounds are lycopene, flavonoids, phytoestrogens, acetogenins, organosulfurs, resveratrol, L-ascorbic acid, and tocopherols. These are found in a vast array of fruits and vegetables, and are important components in promoting a healthy metabolism and providing antioxidant, anti-tumor, and anti-thrombotic activities. These compounds will each be discussed below.

Lycopene is a carotenoid antioxidant found in tomatoes, gac, and pink grapefruit, which inhibits tumor cell growth and is thought to prevent prostate cancer. Many types of cereals, nuts, oils, fruits, vegetables, and red wine contain phenols of many varieties, including flavonoids. Phytoestrogens may be beneficial in reducing the risk of cardiovascular disease, and are found in

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flaxseed oil, vegetables, grains, and soy (Kris-Etherton, Hecker et al. 2002). Acetogenins are

polyketides that have demonstrated anti-inflammatory, anti-tumor and antioxidant activities, and

are found in many types of fruits, including *Annona cherimola*, guanabana, and other

magnoliales (Chang, Chen et al. 1998; Chen, Chang et al. 1999; Gupta-Elera, Garrett et al.

2010).

Organosulfur compounds, found in garlic and onions, have been shown to possess both

cardioprotective and anticarcinogenic properties (Kris-Etherton, Hecker et al. 2002; Stan, Kar et

al. 2008). Resveratrol has recently become a well-known potent antioxidant, and is found in red

grapes, blueberries, cranberries, and other types of *Vaccinium* berries (Rimando, Kalt et al.

2004). It is also known for its anti-thrombotic, anti-inflammatory, anticarcinogenic, and lifespan

elongation effects, as well as its positive role in protection against insulin resistance (Kris-

Etherton, Hecker et al. 2002; Baur, Pearson et al. 2006; Lagouge, Argmann et al. 2006; Hung,

Chen et al. 2010).

L-Ascorbic acid (vitamin C) is an important essential nutrient found in a wide variety of

fruit, vegetable, herb, and animal sources, including citrus fruits, berries, spices, leafy greens,

cabbage, tomatoes, and potatoes. Known for its role in producing mature collagen and its

usefulness in fighting the common cold, vitamin C was also linked to cancer treatment by Dr.

Linus Pauling, who observed improved outcomes in cancer patients that were given very high

doses of the vitamin as a supplement to their treatments. Since then, vitamin C has been the

focus of many studies involving to supplementation, cancer prevention, and epidemiology

(Block 1991; Packer and Colman 1999; Doyle, Kushi et al. 2006).

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Tocopherols and tocotrienols together make up the vitamin E class of compounds. Vitamin E is found in many types of raw oils, nuts, rice bran oil, barley, and leafy green vegetables, with α-tocopherol having the highest bioavailability (Brigelius-Flohe and Traber 1999). Vitamin E is an important bioactive food component, and is well-known for its protection against the harmful effects of UV light, its anti-inflammatory properties, its role in reducing the risk of prostate cancer, and its ability to inhibit cognitive decline (Reiter 1995; Packer and Colman 1999; Emerit, Edeas et al. 2004; Aggarwal and Shishodia 2006; Masaki 2010).

3. The Processes of Aging

3.1 Senescence

The English *senescence* comes from the latin *sĕnex*, meaning old or aged, and refers to the general biological processes that occur in an organism, following maturation, that eventually lead to the functional decline and death of the organism. These processes include decreased efficiency, loss of function, and decreased immune function.

These phenomena have been investigated at the cell, tissue, and organismic levels, and research has expanded in these areas in recent years. On the vertebrate level, aging has been described in terms of decreasing levels of collagen, elastin, and bone mineral content, prostate pathologies, and neurodegenerative diseases. On the cellular level, aging is the result of telomere shortening, accumulation of waste, genetic mutations, and general cellular wear and tear, all of which ultimately culminate in either apoptosis or necrosis (Bianchi-Frias, Vakar-Lopez et al. 2010; Freeman 2010; Hung, Chen et al. 2010; Pena Ferreira, Costa et al. 2010).

One of the first inquiries into the processes involved in cellular aging was performed in the early 1960s by Leonard Hayflick. In his experiments, Hayflick demonstrated that a

population of normal human fetal cells, when grown in culture, normally divides between 40 and 60 times before entering the senescence phase. In this phase, telomeres shorten, cell division eventually ends, and apoptosis results (Hayflick 1965). This observed limit of cell division came to be known as the "Hayflick limit". This limit of cell divisions was further investigated in terms of oxidative stress and aging, as incubation with alpha-tocopherol resulted in an extended lifespan of healthy human cells (WI-38 cells) to over 100 divisions (Packer and Smith 1974). These results support the free-radical theory of aging.

3.2 Free Radical Theory of Aging

In his investigations regarding the "biological clock" of life, Denham Harman first proposed a free-radical theory of aging in 1956 (Harman 1956). This theory of aging places oxidative stress as the main mechanism by which cells senesce, given that metabolic rate is linked to oxygen consumption. Although the original theory focused on the effects of superoxide, it has grown to include investigating the effects of all types of free radicals, including reactive oxygen species and reactive nitrogen species. Harman later expanded his theory to address mitochondrial production of reactive oxygen species (Harman 1972).

Much of the research involving bioactive foods centers around the free radical theory of aging, and how the antioxidant compounds in these bioactive foods act to quench the radicals that damage cellular components and processes. In order to understand these interactions more fully, it is important to understand free radical chemistry.

4. Free Radicals, Aging, and Cancer

4.1 What are Free Radicals?

Radicals are unpaired valence electrons found in various types of biological and chemical molecules. These compounds can either have one extra electron (giving them a slight negative

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charge), or be one electron deficient (giving them a slight positive charge). Either way, most compounds that have unpaired radicals are highly chemically reactive.

There are two main classes of free-radical compounds: reactive oxygen species (ROS) and reactive nitrogen species (RNS). The more common of the two groups is ROS, which are derivatives of O_2, with superoxide radical ($O_2^{\bullet-}$) being the most common. Other examples of ROS include hydrogen peroxide, (H_2O_2), alkoxyl/peroxyl radical ($RO^{\bullet}/ROO^{\bullet}$), and peroxynitrite ($ONOOH/ONOO^-$)(Liu, Chen et al. 2008). Recently, ROS have been linked to a variety of chronic diseases, including Alzheimer's disease, Parkinson's disease, and cancer (Markesbery 1997; Garrett, Murray et al. 2010). ROS have also been linked to p53 function (Liu, Chen et al. 2008), diabetes (Baynes and Thorpe 1999; Singh, Mahadi et al. 2009), neurodegenerative diseases (Emerit, Edeas et al. 2004), and cognitive decline (Reiter 1995; Pratico, Clark et al. 2002).

