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Antioxidant Assessment in Western Maine Elderly Women Following 30 Days of Wild Blueberry Consumption

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ANTIOXIDANT ASSESSMENT IN WESTERN MAINE ELDERLY WOMEN FOLLOWING 30 DAYS OF WILD BLUEBERRY CONSUMPTION

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ANTIOXIDANT ASSESSMENT IN WESTERN MAINE ELDERY WOMEN FOLLOWING 30 DAYS OF BLUEBERRY CONSUMPTION

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An Abstract of the Thesis Presented in Partial
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Increased fruit and vegetable consumption is the single most protective characteristic of a diet against disease. While the exact mechanism by which this protection is offered remains unclear, the leading theory is centered on the antioxidant content of fruits and vegetables. Recent studies have shown that certain fruits and vegetables have significantly higher antioxidant contents than others. Wild North American blueberries (*Vaccinium angustifolium*) have one of the highest antioxidant contents of all fruits and vegetables tested. This thesis investigates the relationship between the consumption of blueberries and blood antioxidant levels in 24 elderly women. In addition, fruits and vegetables with similar Oxygen Radical Absorbance Capacity (ORAC) were grouped together in the development of an ORAC-based food frequency questionnaire.

Twenty-four, independently living women, over the age of 60, were recruited from a fitness center. These women were randomly assigned to either a control group (n=12) or a blueberry-consuming group (n=12). Dietary data was collected via an interviewer assisted Tufts Food Frequency Questionnaire. Baseline blood antioxidant tests were collected on the morning before the blueberry-consuming group was given their 30 day supply of frozen wild North American blueberries. Blood antioxidant tests included total lymphocyte antioxidant content (Spectrox®), plasma ORAC with protein, non-protein plasma ORAC (ORAC ac), and Ferric-Reducing Ability of Plasma (FRAP). In addition, bilirubin and uric acid analyses were performed as methods of identifying contributing antioxidant characteristics. Immediately following the consumption of one cup of blueberries each day for 30 days, all blood tests were repeated.

Those women assigned to the blueberry consuming group experienced both a significant increase in overall FRAP scores (+28 uM Trolox equivalents/mL, p=0.036) as well as a significant increase in non-protein plasma ORAC scores (+234.5 uM Trolox equivalents/mL, p=0.048), with no significant changes observed in the control group. Additionally, women assigned to the control group experienced a significant decline in plasma ORAC scores (-1153 uM Trolox equivalents/mL, p=0.038), with no concomitant changes in the blueberry consuming group. Absence of increases in plasma uric acid and bilirubin levels among both groups showed no antioxidant interference from this parameter, as further validation that the increases were due to the antioxidants coming from the blueberries.

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INTRODUCTION

BACKGROUND

The decline in health associated with aging is at least partially driven by the presence of free radicals. Antioxidants ameliorate both cellular and biochemical damage caused by free radicals. Fruits and vegetables are the overall richest sources of antioxidants. There is consistent and substantial evidence that diets high in fruits and vegetables are protective against a number of age related diseases (Kohlmeier et. al., 1995, Ames et. al., 1995). Cancer, heart disease, and cerebrovascular accident incidence are all inversely related to fruit and vegetable consumption (Gillman et. al., 1995, Steinmetz and Potter, 1991, Rimm et. al., 1996). Dietary analysis of Americans over the past two decades has consistently revealed inadequate fruit and vegetable intake (Block, 1991, Ames, 1998). Therefore, it is understood that one of the major public health objectives, identified by epidemiologists as having the greatest impact on disease rates in the United States, is increased fruit and vegetable consumption (Ames et. al., 1993).

Fruits and vegetables, however, vary widely in both their antioxidant potential and their ability to prevent disease. Americans might benefit more from the few fruits and vegetables that they eat, if those fruits and vegetables consumed are at least rich sources of disease mitigating antioxidants. In order for more specific dietary recommendations to be made, more information is needed on the antioxidant protection elicited by different fruits and vegetables. Additionally, fruits and vegetables with relatively low vitamin and mineral contents may offer comparatively high antioxidant-

related protection (Steinmetz and Potter, 1996). Therefore, it is understood that compounds other than vitamins and minerals are responsible for a considerable percentage of the putative health benefits resulting from regular fruit and vegetable consumption.

Blueberries have an exceptionally high total antioxidant value. Wild North American blueberries, in fact, have the highest total antioxidant capacity (as measured by oxygen radical absorbance capacity, ORAC) of any fruit or vegetable tested thus far (Cao et. al., 1996, Wang et. al., 1996). Blueberries contain minimal amounts of vitamins and minerals (Bushway et. al., 1983). The total antioxidant capacity is, therefore, most heavily influenced by the abundance of phytonutrients present, most notably anthocyanins (Prior et. al., 1998). Although it is well known that blueberries have a high antioxidant potential and that consumption of large, pharmacological doses of anthocyanins and blueberry extracts produce changes in serum antioxidant capacity, less is known on how the regular consumption of more moderate amounts of blueberries affects similar parameters (Mazza et. al., 2000).

OBJECTIVES

The objective of this research was to determine if there are protective effects with respect to increased physiological antioxidant capacity produced by the regular consumption of Wild North American Blueberries. This study examined both serum and lymphocyte antioxidant potential by a variety of assessment methods, as well as other serum indices that might have been associated with any changes. In addition, it is hoped that this study will provide researchers with the necessary insight to develop an ORAC-

based food frequency questionnaire. This questionnaire would score fruits, vegetables, and other foods by ORAC potential. The development of such a dietary assessment tool could potentially allow dietitians and epidemiologists to more accurately assess dietary antioxidant levels and might help identify the inadequate dietary intake that is closely associated with a variety of diseases.

CHAPTER 1

REVIEW OF LITERATURE

ANTIOXIDANTS

Overview

Antioxidants are those compounds that have the ability to donate electrons to molecules or atoms with single, unpaired electrons in their outer orbit. In biological systems, these molecules, and their ability to intervene in oxidation-reduction reactions, spare essential compounds from oxidative changes and subsequent loss of physiological function. Those highly charged molecules that possess unpaired, outer-orbital electrons and promote oxidative changes in other atoms are commonly known as free radicals.

Free radicals are ubiquitous and are produced in large quantities in aerobic respiration within the mitochondria (Vendemiale et. al.,1999). Free radicals are also produced endogenously in other oxidative processes. Exogenous sources of free radicals vary widely from dietary peroxides, in the form of oxidized fatty acids generated in the process of frying or cooking unsaturated fats and oils, to a variety of molecules found in air pollution.

Individual free radicals initiate oxidative chain reactions that ultimately effect many other molecules, altering cellular biochemistry. These reactions are deleterious to the health of the cell, limiting its ability to produce energy and correctly transcribe genetic codes. Lipids that have polyunsaturated characteristics are most susceptible to free radical oxidation. The effects of free radical-based oxidation accumulate over the

lifespan and ultimately lead to premature aging, disease, and death (Vendemiale et. al., 1999).

Antioxidants ameliorate the destructive action of free radicals, largely by means of reduction-based reactions (Hu et. al., 2000, Vendemiale et. al., 1999). The body's total antioxidant pool is comprised of two major components: endogenous and exogenous antioxidants. Antioxidants are produced endogenously in the form of enzymes and other proteins. These antioxidants include superoxide dismutase, glutathione, glutathione reductase, glutathione peroxidase, catalase, uric acid, albumin, melatonin, and bilirubin, to name only a few. Exogenous antioxidants are usually in the form of dietary micronutrients. Vitamins, specific trace minerals, and phytonutrients are examples of dietary antioxidants.

Vitamins were the first nutrients to receive attention as antioxidants. Vitamins C and E, in particular, were initially studied for their use as preservatives. It was discovered that these vitamins were able to prevent oxidative changes in the taste, color, and nutritive value of foods. Later, vitamins A and D, and the carotenoid precursors to vitamin A, were also identified as nutrients with antioxidant capacity. However, subsequent research in the field of antioxidants has discovered an enormous number of compounds found in plants that have far greater antioxidant activity than any vitamin. These non-vitamin antioxidants have been generally categorized as phytonutrients. Phytonutrients are grouped into classes of molecules with similar chemical structures and characteristics.

Polyphenols/Flavonoids

Polyphenolic compounds are characterized by their multiple-ringed chemical structure. This array of phenolic rings, and the frequent accompaniment of attached, potential electron-donating groups, is responsible for the group's high antioxidant activity (Van Acker et. al., 1996). Flavonoids, quinones, and xanthones are subclasses of polyphenols. Flavonoids are those polyphenolic molecules that have a characteristic C6C3C6 carbon skeleton, containing two benzene rings (Figure 1) (Von Elbe and Schwartz, 1996). While most plants have at least minimal flavonoid contents, a number of species have been identified as having exceptionally high levels. Green tea, black tea, citrus peels, thyme, and a variety of berries are among the richest sources of flavonoids and have more recently received attention for their potential role in preventing disease (Van Acker et. al., 1996). Earlier research suggests that flavonoid consumption by humans varies widely, with average per capita consumption in the United States estimated at a few hundred milligrams per day (Hollman et. al., 1999).

Anthocyanins

Anthocyanins are a subgroup of flavonoids. They are one of the more broadly distributed pigment groups in the plant kingdom (Von Elbe and Schwartz, 1996). Historically, anthocyanins have served as effective food colorants. In recent years, however, anthocyanins have received attention as powerful antioxidants; extremely effective free radical scavengers with a high affinity for both hydrogen ions and singlet oxygen molecules (Van Acker et. al., 1996, Cao et. al. 1997). In fact, anthocyanins are one of the most potent antioxidants known to science thus far, with oxygen radical

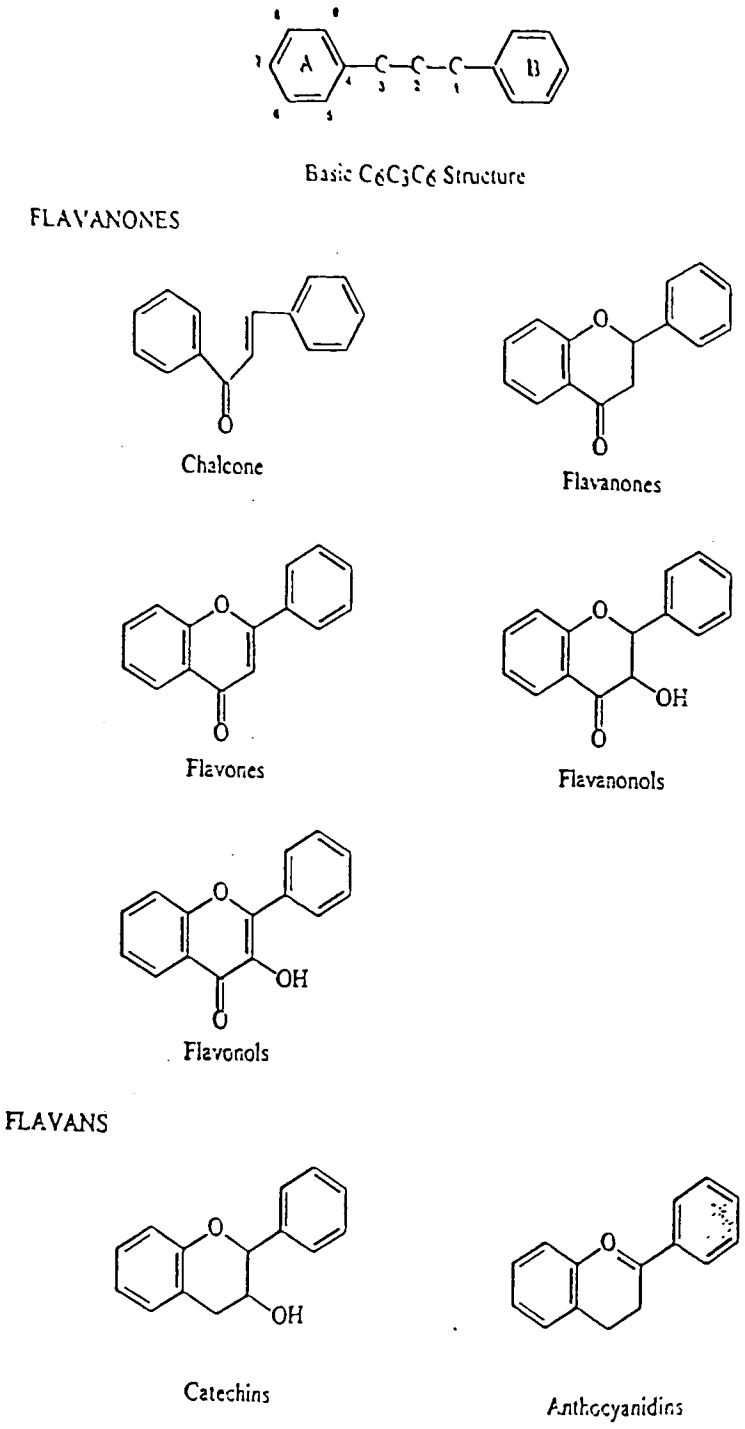


Figure 1. Basic Flavonoid Structure and Subclasses of Flavonoids.

absorbance capacities (ORAC) seven to ten times as great as similar molar concentrations of vitamins E and C (Wang et. al., 1997). Although more than 250 different anthocyanins have been discovered in plants, there are only six that commonly occur in food. All anthocyanins share a common 2-phenylbenzopyrylium of flavylium salt base structure (Figure 2).

Anthocyanins are distinguished from one another by the order, number, and substitution of attached sugars and hydroxyl or methoxy groups (Von Elbe and Schwartz, 1996). It is this abundance of attached hydroxyl groups that is largely responsible for the class's overall superior antioxidant potential (Cao et. al., 1997). Anthocyanin sugar moieties that are hydrolyzed produce a non-sugar product or aglycone known as an anthocyanidin. Although the glycoside version (anthocyanin complex) may have greater substitution, anthocyanidin antioxidant capacity remains high and is similar to that of the anthocyanin. While both anthocyanins and anthocyanidins occur in processed foods, only anthocyanins are present in nature. The absence of free-existing anthocyanidins in nature is attributed to the molecule's general insolubility in water (Von Elbe and Schwartz, 1996).

Anthocyanins occur with the highest concentration in fruits and vegetables that are a deep red, blue, or purple. Within this range, there are an infinite number of variations and combinations of these colors that anthocyanins and anthocyanidins produce when exposed to visible light. Anthocyanins move from redness to blueness in color with the increased presence of attached hydroxyl groups (Figure 3). Therefore, in general, the darker blue and violet-producing anthocyanins have greater substitution and consequently a higher antioxidant potential than the more red-colored anthocyanins (Von

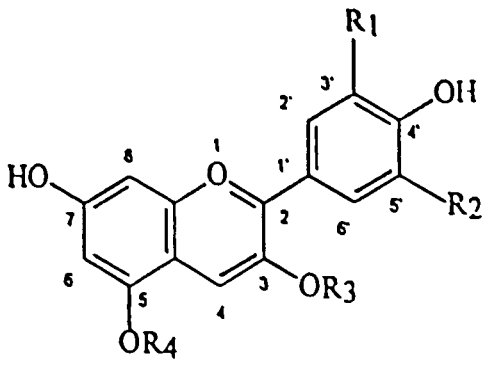


Figure 2. Basic Anthocyanin Structure.

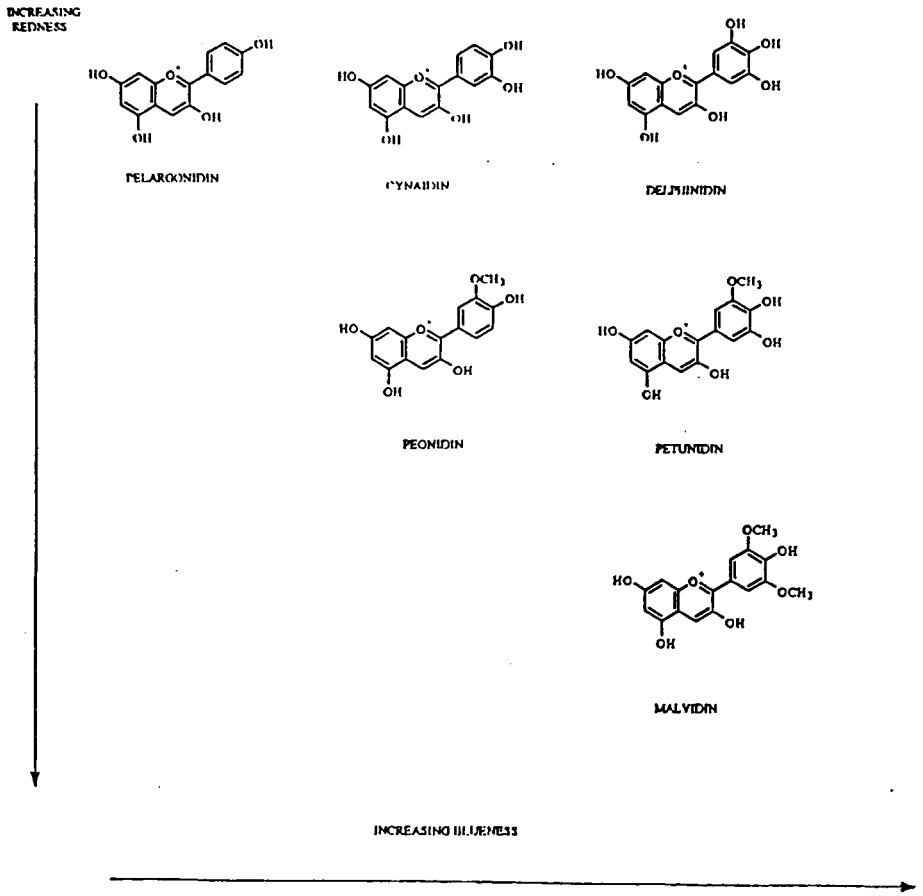


Figure 3. Change in Anthocyanin Color with Changes in Substitution.

Elbe and Schwartz, 1996). Anthocyanin content among plants varies from 20mg per 100g of fresh weight to more than 600mg per 100g of fresh weight. Individual species of plants always contain mixtures of several different anthocyanins. In addition, it is important to note that both the mixture and overall anthocyanin concentration vary widely within each species, depending on the cultivar, plant maturity at harvest, geographic location, and the relative weather patterns during the growing season (Mazza and Miniati, 1993).

