AN ABSTRACT OF THE THESIS OF

Edward E. Morris for the degree of Master of Science in Food Science and Technology presented on March 7, 2003.
Title: Antioxidant Potential of Yeast Containing Beer.

Abstract approved: _____
Mark A. Daeschel

It has been suggested that cellular damage from oxygen radicals is one of the processes leading to cardio-vascular disease and cancer. Natural antioxidants prevent uncontrolled oxidative reactions by decreasing molecular oxygen levels, scavenging chain-initiating and chain-propagating free radicals, chelating metals, or decomposing peroxides. Beer is rich in antioxidants, derived both from malt and hops, consisting mostly of flavanoids and phenolic secondary plant metabolites. Much research has been conducted concerning antioxidant activity of beer in relationship to flavor stability. Yeast cells possess both enzymatic and non-enzymatic antioxidant systems to defend against oxygen radicals, in addition to scavenging and absorbing molecular oxygen for cell synthesis. It is well known that bottle-conditioned beer has a longer shelf life than conventional beer in terms of flavor stability and freshness. This is likely due to a complex relationship between the yeasts inherent ability to scavenge oxygen species, produce SO_2, chelate transition metals and employ other methods to defend against molecular oxygen. The objective of this research was to determine whether bottle-

conditioned beer (which contains live yeast) has a higher antioxidant activity compared to that of conventional beer.

Initial experiments were conducted to establish a baseline of antioxidant potentials. The first experiment consisted of determining the antioxidant potential of commercially available beers and how those values compared to common foods and drinks. Next, live yeast was added to commercially available artificially carbonated beer, to determine whether the presence of live yeast alone had an impact on antioxidant potential. Lastly, in the first set of beer trials wort was prepared, brewers yeast added, and then allowed to complete primary fermentation. The beer was bottle-conditioned (naturally carbonated) by the addition of a second yeast strain and either a 'high' or 'low' level of sugar, or artificially carbonated. Treatments consisted of using three different yeasts. A control was prepared by artificially carbonating beer without live yeast. Antioxidant potentials were determined using Ferric Reducing Antioxidant Potential (FRAP) analysis. Results indicated that there was an increase in the level of antioxidant activities between the bottle-conditioned beers compared to the control beer, whether or not the high or low level of yeast was present.

In the second set of beer trials wort was prepared, brewers yeast added, and then allowed to complete primary fermentation, as in the first set of beer trials. The beer was bottle-conditioned by the addition of a second yeast strain and sugar, or artificially carbonated followed by the addition of a second yeast but no sugar. Treatments consisted of using three different yeasts. A control was prepared by

artificially carbonating beer without live yeast. Dissolved oxygen, free and total sulfite, yeast viability, and antioxidant capacities were again determined. Results indicated that there was an increase in the level of sulfite, a decrease in dissolved oxygen, yeast remained viable for a longer period of time, and antioxidant activities were higher in the bottle-conditioned beers when compared to the control beer. Furthermore, while the differences were not as great, the same trends were observed for all parameters when comparing the artificially carbonated beers containing live yeast to the control beer. The elevation in antioxidant activities of beer with live yeast present (live beer) was significant.

The third and final phase of experiments focused on the contribution of antioxidant potential specifically from the yeast. Yeast cells for each strain were cultivated in beer wort, harvested, washed, and cell extracts prepared. The crude yeast extracts were subjected to heat treatment, size fractionation followed by heat and protease treatments, glutathione determination, and lipid extraction, and then analyzed for antioxidant activity. Results indicated a complex interaction between many different yeast components that contributed to the total antioxidant activity provided specifically from yeast. Rather than one single compound, the yeast contributed heat stable components, consisting of proteins and enzymes, molecules with high, medium, and low molecular weights, and active lipid portions.

The overall results suggest, that while the malt and hops components likely play the major role in antioxidant activity of beer, beer containing live yeast has a

significant increase on that antioxidant activity. Consequently, the flavor stability and health benefits from beer containing live yeast would be increased.

ANTIOXIDANT POTENTIAL OF YEAST CONTAINING BEER

By
Edward E. Morris

A THESIS

Submitted to
Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented March 7, 2003
Commencement June 2003

Master of Science thesis of Edward E. Morris presented on March 7, 2003.