Reactive nitrogen species are species of free radical compounds derived from nitric oxide ($NO^{\bullet}$), and include peroxynitrite ($ONOO^-$), nitrogen dioxide ($^{\bullet}NO_2$), and dinitrogen trioxide (N_2O_3). These and other RNS, similar to ROS, have been shown to cause damage to lipids, amino acids, nucleic acids, and other small molecules (O'Donnell, Eiserich et al. 1999).

In a recent review, the signaling, cytotoxic, and pathogenic characteristics of nitric oxide and peroxynitrite were discussed in detail (Pacher, Beckman et al. 2007). Nitric oxide is an important signaling molecule because of its unique chemical properties, which include its rapid cellular diffusion (its diffusion coefficient in water is slightly higher than oxygen and carbon dioxide), and its propensity to quickly produce oxygen radicals. Peroxynitrite is another well-known RNS formed by the reaction of hydrogen peroxide and nitric oxide. Although

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peroxynitrite itself does not contain free radicals, it is a powerful oxidant, and its decomposition in the phagosome results in the formation of H_2O_2 and NO_2^- (Pacher, Beckman et al. 2007).

Similar to ROS, not all RNS are detrimental, as both are important components of the innate immune system. A study performed by Iovine et al. demonstrated that murine macrophages significantly upregulated RNS production following exposure to *Campylobacter jejuni,* and were much more effective at eliminating it when compared to mutant macrophages unable to produce RNS (Iovine, Pursnani et al. 2008). An earlier study by Neu et al. also demonstrated that prolonged inhibition of nitric oxide synthesis in human umbilical vein epithelial cells supports neutrophil adhesion, although it also lead to an increase in intracellular oxidative stress in those cells (Neu, Smith et al. 1994).

4.2 Intracellular Oxidative Stress

The amount of oxidative stress formed within the cell is highly dependent on the rate at which $O_2^{\bullet-}$ and H_2O_2 are made (Messner 2002; Imlay 2003). During normal metabolic processes, baseline levels of free radicals are produced as by-products of several chemical reactions, including mitochondrial aerobic metabolism, but potential damage caused by these stresses are constantly prevented by constitutive antioxidant mechanisms. (Sastre 2003).

As mentioned, both RNS and ROS are produced as part of the innate immune system to attack and kill pathogens. These ROS and RNS are released nonspecifically, however, which can be harmful for surrounding tissues as chemical damage may result (Rice-Evans and Gopinathan 1995). For this reason, the consequences of oxidative stress have recently been investigated with regards to inflammation, disease, and cancer development (Schmid-Schonbein 2006).

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5. Cancer

5.1 Cancer Epidemiology

Recent estimates state that as many as 90-95% of cancer cases are attributed to lifestyle factors, while the remaining 5-10% are due to genetics (Anand, Kunnumakara et al. 2008). As previously mentioned, the main lifestyle-related risk factors are alcohol consumption, tobacco use, diet, obesity, infectious agents (as in the case with several viruses like human papillomavirus), radiation, and environmental pollutants (asbestos or benzene exposure in the workplace, for example). From these data, cancer is a largely preventable condition if certain lifestyle behaviors are managed properly.

Among men in the United States, prostate cancer is the most common cancer with an estimated 217,730 new cases in 2010, accounting for 28% of all new cancers among men. Lung and colorectal cancer follow, accounting for 15% and 9% of all new cases, respectively. Among women, breast cancer is most common, with an estimated 207,090 new cases in 2010 (28% of all new cases). Similar to cancers in men, lung cancer and colorectal cancer are the next most prevalent, accounting for 14% and 10% of all new cases in women, respectively (Jemal, Siegel et al. 2010).

Currently, cancer accounts for nearly 1 in 4 deaths in the United States. Five-year relative survival rates from cancer have increased in recent years, up from 50% between 1975-77 to 68% between 1999 and 2005. While these rates vary greatly among cancer types, progression upon diagnosis, and demographic factors, the improvements seen in survival rates may be attributed largely to more effective diagnoses, earlier diagnoses, and more effective treatments once cancer is diagnosed. The present paper focuses on the role of bioactive foods in cancer prevention and treatment; for a more exhaustive review of cancer epidemiology data and figures,

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the reader is referred to literature which cover these topics in greater detail (Bray and Møller 2005; Schmid-Schonbein 2006; AICR 2007; Anand, Kunnumakara et al. 2008; ACS 2010; Jemal, Siegel et al. 2010).

6. Bioactive Foods in Cancer Treatment

6.1 Premise of Antioxidant Therapy

The principal treatments used for combating cancer today include surgery, radiation, and chemotherapy. Surgery is required when the cancer exists as a solid tumor mass that can be physically removed. Radiation is used to damage and/or kill cancer cells using a beam of high-energy particles. Both of these are local treatments because they affect only the site of treatment, where chemotherapeutic agents are systemic treatments because they can potentially affect many cells in the body.

Most chemotherapeutics developed for cancer are antineoplastic agents, which inhibit the proliferation of rapidly-dividing cells. While these agents target cancer cells, they may also affect several other tissues in the body that rely on constant cell turnover to function properly, including blood cells and cells of the digestive tract. Consequently, chemotherapy can have undesirable side effects such as mucositis and myelosuppression, leading to immunosuppression due to low white blood cell count. These side effects can lead to susceptibility to opportunistic infections, loss of appetite, and weight loss, all of which increase the difficulty of treatment and decrease the chance of recovery (Doyle, Kushi et al. 2006). Such symptoms underscore make the need for correct balance in the die, which is but vital to sustaining health during cancer treatment.

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One of the leading causes of complications associated with cancer treatments is malnutrition (Doyle, Kushi et al. 2006; Gupta, Lis et al. 2009). Studies have shown that chemotherapy may cause a decrease in the amount of nutrients absorbed from food. One study showed that rectal carcinoma patients treated with a combination of chemotherapy and radiation showed a decrease in serum levels of α-tocopherol (Dvořák, Melichar et al. 2009). A second study demonstrated that plasma concentrations of the carotenoids lutein, α-carotene, and β-carotene are decreased in patients being treated for head and neck squamous cell carcinomas when compared to healthy controls (Sakhi, Bohn et al. 2010).