In addition to possessing an exceptional antioxidant capacity, anthocyanins are known for their ability to impart antimicrobial and enzyme-inhibiting effects on biological systems (Hollman and Katan, 1999). Researchers exhibited noncompetitive inhibition of lipid peroxidation with the administration of anthocyanins (Narayan et. al., 1999). In addition, anthocyanins have demonstrated an ability to inhibit nitric oxide production by macrophages (Wang and Mazza, 2002). These two studies reflect the rationale for current research investigating the potential use of anthocyanins in the treatment and prevention of inflammatory-related disease.

Absorption of anthocyanins following single, pharmacological doses of isolated anthocyanins and specific, anthocyanin-rich fruit extracts has been demonstrated in previous research (Cao and Prior, 1999, Cao et. al. 2001, Mazza et. al. 2002). Moreover, volunteers in a most recent study experienced significant increases in both serum anthocyanin levels and ORAC scores following a single dose (100g) of freeze dried lowbush blueberry powder (Mazza et. al. 2002). Although this study was limited by its small sample size, these findings suggest that a similar study involving a larger number

of subjects might confirm the relationship between dietary anthocyanins and serum ORAC scores with a higher level of significance.

BLUEBERRIES

Wild North American blueberries (*Vaccinium augustifolium*) have the highest total antioxidant capacity of any commonly eaten fruit or vegetable tested by the United States Department of Agriculture (USDA) (Wang et. al., 1996). Wild lowbush blueberries have an oxygen radical absorbance capacity (ORAC) of 22.34 umol Trolox equivalents/g (Table 1). This ORAC score is approximately five times greater than that of a green grape and ten times as great as the banana (Wang et. al., 1996). Previous research indicates that blueberries have a relatively low vitamin and mineral content (Bushway et. al., 1983). As stated earlier, however, blueberries have exceptionally high flavonoid and polyphenol levels, specifically anthocyanins (Prior et. al., 1998). This abundance of anthocyanins produces an exceptional antioxidant potential. It should be noted that there are other fruits with high anthocyanin contents that have not been tested for total antioxidant capacity. These fruits, such as the bilberry (*Vaccinium myrtillus*), elderberry (*Sambucus nigra*), black currant (*Ribes nigrum*), and pomegranate (*Punica granatum*) may, in fact, have equally high or higher antioxidant capacities if tested in the future.

Five different anthocyanin aglycones or anthocyanidins are found in blueberries (Mazza and Miniati., 1993). Malvidin, delphinidin, cyanidin, petunidin, and peonidin are found in all *Vaccinium* species, with significant differences in both the types

#	ITEM	ORAC (umol Trolox equiv./g)
1	Blueberry	22.34
2	Blackberry	20.36
3	Garlic	19.39
4	Kale	17.70
5	Strawberry	15.36
6	Spinach	12.10
7	Raspberry	12.27
8	Brussell Sprouts	9.81
9	Plum	9.49
10	Alfalfa Sprouts	9.31
11	Broccoli Florets	8.88
12	Beets	8.41
13	Orange	7.50
14	Grape, Red	7.39
15	Pepper, Red	7.13
16	Cherry	6.70
17	Kiwifruit	6.02
18	Beans, Baked	5.03
19	Grapefruit, Pink	4.83
20	Beans, Kidney	4.60
21	Onion	4.49
22	Grape, White	4.46
23	Corn	4.02
24	Eggplant	3.86
25	Cauliflower	3.77
26	Peas, Frozen	3.64
27	Potatoes	3.13
28	Potatoes, Sweet	3.01
29	Cabbage	2.98
30	Leaf Lettuce	2.62
31	Cantelope	2.52
32	Banana	2.21
33	Apple	2.18
34	Tofu	2.13
35	Carrots	2.07
36	Beans, String	2.01
37	Tomato	1.89
38	Zucchini	1.76
39	Apricots	1.64
40	Peach	1.58
41	Squash, Yellow	1.50
42	Bean, Lima	1.36
43	Lettuce	1.16
44	Pear	1.14
45	Watermelon	1.04
46	Melon, Honeydew	0.97
47	Celery	0.61
48	Cucumber	0.54

Table 1. Fruit and Vegetable Oxygen Radical Absorbance Capacity (ORAC) Scores.

of anthocyanins and total anthocyanin content among subspecies (Kalt et. al., 1999). Wild North American lowbush blueberries have considerably higher total anthocyanin levels per 100g of fresh weight than both wild and cultivated highbush blueberries (*Vaccinium corymbosum*), as the majority of anthocyanins occur in the fruit's skin and lowbush blueberries have a greater amount of surface area per volume of fruit (Kalt et. al., 1999). Anthocyanin concentrations are highest in the skin, as they play a vital role in protecting the fruit's inner tissues from harmful overexposure to ultraviolet light. Hence, wild highbush blueberries have greater anthocyanin levels than cultivated highbush blueberries, and bilberries (*Vaccinium myrtillus*) have greater levels than any type of blueberry. In addition, lowbush blueberries contain a higher percentage of their anthocyanins in the form of cyanidin than highbush blueberries (Table 2). While it is unclear how significant individual anthocyanin distribution is in determining the different subspecies antioxidant capacities, this anthocyanin has a higher individual antioxidant score (Table 3) (Kalt et. al., 1999). Overall, the higher total anthocyanin level, in conjunction with the possible significance of differences in specific anthocyanin distributions, distinguishes lowbush blueberry antioxidant capacities from that of highbush varieties (Kalt et. al., 1999).

These differences in antioxidant potential are significant with respect to assessing blueberry consumption. Highbush blueberries are more often the only variety of blueberry available as a whole, relatively unprocessed fruit in grocery stores. These berries are usually available in both frozen and fresh versions. Traditionally, lowbush blueberries have been used predominantly in processed foods (jams, jellies, pies, fillings, etc.) and as an ingredient in assorted pastry products and flavored yogurt (Kalt et. al.,

Anthocyanidin	Bilberry	Lowbush blueberry[z]	Highbush blueberry[y]
	% of total anthocyanidins		
Cyanidin	31.25	14.19	5.81
Delphinidin	42.75	37.63	41.42
Malvidin	5.10	24.20	32.08
Peonidin	6.47	6.81	1.29
Petunidin	14.15	16.23	19.40

[z]Mix of wild clones.
[y]Mean value of cultivars Bluecrop, Coville and Jersey.

Table 2. Anthocyanidin Contributions Among Different Blueberry Species.

Anthocyanidin	ORAC score uM Trolox equivalents/umol
Cyanidin	2.24
Malvidin	2.01
Delphinidin	1.81
Peonidin	1.69
Pelargonidin	1.54

Table 3. Oxygen Radical Absorbance Capacity (ORAC) Scores of Individual Anthocyanidins.

2000). Until recently, unprocessed or fresh market lowbush blueberries have only been available to consumers in the local markets and grocery stores of the areas where the berries are harvested. These areas are generally limited to the Northeastern United States and Eastern Canada. Wild North American blueberries have very specific climate and soil requirements that are unique to Northeastern North America.

Fruit processing significantly affects the antioxidant capacity of blueberries and blueberry products (Kalt et. al., 2000). Anthocyanins are generally unstable molecules, with the greatest loss of stability under basic pH and high temperatures (Von Elbe and Schwartz). In addition, water activity (aw), and oxygen concentrations are also critical factors influencing anthocyanin half life in processing and storage (Kalt et. al., 2000). Therefore, conditions in a typical baking environment (presence of sodium bicarbonate and high temperatures) are detrimental to the fruit's antioxidant capacity in the finished product. In addition, the process of concentrating blueberry purees and juices into other products also reduces the overall antioxidant value. Although freezing is a mild form of processing, blueberries flash frozen in environments with low oxygen concentrations still experience significant losses in antioxidant potential. Kalt et. al. assessed a variety of commercially-prepared, lowbush blueberry products. Samples of individually flash-frozen lowbush blueberries had 41.0 to 25.7% lower ORAC scores than those of equal amounts of fresh berries. While these losses were substantial, frozen blueberries and fresh blueberry purees retained higher levels of un-oxidized anthocyanins than all other forms of processed blueberries and blueberry products (Table 4) (Kalt et. al., 2000).

Category	Product	Dry Weight %	ORAC mmol Trolox e1/100 g FW	mmol Trolox eq/100 g DW
Fresh	Fresh	17.7	6.34	52.9
Frozen	IQF 1	15.5	4.60	31.2
	IQF 2	12.2	4.80	39.3
	60% Blue, IQF	16.9	9.33	52.9
	80% Blue, IQF	12.6	8.53	50.7
	Flour coated fruit	26.7	5.99	22.3
Puree	Puree	12.8	5.37	42.0
Baked	Fresh Pie	28.5	5.38	19.1
	Pie Filling, rebaked	35.8	2.20	6.13
	Muffin	46.9	2.83	6.04
Canned	Pie Filling	36.6	2.37	6.49
	Fruit	25.4	4.76	18.7
	Light syrup	21.4	3.03	14.2
	Jam	27.3	2.88	10.6
Dried	Intermediate moisture	66.8	17.1	25.5
	Low moisture	83.2	12.6	15.1
	Cereal fruit	92.2	2.74	2.97
	Sugar infused fruit	71.8	8.11	11.3
	Blended powder	90.0	6.40	7.44
Sorbet	Sorbet	23.7	2.24	9.54
Juice	Concentrate	49.5	5.54	29.4
	Juice pomace	46.2	3.40	7.41

Table 4. Oxygen Radical Absorbance Capacity (ORAC) Scores of Commercially Prepared Blueberry Products.

FRUIT AND VEGETABLE CONSUMPTION

There is an abundance of consistent scientific evidence that high levels of fruit and vegetable consumption decreases a population's risk for a variety of degenerative diseases (Ames et. al., 1995, Ames 1999, Steinmetz and Potter, 1991). Recently, researchers revealed that men consuming fruit on a daily basis have a significant reduction in overall mortality, independent of known cardiovascular disease risk factors. Daily fruit consumption was positively associated with long term survival in middle-aged men when smoking, hypertension, and cholesterol were accounted for with the use of multivariate analysis (Strandhagen et. al., 2000). These results are consistent with previous research.

Stahlen et. al. found subjects with higher plasma antioxidants from increased fruit and vegetable consumption experienced lower incidence of cancer after a 12 year follow-up period (Stahlen et. al., 1991). It is also known that high dietary fruit and vegetable consumption is most protective against epithelial cell-based cancers (Steinmetz and Potter, 1991). In addition, new evidence in the etiology of atherosclerosis suggests that fruit and vegetable consumption (because of the concomitant antioxidant and fiber intake) may be more preventive than reducing dietary fat and cholesterol levels (Castelli, 1998).

While the exact mechanism behind this protective effect is not fully understood, there is general agreement among researchers that it is most likely due to the antioxidants in fruits and vegetables (Steinmetz and Potter, 1991, Lampe 1999). Strain et. al. showed that there is a dose-response relationship between plasma concentrations of antioxidants and frequency of fruit and vegetable consumption in middle-aged men living in the

United Kingdom (Strain et. al., 2000). Zino et. al. had similar findings with subjects in a study of a New Zealand population (Zino et. al., 1997). In an investigation into the effects of fruit and vegetable consumption on oxidative biochemical damage, the results of Thompson et. al. were more supportive of the mechanism by which fruits and vegetables offer protection against disease. Over a 14 day dietary intervention period, where an experimental group of women increased daily fruit and vegetable consumption from 5.8 servings to 12.0 servings, increased fruit and vegetable consumption significantly reduced markers of cellular oxidation. The experimental group had a greater overall reduction in markers of cellular oxidation than controls. Most notably, levels of 8-hydroxydeoxyguanosine (8-OHdG), a byproduct of oxidized DNA isolated from lymphocytes, fell 32% over the 14 days in the increased fruit and vegetable consuming-women versus 5% in the controls (Thompson et. al., 1999).

ANTIOXIDANT SUPPLEMENTS

While fruits and vegetables have undisputedly demonstrated protective effects, the efficacy of antioxidant supplement use and the role of supplements in disease prevention, in general, remain uncertain. To date, there have been diverse findings in a wide array of efforts made to demonstrate a relationship between supplemental vitamins, antioxidants, phytonutrients, and a variety of diseases. The nature of these investigations has varied greatly in design.

In general, human research with antioxidant supplements has failed to demonstrate a significant change in mortality, disease incidence, or free radicals with regular supplementation (Prieme et. al., 1997). Vitamins E and C, Co Q10 (ubiquinone),

beta carotene, and melatonin, as well as multivitamin supplements, do not offer any significant protection against premature mortality or disease incidence in comparison to controls (Van Poppel et. al., 1995, ATBC Study Group, 1994, Omenn et. al., 1996, McKay et. al., 2000). Eighty individuals, ages 50 to 87 years, were supplemented with a multivitamin (containing 100% of the Daily Recommended Intake of the antioxidants A, C, and E for adults) over an eight week period. This intervention produced no significant changes in total blood antioxidant levels (McKay et. al., 2000). In fact, two major antioxidant intervention trials have actually found that subjects taking beta-carotene supplements were at an increased risk for developing lung cancer and heart disease in comparison to un-supplemented subjects (ATBC Study Group, 1994, Omenn et. al., 1996).

The Alpha-Tocopherol, Beta Carotene Prevention Study Group (1994) observed that among 30,000 male smokers living in Finland, those men who had received 20 mg beta-carotene supplements were 36% more likely to develop lung cancer in the follow up period than those men who did not receive the supplement. Omenn et. al. also discovered a similar surprise in the Beta-Carotene and Retinol Efficacy Trial (1996). Again, smokers, as well as subjects who had been exposed to asbestos, were given either a 30 mg beta-carotene supplement or a placebo. Those receiving a beta-carotene supplement experienced a 28% greater incidence of lung cancer, a 26% greater mortality from heart disease, and a 17% higher overall mortality rate than the control group. These findings raise alarming questions about both the safety and efficacy of antioxidant supplement use.

The results of animal studies are mixed. Although numerous studies have found that antioxidant-supplemented animals do not experience an increase in longevity or disease incidence (Holloszy, 1998, Lipman et. al., 1998), certain antioxidants, such as alpha-lipoic acid, have been shown to reduce inter- and intracellular biomarkers of oxidative damage (Hagen et. al., 1999, McGahon et. al., 1999, Suh et al., 2001, Bondy et. al., 2002, O'Donell et. al., 1998).

More specific inquiries into the effect that supplements have on the antioxidant capacities of different physiological compartments have yielded similar differences. Compromised electron transport chain activity is an indicator of long term oxidative stress and is positively associated with biochemical aging. Supplementation with a variety of antioxidants produced no improvement in cerebral cortex electron transport chain activity (Sharman and Bondy, 2001). There was no reduction in oxidative stress markers or improvements in psychomotor performance when rat diets were supplemented with vitamin E, glutathione, melatonin, or strawberry extract (Shukkit-Hale et. al., 1999). However, in a follow up study, rats did experience psychomotor-related improvements when their diets were fortified with different fruit and vegetable extracts, most notably freeze-dried blueberry extract (Joseph et. al., 1999).

PLANT EXTRACT AND NUTRACETICAL RESEARCH

Research examining the effect of fruit, vegetable, and herbal extracts on exogenous and endogenous antioxidant defense systems, as well as other parameters of physiological health, has largely demonstrated positive effects (Youdim and Deans, 1999, Joseph et. al., 1998, Joseph et. al., 1999, Facino et. al., 1999). Youdim and Deans

investigated the effect of a thyme oil supplemented diet on the antioxidant status and omega 3 fatty acid content in aging rat brains. This study showed that omega 3 fatty acid content of rat brains fed the thyme oil diet were protected against lipid peroxidation and that antioxidant enzyme activities were increased significantly in the thyme oil fed rats (Youdim and Deans, 1999).

In what may be the most interesting animal research in this area as of yet, researchers added blueberry (18.6g/kg), spinach (9.1g/kg), or strawberry (14.8g/kg) dried aqueous extracts to the diets of 19-month old Fischer 344 rats for 8 weeks.
All three extract fed groups experienced greater striatal dopamine release with the blueberry fed group experiencing the greatest release in comparison to the controls. GTPase activity from striatal slices were significantly higher in both the spinach and blueberry fed groups. Again, the blueberry fed group scored the highest of all groups. Calcium recovery from striatal synaptosomes in the presence of oxidative stress was enhanced in only the blueberry fed rats. In addition, the blueberry fed group was the only group to experience improved motor performance on two tests which rely heavily on balance and coordination (Joseph et. al., 1999). These aforementioned studies, while limited in application because of their use of animals, obviously warrant similar investigative approaches to examine the role of such dietary additions in human performance.

HUMAN SERUM ANTIOXIDANT RESEARCH

Yeum et. al. demonstrated that there was a linear relationship between the quantity of fruits and vegetables consumed and the level of carotenoid antioxidants in

subjects (Yeum et. al., 1996). Women fed 12 servings of fruits and vegetables per day for 14 days had a significant reduction in the amount of oxidative damage to DNA and lipids, in comparison to a control group who consumed 5.8 servings per day (Thompson et. al., 1999). Volunteers that were fed diets containing ten servings of commonly-eaten fruits and vegetables each day experienced significant increases in plasma antioxidant capacity (Cao et. al., 1998). Villeponteau et. al. observed a significant decrease in the biomarkers of oxidative stress in volunteers consuming a concord grape extract combined with a mixture of antioxidants. In comparison to controls, subjects receiving the proprietary blend of concord grape extract and selected micronutrients reduced oxidative stress levels by 27% ($p<0.019$) (Villeponteau et. al., 2000). Stewart et. al., however, found that children fed a fruit and vegetable concentrate-fortified supplement had no significant changes in oxidative stress levels after 21 days of supplementation and were not significantly different from a control group of similar children (Stewart et. al., 2002).

Only a few studies to date have attempted to examine the effects of diets high in specific antioxidant-rich fruits and vegetables on different biochemical markers in human populations. Subjects fed Brussels sprouts (300g/day) showed marked decreases in the amount of free radical-damaged DNA, as measured by 8-hydroxydeoxyguanosine excreted in the urine (Verhagen et al., 1995). Kaplan and Aviram discovered that subjects consuming moderate amounts of pomegranate juice also experienced significant reductions in other signs of oxidative cellular damage (Kaplan and Aviram, 1999).

In research most closely related to this investigation, Cao et. al. assessed the antioxidant capacity of elderly women fed beverages containing strawberries (240g), red wine (300ml), spinach (294g), or vitamin C (1250mg) (Cao et al., 1998). Subjects were

admitted into the research clinic the evening prior to the day of testing. The clinical nature of this study ensured that subjects fasted overnight and that meals during each day of testing were low in antioxidant value. This procedure helped reduce the possibility of dietary interference from other foods. Total antioxidant capacity of serum and urine samples was measured using oxygen radical absorbance capacity (ORAC), Trolox equivalent antioxidant capacity (TEAC), and ferric reducing ability (FRAP) assays.