APPROVED:

Major Professor, representing Food Science and Technology

Head of Department of Food Science and Technology

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Edward E. Morris, Author

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Mark Daeschel, my advisor, for his tireless support and belief in me, and also his understanding and friendship. I would also like to thank my committee members, Phil McFadden, Ron Wrolstad, and Willie 'Skip' Rochefort for their patience and advice. In addition, thanks to Balz Frei, Deborah Hobbs, and the Linus Pauling Institute, for their support, assistance, and time. I would further like to thank all the members of the Daeschel Lab, for their friendship, support, and encouragement. And last but not least, thanks to my wife Susie, my daughters Michaela and Matilda, and my mother Jane, for always being there for me and allowing me to never lose sight of the things that really matter.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
Composition of Beer	2
Overview of the Brewing Process	6
Health Benefits of Antioxidants	7
Antioxidant Activity of Beer	11
Yeast and Antioxidant Activity	16
Measurement of Antioxidant Activity	19
Yeast in Brewing	20
MATERIALS AND METHODS	22
Yeast and Media	22
Isolation and Enrichment	22
Yeast Extracts	23
Yeast Fractions	23
Yeast Lipid Extraction	24
Brewing Formulation and Treatments	25
Analysis	26
Dissolved Oxygen	27
Viable Yeast	27
Sulfur Dioxide	28
Antioxidant Capacity	29

TABLE OF CONTENTS (continued)

	Page
RESULTS AND DISCUSSION	29
Initial Experiments	34
Survey Studies	34
Yeast Addition to Commercial Beer	36
First Set of Beer Trials	38
Second Set of Beer Trials	40
Dissolved Oxygen	40
Yeast Viability	41
Sulfur Dioxide	43
Antioxidant Potential	47
Yeast Extracts	51
Heat Treatment	51
Size Fractionation	53
Glutathione Concentration	55
Lipid Extraction	57
CONCLUSION	59
BIBLIOGRAPHY	61
APPENDICES	65

LIST OF FIGURES

Figure		Page
1.	Overview of the Brewing Process	6
2.	Overview of All Experiments	33
3.	Antioxidant Activity of Some Common Foods and Drinks	35
4.	Antioxidant Activity of Various Types of Commercial Beers	36
5.	Artificially Carbonated Beer with Yeast Added	37
6.	Antioxidant Capacity of Artificially Carbonated and Bottle-Conditioned Beers at High (~1.1 x 10^6 cells/mL) and Low (~5.66 x 10^5 cells/mL) Levels of Live Yeast Cells	39
7.	Dissolved Oxygen for second set of beer trials at 2, 3, 4, 5, and 6 months	41
8.1.	Free and Total Sulfite at 1 Month	44
8.2.	Free and Total Sulfite at 2 Months	45
8.3.	Free and Total Sulfite at 3 Months	45
8.4.	Free and Total Sulfite at 4 Months	46
8.5.	Free and Total Sulfite at 5 Months	46
8.6.	Free and Total Sulfite at 6 Months	47
9.	Antioxidant Capacity of Artificially Carbonated and Bottle-Conditioned Beers	48
10.1.	Antioxidant Capacity of California Ale Yeast Treatments	49
10.2.	Antioxidant Capacity of San Francisco Lager Yeast Treatments	50
10.3.	Antioxidant Capacity of Belgian Wit Yeast Treatments	50

LIST OF FIGURES (continued)

Figure		Page
11.	Antioxidant Capacity of Crude Yeast Extracts-Heat Treated	52
12.1.	Antioxidant Capacity from Different Molecular Weight Fractions of Crude Yeast Extracts-Control	53
12.2.	Antioxidant Capacity from Different Molecular Weight Fractions of Crude Yeast Extracts-Boiled 60 Minutes	54
12.3.	Antioxidant Capacity from Different Molecular Weight Fractions of Crude Yeast Extracts- Treated with 10mg/mL Protease	55
13.	Antioxidant Capacity of Crude Yeast Extracts with Glutathione Reductase (Enzyme) and NADPH	57
14.	Antioxidant capacity of lipid extracted crude yeast extracts	58

LIST OF TABLES

Table		Page
1.	Average Composition of Beer	4
2.	Average Nutritional Value per 12 ounces of Beer	5
3.	Yeast Viability	42