6.2 Bioactive Food Components in Research

Bioactive foods may have the potential to decrease both the initiation and progression of cancer due to their rich antioxidant and nutrient contents, as previously discussed, and evidence is growing that may help define the role of bioactive foods in the prevention of cancer development and progression. Multiple studies have found correlations between plasma levels of bioactive food components (i.e. caroteniods, tocopherols, etc.) and the likelihood of survival of patients undergoing chemotherapy (Garg, Yoo et al. 2009; McCann, Thompson et al. 2009; Sakhi, Bohn et al. 2010). Research has also shown a positive correlation between the amount of plant material consumed and a patient's chance of recovery. Two studies in particular followed the dietary intake of women with breast cancer as they went through treatment. These studies found an increased chance of survival in those women who had a high intake of plant-based foods and/or supplements containing bioactive food components when compared to those who consumed diets containing less of these components (Pierce, Faerber et al. 2002; McCann, Thompson et al. 2009).

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6.2.1 Resveratrol

Resveratrol is a stilbene polyphenol that has recently garnered much attention for its anti-inflammatory, antitumorigenic and antioxidant properties (Alarcón de la Lastra and Villegas 2005; Ndiaye, Philippe et al. 2011). Found in high concentrations in *Vitis* and *Vaccinium* fruits, studies showing that resveratrol extends lifespan in *S. cerevisiae, C. elegans, Drosophila*, and murine models (through SIR2 activation) has given support to reports that regular consumption of red wine may increase longevity in humans (Alarcón de la Lastra and Villegas 2005; Bass, Weinkove et al. 2007; Miller, Harrison et al. 2010). The results from these studies may not be so easily applied to human models, however, due to resveratrol's low potency and poor bioavailabilty after metabolism (Hsieh, Huang et al. 2010). Further research is needed to more fully elucidate the extent to which resveratrol may affect longevity and aging in humans.

In addition to its potential health-promoting effects, resveratrol may directly influence the development and progression of cancer. A recent report demonstrated that resveratrol-induced apoptosis in colorectal cancer cells is mediated by adaptive response gene ATF3 *in vitro*, supporting the idea that ATF3 may play an antitumorigenic role in colorectal tumorigenesis (Whitlock, Bahn et al. 2011). Another recent report demonstrated the ability of resveratrol and resveratrol analogs to elicit blocks in the cell cycle of cultured human prostate cells, as well as the resulting increase in p53 and p21, providing further evidence of resveratrol's antitumorigenic abilities (Hsieh, Huang et al. 2010).

6.2.2 Carotenoids

Carotenoids, particularly lycopene, are known to be powerful antioxidants linked to oxidation-preventing mechanisms. In one case-control study, carotenoid plasma levels were

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measured to compare 118 non-Hispanic Caucasian men suffering from non-metastatic prostate cancer with 52 healthy men in southeast Texas. Results showed that the risk for men with high levels of α-carotene, trans-β-carotene, β-cryptoxanthin, and lutein and zeaxanthin in their plasma was less than half that of those with low levels of these compounds. No correlation was found between carotenoid plasma levels and the stage of aggressive disease in these patients. This study suggests that high plasma levels of carotenoids may help reduce prostate cancer development, but not its progression (Chang, Erdman et al. 2005).

6.2.3 Vitamin D

Another well-known bioactive food component is vitamin D. Although vitamin D deficiency is mainly associated with bone-related diseases, interest has risen in vitamin D deficiency as a risk factor for different cancer types, mainly colon, breast, ovarian and prostate cancer. A case-control study concluded that patients with plasma levels of 25(OH)D (the main form of circulating vitamin D and main marker for vitamin D deficiency) below 30 ng/ml had about twice the risk of developing colon cancer (Feskanich, Ma et al. 2004), while another revealed a doubling of colon cancer incidence for patients with less than 20ng/ml of vitamin D (Tangrea, Helzlsour et al. 1997). The association of 25(OH)D levels in different stages of colon cancer was investigated, and results suggested that vitamin D metabolites may have protective effects in all the stages of colon carcinogenesis (Braun, Helzlsour et al. 1995).

Low levels of vitamin D have also been associated with breast cancer risk. Studies suggested that women in the lowest quartile of serum 25(OH)D had a five times greater risk of breast cancer than those in the highest quartile (Janowsky, Lester et al. 1999), while other case

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studies suggested that low 25(OH)D plasma levels were associated with a more rapid

progression of metastatic breast cancer (Brenner, Russell et al. 1998).

In a study including 19,000 men with prostate cancer, those with 25(OH)D levels below

16 ng/ml had a 70% higher incidence rate of prostate cancer than those with higher levels of

vitamin D, and the incidence of prostate cancer for younger men was 3.5 times higher if their

levels were below 16 ng/ml (Ahonen, Tenkanen et al. 2000). These studies suggest that vitamin D

levels have significant effects on the initiation of colon and prostate cancer, and in the

progression of metastatic breast cancer.

6.2.4 Vitamins A, E, and C

Additional studies on vitamins A, C, and E have shown inverse correlations between the

presence of these vitamins and cancer incidence. In a study investigating the correlation of

vitamin A, C and E in 5,454 colon cancer patients, results showed that the inverse correlation

between vitamin intake and colon cancer incidence was statistically significant (Park,

Spiegelman et al. 2010). Also, a case-control study that examined the association of antioxidant

vitamins A, C, and E and beta-carotene for 144 cervical cancer patients in South Korea, showed

that total intakes of vitamins A, C and E and beta-carotene were inversely correlated with

cervical cancer risk (Kim, Kim et al. 2010).

Vitamin E is a fat-soluble antioxidant known to quench oxygen radical species formed

during fat oxidation, while vitamin A (retinol) is known for its role in vision and the production

of retinoic acid. Both of these vitamins are well-known components in bioactive foods. A study

involving 26 patients with gastroesophageal cancer examined vitamin A and E plasma levels

compared to the plasma levels of healthy individuals in Eastern Anatolia. Contrastingly, these

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results showed that the difference in the plasma levels of vitamin A and E between healthy and cancer patients was not statistically significant (Yilmaz, Dursun et al. 2010).

In contrast to vitamin E, vitamin C is a water-soluble vitamin that acts as a powerful antioxidant and is highly concentrated in citrus fruits. To better understand the role of citrus fruit in cancer risk, 955 patients with oral and pharyngeal cancer, 395 with esophageal, 999 with stomach, 3,634 with large bowel, 527 with laryngeal, 2,900 with breast, 454 with endometrial, 1,031 with ovarian, 1,294 with prostate, and 767 with renal cell cancer were studied in Switzerland and Italy. Results showed that there was a statistically significant inverse correlation between citrus fruit consumption and the risk of cancer for the digestive tract and the larynx (Foschi, Pelucchi et al. 2010).