Dietary treatments raised both urine and serum antioxidant capacities. During the four hour post-prandial period, serum antioxidant capacity increased by 7 to 25% ($p<.04$) across all treatments. Urine ORAC values increased by 9.6, 27.5, and 44.9% ($p<.05$) for strawberry, spinach, and vitamin C treated groups, respectively (Cao et. al., 1998). This researcher's findings were significant in spite of an extremely small sample size ($n=8$). The increases in urine and serum antioxidant capacities could not be accounted for by the food's vitamin content alone. This study helped to define the importance of the antioxidant contributions made by non-vitamins in fruits and vegetables.

Most recently, Mazza et. al. fed five male subjects freeze-dried wild blueberry powder (100g) mixed with 500ml of water. In comparison to control meals administered seven days prior, blueberry extract consumption produced serum ORAC (acetone treated) assays that were significantly higher four hours post prandial, 58.9 vs 50.7 Trolox equivalents ($p<.04$) (Mazza et. al., 2002). This current investigation is the first to examine the effect of the regular consumption of one fruit or vegetable, with a high antioxidant value, on a population's serum antioxidant capacity.

SERUM ANTIOXIDANT CAPACITY

Serum antioxidant capacity is the sum of all circulating endogenous and exogenous antioxidants. While the previously mentioned research has demonstrated the effect of dietary or exogenous antioxidants, it is important to consider the numerous other factors that influence an individual's antioxidant capacity. Several endogenous antioxidants are significantly affected by medications, sunlight, sleep, stress, and other lifestyle characteristics. Medications that challenge phase I and phase II detoxification enzymes reduce the body's ability to neutralize reactive species. Melatonin is a relatively strong antioxidant that is largely produced endogenously, although a limited number of foods contribute significant amounts. Serum antioxidant capacity is influenced by melatonin secretion. Melatonin production is significantly affected by circadian rhythms, exposure to direct light and darkness, and psychological stress. Katz et. al. observed increased oxidation of melatonin, as measured by the urinary presence of the melatonin metabolite 6-sulfatoxymelatonin, in pregnant women within stressful workplaces. Urinary 6-sulfatoxymelatonin levels increased 81% in women within the workplace in comparison to non-workplace environments (Katz et. al., 1995).

Psychological stressors have also exhibited effects on other serum antioxidants. Schmidt et. al. found that exercising Marines in a stressful environment had greater signs of oxidative stress than controls (Schmidt et. al., 2002). In addition, it is possible that endogenously produced antioxidant levels decrease in stressful environments. Specific minerals and trace minerals play critical roles in a variety of antioxidant systems. While selenium has antioxidant and antioxidant-sparing potential by itself, zinc, copper, and manganese are essential components to the formation of endogenous antioxidant proteins.

Pizent et. al. discovered that prisoners of war had suppressed levels of both zinc and copper in comparison to non-combat military personnel (Pizent et. al., 1999).

The fat-soluble vitamin D might also influence serum antioxidant capacity. Although vitamin D is found in a number of fortified foods, endogenous production from exposure to ultraviolet light is the largest contributing source in most populations with access to adequate sunlight. Numerous studies have documented the seasonal fluctuations in circulating vitamin D levels in a variety of populations at higher latitudes (Woitge et. al., 2000, Perry et. al., 1999, Rapuri et. al., 2002, Melin et. al., 2001). Those subjects with inadequate exposure to ultraviolet light in the winter months experience significant declines in serum 25-hydroxyvitamin D (25OHD) levels.

SERUM ANTIOXIDANT ASSAYS

There are numerous antioxidants that contribute to total serum antioxidant capacity. Serum antioxidants include macromolecules (albumin, ceruloplasmin), enzymes (glutathione peroxidase, superoxide dismutase), and a potentially large number of individual, single molecule antioxidants (carotenoids, vitamin D, ascorbic acid, tocopherols, biliruin, uric acid, anthocyanins). The difficulty in assessing serum antioxidant capacity lies in the diverse action of each different antioxidant. Also, antioxidants are carried or held in different compartments of human serum; lipid or aqueous-soluble. Several assays have been developed thus far for measuring one or more of the different types of antioxidants.

ORAC

In 1992, the oxygen radical absorbance capacity (ORAC) assay was developed (Cao et. al., 1993), offering considerable advantages to other assays widely used at that time. The Trolox equivalent antioxidant capacity assay (TEAC), developed by Miller et. al., and the total peroxyl radical trapping parameter (TRAP), developed by Wayner et al., had been the most popular assays prior to the development of the ORAC (Wayner et. al., 1985, Miller et. al., 1993). Each of these previously used antioxidant assays used either the free radical absorbance inhibition time (TRAP) or percentage (TEAC) (Cao and Prior, 1998).

To date, the ORAC assay is the only method that takes free radical action to completion, combining both inhibition time and inhibition percentage of the free radical species by antioxidants within a sample. These two measurements are simultaneously quantified through an area-under-curve technique (Cao and Prior, 1998). The ability of this assay measures both the percentage of free radicals that are inhibited and the length of time required for inhibition to take place, making the ORAC assay the most comprehensive measurement of antioxidant potential available (Cao and Prior, 1999).

ORAC assays have been conducted on a wide variety of biological specimens ranging in complexity from tea to animal tissue (Cao et. al. 1996, Wang et. al. 1996, Cao, Giovanni, and Prior, 1996). In addition, ORAC assays can be made more specific by utilizing different extraction techniques. Serum proteins can be extracted with perchloric acid (PCA) or acetone to isolate nonprotein fractions. Previously, perchloric acid (1:1, serum:PCA, at 0.5 M) was most commonly used to precipitate proteins for separation. More recently, however, acetone (1:1, 0.5M) has been used as a more effective method of

extracting both hydo- and lipophillic protein fractions, while still capturing lipid soluble antioxidants (Cao and Prior, 1998). Although total plasma ORAC may be more reflective of an individual's serum antioxidant capacity, the non-protein fraction ORAC is most reflective of the impact of an individual's dietary antioxidant intake. The non-protein fraction eliminates the antioxidant activity of endogenously produced enzymes, which vary greatly within human subjects.

FRAP

The ferric reducing ability of plasma (FRAP) assay measures the reduction of ferric-to-ferrous iron within a sample of antioxidants (Benzie and Strain, 1996). Although the FRAP assay is relatively simple and inexpensive, it does not truly measure a sample's antioxidant capacity. The FRAP assay measures the ferric reducing ability of a substance, instead of subjecting the substance to a known concentration of oxidant or free radical. There is a distinct difference between a serum antioxidant capacity and the ferric reducing ability of serum. It is important to note that the FRAP assay is unable to detect the antioxidant capacities of serum proteins and the sulfur-containing antioxidants (Cao and Prior, 1998). Nevertheless, the FRAP assay can be used for correlation with ORAC assays. FRAP assay results have, historically, correlated more closely with non-protein serum fraction ORAC assay results (Cao and Prior, 1998). This difference in correlations might possibly be explained by the presence of sulfur-containing antioxidant enzymes in total serum that are extracted by perchloric acid or acetone in the production of a non-protein fraction. In addition, FRAP assays can be used as a method of quality control. Cao and Prior found that within-run critical values (CV) of antioxidant

measurements of human serum were 0.6% with the FRAP assay, compared to 3.5% and 2.5% with the ORAC and TEAC assays, respectively (Cao and Prior, 1998).

Bilirubin

In vitro, bilirubin is a known antioxidant. Although bilirubin's role as an antioxidant is poorly understood, it is necessary to measure serum levels in conjunction with other serum antioxidant assays. Significant increases in serum antioxidant assays can be more definitively attributed to dietary constituents if there is no concomitant rise in serum bilirubin levels.

Uric Acid

Uric acid represents a major component of an individual's total serum antioxidant capacity. As much as 29% of total antioxidant activity can be attributed to uric acid (Cao and Prior, 1998). Post prandial serum urate levels rise significantly and may change with dietary intervention (Cao and Prior, 1998). Research that attempts to measure total serum antioxidant capacity with dietary change must also examine serum uric acid levels. Validation that changes in serum antioxidant capacity are at least partially a result of a dietary intervention, rather than endogenous antioxidant production or metabolic changes, can be supported by the lack of change in serum uric acid levels.

Functional Intracellular Analysis: Spectrox®

Lymphocyte antioxidant capacity is a lesser known and distinctly unique antioxidant assay. Functional Intracellular Analysis (FIA), (Spectracell Laboratories, Houston, Texas) utilizes lymphocyte growth rates in a specifically developed growth medium, Clayton Foundation Biochemical Institute 1000 (CFBI 1000), to determine cellular nutrient levels. An *in vitro* growth assay that examines growth responses to

known concentrations of nutrients, FIA technology attempts to reflect nutritional status at the tissue level (Bucci, 1994). Lymphocyte lifespan is approximately 3-12 months. Therefore, lymphocyte nutrient levels should not be significantly influenced by short-term dietary changes and should represent an individual's nutritional status over the past several months (Boerner, 2001).

FIA Spectrox measures lymphocyte growth rates in response to the addition of cumene hydroperoxide to the growth medium. Cumene hydroperoxide (CuOOH) is a free radical-generating compound that challenges the antioxidant defense systems. Inhibition of lymphocyte growth in the presence of cumene hydroperoxide is compared to growth in a 100% medium. At high concentrations of cumene hydroperoxide, cell growth of lymphocytes is completely inhibited. At lower concentrations, however, resistance to oxidative damage from free radicals and subsequent cell growth inhibition varies significantly between individuals (Boerner, 2001).

The results of prior Spectrox®-based research have shown an ability to detect changes in lymphocyte antioxidant capacity with dietary intervention over three to five months (Ellithorpe). Although Spectrox® results related linearly with lymphocyte glutathione levels (R2=0.604), another FIA antioxidant assay, there is no data comparing FIA Spectrox® assay results to those of other serum antioxidant capacity assays (Boerner, 2001).

DIETARY ASSESSMENT METHODS

There are numerous dietary assessment tools currently available. Diet history, twenty-four recall, three and seven day food records, and food frequency questionnaires

are all used in efforts to capture accurate dietary information. Dietary assessment tools and methods vary greatly in validity, ease of administration, user friendliness, and the time required for data entry and assessment. In general, diet history and twenty-four hour recall methods are most useful for those efforts attempting to determine general diet trends and characteristics in large numbers of subjects. These methods inherently miss a significant number of specific dietary constituents, as there is no list of food items available to prompt a more detailed recall. Three and seven day food records do offer greater insight into the details of an individual's diet, but are relatively burdensome to subjects and require more time for data entry. Overall, food frequency questionnaires are more effective at capturing specific dietary items (i.e. fruits and vegetables) and can be used in conjunction with established data bases for a thorough data analysis of a vast number of specific dietary nutrients (i.e. antioxidants) (Willett et. al., 1985, Rimm et. al., 1992).

Food Frequency Questionnaires

Fruit and vegetable consumption patterns range widely in both the quantity and types of fruits and vegetables consumed. Food frequency questionnaires enable subjects to recognize and recall specific items more accurately than other dietary assessment methods. While the results of research examining the efficacy of food frequency questionnaires have been mixed, food frequency questionnaires have been shown to be more effective at assessing micronutrients than macronutrients (Schaefer et. al., 2000, Jacques et. al., 1993).

Over the past several years, food frequency questionnaires have been developed to be more effective at capturing data on a large number of antioxidants (Willett, 1985,

Michaud et. al., 1998, Ritenbaugh et. al., 1996). Food frequency questionnaires reflect carotenoid and vitamin C intake more accurately than vitamins A and E (Boeing et. al., 1997, Munger et. al., 1992, McCarty et. al., 1997). The Tufts Elderly Health and Food Frequency Questionnaire and the Willett Food Frequency Questionnaire were developed specifically for assessing various dietary antioxidants. The Tufts Elderly Health and Food Frequency Questionnaire, however, was more appropriately designed for use in an elderly population. This food frequency questionnaire has a database that contains both vitamin and non-vitamin antioxidants (carotenoids). In previous attempts to assess validity and reproducibility, the Tufts Elderly Health and Food Frequency Questionnaire produced relatively high correlations with plasma carotenoid concentrations (Tucker et al., 1999).

Fruit and vegetable consumption assessment through the use of limited food frequency questionnaires has thus far failed to produce exceptionally high levels of correlation with 24-hour recall and comprehensive food frequency questionnaires (Thompson et al., 2002). The New England Regional 172 (NE-172) Fruit and Vegetable Questionnaire is currently being developed at the University of Rhode Island for assessment of carotenoid consumption. This questionnaire could offer distinct advantages over broader food frequency questionnaires when examining specific dietary constituents, such as carotenoids. This specific questionnaire is designed to capture data on those food items that contribute the most significant amount of antioxidants to the diet overall, through the use of a relatively comprehensive carotenoid database (Fey-Yensan, 2003).

CHAPTER 2

MATERIALS AND METHODS

METHODOLOGY

In January of 2001, the Wild Blueberry Association of North America (WBANA) and the United States Department of Agriculture (USDA) were approached in an effort to determine if there was interest in this research area. The overall response by both organizations reflected a keen interest in the topic and each expressed that there was potential for financial assistance. After forming and submitting an initial proposal to both the WBANA and the USDA, it was learned that each would agree to provide grants for this research. Upon confirmation of financial support, a consent form was developed for potential subjects involved in this study (Appendix A). The consent form was presented before the University of Maine Human Research Consent and Review Committee. The consent form was later approved by the committee (Appendix B).

Subject Recruitment

Twenty-eight women, between the ages of 60 and 90, were recruited from the greater Farmington Area in Western Maine (Appendix C). The women were recruited through the use of bulletin notices, phone calls, and by word of mouth. This geographic area and the University of Maine at Farmington were chosen as the site for this research for a variety of reasons. One of the primary researchers involved in this study lived in Farmington and was familiar with the area and its resources. Subject recruitment was greatly enhanced by the researcher's ties with different community organizations. The

primary researcher was also employed as an instructor at the University of Maine at Farmington and was therefore allowed to use a number of the university's facilities in this investigation. These facilities included the University of Maine at Farmington Health and Fitness Center offices, for subject recruitment, screening, and food frequency interviews, the Ricker Chemistry Laboratory, for sample treatment and storage, and the University of Maine at Farmington Student Health Center, for blood sampling and blueberry distribution. The close physical proximity of all of these facilities aided immensely to the logistical planning and ease of subject accessibility of this study (Appendix D).

Subject Screening

Subjects were accepted for participation upon verbal confirmation that they were not taking any medications from a list of drugs that are known to affect the body's endogenous antioxidant pool or ability to detoxify reactive species (Appendix E). These medications are generally known as hepatic or cytochrome P-450 challenging substances and include most cholesterol-lowering medications (Lipitor, Mevacor), anti-depressants (Paxil, Zoloft), and hydrochloric acid release-inhibiting drugs (Prilosec, Zantac). In addition, medications used for managing hypertension also eliminated potential subjects from eligibility, as these medications are generally either diuretics (which increase urinary antioxidant losses) or vasorelaxants (which require hepatic detoxification and hence rely on the body's endogenous antioxidant enzymes). Lastly, women were asked to discontinue taking any vitamin or multivitamin supplements from this point in time (one month prior to the intervention period) to the completion of the study (final blood

draw). Women were allowed to continue taking individual calcium supplements, as the majority had received physician instructions to do so.

Upon agreement to participate, subjects were asked to answer a medical questionnaire and sign the study's consent form (Appendices F). Fourteen of the women were randomly selected as the treatment group of the study and 14 subjects as controls through a lottery-draw method. Subjects were then scheduled for a dietary interview. Each woman was given an interviewer-assisted Tufts Elderly Health and Food Frequency Questionnaire (Appendix G). This questionnaire was selected for two major reasons. As stated earlier in this paper, the Tufts Elderly Health and Food Frequency Questionnaire was developed to examine the intake of a variety of antioxidants in an elderly population's diet. In addition, as members of the New England Regional-172 Research Group, Tufts University and the University of Maine have similar interests in this research area and there was a cooperative effort by Dr. Katherine Tucker of Tufts University and the researchers initiating this effort. Dr. Tucker agreed to provide the food frequency questionnaires and assess the data for a reduced fee.

In February of 2001, questionnaires were administered in either the subject's home or in offices within the University of Maine at Farmington Health and Fitness Center. After all subjects had completed the questionnaire, blood sample draws were scheduled for three consecutive mornings (February 21, 22, and 23) at the University of Maine at Farmington Student Health Center. The women were instructed to continue eating their usual diet and to fast for the twelve-hour period of time (overnight) preceding their scheduled blood draw. In addition, those women randomly selected to the blueberry intervention group were notified and instructed to bring a cooler with them so that they

could keep the blueberries frozen while returning home. Four subjects (two members of the control and two members of the treatment arm) dropped out of the study due to illness, scheduling difficulties, and general attrition.

Upon arrival at the health center, women were given a New England Regional Research 172 Fruit and Vegetable Screener (Appendix H). This fruit and vegetable questionnaire was given by a research assistant as part of a master's degree requirement. The questionnaire was administered while women waited for scheduled blood drawing. Incomplete data was collected with the NE-172 Fruit and Vegetable Screener, as not all subjects were given the assessment.

Serum Sample Acquisition

Twenty-four milliliters of blood was drawn from each subject by a trained and licensed phlebotomist. Two different samples were collected by venapuncture. The first sample was drawn into a patented 24 mL Spectracell Technologies tube containing an anticoagulant and antioxidant. Each Spectrox sample tube was inverted a complete 180 degrees four times to mix the drawn blood with the tube contents. All tubes were properly labeled with the date the sample was drawn and the subject's identification number. These samples were shipped via overnight delivery to Spectracell Laboratories, Houston, TX. The second sample was drawn into a 12mL heparinized, vacuum sealed, Tiger Top glass tube. Again, all tubes were labeled accordingly. The samples were placed in ice for 30 minutes and then centrifuged at high speed (30 revolutions per second) for 30 minutes. Afterwards, the centrifuged samples were carefully transported on ice, by foot, to the local campus chemistry laboratory (across the street) for further preparation.

Sample Preparation

Surface plasma was removed from each sample by means of a manual pipette. The plasma from each sample was subdivided into five 2.0 mL Corning Cryogenic Internal Thread Vials. The subdivided plasma consisted of four 0.75 mL and one 1.0 mL samples. Two of the 0.75 mL plasma samples were individually treated with 0.75mL of perchloric acid (PCA 0.5M). The PCA treated plasma was fractioned for ORAC, vitamin C, and uric acid analysis. Two other untreated 0.75 mL plasma samples were allocated for bilirubin, FRAP, and vitamin E analyses. The final 1.0 mL was treated with 0.10mL of trifluoroacetic acid (TFA, 0.5M). The TFA treated sample was designated for assessing serum anthocyanin content. Once more, all cryogenic vials were labeled with the subject's identification number, the date, and the method in which the plasma had been treated. All plasma samples were stored at -40 C.

Blueberry Distribution

After subjects had successfully completed the blood draw, those women randomly assigned to the experimental group were given a 30 day supply of frozen, wild, North American lowbush blueberries (*Vaccinium augustifolium*). These blueberries had arrived in Farmington approximately two weeks prior to the initial blood draw. The blueberries were selected from the year 2000 harvest's research sample lot. These blueberries represented those harvested in different locations within Maine, Nova Scotia, and New Brunswick. The berries from different locations were then mixed and used to create this research batch. This mixture was chosen for use in research in an attempt to provide a broad representation of wild, North American blueberries. These efforts were made to provide uniform and consistent characteristics in the berries used for research, as

differences in geographic location and harvest methods can affect the fruit's antioxidant capacity. The blueberries were delivered frozen, by truck, from Cherryfield, Maine. They were transported in 15 pound boxes that were contained within larger Styrofoam insulated containers. All of the delivered blueberries were divided into allotments consisting of two-gallon Ziploc Freezer bags.

Each woman was given two of the two-gallon containers of wild, North American blueberries, as this represented exactly 31 cups of berries. The women were given the one extra cup of berries as replacement for the possible spillage or loss of berries during the intervention period. The women were instructed to keep the blueberries frozen throughout the 30 day trial period. The subjects were instructed to eat exactly one cup of blueberries each day, with ½ cup before 10 am and ½ cup after 5pm. The blueberries for the next day could be thawed out at room temperature the night before, but they were not to be cooked in any way (baking, microwave, etc.). Numerous recipes and ideas for incorporating the blueberries into a meal plan were given to the women as well. Those women not selected as part of the experimental group were informed that they, too, would receive a 30 day supply of frozen blueberries upon completion of the study.

Trial Period

Women in the experimental group were given the appropriate phone numbers of the primary researchers involved and instructions to call if there were any questions or concerns at any time. No subjects called at any time during the study. In addition, each subject received two phone calls during the 30 day experimental period. After 10 days, the women were called in an effort to increase study compliance and to see if they needed any assistance. The second phone call was made at the end of twenty days to all subjects.

All women were reminded of the second blood draw and were again instructed to fast for the twelve-hour period prior to their scheduled appointment. In addition, members of the experimental group were asked to continue eating the blueberries and were again assisted with recipes or new ideas if needed.

At the end of 30 days, subject blood draws were again collected by venapuncture. All methods used in the initial blood draw were repeated. Frozen blueberries were distributed to those subjects within the control group. It should be noted that there was a snow storm in the evening prior to the final blood draw. Although many of the women were concerned about the conditions of the road and general safety of traveling to and from the student health center, all scheduled subjects reported on time. Special arrangements were made earlier that morning for subjects to car pool with one another and in certain cases, transportation was provided by the researcher.

Sample Analysis

All frozen plasma samples were packed on dry ice and shipped, via overnight delivery, to the Arkansas Children's Nutrition Center, Little Rock, Arkansas. Upon delivery, frozen samples were again stored at -40 C. Later, research assistants under the supervision of Dr. Ronald Prior (Director, Arkansas Children's Nutrition Center, Little Rock, AR), assayed the plasma samples for total plasma ORAC, FRAP, bilirubin, uric acid, and non-protein plasma ORAC (treated with acetone, 1:1, 0.5M). While this study was being conducted, the laboratory of the Arkansas Children's Nutrition Center was being moved. In this process, some of the samples were inadvertently misplaced. The loss of this portion of the plasma samples required researchers to eliminate vitamin C

assays, as it was deemed more essential to use the remaining samples for the ORAC assessment of the plasma without interfering proteins (ORACac).

Blood samples received at Spectracell Laboratories, Houston, Texas, were immediately processed for FIA Spectrox® assessment (Appendix I). Dr. Fred Crawford supervised lymphocyte isolation, lymphocyte addition to CFBI 1000 growth medium, cumene peroxide addition, and measurement of cell growth inhibition. Completed Tufts Food Frequency Questionnaires were mailed to the United States Department of Agriculture Human Nutrition Research Center on Aging, Tufts University, Boston, Massachusetts. Questionnaire data was entered under the supervision of Dr. Katherine Tucker.

CHAPTER 3

RESULTS

Serum antioxidant capacity, which was measured as ORAC total plasma, ORAC acetone treated (non-protein) plasma, and FRAP, as well as serum uric acid and bilirubin, at the first blood draw (day 0) following a 12 hour fast, are presented in tables 5-7. No significant differences were observed between control and blueberry treatment groups at day 0. Groups were compared statistically using two-tailed, paired t tests.

Table 5

Baseline Serum Antioxidant Values

	FRAP* uM Trolox equivalent	ORAC** uM Trolox equivalent	ORAC ac*** uM Trolox equivalent
Control Group	234.7	11898.8	903.2
Blueberry Group	216.3	11726.9	747.3
Difference	18.4	171.8	155.9
Probability	0.275	0.805	0.750

*FRAP represents the ferric-reducing ability of plasma

**ORAC represents the oxygen radical absorbance capacity

** ORAC ac represents the non-protein portion of plasma sample

Table 6

Baseline Lymphocyte Antioxidant Values

	Spectrox® score*
Control Group (n=12)	65.5
Blueberry Group (n=12)	61.5
difference	4.0
probability	0.679

*Spectrox ® is a patented Spectracell Laboratories antioxidant assay. Scores represent a percent of standard reference value for complete in vitro antioxidant protection against known concentrations of cumene hydroperoxide (a free radical-generating, reactive species).

Table 7

Baseline Bilirubin and Uric Acid Scores

	Bilirubin mg/dL	Uric acid mg/dL
Control Group (n=12)	0.49	6.06
Blueberry Group (n=12)	0.47	6.37
difference	0.02	0.21
probability	0.837	0.782

Dietary data associated with antioxidant values, as collected by the Tufts Elderly Health and Food Frequency Questionnaire, are presented in tables 8 and 9. No significant differences were observed between control and blueberry group dietary antioxidant data at day 0. Groups were compared with two-tailed, paired t-tests.

Table 8

Dietary Antioxidant Levels: Vitamins

	Vitamin C mg/day	Vitamin D mcg/day	Vitamin E mg/day	Vitamin A mcg/day	Total A Activity mcg/day, retinol equival.
Controls	197.1	7.3	10.8	648.6	1748.0
Blueberry	201.5	8.3	9.4	693.1	1891.3
Difference	4.4	1.0	1.4	45.5	143.3
Probability	0.907	0.602	0.669	0.833	0.716

Table 9

Dietary Antioxidant Levels: Carotenoids

	beta carotene mcg/day	alpha carotene mcg/day	cryptoxanthein mcg/day	lycopene mcg/day	lutein mcg/day
Controls	6355.5	687.7	77.5	5101.5	3205.6
Blueberry	6295.0	1095.3	107.6	4444.7	4260.9
Difference	60.5	407.6	30.1	656.8	1055.3
Probability	0.972	0.189	0.409	0.728	0.241

Upon completion of 30 days, serum antioxidant capacities, uric acid, and bilirubin levels were measured again following a 12 hour fast. Serum antioxidant capacities were measured as ORAC total plasma (oxygen radical absorption capacity), ORAC acetone treated (non-protein) plasma, and FRAP (ferric reducing ability of plasma). The change in each subject's serum parameters from the first blood draw (day 0) to the second blood draw (day 30) are presented in tables 10-15.

Statistical comparison of the results of the acetone treated serum ORAC assay and the FRAP assay (neither of which recognizes protein-based antioxidant contributions to the serum antioxidant capacity), by means of a Pearson's correlation test, revealed a strong positive relationship in the blueberry consuming group. The statistical analysis resulted in a correlation coefficient (r) of 0.72 for the blueberry consuming group and 0.3397 for the control group.

Mean oxygen radical absorbance capacity (ORAC) values significantly decreased in the control group over thirty days, while the blueberry consuming group experienced no significant changes.

Table 10

Initial and Final Total Plasma ORAC* Scores

Subject	Initial ORAC	Final ORAC		
Controls	uM trolox equ/mL	uM trolox equ/mL		
1	11595	9339		
2	12898	12092		
3	11037	11012		
4	12946	12173		
5	11757	10661		
6	13506	9956		
7	16165	11148		
8	10057	10525		
9	10746	10515		
10	10397	10777		
11	10971	9746		
12	10710	11012	difference	p
mean	11899	10746	-1153	0.038
std. dev.	1732	846		
Blueberry				
1	13448	9886		
2	12738	12329		
3	8885	10818		
4	10038	12863		
5	14606	9792		
6	12463	12389		
7	10922	12283		
8	13222	12783		
9	11343	12503		
10	10458	13861		
11	10617	10598		
12	11983	11783	difference	p
mean	11727	11824	97	0.890
std. dev.	1643	1272		

• ORAC represents oxygen radical absorbance capacity of plasma samples

Acetone-treated serum (1:1, 0.5M) ORAC values significantly increased in the blueberry consuming groups, with no concomitant changes in the control group. The acetone treatment removes endogenous antioxidant enzymes and other proteins with potential antioxidant value from the serum.

Table 11

Initial and Final ORACac* Scores

Subject	Initial ORACac	Final ORACac		
Controls	uM trolox equ/mL	uM trolox equ/mL		
1	994.2	987.6		
2	957.0	896.8		
3	773.7	672.0		
4	749.4	893.4		
5	392.1	1214.8		
6	751.6	857.0		
7	1245.3	842.0		
8	1132.3	1232.2		
9	737.0	640.5		
10	1101.7	1237.5		
11	1041.4	1109.2		
12	963.0	845.5	difference	p
mean	903.2	952.4	49.2	0.566
std. dev.	232.	206.6		
Blueberry group				
1	296.3	953.5		
2	829.2	1017.9		
3	795.3	866.1		
4	386.4	1074.4		
5	986.8	1084.4		
6	1597.9	1266.2		
7	443.7	853.5		
8	734.8	843.5		
9	1085.5	960.8		
10	858.5	891.9		
11	820.3	913.0		
12	132.8	1056.3	difference	p
mean	747.3	981.8	234.5	0.048
std. dev.	396.1	124.3		

*ORACac represents the oxygen radical absorbance capacity of acetone treated plasma

The mean ferric reducing ability of plasma (FRAP) values in the blueberry consuming group were significantly higher after 30 days. There were no significant changes in the control group upon completion of 30 days.

Table 12

Initial and Final FRAP* Scores

Subject	FRAP initial	FRAP final		
Controls	uM/L	uM/L		
1	228	230		
2	214	229		
3	206	244		
4	231	225		
5	260	218		
6	208	232		
7	300	265		
8	203	184		
9	217	221		
10	251	268		
11	199	215		
12	299	316		
mean	234	237	difference	p
std. dev	36	33	3	0.718
Blueberry group				
1	205	242		
2	185	190		
3	232	275		
4	167	274		
5	201	216		
6	296	283		
7	250	243		
8	268	317		
9	231	205		
10	205	204		
11	224	261		
12	131	220		
mean	216	244	difference	p
std. dev.	45	39	28	0.036

*FRAP represents the ferric reducing ability of plasma

There were no significant changes in either group for both uric acid and bilirubin levels following the 30 day trial period (Tables 7 and 8). These results serve as further validation that the increases in plasma ORAC ac and FRAP scores are resulting from dietary antioxidants and not endogenously produced antioxidants.

Table 13

Initial and Final Uric Acid Scores

Subject controls	Initial uric mg/dL	Final uric mg/dL		
1	3.74	4.67		
2	6.69	7.39		
3	5.00	5.91		
4	3.42	3.75		
5	11.46	8.60		
6	5.28	4.59		
7	9.00	6.67		
8	6.50	5.88		
9	6.49	6.20		
10	8.91	6.86		
11	5.50	5.20		
12	0.71	3.20	difference	p
mean	6.06	5.74	-0.28	0.49
std. dev.	2.86	1.55		
Blueberry group	Initial uric mg/dL	Final uric mg/dL		
1	5.20	4.56		
2	8.44	8.35		
3	3.48	3.88		
4	3.33	1.68		
5	4.33	3.95		
6	10.21	9.63		
7	8.86	7.40		
8	7.27	6.08		
9	7.96	6.48		
10	9.05	10.40		
11	4.25	3.46		
12	4.00	4.09	difference	p
mean	6.37	5.83	-0.54	0.06
std. dev.	2.50	2.69		

Table 14

Initial and Final Bilirubin Levels

Subject	Initial bilirubin	Final bilirubin		
Controls	mg/dL	mg/dL		
1	0.70	0.63		
2	0.24	0.44		
3	0.30	0.52		
4	0.48	0.46		
5	0.70	0.76		
6	0.63	0.43		
7	0.50	0.42		
8	0.40	0.25		
9	0.50	0.50		
10	0.37	0.32		
11	0.50	0.56		
12	0.50	0.38	difference	p
mean	0.49	0.47	-0.02	0.745
Std. dev.	0.14	0.14		
Blueberry				
group				
1	0.51	0.55		
2	0.43	0.29		
3	0.90	0.82		
4	0.25	0.34		
5	0.50	0.30		
6	0.60	0.43		
7	0.50	0.34		
8	0.43	0.77		
9	0.42	0.46		
10	0.50	0.45		
11	0.30	0.40		
12	0.32	0.35	difference	p
mean	0.47	0.46	-0.01	0.769
std. dev.	0.17	0.17		

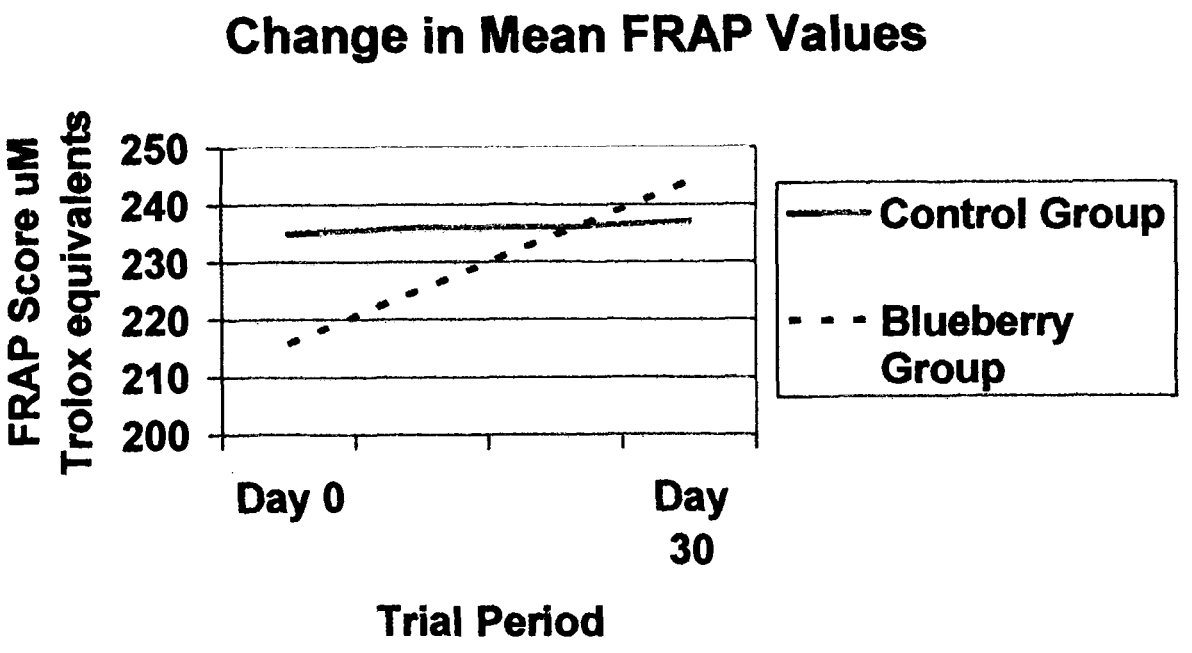
There were no significant changes observed in lymphocyte antioxidant capacity of either the intervention or control group.

Table 15

Initial and Final Spectrox®* Scores

Subject	Initial Spectrox	Final Spectrox		
Controls	% standard	% standard		
1	29.4	34.4		
2	57.5	95.0		
3	35.0	93.8		
4	85.6	86.9		
5	88.1	60.0		
6	63.1	90.6		
7	71.9	90.6		
8	65.6	71.9		
9	85.6	95.0		
10	44.4	23.8		
11	95.0	92.5		
12	64.4	95.0	difference	p
mean	65.5	77.5	12.0	0.117
std. dev.	21.3	25.1		
Blueberry				
group				
1	21.3	74.4		
2	63.1	21.1		
3	95.0	86.9		
4	51.2	75.6		
5	74.4	65.6		
6	89.4	95.0		
7	88.8	83.8		
8	60.0	24.4		
9	25.0	63.1		
10	74.4	77.5		
11	64.4	91.3		
12	30.6	85.0	difference	p
mean	61.5	70.3	8.8	0.350
Std. dev.	25.2	24.2		

*Spectrox® represents lymphocyte antioxidant capacity as measured by resistance to free radical oxidation.