Several additional studies have also demonstrated that the overall nutrient state of an individual is positively correlated with the probability of both surviving cancer treatment and experiencing remission (Doyle, Kushi et al. 2006; Garg, Yoo et al. 2009; Gupta, Lis et al. 2009). Obesity, which can be prevented through modifying diet and activity, has been linked to increased probability of recurrence and incidence of secondary cancers in cancer patients. Indeed, studies have shown the importance of alternative feeding routes to help sustain nutrition during treatment (Doyle, Kushi et al. 2006; Bower and Martin 2009; Garg, Yoo et al. 2009).

7. Conclusion

It is becoming increasingly evident that lifestyle is a critical component of the prevention of many diseases, including cancer. As discussed, evidence of the direct correlation between healthy lifestyle habits and the low incidence of cancer is ever-increasing. As the breadth of scientific research continues to grow, additional light will be shed on the importance of these

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behaviors in the prevention of cancer and other types of chronic diseases. The consumption of bioactive foods plays an important role in these lifestyle habits.

It is also becoming evident that patients with cancer who consume high amounts of bioactive foods during treatment have a higher chance of survival. Additional research is needed to elucidate the precise mechanisms of this interesting interaction. While some have speculated that the consumption of these foods may help boost the immune system, stemming off secondary infections, others have suggested that such a diet may help maintain proper nutritional levels, making the patient stronger overall. Whatever the case may be, the evidence is clear: eating a diet rich in bioactive foods helps prevent cancer and maintains more favorable health conditions through cancer treatment, thereby reducing the risk of secondary cancers and secondary infections (Doyle, Kushi et al. 2006).

As additional research discoveries are made connecting healthy behavioral habits and cancer prevention, the onus of responsibility shifts toward the proper dissemination of this knowledge through educational outreach. Researchers, educators, government agencies, and non-governmental organizations alike have the increasing responsibility of providing accurate, useful information to people of the world so that the formidable burden of cancer may be lessened for each successive generation.

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CHAPTER VI: CHERIMOYA PAPER

The Antioxidant Properties of the Cherimoya (*Annona cherimola*) Fruit

The antioxidant properties of the cherimoya (*Annona cherimola*) fruit

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ARTICLE INFO

ABSTRACT

Annona cherimola is an exotic fruit from the genus *Annona*, native to the Andean highlands in western South America. The cherimoya skin, flesh and juice were isolated and analyzed for antioxidant content using the oxygen radical absorbance capacity (ORAC) assay. The juice showed the highest antioxidant activity, while the flesh exhibited the lowest. The cherimoya skin, flesh and juice extracts were also incubated with Raji (Burkitt's Lymphoma) and HT-29 (colon cancer) cell lines, and the antioxidant uptake of cells was measured. Both cell lines, when subjected to cherimoya juice, showed the highest antioxidant uptake. The cells were then exposed to AAPH, a radical initiator, to simulate the conditions of cells under oxidative stress, and then subjected to cherimoya skin, flesh and juice extracts. Both cell lines absorbed more antioxidants after being pre-exposed to AAPH, indicating that cells under stress have the ability to import antioxidants.

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1. Introduction

Annona cherimola, commonly known as cherimoya, is a species of fruit found in different subtropical areas around the world, mainly in northern Peru and southern Ecuador. Cherimoya is known to have been cultivated during the times of the Incan Empire, dating back to 1200 BC. Anthropological evidence suggests that the cherimoya fruit was an active ingredient in the Incan diet (Bonavia, Ochoa, Tovar, & Palomino, 2004). The cherimoya fruit is known for its exceptional taste, its use in traditional medicine as an antimicrobial and insecticide, and as an effective treatment for digestive disorders such as stomachache and pancreatic ulcers (Amoo, Emenike, & Akpambang, 2008).

Considerable evidence suggests that oxidative stress leads to the development of conditions such as cancer and heart disease the two leading causes of death in the United States (Tribble & Frank, 1994; Xu, Kochanek, Murphy, & Tejada-Vera, 2010). Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds antioxidant capacity, which leads to oxidative damage. Partially reduced forms of oxygen, such as superoxide and hydrogen peroxide, that are produced during aerobic metabolism have the potential of causing oxidative injury to macromolecules such as nucleic acids, proteins and lipids. Oxygen radicals are capable of initiating radical chain-reactions that propagate oxidative injury (O'Neill, Standage, Hughes, & Murray, 2001). DNA damage is an early event in carcinogenesis, and oxidation is known to induce 2′-4′-oxidative DNA damage which may lead to the formation of DNA–protein cross-links, alterations in the carbohydrate-phosphate backbone, or direct modification of purine and pyrimidine bases. Tumors may develop if alterations in DNA lead to the activation of oncogenes or the inactivation of tumor suppressor genes.

Dietary antioxidants found in bioactive food components exert regulatory effects on cell proliferation and cytotoxicity. Antioxidants such as vitamin C, vitamin E, soy sterols, and polyphenols help protect the cell against oxidant-induced proliferation, lipid peroxidation, glutathione peroxidase activation (Garcia-Alonso, Ros, & Periago, 2006; Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002), and also contribute to thyroid regulation and glucose homeostasis (Panda, Kar, & Patil, 2009). Evidence indicates that *A. cherimola* is rich in antioxidants such as vitamins A and C, making it a beneficial health supplement (Garcia, Zepeda-Vallejo, Gallegos, Gonzales, & Madrigal, 2008).

Interest in this fruit has also risen due to its high concentration of acetogenins, which are polyketides that act as secondary metabolites in plants. Research has accredited beneficial effects to acetogenins, including the induction of cytotoxic, anti-inflammatory and anti-tumorous activities, particularly in mammary, pancreatic, prostatic, and colon cancer cells (Chen, Chang, Chiu, Wu, & Wu, 1999). Analysis of twelve acetogenins in *Annona cornifolia*, a fruit related to *A. cherimola*, revealed through DPPH radical-scavenging activity that acetogenins contain antioxidant capacity, comparable to that of ascorbic acid (Santos Lima, Pimenta, & Boaventura, 2010). Acetogenins are thought to exert their effects through the inhibition of the ubiquinone oxido-reductase complex in the mitochondrial electron transport chain and the inhibition of NADH oxidase in the plasma membrane. Acetogenins contained in the stems of *A. cherimola* (Chen et al., 1999) and the seeds of *Annona reticulata* (Chen, Chang, Chiu, Wu, & Wu, 1998) have been isolated and identified, however, antioxidant properties of these

acetogenins have yet to be assessed in order to evaluate their potential for use in dietary supplements.