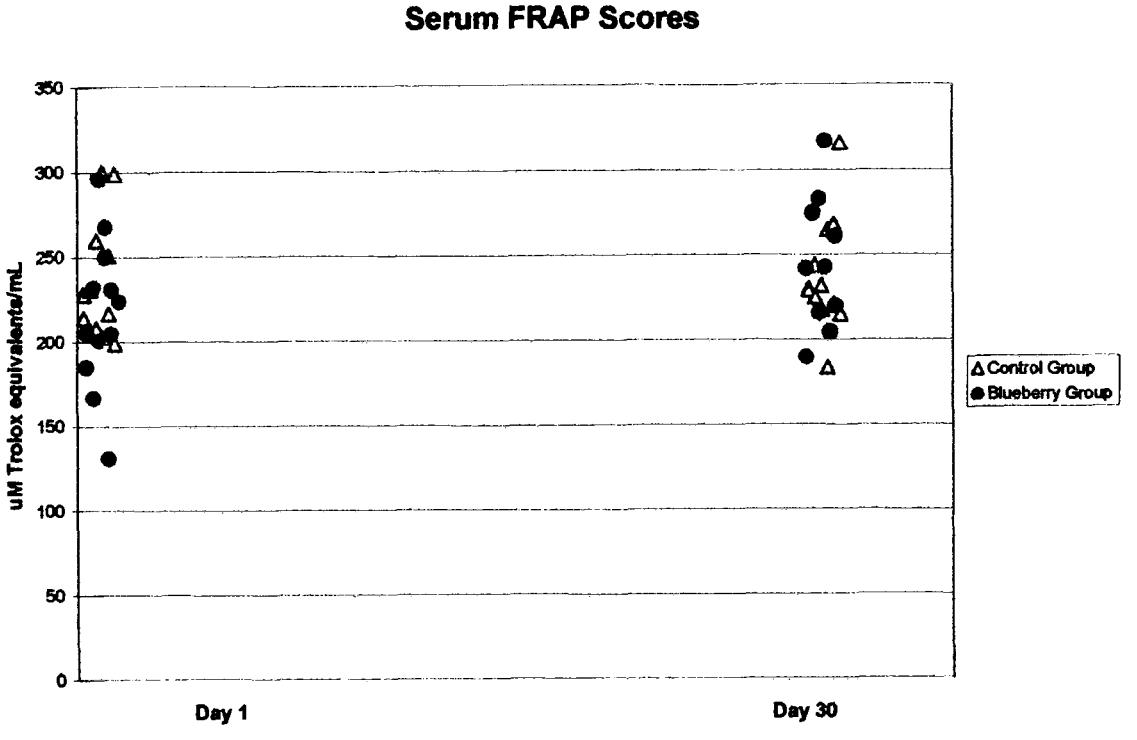
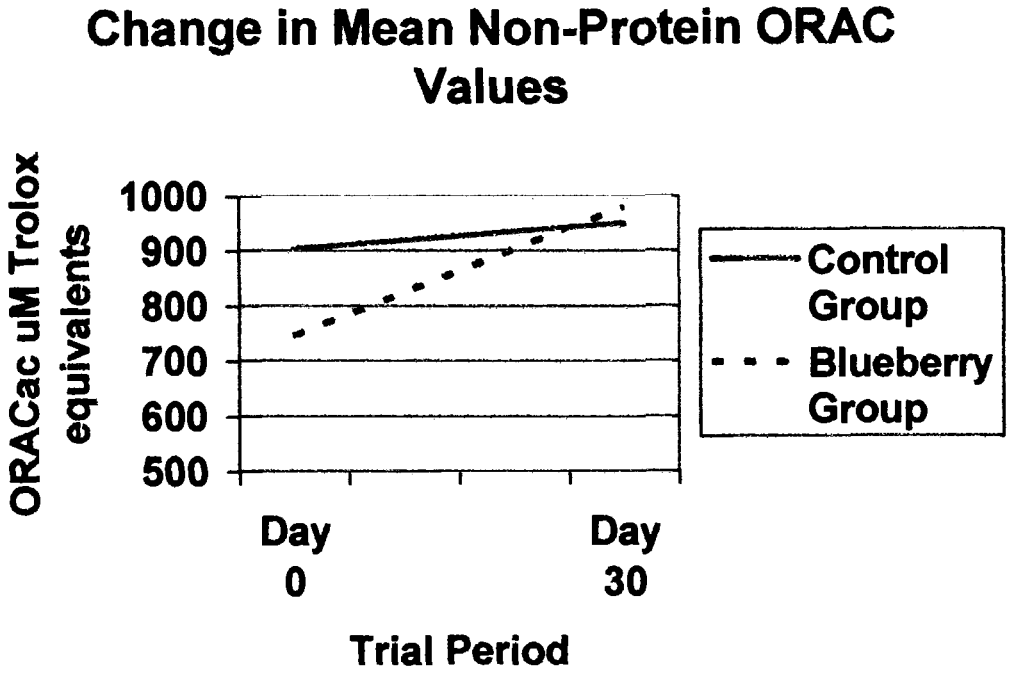


Figure 4. Change in Mean FRAP Values



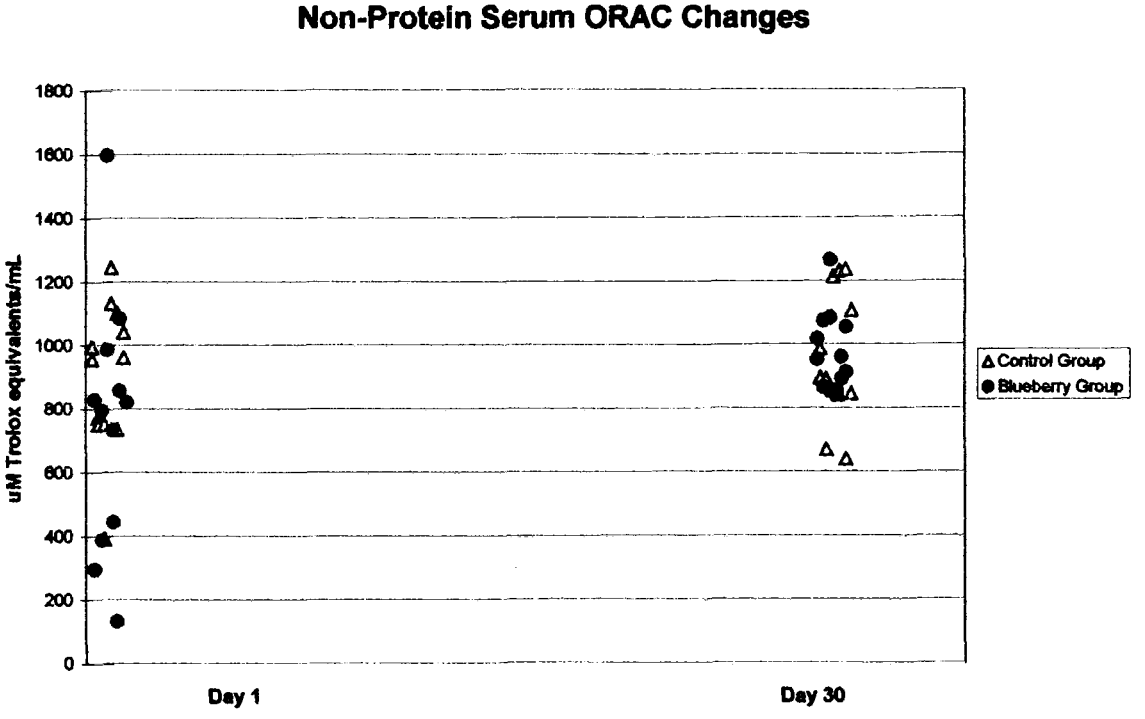


Figure 5. Change in Mean Non-Protein ORAC Values

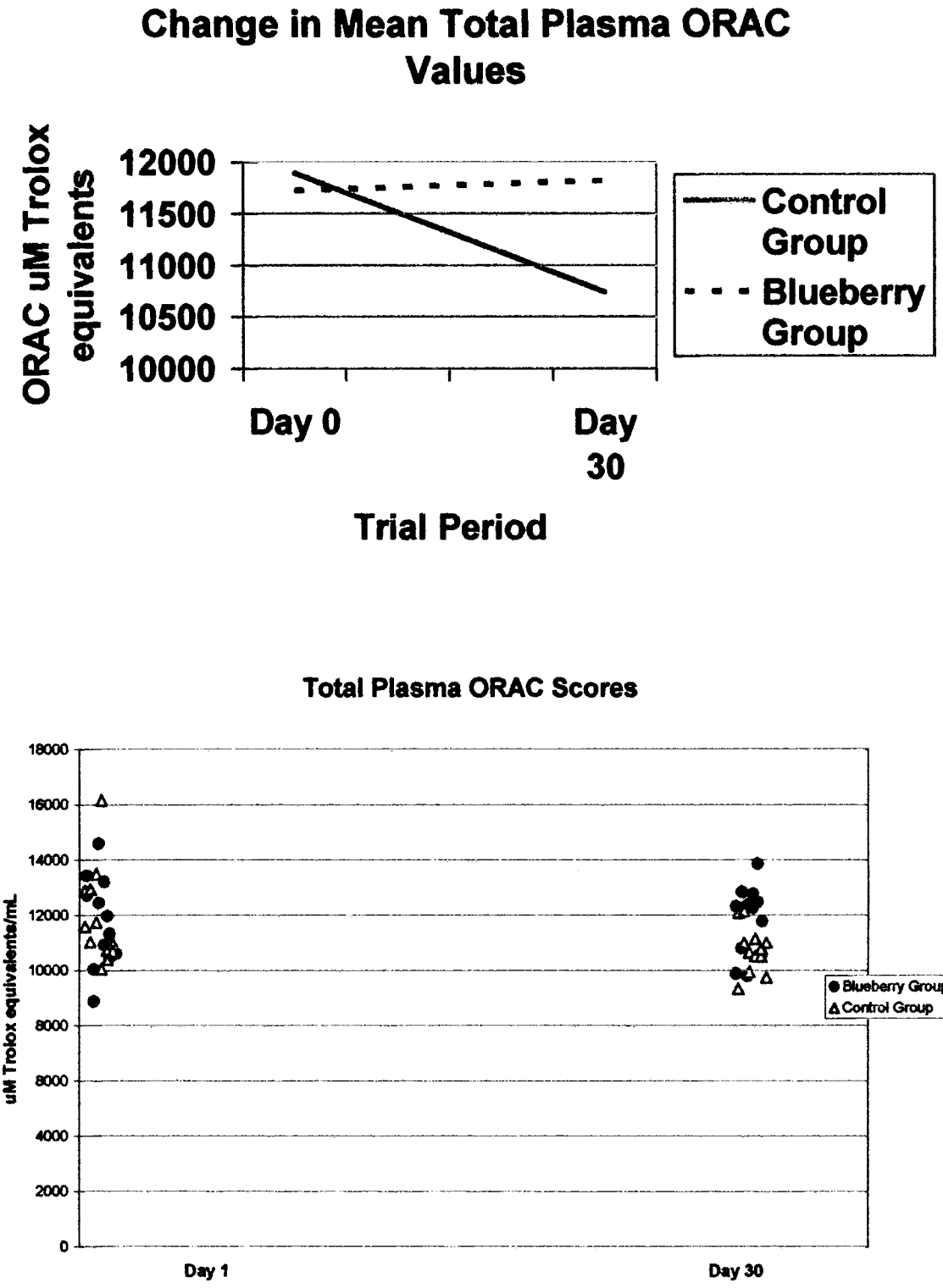


Figure 6. Change in Mean Total Plasma ORAC Values

CHAPTER 4

DISCUSSION

Previous studies examining the antioxidant capacity of specific foods indicated that blueberries have the highest ORAC score of any commonly eaten fruit or vegetable in the United States (Wang et. al, 1996, Prior et. al, 1998). This discovery led us to inquire into the possible health effects of regular blueberry consumption. While the results of a limited number of animal studies suggested that a diet enriched with blueberries or blueberry extract enhanced certain parameters of physiological health, little was known with respect to the impact that similar dietary interventions would produce in a human population (Joseph et. al, 1999). Although Mazza et. al. (2002) has since demonstrated an increase in post-prandial serum ORAC scores in five male subjects given pharmacological doses of freeze-dried wild blueberry extract, no other study has attempted to investigate the relationship between prolonged, whole-fruit intake and fasting serum antioxidant status.

Our efforts inherently examined the bioavailability of blueberry antioxidants as well as the effect on serum antioxidant capacity. It is important to keep in mind that the antioxidants found in blueberries are transitory in nature. The water soluble characteristics of the antioxidants influence and limit the duration of their effect on serum antioxidant capacity. Therefore, increases in fasting serum antioxidant capacity should be interpreted as significant changes, possibly reflecting changes in tissue concentrations. This investigation attempted to assess specific tissue antioxidant changes by analyzing

changes in lymphocyte antioxidant potential through Spectrox® Intracellular Functional Analysis.

There were no significant changes observed in the Spectrox® scores of either the control or blueberry-consuming groups (Table 15). Although it was hypothesized that 30 days of increased dietary antioxidant consumption would improve the intervention group's lymphocyte resistance to oxidative stress, it is uncertain that 30 days of intervention time was sufficient to elicit these expected changes. Lymphocyte life spans are typically three to six months. It is possible that considerably more time is required to alter this tissue measure.

Total plasma antioxidant capacity decreased significantly ($p<.05$) in the control group (-1153 uM Trolox equivalents/mL, $p=0.038$), with no significant changes observed in the blueberry consuming group (Table 10, Figure 6). The control group's loss of antioxidant capacity can only be explained by a change in the group's endogenous antioxidant levels. Decreases due to a common stress or change in environment are the leading candidates for potential causes of this effect. It is possible that psychological stress from a recent storm and the potential hazards of travel in inclement weather may have affected the subjects' final serum ORAC scores. On March 21st, there was a mild snowstorm during the evening prior to the final blood draw. The psychological stress experienced by this elderly population over road conditions and planned transportation might have reduced specific circulating antioxidant proteins by generating additional free radical species. Katz and Schmidt (1999) have previously demonstrated the antioxidant-diminishing effect of acute psychological stress and stressors. Again, this reduction may

have been masked in the blueberry consuming group by their additional consumption of exogenous or dietary antioxidants.

Additionally, it is remotely possible that these decreases might reflect the seasonal effects of inadequate ultraviolet light exposure. Woitge et. al. found significant decreases in circulating vitamin D levels in an elderly population at a latitude dramatically lower than the geographic area encompassed by the subjects involved in this study. The decrease in circulating vitamin D levels, and a subsequent decrease in serum antioxidant capacity expressed in the control group, may have been masked by the intervention group's additional intake of dietary antioxidants. All other lifestyle characteristics seem to have remained constant over this period of time.

Lastly, it is possible that the control group was aware that they were in fact the control group and did not receive the blueberries or their protective effect. Could the group's expected results (lower disease resistance) have actually caused psychological stress that resulted in lower ORAC scores? It is unlikely, yet possible.

The non-protein fraction of the serum samples reflected significant changes ($p<.05$) in the ORAC score of the blueberry-consuming group (Table 11, Figure 5). The acetone-treated serum ORAC assay (ORAC ac) increased by 234.5 uM/Trolox equivalents in the blueberry-consuming group ($p=0.048$) with no concomitant changes in the control group. This change in the blueberry-consuming group's antioxidant capacity represents a 31.4% increase from the average ORAC score at day 0. Again, this assay's strength of assessment rests in its ability to detect only the non-protein serum antioxidants. The absence of these potentially interfering proteins may have enabled a true observation of dietary antioxidants. As mentioned previously, changes in

endogenous antioxidant proteins due to environmental or behavioral factors might have masked the otherwise significant increases in serum ORAC values of the blueberry group from increased dietary antioxidant consumption.

The mean FRAP value for the blueberry-consuming group increased significantly (27.92 uM Trolox equivalents/mL, p=0.036), while there was no significant change in the control group (Table 12, Figure 4). This increase represents a 12.9% change from day 0. The FRAP assay, like the acetone-treated plasma ORAC assay, neglects, in its assessment, the antioxidant contributions made by proteins. In addition, the FRAP test does not detect the sulfur-containing antioxidants. Therefore, it is expected that there is the observed correlation between the ORAC ac and FRAP changes in the intervention group. Furthermore, comparison of the ORAC ac and FRAP assay results by means of a Pearson's correlation test resulted in a correlation coefficient of 0.72. This result indicates there was in fact a strong, positive, linear relationship.

There were no significant changes observed in either the bilirubin or uric acid assays from day 0 to day 30 in both the blueberry-consuming and control groups. These two assays were included as a means of detecting any possible dietary or endogenous antioxidant interference that might affect serum antioxidant capacity. The lack of change in these parameters is, therefore, validation that the observed changes in plasma antioxidant capacity was due to the dietary intervention being investigated and not an increase in post-prandial uric acid production or circulating bilirubin levels. Although the probability of chance (p-value) associated with the experimental group's change in mean uric acid values approached the critical value (p=.06), the change was still

statistically insignificant and could not have been solely responsible for the dramatic changes in the group's plasma antioxidant capacities.

These results suggest that the regular consumption of wild blueberries can significantly raise an individual's serum antioxidant capacity and possibly reduce their risk for a number of diseases. Furthermore, while blueberries have an exceptionally high antioxidant value, this study also supports the antioxidant-based theory of how a diet rich in a variety of fruit and vegetables offers protection against disease. Additionally, this research might serve as a foundation for future investigations into the magnitude of serum antioxidant increases elicited by different fruits and vegetables. It stands to reason that, since different fruits and vegetables have unique ORAC scores, dietary consumption of specific food items can significantly alter an individual's antioxidant intake. If a dose response in serum antioxidant changes can be demonstrated in response to fruits and vegetables with known antioxidant values, an ORAC-based food frequency questionnaire could be used to assess a population's dietary antioxidant status.

To date, there is no total antioxidant capacity-based food frequency questionnaire, or any other dietary assessment tool designed to capture the total antioxidant contributions of an individual's diet, in existence. We developed a preliminary ORAC-based food frequency questionnaire on the fruit and vegetable ORAC scores determined by Wang et. al. in 1996. This food frequency questionnaire uses a simple formula for each food item. Each different fruit or vegetable is given a total antioxidant score by multiplying that item's average serving size (measured in grams) by the previously determined ORAC score of the fruit or vegetable (measured as uM Trolox equivalents/gram) (see Appendix J). While this questionnaire is limited to fruits and

vegetables, further antioxidant research will undoubtedly determine the total antioxidant values for more food items. The development of a more extensive database of these antioxidant values is essential for improving our understanding of the relationship between diet and disease.

Research is currently being conducted on the bioavailability and dietary factors influencing the absorption of anthocyanins from blueberry beverages. Dr. Ronald Prior and Dr. Richard Cook are examining urine and serum anthocyanin metabolite levels post-prandially. This bioavailability study will provide additional insight into the mechanism by which blueberries can produce changes in serum antioxidant capacity and may support our findings.

The extrapolation of our recent findings to other populations is limited since this research was based on an unusually homogeneous population. Future work might incorporate a larger and more heterogeneous sample. While this variation in physiology may prove to require a dramatically larger number of subjects, it is necessary to solidify the relevance of these results. Also, it is recommended that future research in this area attempt to measure serum antioxidant capacities at regular intervals within the trial period. This work is also limited by its use of only two endpoints. While we assume that there is a linear progression in the changes demonstrated over the 30 day period, it is likely that if tissue levels change serum levels are dependent upon tissue uptake. Lastly, additional efforts should examine more potential cellular changes with respect to antioxidant capacity or anthocyanin storage. Although the lymphocyte-based Spectrox® assay failed to detect changes in our blueberry-consuming group, it may require introspection into another tissue compartment for a greater amount of dietary exposure.

While this investigation has been limited in a number of ways, the preceding findings are significant in many respects. This is the first time that research has demonstrated an increase in serum antioxidant capacity from the regular consumption of a single, whole, non-pharmacological dose of fruit. Additionally, this work quantified these significant changes in an extremely small number of subjects. This outcome suggests that if a similar study were conducted, utilizing a larger sample size, it would most likely produce significant serum antioxidant increases and would support our findings. This study also exhibited the importance of antioxidant-rich fruits and vegetables over fruits and vegetables in general. These results were observed in a population with a varied consumption pattern of fruits and vegetables. As the only dietary intervention made in the experimental group, the addition of one cup of blueberries each day superceded this variance and produced statistically significant differences in two different serum antioxidant assays, and possibly a third. Hopefully this study will yield future inquiries into the relationship between dietary antioxidants and physiological parameters of health. Most importantly, this research was significant because it extended our knowledge of the role of fruits, vegetables, and all botanicals have in health and disease.

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APPENDICES

APPENDIX A

STUDY PARTICIPANT CONSENT FORM

Project NE-172 2000

INFORMED CONSENT

You are being invited to participate in a study that assesses your antioxidant status. Antioxidants are obtained in the fruits and vegetables we eat and help protect us from disease. We will determine your antioxidant levels from the food you consume regularly and from two samples of your blood. You may be selected, at random, to eat one cup of Wild Maine blueberries (supplied to you at no cost) each day for a month. These blueberries can be eaten in a variety of different ways. You will be provided with ideas of how to incorporate them into your meals so that it will be easier for you to eat them every day. If you are allergic to blueberries, you cannot participate in that portion of the research and must identify your condition to us now.

Initially, a researcher will ask to see your current medications. You will be asked to provide either a list or the actual medicine containers. To begin the project, you will be asked to fill out a food frequency questionnaire (for example, how many times per day, week, or month do you eat broccoli and what is the usual serving size) and an informational questionnaire with the help of an interviewer. That process should take no longer than one hour. You will also be asked to have a blood test taken at that time or soon after that as arranged by the interviewer. A venous blood sample will be required and three tubes of blood will be drawn once the needle is inserted for a total or approximately 12 milliliters. At the end of 30 days from the initial blood test, a follow-up blood test identical to the first one will be conducted. No cost will be incurred by you for any of the blood work. If you are selected for the blueberry part of this research, you will also be required to maintain a simple journal that records the time and manner in which the blueberries are eaten each day. Following completion of the study, you will be offered a free consultation of your results with a Registered Dietitian.

Participation in the study is voluntary and you may withdraw at any time. Results of the research will be confidential and only aggregate data (results for the whole group) obtained in this research will be made available for public review. A code number, rather than your name, will be used in all phases of data analysis. Personal information obtained will not be shared with any other agencies or organizations. All personal information that you provide or that is produced by this research will be kept in a locked office at all times. The data will be destroyed after the publication of this research. There is no more risk in participating in this study than that in everyday living or from having a blood test drawn by a qualified medical specialist (for example, a possible "black and blue").

You will receive a copy of this consent form to keep for reference. Please contact either of the following individuals if you have any questions:

Richard A. Cook, Ph.D.
Project Director
207-581-3117 (work)
207-848-2771 (home)

John Bagnulo, M.P.H., R.D.
Research Investigator
207-581-3117 (work)
207-778-2064 (home)

Name: _____ Date: _____ Project No. _____

APPENDIX B

UNIVERSITY OF MAINE HUMAN RESEARCH CONSENT AND REVIEW COMMITTEE APPROVAL LETTER

UNIVERSITY OF MAINE – APPLICATION FOR APPROVAL OF RESEARCH WITH HUMAN SUBJECTS

PRINCIPAL INVESTIGATOR: RICHARD Cook PhD
CO-INVESTIGATOR(S): JOHN BAGNULO MPH, RD
FACULTY SPONSOR (if any): _____
TITLE OF PROJECT: ANTIOXIDANT ASSESSMENT OF THE ELDERLY BY USE OF A MODIFIED FOOD FREQUENCY QUESTIONNAIRE IN CONJUNCTION WITH BIOMARKERS
PROJECT START DATE: _____
PI DEPARTMENT: FOOD SCIENCE AND HUMAN NUTRITION
MAILING ADDRESS: 5749 MERRILL HALL TELEPHONE: 581-3117
FUNDING AGENCY (if any): _____ CONTRACT/GRANT #: _____
STATUS OF PI (circle one):

(FACULTY)/STAFF/GRADUATE/UNDERGRADUATE/OTHER _____

1. If PI is a student, is this research to be performed:

   ____ for an honors thesis? ____ for a master's thesis?
   ____ for a doctoral dissertation? ____ for a course project?
   ____ other (specify) _____

2. Does this application modify a previously approved project? NO. If yes, please give title (and assigned number, if known) of previously approved project: _____

3. Is this project exempt from further review requirements? NO. If yes, please give the number of the exemption category (see Policies and Procedures, Section III B2a): ____.

4. Is expedited review requested? NO. If yes, please give the number of the review category (see Policies and Procedures, Section III C5a): _____.

See instructions on reverse for completing application.

SIGNATURES: All procedures performed under the project will be conducted by individuals qualified and legally entitled to do so. No deviation from the approved protocol will be undertaken without prior approval of the Board.

Faculty Sponsors are responsible for oversight of research conducted by their students. By signing this application page, the Faculty Sponsor ensures that the conduct of such research will be in accordance with the University of Maine's *Policies and Procedures for the Protection of Human Subjects of Research.*

6/9/00 Richard A. Cook
Date Principal Investigator

_____
Faculty Sponsor

John D Bagnulo
Co-Investigator

_____
Co-Investigator

****

FOR BOARD USE ONLY Application # 2000-07-01 Date received 7/7/00
ACTION TAKEN:

____ Exempt; category _____.
__√__ Approved ~~as submitted~~ with modifications. Date of next review: 7/01.
____ Modifications required. (See attached statement.)
____ Not approved. (See attached statement.)

modifications accepted 9/12/00

SIGNATURES:

7/24/00
Date

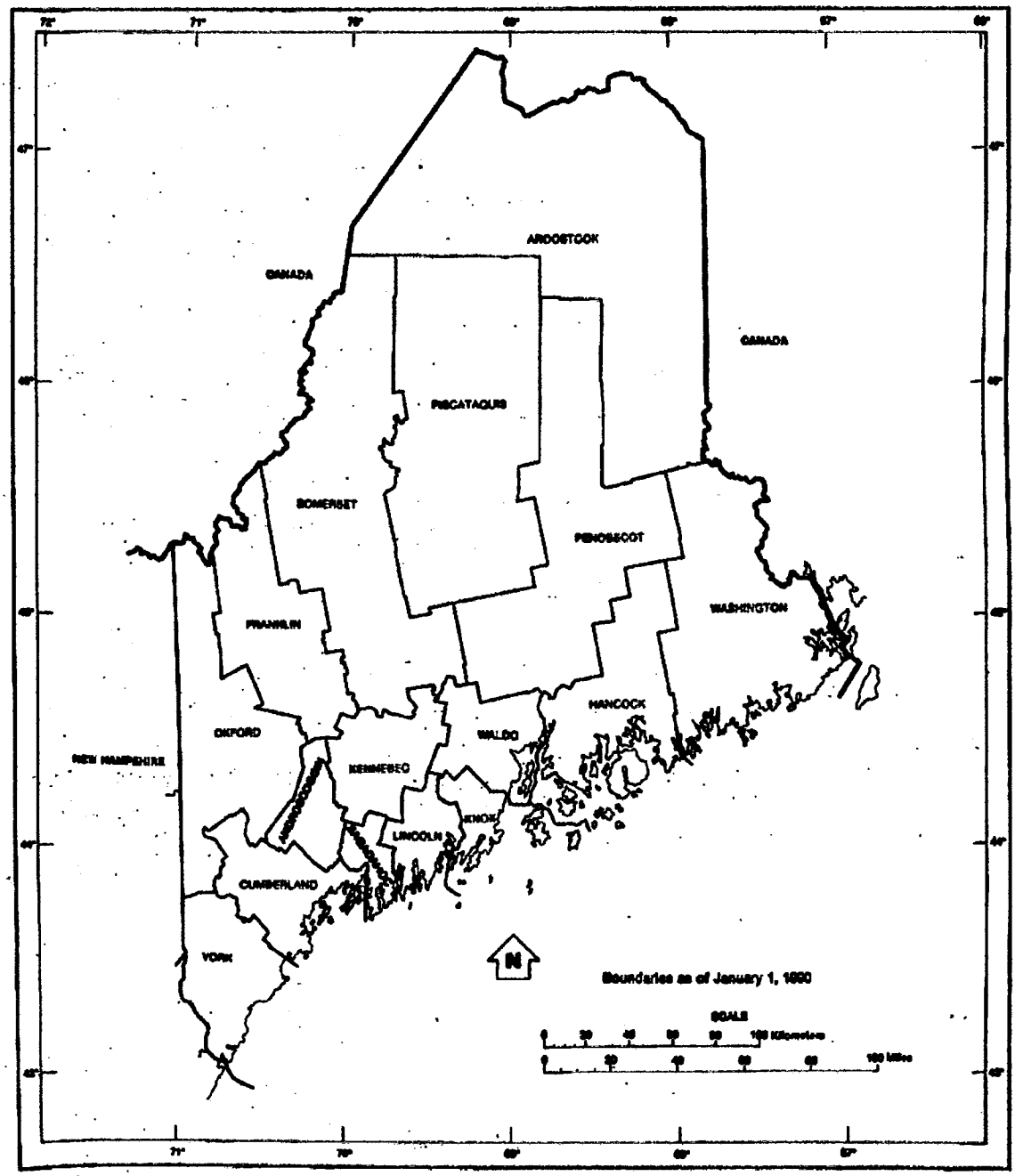
_____ _____

5/98

APPENDIX C

MAP OF MAINE

Counties



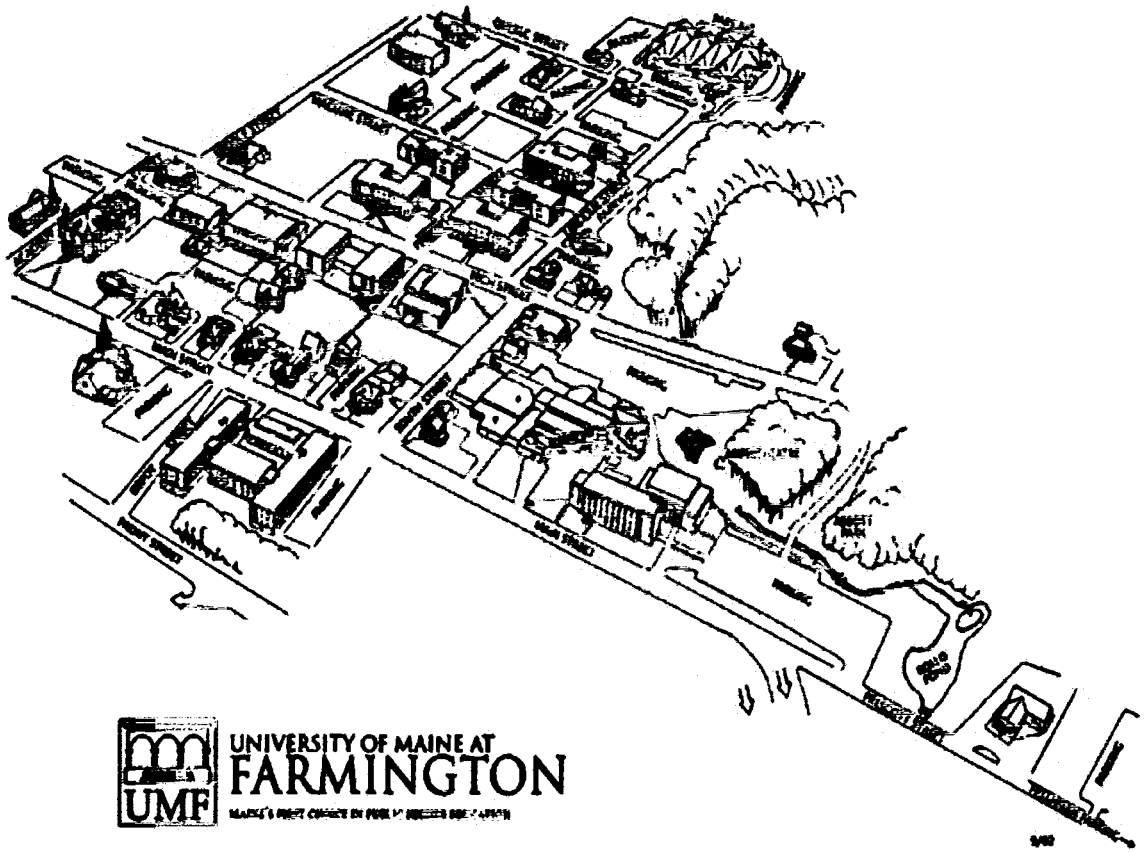
U.S. DEPARTMENT OF COMMERCE Economics and Statistics Administration Bureau of the Census

MAPS

MAINE G-1

APPENDIX D

MAP OF THE UNIVERSITY OF MAINE AT FARMINGTON



UNIVERSITY OF MAINE AT
FARMINGTON

APPENDIX E

HEPATIC DETOXIFICATION INTERFERING MEDICATIONS

Lipitor
Mevacor
Paxil
Zoloft
Wellbutrin
Lotensin
Prilosec
Zantac
Diamox
Non-steroidal Anti-inflammatory Drugs
Aspirin
Ibuprofen
Tylenol
Celebrex
Pravacol
Codeine
Antibiotics
Acetaminophen
Naproxen
Digoxin
Phenylbutazone
Propanolol
Brompheniramine
Tetracycline
Inoxidil
Minoxidil
Steroids

APPENDIX F

MEDICAL QUESTIONNAIRE

Project NE-172 2000 Name: _____ Project No._____
Medical Questionnaire

Are you presently taking any medications? _____
If yes, please list, including name, dosage, and frequency:

Do you have any medical conditions or illnesses that might prevent you from participating in this research? _____ Please identify:

Are you allergic to blueberries? _____

Do you exercise regularly? _____ If yes, please describe type of exercise and frequency:

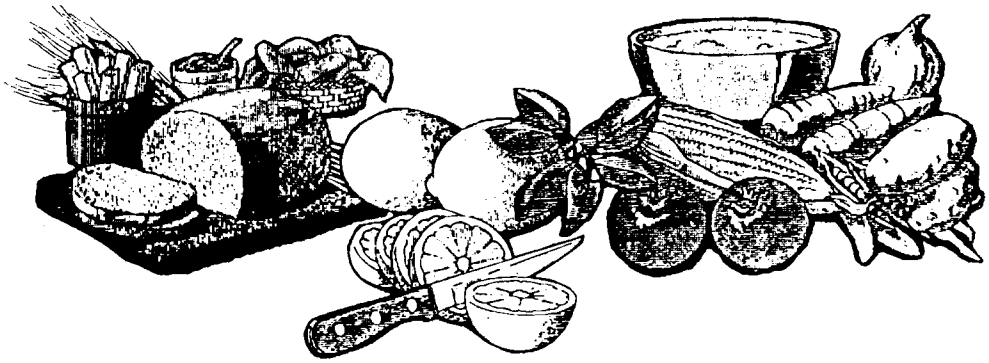
Are you currently taking any vitamin, mineral, or other dietary supplements? _____
If yes, please list type, amount, and frequency:

APPENDIX G

TUFTS ELDERLY HEALTH AND FOOD FREQUENCY QUESTIONNAIRE

FOOD QUESTIONNAIRE

TUFTS ELDERLY HEALTH AND NUTRITION STUDY



NAME AND ADDRESS:
PLEASE DO NOT WRITE
OUTSIDE THE BOXED AREA.

DATE		
MO	DAY	YR

SEX
○ MALE
○ FEMALE

INTERVIEWER ID
○ 1
○ 2
○ 3
○ 4
○ 5
○ 6
○ 7

IDENTIFICATION NUMBER

7218

PLEASE MAKE NO MARKS IN THIS AREA

Form N-1 (Revised 08/93)

1. During the last _____:

	SELDOM/NEVER	SOMETIMES	OFTEN/ALWAYS
a. How often did you add salt to your food?	○	○	○
b. How often did you add pepper to your food?	○	○	○
c. How often did you eat the skin on chicken?	○	○	○
d. How often did you eat the fat on meat?	○	○	○

2. a. **If you ate poultry, it was usually...** ○ Light meat ○ Dark meat ○ Both

b. **If you ate hamburger or beef, it was usually...** ○ Regular ○ Lean ○ Extra lean

c. **If you ate tuna, it was usually...** ○ Oil pack ○ Water pack ○ Either one ○ Don't know

3. **What kinds of fat did you *usually* use in cooking (to fry, stir-fry, saute or bake)? Specify only one or two.**

○ Stick margarine ○ Butter ○ Soft tub margarine ○ Olive oil

○ 1/2 butter, 1/2 margarine ○ Low-calorie margarine ○ Whipped butter ○ Other vegetable oil

○ Lard, fatback, baconfat ○ Pam or no oil ○ Don't know or don't cook

4. **What kinds of fat did you *usually* add to vegetables, potatoes, etc.? Specify only one or two.**

○ Stick Mmargarine ○ Soft tub margarine ○ Low-calorie margarine ○ Other vegetable oil

○ Butter ○ Whipped butter ○ 1/2 butter, 1/2 margarine

○ Lard, fatback, baconfat ○ Don't add fat ○ Olive oil

5a. **Do you add oil or fat to rice when you cook it?** ○ Yes ○ No

5b. **What kinds of fat did you usually add to rice during cooking?**

○ Don't add oil or fat ○ Butter ○ Soft tub margarine ○ 1/2 butter, 1/2 margarine

○ Stick margarine ○ Low-calorie margarine ○ Whipped butter ○ Olive oil

○ Other vegetable oil ○ Lard, fatback, baconfat ○ Don't know or don't use

6a. **Do you add oil or fat to beans when you cook them?** ○ Yes ○ No

6b. **What kinds of fat did you usually add to beans during cooking?**

○ Don't add oil or fat ○ Butter ○ Soft tub margarine ○ 1/2 butter, 1/2 margarine

○ Stick margarine ○ Low-calorie margarine ○ Whipped butter ○ Olive oil

○ Other vegetable oil ○ Lard, fatback, baconfat ○ Don't know or don't use

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7. About how often did you eat foods from the following restaurants during _____?