This project explores the potential health benefits of cherimoya consumption by examining the antioxidant activity of its individual components, including skin, flesh and juice using the oxygen radical absorbance capacity (ORAC) assay. The cherimoya extracts were also incubated with Raji (Burkitt's Lymphoma) cells and HT-29 (colon cancer) cells to measure antioxidant uptake. Incubations with cherimoya juice extract exhibited the highest antioxidant uptake in both cell lines. Cells were stressed by exposure to 2,2′-azobis(2-aminidopropane) dihydrochloride (AAPH) and then exposed to cherimoya extracts to measure their antioxidant uptake. Results showed that cells under oxidative stress import more antioxidants when incubated with cherimoya skin, flesh, and juice extract, thus suggesting the potential benefits of consuming antioxidants following exercise.

2. Materials and methods

2.1. Chemicals

2,2′-azobis(2-aminidopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). RPMI 1640 medium was purchased from Hyclone (Logan, UT). Cosmic Calf Serum was purchased from Hyclone (Logan, UT). L-glutamine was obtained from Fisher Scientific (Pittsburgh. PA). Fluorescein-sodium salt was obtained from Sigma-Aldrich, Inc. (Milwaukee, WI). Hank's Balanced Solution was purchased from Hyclone (Logan, UT). Trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid) was purchased from Enzo Life Sciences, Inc. (Plymouth, PA). Phosphate buffered saline (PBS) was purchased from Sigma-Aldrich, Inc. (Milwaukee, WI).

2.2. Materials

Costar 96- and 24-well clear culture plates were purchased from Corning Inc. (Lowell, MA). Raji cells (Burkitt's Lymphoma cell line) and colon cancer cells (HT-29) were obtained from the American Type Culture Collection (ATCC).

2.3. Equipment

All fluorescence readings were taken using the BMG FLUOstar Optima (BMG Laboratories) microplate reader (serial #413-0225). All data were analyzed using Microsoft Excel and Apple Numbers.

2.4. Burkitt's Lymphoma cell culture

2.4.1. Growth medium
RPMI 1640 Medium was supplemented with 10% Fetal Bovine Serum and 2 mM of L-glutamine. Cells were kept in an incubator with 5% carbon dioxide at 37 °C.

2.4.2. Changing media
Cells were placed in a conical vial and centrifuged at 450 g for 15 min. Spent media was discarded and new RPMI media was introduced. The conical vial containing new media was vortexed and placed in incubation plates. Cell count and viability were determined via trypan blue staining using a hemocytometer. Cell viability was maintained above 90% and cell counts were maintained between 900,000 and 1,100,000 cells/mL.

2.5. HT-29 (colon) cancer cell culture

2.5.1. Growth medium
McCoy Medium was supplemented with 10% Fetal Bovine Serum and 1.5 mM L-glutamine. Cells were kept in an incubator with 5% carbon dioxide at 37 °C.

2.5.2. Changing media
Used media was discarded and cells were washed with 5 mL of Hank's solution. One mL of 0.05% trypsin-EDTA at 37 °C was added to the flask and evenly dispersed over the surface. The flask was placed in the 37 °C incubator and observed until the cells were detached from the walls of the flask. After centrifugation at 450 g for 15 min, the trypsin was discarded, the pellet of cells was resuspended in 5 mL of McCoy media, re-seeded in a flask. The new media introduced was used to rinse the surface of the flask and was pipetted up and down to dissociate cell clumps. A cell count and viability test was performed via trypan blue using a hemocytometer. Cell viability was maintained above 90% and cell counts were maintained between 900,000 and 1,100,000 cells/mL.

2.6. Fruit sample

All the cherimoya fruits were purchased at a local grocery store in Orem, UT, and were stored in a refrigerator at 3 °C.

2.7. Sample preparation

Cherimoya skin and flesh were excised from the fruit and homogenized by weighing 0.4 g of sample and diluting it in 10 mL of distilled water. Juice was extracted through the compression of the cherimoya flesh. Juice was then homogenized to reduce possible interference from fruit constituents, according to recent methodological recommendations (Perez-Jimenez et al., 2008). Samples were centrifuged at 450 g for 15 min, filter-sterilized and kept in a freezer at −20 °C until used.

2.8. Oxygen radical absorbance capacity (ORAC) assay

All samples were analyzed using a modified ORAC assay based on published methods (Garrett, Murray, Robison, & O'Neill, 2010). The ORAC assay measures oxidative degradation of a fluorescent compound (fluorescein sodium salt) after being exposed to an oxygen radical initiator, AAPH (2,2′-azobis(2-aminidopropane) dihydrochloride). All readings were taken in Cole-Parmer black bottom 96-well plates, with 133 μL Fluorescein (0.1 μM), 50 μL AAPH (4 μM), and 13 μL of antioxidant sample or PBS in each well. Readings were taken every 2 min for 1 h, followed by readings every 15 min for 3 h. Measurements were taken by the BMG FLUOstar Microplate reader at 37 °C; 485 nm excitation and 590 nm emission filters were used for fluorescence readings. All fluorescent measurements were expressed relative to the initial reading. The area under the curve (AUC) was calculated by using the difference of area under the fluorescein decay curves between the sample curve and the fluorescein + AAPH curve. The AUC measures the total antioxidant activity in the sample minus the fluorescein + AAPH antioxidant curve.

2.9. Standard curve data

Trolox, a water-soluble analogue of vitamin E, was used as a control standard. Trolox standards were run at concentrations of 50 μM, 25 μM, 12.5 μM, and 6.25 μM to obtain standard curves for each trial. Data from each standard curve were used to convert raw net area under the sample curve (net AUC) values to Trolox Equivalents per liter (TE/L). Subsequent calculations converted TE/L to TE/100 g.

2.10. Antioxidant concentration of samples only

Fifty μL of PBS was inserted in the first and last columns of the 96-well plate and 133 μL of fluorescein was inserted into rows 2–11. The cherimoya skin, flesh and juice extract were placed in a 37 °C water bath for 10 min. 13 μL of skin sample was added to rows 2–4, 13 μL of flesh sample was added to rows 5–7 and 13 μL of juice sample was

added to rows 8–9. Mixtures were pipetted up and down to ensure homogeneity of the mixture. After the fluorescein was measured twice in a two minute interval using the FLUOstar Optima fluorescence microplate reader, 50 μL of AAPH was placed in rows 2–10. Fluorescein levels were recorded in 2 minute intervals for 1 h, followed by 15 minute intervals for 3 h.