TYPE OF RESTAURANT	NUMBER OF VISITS						
	NEVER IN PAST YEAR	1-4 TIMES PAST YEAR	5-11 TIMES PAST YEAR	1-3 TIMES A MONTH	ONCE A WEEK	2-4 TIMES A WEEK	ALMOST EVERY DAY
Fried Chicken	○	○	○	○	○	○	○
Burgers	○	○	○	○	○	○	○
Pizzas	○	○	○	○	○	○	○
Chinese food	○	○	○	○	○	○	○
Mexican food	○	○	○	○	○	○	○
Fried fish	○	○	○	○	○	○	○
Other restaurants	○	○	○	○	○	○	○

8. This section is about your *usual* eating habits. Your answers should reflect your food intake during _____?

TYPE OF FOOD	AVERAGE USE									USUAL SERVING SIZE
	NEVER OR LESS THAN ONCE PER MONTH	1 Per MONTH	2-3 Per MONTH	1 Per WEEK	2 Per WEEK	3-4 Per WEEK	5-6 Per WEEK	1 Per DAY	2+ Per DAY	
FRUITS AND JUICES										
Apples, applesauce, pears	○	○	○	○	○	○	○	○	○	
Bananas	○	○	○	○	○	○	○	○	○	
Peaches, apricots (canned, frozen or dried)	○	○	○	○	○	○	○	○	○	
Peaches, apricots, nectarines (fresh, in season)	○	○	○	○	○	○	○	○	○	
Cantaloupe (in season)	○	○	○	○	○	○	○	○	○	
Watermelon (in season)	○	○	○	○	○	○	○	○	○	
Strawberries (fresh, in season)	○	○	○	○	○	○	○	○	○	
Mangoes	○	○	○	○	○	○	○	○	○	
Oranges	○	○	○	○	○	○	○	○	○	
Grapefruit	○	○	○	○	○	○	○	○	○	
Any other fruit, including berries, fruit cocktail	○	○	○	○	○	○	○	○	○	
Orange juice or grapefruit juice	○	○	○	○	○	○	○	○	○	
Other 100% fruit juices or nectar (apple juice, guava, mango)	○	○	○	○	○	○	○	○	○	

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PLEASE MAKE NO MARKS IN THIS AREA

TYPE OF FOOD	AVERAGE USE									USUAL SERVING SIZE
	NEVER OR LESS THAN ONCE PER MONTH	1 Per MONTH	2-3 Per MONTH	1 Per WEEK	2 Per WEEK	3-4 Per WEEK	5-6 Per WEEK	1 Per DAY	2+ Per DAY	
Fortified fruit drinks (Hi-C, Tang)										
Non-fortified fruit drinks										
BREAKFAST FOODS										
High fiber, bran or granola cereals, shredded wheat										
Highly fortified cereals, such as Product 19, Total or Most										
Other cold cereals, such as corn flakes, Rice Krispies										
Cooked cereals										
Milk on cereal										
Sugar added to cereal										
Eggs										
Bacon										
Sausage										
VEGETABLES										
String beans, green beans										
Peas										
Tomatoes, tomato juice										
Broccoli										
Cauliflower or brussel sprouts										
Spinach (raw)										
Spinach (cooked)										
Mustard greens, turnip greens, collards										
Cole slaw, cabbage, sauerkraut										
Carrots, or mixed vegetables containing carrots										
Green salad										
Avocado, raw										

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TYPE OF FOOD	AVERAGE USE									USUAL SERVING SIZE
	NEVER OR LESS THAN ONCE PER MONTH	1 Per MONTH	2-3 Per MONTH	1 Per WEEK	2 Per WEEK	3-4 Per WEEK	5-6 Per WEEK	1 Per DAY	2+ Per DAY	
Winter squash (including butternut, hubbard)										
Regular salad dressing & mayonnaise, including on sandwiches, macaroni and potato salad										
Diet salad dressing & mayonnaise										
Any other vegetables, including cooked onions, summer squash										
Butter, margarine or other fat added to vegatables										
RICE, BEANS, AND STARCHY VEGETABLES										
Rice with meat (pork, beef)										
Rice with chicken										
Rice with pigeon peas										
Rice with beans										
Rice, plain										
Chili with beans										
Beans, such as baked beans, pinto, kidney, lima, lentils (other than cooked in rice)										
Corn										
Root crops (including tannier, cassava, breadfruit)										
Green plantains: boiled/baked										
Green plantains: fried										
Ripe plantains: boiled/baked										
Ripe plantains: fried										
Sweet potatoes										
French fries and fried potatoes										
Other potatoes, including boiled, baked, mashed & potato salad										
Butter, margarine or other fat added to potatoes										

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TYPE OF FOOD	AVERAGE USE									USUAL SERVING SIZE
	NEVER OR LESS THAN ONCE PER MONTH	1 Per MONTH	2-3 Per MONTH	1 Per WEEK	2 Per WEEK	3-4 Per WEEK	5-6 Per WEEK	1 Per DAY	2+ Per DAY	
MEAT, FISH, POULTRY, LUNCH ITEMS										
Hamburgers, cheeseburgers, other ground beef (including meat loaf, tacos, casseroles)										
Beef (steaks, roasts, etc. including on sandwiches)										
Beef stew or pot pie with carrots or other vegetables										
Liver, including chicken livers										
Pork, including chops, roasts										
Fried chicken										
Chicken or turkey (roasted, stewed or broiled, including on sandwiches)										
Fried fish or fish sandwich										
Tuna, tuna salad, tuna casserole										
Shell fish (shrimp, lobster, crab, oysters, etc.)										
Other fish (broiled or baked)										
Spaghetti, lasagna, other pasta with tomato sauce										
Pizza										
Mixed dishes with cheese (such as macaroni and cheese)										
Meat pies, eggrolls, fritters										
Hot dogs										
Ham, bologna, salami and other lunch meats										
Homemade soups with meat/ chicken										
Vegetable & tomato soups, including canned vegetable beef, minestrone										
Other soup										
BREADS, SNACKS, SPREADS										
Biscuits, muffins, burger rolls (including fast foods)										

7218

PLEASE MAKE NO MARKS IN THIS AREA

TYPE OF FOOD	AVERAGE USE									USUAL SERVING SIZE
	NEVER OR LESS THAN ONCE PER MONTH	1 Per MONTH	2-3 Per MONTH	1 Per WEEK	2 Per WEEK	3-4 Per WEEK	5-6 Per WEEK	1 Per DAY	2+ Per DAY	
White breads (including sandwiches, bagels, crackers, tortillas)										
Dark breads, such as wheat, rye, pumpernickel (including sandwiches, bagels, crackers)										
Corn bread, corn muffins, corn tortillas										
Potato chips. corn chips										
Popcorn										
Peanuts, peanut butter										
Margarine on bread or rolls										
Butter on bread or rolls										
Gravies made with meat drippings, or white sauce										
DAIRY PRODUCTS										
Regular cottage cheese										
Low fat cottage cheese or low-fat cheeses										
Other cheeses and cheese spreads										
Flavored yogurt										
Plain yogurt										
SWEETS										
Ice cream										
Sherbet or jello										
Frozen yogurt, ice milk										
Doughnuts, cookies, cake, pastry										
Pies										
Pudding, custard, cheesecake										
Chocolate candy										
Other candy, jelly, honey, brown sugar										

PLEASE DO NOT WRITE IN THIS AREA

TYPE OF FOOD	AVERAGE USE									USUAL SERVING SIZE
	NEVER OR LESS THAN ONCE PER MONTH	1-3 Per MONTH	1 Per WEEK	2-4 Per WEEK	5-6 Per WEEK	1 Per DAY	2-3 Per DAY	4-5 Per DAY	6+ Per DAY	
BEVERAGES (Please note that the categories for these columns are different.)										
Whole milk and beverages with whole milk (not incl. on cereal/ coffee)	○	○		○		○		○		
2% milk and beverages with 2% milk (not including on cereal/coffee	○	○		○		○		○		
Skim milk, 1% milk or buttermilk (not including on cereal/coffee)	○	○		○		○		○		
Regular soft drinks	○	○		○		○		○		
Diet soft drinks	○	○		○		○		○		
Beer	○	○		○		○		○		
Wine	○	○		○		○		○		
Liquor	○	○		○		○		○		
Coffee (regular or decaffeinated)	○	○		○		○		○		
Tea (hot or iced)	○	○		○		○		○		
Lemon in tea	○	○		○		○		○		
Non-dairy creamer in coffee or tea	○	○		○		○		○		
Whole milk in coffee or tea	○	○		○		○		○		
2% milk in coffee or tea	○	○		○		○		○		
Skim, 1% or buttermilk in coffee or tea	○	○		○		○		○		
Cream (real) or Half-and-half in coffee or tea	○	○		○		○		○		
Sugar in coffee or tea	○	○		○		○		○		

9.

SUMMARY QUESTIONS	AVERAGE USE								
	LESS THAN ONCE PER WEEK	1-2 Per WEEK	3-4 Per WEEK	5-6 Per WEEK	1 Per DAY	1 1/2 Per DAY	2 Per DAY	3 Per DAY	4+ Per DAY
a. How often did you use fat or oil in cooking?	○	○	○	○	○	○	○	○	○
b. Not counting salad or potatoes, how many servings of vegetables did you eat?	○	○	○	○	○	○	○	○	○
c. Not counting juices, how many servings of fruit did you eat?	○	○	○	○	○	○	○	○	○

7218

PLEASE MAKE NO MARKS IN THIS AREA

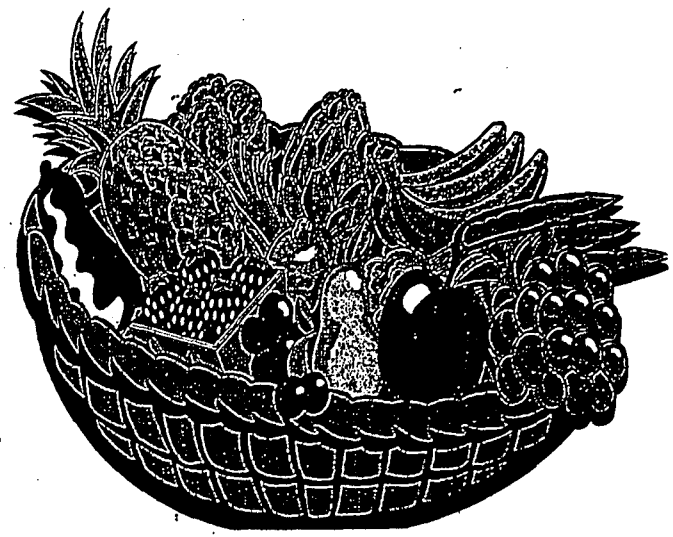
Printed in U.S.A. R7649-PFI-54

APPENDIX H

NE-172 FRUIT AND VEGETABLE SCREENER

Eating Habits Screener

- Participant name_____
- What is your birth date? ___/___/__
- Are you: Male_____ Female_____
- How many servings of fruit do you usually eat each day? _____
  One serving of fruit are portions such as
  1 medium size piece of fruit
  ½ grapefruit
  ½ cup cut up fruit or berries
  6 ounces of juice
- How many servings of vegetables do you usually eat each day? _____
  One serving of a vegetable would be
  ½ cup cooked or raw vegetables
  1 cup of leafy raw vegetables like lettuce or spinach.

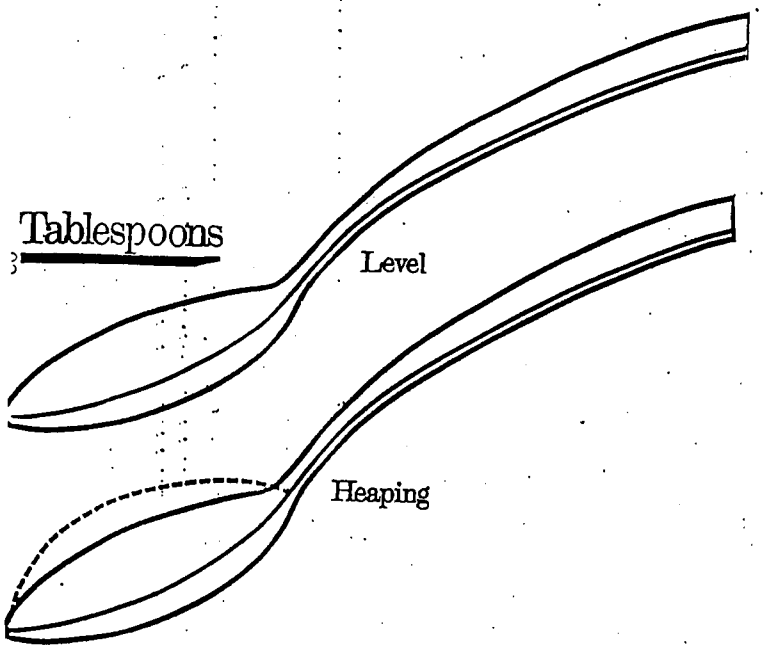


Some tips to help you fill out this survey:

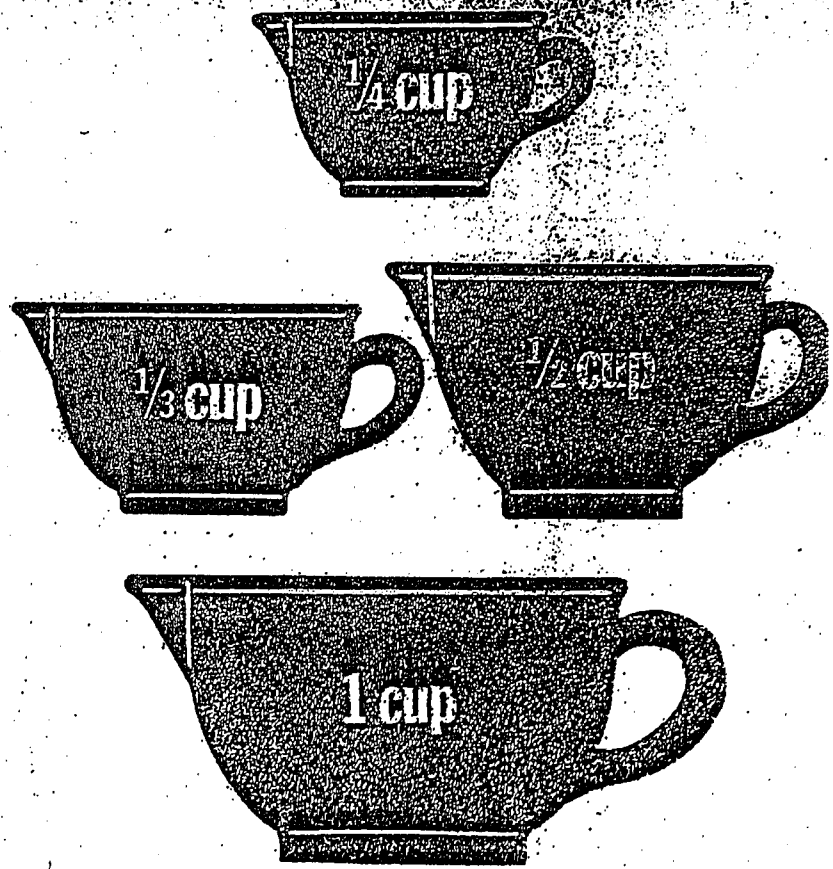
- **Please think about your typical food intake during the past year.**
- **You will need to average foods that are eaten seasonally.**
- **Please refer to the portion sheet to complete the amount or size information.**
- **If you answer " never or less than once a month" you should not enter a portion size.**
- **We have identified small, medium and large for the sizes of the fruit. If you eat more than one Piece of fruit at a time, than you will need to write that in the "other portion" section.**

Serving Spoons

Tablespoons



Measuring Cups



University of Rhode Island, University of New Hampshire, University of Maine/USDA NE-172 12/2

Practice Page

Here is an example for you to practice with:

All about Mary:

- Mary usually packs a 4-ounce can of sliced peaches in her lunch every day for work.
- She does not eat cantaloupe.
- In the summer she eats about 2 cups fresh blueberries per week, but not during the rest of the year.

FRUITS	NEVER OR LESS THAN ONCE A MONTH	2-3 TIMES MONTH	ONCE A WEEK	2 - 3 TIMES A WEEK	4 - 6 TIMES A WEEK	ONCE A DAY	2 OR MORE TIMES A DAY	MOSTLY F=FRESH C=CANNED FRZ=FROZEN	PORTION SIZE
								Circle your Answer	Using the pictures provided, circle your usual portion size or write in your usual portion size.
Peaches					√			F [C] FRZ	½ peach 1 small 1 medium 1 large ¼ cup slices [½ cup slices] 1 cup slices Other portion
Cantaloupe	√							F	1/8 melon ¼ melon ½ melon ½ cup Other portion:
Blueberries		√						[F] C FRZ	1 Tablespoon 1/4 cup 1/2 cup [1 cup] Other portion:

FRUITS	NEVER OR LESS THAN ONCE A MONTH	2-3 TIMES MONTH	ONCE A WEEK	2 - 3 TIMES A WEEK	4 - 6 TIMES A WEEK	ONCE A DAY	2 OR MORE TIMES A DAY	MOSTLY F=FRESH C=CANNED FRZ=FROZEN	PORTION SIZE
	↓ once a month							Circle your Answer	Using the pictures provided, circle your usual portion size or write in your usual portion size.
Apples								F C	1/ 2 Apple Small Medium Large Other portion:
Apricots								F C FRZ	¼ cup slices ½ cup slices 1 cup slices Other portion:
Bananas								F	½ Banana Small Medium Large Other portion:
Blueberries								F C FRZ	1 Tablespoon ¼ cup ½ cup 1 cup Other portion:
Blackberries								F C FRZ	1 Tablespoon ¼ cup ½ cup 1 cup Other portion:
Cantaloupe								F	1/8 melon ¼ melon ½ melon ½ cup Other portion:

FRUITS	NEVER OR LESS THAN ONCE A MONTH	2-3 TIMES MONTH	ONCE A WEEK	2 - 3 TIMES A WEEK	4 - 6 TIMES A WEEK	ONCE A DAY	2 OR MORE TIMES A DAY	MOSTLY F=FRESH C=CANNED FRZ=FROZEN	PORTION SIZE
	Once A Month							Circle your Answer	Using the pictures provided, circle your usual portion size or write in your usual portion size.
Green Grapes								F C	¼ cup ½ cup 1 cup Other portion:
Red Grapes								F C	¼ cup ½ cup 1 cup Other portion:
Pink Grapefruit								F C	½ 1 whole Other portion:
White Grapefruit								F C	½ 1 whole Other portion:
Grapefruit Juice								F C FRZ	½ cup 1 cup 1 ½ cups Other portion:

FRUITS	NEVER OR LESS THAN ONCE A MONTH	2-3 TIMES MONTH	ONCE A WEEK	2 - 3 TIMES A WEEK	4 - 6 TIMES A WEEK	ONCE A DAY	2 OR MORE TIMES A DAY	MOSTLY F=FRESH C=CANNED FRZ=FROZEN	PORTION SI ZE
	Once Month							Circle your Answer	Using the pictures provided, circle your portion size or write in your usual portion size.
Honeydew Melon								F C	1/8 melon ¼ melon ½ melon 1/2 cup Other portion:
Kiwi								F C FRZ	¼ kiwi ½ kiwi 1 whole Other portion:
Mangos								F C FRZ	¼ Mango ½ Mango 1 whole Other portion:
Nectarines								F C FRZ	½ Nectarine Small Medium Large Other portion:
Orange								F C FRZ	½ Orange Small Medium Large Other portion:

FRUITS	NEVER OR LESS THAN ONCE A MONTH	2-3 TIMES MONTH	ONCE A WEEK	2 - 3 TIMES A WEEK	4 - 6 TIMES A WEEK	ONCE A DAY	2 OR MORE TIMES A DAY	MOSTLY F=FRESH C=CANNED FRZ=FROZEN	PORTION SIZE
	Once A Month							Circle your Answer	Using the pictures provided, circle your portion size or write in your usual portion size.
Orange Juice								F C FRZ	½ cup 1 cup 1 ½ cups Other portion:
Peaches								F C FRZ	½ peach Small Medium Large ¼ cup ½ cup 1 cup 2 cups Other portion:
Pears								F C FRZ	½ pear Small Medium Large ¼ cup ½ cup 1 cup 2 cups Other portion:
Plums								F C FRZ	½ 1 whole Other portion:

FRUITS	NEVER OR LESS THAN ONCE A MONTH	2-3 TIMES MONTH	ONCE A WEEK	2 - 3 TIMES A WEEK	4 - 6 TIMES A WEEK	ONCE A DAY	2 OR MORE TIMES A DAY	MOSTLY F=FRESH C=CANNED FRZ=FROZEN	PORTION SIZE			
	Once A Month							Circle your Answer	Using the pictures provided, circle your portion size or write in your usual portion size.			
Strawberries								F C FRZ	¼ cup	½ cup	1 cup	1 ½ cups
									Other portion:			
Watermelon								F	¼ cup	½ cup	1 cup	1 ½ cups
									Other portion:			
Fruits or 100% Fruit Juice: Name								F C FRZ	½	Small	Medium	Large
									¼ cup	½ cup	1 cup	1 ½ cups
									Other potion:			
Fruits or 100% Fruit Juice: Name								F C FRZ	½	Small	Medium	Large
									¼ cup	½ cup	1 cup	1 ½ cups
									Other portion:			
Fruits or 100 % Fruit Juice: Name								F C FRZ	½	Small	Medium	Large
									¼ cup	½ cup	1 cup	1 ½ cups
									Other portion:			

VEGETABLES	NEVER OR LESS THAN ONCE A MONTH	2-3 TIMES MONTH	ONCE A WEEK	2 - 3 TIMES A WEEK	4 - 6 TIMES A WEEK	ONCE A DAY	2 OR MORE TIMES A DAY	MOSTLY F=FRESH C=CANNED FRZ=FROZEN	PORTION SIZE
	once a month							Circle your Answer	Using the pictures provided, circle your portion size or write in your usual portion size.
Beets								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Broccoli								F C FRZ	½ cup 1 cup 1 ½ cups 1 stalk Other portion:
Brussels Sprouts								F C FRZ	1/2 cup 1 cup 1 ½ cup Other portion:
Cooked Carrots								F C FRZ	1/ 2 Small Medium Large ¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Raw Carrots								F	¼ cup ½ cup 1 cup 1 ½ cups Other portion:

VEGETABLES	NEVER OR LESS THAN ONCE A MONTH	2-3 TIMES MONTH	ONCE A WEEK	2 - 3 TIMES A WEEK	4 - 6 TIMES A WEEK	ONCE A DAY	2 OR MORE TIMES A DAY	MOSTLY F=FRESH C=CANNED FRZ=FROZEN	PORTION SIZE
	Once A Month							Circle your Answer	Using the pictures provided, circle your portion size or write in your usual portion size.
Celery								F	¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Cooked Collard Greens								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Corn								F C FRZ	½ cup 1 cup 1 ½ cups ½ ear 1 ear Other portion:
Cucumber								F	½ cucumber 1 whole ½ cup slices 1cup slices Other portion:
Green/String Beans								F C FRZ	¼ cup 1/2 cup 1 cup 1 ½ cups Other portion:

VEGETABLES	NEVER OR LESS THAN ONCE A MONTH	2-3 TIMES MONTH	ONCE A WEEK	2 - 3 TIMES A WEEK	4 - 6 TIMES A WEEK	ONCE A DAY	2 OR MORE TIMES A DAY	MOSTLY F=FRESH C=CANNED FRZ=FROZEN	PORTION SIZE
	Once a Month							Circle your Answer	Using the pictures provided, circle your usual portion size or write in your usual portion size.
Green Peas								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Cooked Kale								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Legumes/Beans (Such as kidney, garbonzo, and black eyed peas								F C	¼ cup 1/2 cup 1 cup 1 ½ cups Other portion:
Lettuce (Iceberg or head lettuce)								F	¼ cup ½ cup 1 cup 1 ½ cups A few leaves 1/8 head ¼ head Other portion:

VEGETABLES	NEVER OR LESS THAN ONCE A MONTH	2-3 TIMES MONTH	ONCE A WEEK	2 - 3 TIMES A WEEK	4 - 6 TIMES A WEEK	ONCE A DAY	2 OR MORE TIMES A DAY	MOSTLY F=FRESH C=CANNED FRZ=FROZEN	PORTION SIZE
	Once a month							Circle your Answer	Using the pictures provided, circle your portion size or write in your usual portion size.
Lettuce (Romaine or leaf lettuce)								F	1/ 4 cup ½ cup 1 cup 1 ½ cups A few leaves Other portion:
Lima Beans								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Mixed Vegetables								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Green Peppers								F C FRZ	½ pepper 1 whole pepper ¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Orange Peppers								F C FRZ	½ pepper 1 whole pepper ¼ cup ½ cup 1 cup 1 ½ cups Other portion:

VEGETABLES	NEVER OR LESS THAN ONCE A MONTH	2-3 TIMES MONTH	ONCE A WEEK	2 - 3 TIMES A WEEK	4 - 6 TIMES A WEEK	ONCE A DAY	2 OR MORE TIMES A DAY	MOSTLY F=FRESH C=CANNED FRZ=FROZEN	PORTION SIZE
	Once							Circle your Answer	Using the pictures provided, circle your portion size or write in your usual portion size.
Red Peppers								F C FRZ	½ pepper 1 whole pepper ¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Yellow Peppers								F C FRZ	½ pepper 1 whole pepper ¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Pumpkin								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Scallions								F	1 Scallion ¼ cup ½ cup 1 cup Other portion:
Cooked Spinach								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:

VEGETABLES	NEVER OR LESS THAN ONCE A MONTH	2-3 TIMES MONTH	ONCE A WEEK	2 - 3 TIMES A WEEK	4 - 6 TIMES A WEEK	ONCE A DAY	2 OR MORE TIMES A DAY	MOSTLY F=FRESH C=CANNED FRZ=FROZEN	PORTION SIZE
	Once A Month							Circle your Answer	Using the pictures provided, circle your portion size or write in your usual portion size.
Raw Spinach								F	¼ cup ½ cup 1 cup 1 ½ cups A few leaves Other portion:
Summer Squash								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Winter or Butternut Squash								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Zucchini Squash								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Yams or Sweet Potatoes								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:

VEGETABLES	NEVER OR LESS THAN ONCE A MONTH	2-3 TIMES MONTH	ONCE A WEEK	2 - 3 TIMES A WEEK	4 - 6 TIMES A WEEK	ONCE A DAY	2 OR MORE TIMES A DAY	MOSTLY F=FRESH C=CANNED FRZ=FROZEN	PORTION SIZE
	once A Month							Circle your Answer	Using the pictures provided, circle your portion size or write in your usual portion size.
Tomatoes Fresh, raw								F	½ small medium large Other portion
Tomatoes, Canned or cooked								F C FRZ	½ cup 1 cup 1 ½ cups Other portion
Tomato Juice								F C	½ cup 1 cup 1 ½ cups A few leaves Other portion:
Tomato Sauce								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Turnip								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Other Vegetables: Identify								F C FRZ	¼ cup ½ cup 1 cup 1 ½ cups Other portion:
Other Vegetables: Identify								F C FRZ	

OTHER FOODS	NEVER OR LESS THAN ONCE A MONTH	2-3 TIMES MONTH	ONCE A WEEK	2 - 3 TIMES A WEEK	4 - 6 TIMES A WEEK	ONCE A DAY	2 OR MORE TIMES A DAY	MOSTLY F=FRESH C=CANNED FRZ=FROZEN	PORTION SIZE
	✓ Once a Month							Circle your Answer	Using the pictures provided, circle your portion size or write in your usual portion size.
Eggs								Whole eggs Egg Whites Egg Substitute	1 egg 2 eggs 3 eggs 1/2 cup 1 cup 1 ½ cups Other portion:
Ketchup								Homemade Store bought	1 Tablespoon 2 Tablespoons ¼ cup ½ cup Other portion:
Concord Grape Jelly								Homemade Store bought	1 Tablespoon 2 Tablespoons ¼ cup ½ cup Other portion:
Salsa								F C FRZ	1 Tablespoon 2 Tablespoons ¼ cup ½ cup Other portion:
Pizza								F FRZ	1 slice 2 slices ½ pizza 1 pizza Other portion:

APPENDIX I

SPECTROX® ANALYSIS

SpectraCell Laboratories, Inc.

PATIENT DUPLICATE

LABORATORY REPORT

John Bagnulo, MPH, R.D.
134 Anson Street
Farmington, ME 04938-

Name:
Gender:
Accession Number: C16881
Date of Collection: 03/22/01
Date Received: 03/23/01
Date Reported: 03/30/01
Requisition Number: 032460

Account Number: 29 4301 002

Summary of Test Results

SPECTROX ™ Total Antioxidant Function:

Interpretation: Desired Result: 95.0 Percentile

John F. Crawford, Ph.D.
Laboratory Director

All tests performed at SpectraCell Laboratories, Inc. * 7051 Portwest, Suite 100, Houston, Texas 77024 * Phone: 1-800-227-5227
CLIA # 45D0710715

SPECTROX Total Antioxidant Function

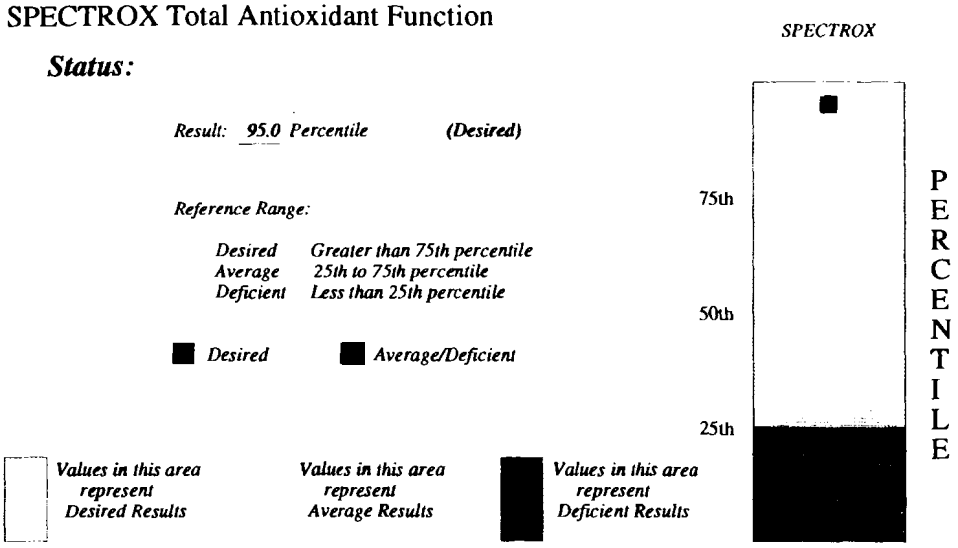
Status:

Result: 95.0 *Percentile* ***(Desired)***

Reference Range:

Desired	*Greater than 75th percentile*
Average	*25th to 75th percentile*
Deficient	*Less than 25th percentile*

■ *Desired* ■ *Average/Deficient*



Interpretation: Desired

SPECTROX values in the upper quartile (75th to 100th percentile) of antioxidant function for apparently healthy persons indicate a desirable status. Since antioxidants are protective nutrients, the most desired status would be the greatest ability to resist an oxidative stress.

SPECTROX measures the lymphocytes' total antioxidant function by addition of a peroxide [oxidative stress] to complete medium. Lymphocyte growth response with peroxide is reported as a percentile of growth responses from a reference range of apparently healthy persons. Greater antioxidant function is indicated by values over the 75th percentile, while deficient antioxidant function is indicated by values less than the 25th percentile.

SPECTROX measures total antioxidant functions and the clinical interpretation of results should consider physiological, pathological, environmental, lifestyle and dietary factors. Please consider that prior, current or additional supplementation with nutrient antioxidants [vitamin C, vitamin E, beta carotene, etc.] reflects only a part of the total antioxidant systems. Supplementation and lifestyle changes may or may not improve overall antioxidant functions, due to the many factors that affect antioxidant status.

FIA ™ Test Description	Test Result (% CONTROL)	Reference Range
Spectrox Total Antioxidant Function	95.0	> 75 %

The reference ranges listed in the above table are valid for male and female patients 12 years of age or older.

APPENDIX J

ORAC-BASED FOOD FREQUENCY QUESTIONNAIRE

Food item	Average *ORAC score (uM Trolox® eq/g)
Blueberries 1/2C Blackberries 1/2C Raspberries 1/2C Cranberries 1/2C Cranberry sauce 1/4C Strawberries 1C	2,107.2
Prunes 1/4C Raisins 1/4C Figs 1/4C	1,720
Spinach (steamed) 1/2C Spinach (raw) 1C Kale 1C Brussel sprouts 1/2C Alfalfa sprouts 1/2C Broccoli 1/2C Collard greens 1C	527.44
Plums 1 medium Beets 1/2C Oranges 1 medium Red peppers 1/2C Red grapes 1C	590.52
Avocado 1/4C Guacamole 1/4C Peanuts 1/4C Peanut butter 2Tblsp	381.04
Cherries 1/2C Kiwi 1 medium Grapefruit ½ medium White grape 1C	368.5

* Oxygen Radical Absorbance Capacity scores for individual food items are determined by the group average. Each food items individual score is determined by multiplying the average serving size of the food (grams) by the individual ORAC score per gram of the food.

Food Item	Average *ORAC score(uM Trolox® eq/g)
Garlic 2 cloves Mushrooms 1/4C Scallions 1/4C Onions 1/4C	402.88
Corn 1/2C Eggplant 1/2C Cauliflower 1/2C Peas 1/2C Potatoes ½ medium Sweet potatoes ½ medium Cabbage 1/2C Tomato sauce 1/4C	216.38
Leaf lettuce 1C Cantaloupe melon 1/4 whole Banana 1 medium Apple 1 medium	121.38
Carrots 1medium String beans 1/2C Tomatoes 1/2C Zucchini 1/2C Apricots 2 medium Peaches 1 medium	137.25
Yellow squash 1/2C Lima beans 1/2C Iceberg lettuce 1C Pear 1 medium	80.4
Watermelon 1C Honeydew melon 1C Celery 1/4C Cucumber 1/4C	37.92

* Oxygen Radical Absorbance Capacity scores for individual food items are determined by the group average. Each food items individual score is determined by multiplying the average serving size of the food (grams) by the individual ORAC score per gram of the food.

Beverages	Average *ORAC score(uM Trolox® eq/g)
Juices (8oz/240ml)	
Prune juice Blueberry juice concord grape juice grape juice Cran grape	4,461.6
Red wine (merlot, cabernet sauvignon)	2,364
Grapefruit juice Tomato juice Orange juice Vegetable juice/V-8 juice	794.4
Apple juice Lemon juice Pineapple juice	583.2

* Oxygen Radical Absorbance Capacity scores for individual food items are determined by the group average. Each food items individual score is determined by multiplying the average serving size of the food (grams) by the individual ORAC score per gram of the food.

BIOGRAPHY

John David Bagnulo was born in Boston, Massachusetts on May 11, 1970. He graduated from Mt. Blue High School, Farmington, Maine in 1988. He attended Boston College, Chestnut Hill, Massachusetts, where he received a Bachelor of Science degree in Economics on May 18, 1992. In that same spring, John was commissioned as an officer in the United States Navy. He served as an ensign within the Physical Training Division of Naval Aviation Schools Command, Pensacola, Florida. In 1994, he attended graduate school at Texas A&M University and worked with ostriches within the Poultry Science Department. In 1995, John attended the University of North Carolina, Chapel Hill, North Carolina. While enrolled there, he worked as a dietetic intern at New Hanover Regional Medical Center, Wilmington, North Carolina. He earned a Masters of Public Health degree and became a certified Registered Dietitian in 1997.

In 1997, he worked as a dietitian at the Canyon Ranch in the Berkshires, Lenox, Massachusetts. He assisted physicians and exercise physiologists in the treatment of clients with a wide variety of conditions, in addition to developing dietary plans for those individuals who were simply interested in improving their nutritional health. This experience was both challenging and rewarding, as it encouraged John to pursue a terminal degree in the field of nutrition. In April of 1999, John left the Canyon Ranch and embarked on his first major adventure. On April 10, 1999, he left Talkeetna, Alaska and snowshoed over 100 miles of wilderness and glacier to the base of Mt. McKinley

(20,500 ft.). Fifteen days later, he stood on McKinley's summit after soloing the West Buttress Route.

John enrolled at the University of Maine in the fall of 1999. He worked as a Graduate Assistant within the Food and Nutrition Science Department until the fall of 2000. He taught part time at the University of Maine at Farmington over the next two years, before earning a full time assistant professor position in May of 2002. John teaches exercise physiology, sports nutrition, community and personal health assessment, and introductory nutrition courses. He delivered an Original Contribution oral presentation at the American Dietetic Association's annual meeting in Philadelphia, Pennsylvania, on October 21, 2002. He is a candidate for the Doctor of Philosophy degree in Food and Nutrition Sciences from the University of Maine in May of 2003.