2.11. Antioxidant uptake cellular model

One mL of cells was placed in each well of the 24-well plate, and each column of the plate was mixed with 100 μL of cherimoya skin, flesh or juice extract. One mL of cells with 100 μL of PBS served as the control. The incubation plate was placed in the incubator for 20 min. The samples from each well were then placed in labeled eppendorf vials that were centrifuged for 15 min at 450 g. The supernatant was discarded. To perform the wash, 1 mL of sterilized PBS was added to each eppendorf vial and vortexed until the cells were suspended in solution. The eppendorf vials were subjected to a 37 °C water bath for 3 min and then to a liquid nitrogen bath for 3 min. The freeze/thaw process was repeated three times. The eppendorf vials were centrifuged for 30 min at 3000 g. The supernatant (cell lysate) was then placed in a 96-well plate to perform the ORAC assay to determine antioxidant uptake in the cells.

2.12. Simulation of oxidative stress: an alternate cellular model

One mL of cells with 100 μL of a 1:16 dilution of AAPH:sterile PBS was placed in each well of the 24-well plate. The incubation plate was placed in the incubator for 10 min. The cells were then placed in eppendorf vials and centrifuged for 15 min at 450 g. The supernatant was discarded and 1 mL of PBS was added to each eppendorf vial and vortexed so the cells were re-suspended. Vials were again centrifuged for 15 min at 450 g. The supernatant was discarded and 1 mL of cell media was added to the eppendorf vials and vortexed until cells were re-suspended. The cells with the media were placed in each well of the 24-well incubation plate, and an additional 100 μL of cherimoya skin, flesh or juice extract was added. The plate was placed in the incubator for 20 min and same procedure outlined earlier was followed to measure the antioxidant uptake of the cells.

2.13. Cytotoxicity

To eliminate the possibility that the cells were exhibiting higher antioxidant uptake because the cell membrane was being damaged by AAPH exposure, Raji cells, with a cell count between 900,000 and 1,100,000 cells/mL and a viability above 90%, were treated with 1:1, 1:2, 1:4, 1:8 and 1:16 dilutions of AAPH in sterile PBS in a 24-well plate. After being exposed to the different concentrations of AAPH, cells were placed in the incubator for 24 h. The cell count and viability for each well containing the different dilutions of AAPH were determined via trypan blue staining using a hemocytometer. The 1:16 dilution of AAPH sterile PBS maintained cell viability and the cell count had increased, showing that the AAPH treatment had not substantially damaged the cell membrane, as to affect the cell integrity.

3. Results

3.1. Raw cherimoya extract

Antioxidant analyses of several cherimoya skin, flesh and juice extracts yielded the following data: The highest measured antioxidant activity was found in the cherimoya juice at 98,085 TE/100 g (stdev = 12,017, N = 48). The next highest activity was in the cherimoya skin at 74,714 TE/100 g (stdev = 6614, N = 48), and the lowest activity was found in the cherimoya flesh at 6004 TE/100 g

(stdev = 1257, N = 48). Measurements on each extract were repeated four times, with 12 replicates for each sample, giving a total of 48 measurements per sample. Data represent mean values for each.

3.2. Raji incubations

Initial assessment of antioxidant activity in a Raji cell lysate following incubation with PBS revealed a final Trolox Equivalent value of 2147 TE/100 mg sample per 1,100,000 cells (N = 36). All of the following data are reported in TE/100 g sample per 1,100,000 cells. Incubations with cherimoya skin, flesh, and juice extracts revealed higher TE values for each sample when compared to PBS controls (4136 for cherimoya skin, N = 36; 2580 for cherimoya flesh, N = 36; 5830 for cherimoya juice, N = 36). Assessments taken from each sample following pre-exposure to AAPH revealed increased antioxidant activity in each sample, including the PBS control (4097, N = 36). Sample data revealed a mean value of 5895 (N = 36) for the cherimoya skin extract, 6939 (N = 36) for the cherimoya flesh extract, and 7527 (N = 36) for the cherimoya juice extract (see Fig. 1).

3.3. HT-29 incubations

Assessment of HT-29 cell lysate antioxidant activity revealed trends similar to those found in the Raji lysates (Fig. 2). Initial measurement of PBS control-incubated HT-29 cell lysate revealed a mean TE value of 1521 (N = 24). Data for cherimoya-incubated HT-29 samples revealed a mean value of 2904 (N = 24) for the cherimoya skin extract, 1760 (N = 24) for the cherimoya flesh extract, and 5644 (N = 24) for the cherimoya juice extract. Pre-exposure to AAPH resulted in higher antioxidant assessments for each sample, including PBS controls: 7769 (N = 24) for PBS control, 10,343 (N = 24) for cherimoya skin extract, 10,433 (N = 24) for cherimoya flesh extract, and 11,929 (N = 24) for cherimoya juice extract. The same trend observed in the Raji cell model was observed in the HT-29 cells, in that the highest overall values were seen in the juice-incubated cell lysate. In contrast to data for Raji cells, however, the flesh and skin-incubated cell lysates were almost equal, where the values for the skin-incubated cell lysates were larger than the flesh-incubated cell lysate in the Raji model.

4. Discussion

4.1. Analysis of raw cherimoya extracts

The current study's antioxidant analyses of raw cherimoya extracts, to our knowledge, represent the first attempt at such quantification using the ORAC assay, although 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) assay analyses have been previously preformed (Vasco, Ruales, & Kamal-Eldin, 2008). When each individual cherimoya component was analyzed for antioxidant activity, the juice exhibited the highest antioxidant activity while the skin contained the lowest. This may be due to the higher concentrations of vitamins A and C found in the fruit flesh (16.63 μg/100 g of vitamin A and 42.38 mg/100 g of vitamin C) (Amoo et al., 2008). Further analysis and testing of the skin and fruit composition are needed to provide additional insight into the compounds present in each part of the fruit that contribute to their overall antioxidant capacities.

4.2. Incubation analysis

Raji cells and HT-29 cells were exposed to cherimoya skin, flesh and juice extracts and the antioxidant capacity of the cell lysates were measured. Antioxidant values in the cellular models were significantly different from the raw antioxidant extract levels themselves. This is likely due to the dynamic cellular processes that allow antioxidant
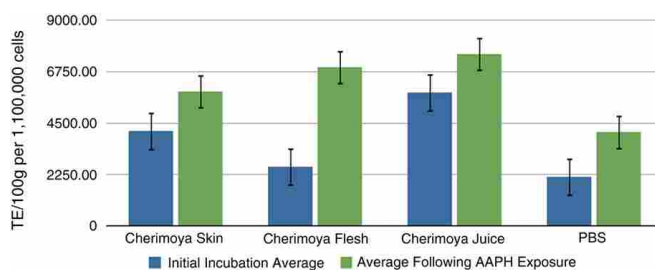
Fig. 1. Changes in antioxidant activity in Raji cell lysates before and after incubation with AAPH. All values were reported as TE/100 g sample per 1,100,000 cells.

compounds from the medium to cross the cellular membrane into the cellular interior. Individual cells are also equipped with their own processes to cope with oxidative stress, resulting in increased antioxidant protection. Both Raji and HT-29 cells exposed to cherimoya juice exhibited higher antioxidant activity in their cell lysates (when compared to those exposed to skin or flesh extract), while cells exposed to cherimoya skin extract had the lowest antioxidant activity of the group. This suggests that the antioxidant compounds in the cherimoya juice are both readily accessible and beneficial to the cells for uptake. By contrast, the cherimoya flesh, for example, either does not contain usable antioxidant compounds or is lacking in antioxidant content.

Student's t-tests performed on data also revealed statistically significant increases in antioxidant capacity in both Raji (*one-tailed* p = 0.016) and HT-29 (*one-tailed* p = 0.0006) models. This suggests that both cell lines are equipped to respond to the oxidative stresses with which they are presented, and encourages further study of more broad and diverse cell lines.

Overall, it was observed that HT-29 cells produced a much greater percent increase in their antioxidant activities following exposure to oxidative stress. The average percent increase for all Raji runs was 82.9% (max = 169%, min = 29.1%, stdev = 62.2), while the average percent increase for all HT-29 runs was 318% (max = 493%, min = 111%, stdev = 169). Student's t-test analysis revealed that the difference in HT-29 cellular response compared to that of the Raji cells was statistically significant (p = 0.013). These results suggest that the HT-29 cell line may be more robust in its ability to respond to oxidative stress and react to protect itself against these stresses. Further testing of this and other cell lines may be beneficial in determining common cellular responses in various cell types. Such analyses may also help to elucidate the mechanisms responsible for responding to oxidative stress in varying cell types. It appears from the data mentioned earlier that the cell lines tested have the ability to sense oxidative damage and correspondingly increase their uptake of available antioxidants. Further

research regarding different levels of oxidative stress exposure, as well as experiments with different classes of antioxidants, may prove beneficial in understanding the mechanisms involved in cellular responses to oxidative stress.

From the PBS results (Figs. 1 and 2), it can be seen that cells exposed to oxidants internally increase their antioxidant capacity. However, cells exposed to fruit extracts substantially increase their intracellular antioxidant levels and these increases are higher than the basal antioxidant levels in the cells, indicating that cells have the ability to import available antioxidants when experiencing oxidative stress. It is interesting to note that while the cherimoya juice is the most potent antioxidant under conditions of oxidative stress, there is a much higher percentage increase in antioxidant activity when exposed to skin and flesh. This is significant because it suggests benefits from the consumption of all components of Cherimoya fruit, including skin, flesh and juice.

5. Conclusion

The first objective of this research was to analyze the antioxidant capacities of the *A. cherimola* fruit, specifically examining its skin, flesh, and juice. Initial analyses suggested that *A. cherimola* contains high antioxidative properties, and that the highest antioxidative potential is found first in the plant's juice, and next in its skin. Further chemical analyses of *A. cherimola* are needed to more fully understand its chemical composition and the antioxidant compounds it contains. Future research may also include studying the plant's stem, leaves, and flowers for antioxidant activity and chemical composition. Regular consumption of *A. cherimola* may contribute to increased antioxidant capacity of human serum, and may thereby help to prevent the development and progression of cardiovascular disease, neurodegenerative diseases, cancer, and other diseases thought to be linked to oxidative stress. Further studies are needed to add insight into the potential role of *A. cherimola* in the prevention of these

Fig. 2. Changes in antioxidant activity in HT-29 cell lysates before and after incubation with AAPH. All values were reported as TE/100 g sample per 1,100,000 cells.

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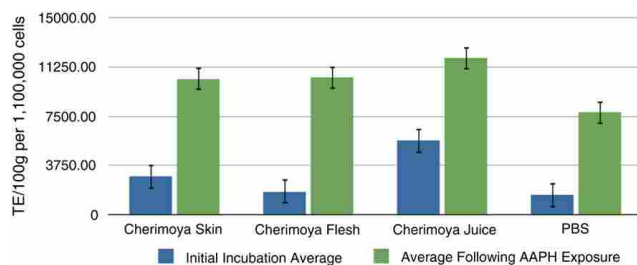
conditions. Additional studies are needed to further elucidate the intracellular mechanisms that may be contributing to the increased antioxidant capacity in these and other cell lines. Further study may also provide insights on the effects of changes of baseline oxidative stress in humans and how these changes affect tolerance to oxidative stress in future stressful environments. Additional insights may be gained into the effects of exercise on oxidative stress and the body's ability to protect its tissues during future stressful events. These insights may also aid in the future study of the development and progression of oxidative stress-induced degenerative conditions, and how oxidative-antioxidant equilibria may increase prevention against such conditions.

As a second focus of this paper, the effects of pre-exposure to oxidative stress on cellular antioxidant uptake were examined, using *A. cherimola* extracts as the source of antioxidants. The data indicate that pre-exposure to oxidative stress may contribute to increased antioxidant uptake in both Raji and HT-29 cell lines. In both cell lines, cell lysate antioxidant capacity was significantly higher when cells were exposed to oxidative stress, suggesting that the presence of oxidative stress contributed to the uptake of antioxidants as part of a cellular response mechanism. These data correlate with similar studies on the effect of pre-exposure to inflammation-inducing environments on tissues (Schmid-Schonbein, 2006), and suggest that the mechanisms that influence these responses should be studied further on the cellular level.

Results show that while cherimoya juice is a potent antioxidant, flesh and skin consumption, along with the juice, will allow a higher antioxidant uptake when cells are placed under oxidative stress. Therefore, when consuming cherimoya, it is beneficial to include all parts of the fruit. Further research may shed light on the use of *A. cherimola* as a useful dietary supplement.

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APPENDIX: PRESENTATIONS AND PUBLICATIONS

PUBLICATIONS

The Role of Oxidative Stress in Prostate Cancer. Gaytri Gupta-Elera, **Andrew R. Garrett**, Richard A. Robison, and Kim L. O'Neill. Submitted to Cancer Prevention Research March 8,2011.

Bioactive Foods in Aging: Role in Cancer Prevention and Treatment. **Andrew R. Garrett**, Gaytri Gupta-Elera, Mary E. Keller, Richard A. Robison, and Kim L. O'Neill. 2011. Submitted for publication to El Sevier on March 1, 2011.

The antioxidant properties of the cherimoya (*Annona cherimola*) fruit. (2010) Gaytri Gupta-Elera, **Andrew R. Garrett**, Andrés D. Martinez, Richard A. Robison, and Kim L. O'Neill.   Food Research International, doi:10.1016/j.foodres.2010.10.038

Developing a Framework for Predicting Relative Antioxidant Capacity Based on Molecular Structure. **Andrew R. Garrett**, Jeffrey W. Collins, Jennifer M. Handy, Jorge A. Chauca, Richard A. Robison, Byron K. Murray and Kim L. O'Neill. Manuscript submitted for publication to the Journal of Functional Foods May 3, 2010.

Measuring Antioxidant Capacity Using the ORAC and TOSC Assays. **Andrew R. Garrett**, Byron K. Murray, Richard A. Robison, and Kim L. O'Neill. Advanced Protocols in Oxidative Stress II in Methods in Molecular Biology (series). Humana Press, 2010, ed. by Donald J. Armstrong. Vol 594, pp. 251-262.

PRESENTATIONS

Antioxidant Levels in Blueberries: Are Organic Better Than Conventional? Ryan D. Kraus, Jacob R. Jensen, **Andrew R. Garrett**, Richard A. Robison, and Kim L. O'Neill. Presented as an oral presentation at the American Society for Microbiology Intermountain Branch Meeting, Ogden Utah, April 9, 2011. Presentation won first-place award at the conference.

Power juices: The role of exotic, anti-oxidant fruit juices in preventing cancer. Timothy C. Michaelis, Andrés D. Martínez, **Andrew R. Garrett**, José M. Peña, Richard A. Robison, and Kim L. O'Neill. Presented as an oral presentation at the American Society for Microbiology Intermountain Branch Meeting, Ogden Utah, April 9, 2011.

Cancer Patients Possess Lower Serum Antioxidant Levels than Age Matched Healthy Subjects. Adam J Grooms, Trevor S. Bluemel, Michael Q. Sanderson, Jacob R Jensen, **Andrew R. Garrett**, Richard A. Robison, and Kim L. O'Neill. Presented as an oral presentation at the American Society for Microbiology Intermountain Branch Meeting, Ogden Utah, April 9, 2011.

The Organic Panic. Jacob R. Jensen, **Andrew R. Garrett**, Ryan D. Kraus, Kylie Measom, Matthew Gillam, Richard A. Robison, and Kim L. O'Neill. Presented as a poster presentation at the American Association for Cancer Research (AACR) meeting April 5, 2011 in Orlando, Florida.

Serum Levels of Antioxidants in college aged, elderly, and cancer patients. Trevor S. Bluemel, Michael Q. Sanderson, Jacob R. Jensen, **Andrew R. Garrett**, Melissa M. Alegre, Richard A. Robison, and Kim L. O'Neill. Presented as a poster presentation at the American Association for Cancer Research (AACR) meeting April 4, 2011 in Orlando, Florida.

Raji antioxidant uptake of exotic fruit juices increases following pre-exposure to oxidative stress. Andrés D. Martinez, **Andrew R. Garrett**, Timothy C. Michaelis, José M. Peña, Richard A. Robison, and Kim L. O'Neill. Presented as a poster presentation at the American Association for Cancer Research (AACR) meeting April 2, 2011 in Orlando, Florida.

The Organic Panic. Jacob R. Jensen, **Andrew R. Garrett**, Ryan D. Kraus, Kylie Measom, Matthew Gillam, Richard A. Robison, and Kim L. O'Neill. Presented as a poster presentation at the Undergraduate Caucus of the American Association for Cancer Research (AACR) meeting April 2, 2011 in Orlando, Florida.

Serum Levels of Antioxidants in college aged, elderly, and cancer patients. Trevor S. Bluemel, Michael Q. Sanderson, Jacob R. Jensen, **Andrew R. Garrett**, Melissa M. Alegre, Richard A. Robison, and Kim L. O'Neill. Presented as a poster presentation at the Undergraduate Caucus of the American Association for Cancer Research (AACR) meeting April 2, 2011 in Orlando, Florida.

Oxidative Stress Promotes Increased Antioxidant Uptake in a Cellular Model. Jorge A. Chauca, **Andrew R. Garrett**, Andres D. Martinez, Evita Giraldez, Jason Loong, Jon Whitaker, José M. Peña, Richard A. Robison, and Kim L. O'Neill. Presented as a poster presentation on April 10, 2010 at the American Society for Microbiology Intermountain Branch Meeting in Provo Utah.

A novel method for assessing antioxidant capacity based on structural characteristics. **Andrew R. Garrett**, Jennifer M. Handy, Jorge A. Chauca, Jeffrey W. Collins, Richard A. Robison, Byron K. Murray, Kim L. O'Neill. Presented as a poster presentation at the American Association for Cancer Research (AACR) meeting April 21, 2009 in Denver Colorado.

Grapes of Wrath: A Fight Against Oxidizers. Jennifer M. Handy, **Andrew R. Garrett**, Jorge A. Chauca, Jeffery W. Collins, Richard A. Robison, Bryon K. Murray, Kim L. O'Neill. Presented as a poster presentation at the American Association for Cancer Research (AACR) meeting April 19, 2009 in Denver Colorado.

Green Tea Health Benefits and Costs--Its About Finding Balance. Jordan G. McDonald, J. McBride, **A. Garrett**, J. Handy, R. Enz, B. K. Murray, R. A. Robison, K. L. O'Neill. Presented at the American Association for Cancer Research (AACR) meeting as an oral presentation April 20, 2009 in Denver Colorado.

A Model for Predicting Antioxidant Capacity From a Chemist's Point of View. J. W. Collins, **A. R. Garrett**, J. M. Handy, J. A. Chauca, R.A. Robison, and K. L. O'Neill. Presented as an oral presentation on March 21, 2009 at the American Society for Microbiology Intermountain Branch meeting, Salt Lake City UT.

Using a Monoclonal Antibody to TK1 to Specifically Identify Malignant Tissue. **Andrew Garrett**, Robert Fuller, Kristie Aamodt, Robert Staples, Byron Murray and Kim O'Neill. Oral presentation at the American Society for Microbiology Intermountain Branch meeting, Grand Junction Colorado, April 21, 2007.

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