ANTIOXIDANT POTENTIAL OF YEAST CONTAINING BEER

INTRODUCTION

It has been suggested that cellular damage from oxygen radicals is one of the processes leading to cardio-vascular disease and cancer. Natural antioxidants prevent uncontrolled oxidative reactions by decreasing molecular oxygen levels, scavenging chain-initiating and chain-propagating free radicals, chelating metals, or decomposing peroxides. Beer is rich in antioxidants, derived both from malt and hops, consisting mostly of flavanoids and phenolic secondary plant metabolites. Much research has been conducted concerning antioxidant activity of beer in relationship to flavor stability. Yeast can also function as an antioxidant because they generate reducing power. Yeast cells possess both enzymatic and non-enzymatic antioxidant systems to defend against oxygen radicals, in addition to scavenging and absorbing molecular oxygen for cell synthesis. It is well known that bottle-conditioned beer has a longer shelf life than conventional beer in terms of flavor stability and freshness. This is likely due to a complex relationship between the yeasts inherent ability to scavenge oxygen species, produce SO_2, chelate transition metals and employ other methods to defend against molecular oxygen. The objective of this research was to determine whether bottle-

conditioned beer (which contains live yeast) has a higher antioxidant activity compared to that of conventional beer.

COMPOSITION OF BEER

Beer is the generic name for any alcoholic beverage made by fermenting malted barley (sometimes with other cereal grains) and flavored with hops. Depending on the style of beer, the composition and nutritional value varies slightly (Tables 1 & 2). The actual brewing process starts with mashing. Mashing is completed by taking malted barley, milling it so the native enzymes and starches are exposed, and adding it to warm water. The water is then heated to different temperatures for various lengths of time in order to promote enzymatic activity, solubilize malt solids, and to convert the starches to sugars. As the temperatures increase, protease enzymes break down complex proteins into polypeptides and amino acids, α- and β-amylase enzymes degrade starch molecules into sugars, and glucanase enzymes degrade glucans to help decrease wort viscosity (22). Eventually, the mash temperature is brought up to 78^0C, which stops all enzymatic activity and decreases viscosity of the liquid for better lautering (separation of the liquid from the spent grains). At this stage the liquid is known as sweet wort. Lautering is the next step, and is the process of draining the sweet wort off of the spent barley hulls and transferring it to the boil kettle. Malt extract is a thick,

sugary syrup prepared by concentrating sweet wort through removal of most or all of the water via low-vacuum evaporation (17). Malt extract is commercially available for small brewers opting to skip the mashing process or improve consistency in experimental batches.

Boiling is the next stage in the process, and takes place typically for 90 minutes. At this time hops are added to the boil kettle. The hops are a very important ingredient in today's beers. Hops contain resins full of α-acids that during the boil are isomerized into iso-α-acids, which contribute the bittering compounds in beer (11). Hops also contain essential oils that contribute to the beer flavor and aroma profile (16). Different hop varieties have different flavor and aroma characteristics, and a combination of varieties is often used. Some of the iso-α-acids will react with proteins and precipitate out. Other compounds in the hops lend to foam stability and cling character (11). Another important characteristic of the hops, besides the flavor and aroma contributions, is the antimicrobial properties. Some hop compounds can inhibit bacterial growth, and one of the compounds- xanthohumol- has been linked to anticancer activity (2). The iso-α-acids are strong antioxidants (2). The boil also adds color and texture to the wort.

The next stage of the brewing process, called pitching, is inoculating the wort with yeast. The yeasts play a very important part in the fermentation process, as they convert the natural malt sugars into alcohol, CO_2, and small amounts of other yeast metabolites. The different yeast strains also impart particular flavor and

aroma characteristics to the finished beer. This fermentation process can last from 2 to 7 days depending on how much fermentable sugar is in the wort and the strain of yeast used (24).

Once the fermentation is complete, the product is known as green beer. It is then transferred to oxygen free tanks for aging and conditioning. This step carries out the maturation of the green beer into beer. Some flavor and aroma characteristics develop, the yeast cells die off and settle to the bottom of the tanks, and the beer is stabilized (24). Next is the clarification. This procedure is done by the larger American breweries and involves either pasteurization or sterile filtering, as a method of biological stabilization. The majority of microbreweries opt to skip this step because the brewmasters feel it strips the beer of valuable flavor and aroma compounds, and alters the viscosity and mouthfeel of the finished product (2). The last step in the entire process is packaging in bottles, cans, kegs, or casks, followed by distribution.

Table 1. Average Composition of Beer

Component	**Percentage**
Water	75-92
Alcohol	2-13
Proteins	3-6
Dextrins	3-6
Mineral Salts	0-2
Carbon Dioxide	0.1-0.4

(Rabin & Forget)

Table 2. Average Nutritional Value per 12 ounces of Beer

Nutritional Property	Amount
Water	92%
Food Energy	150kCal
Protein	1.1g
Carbohydrate	14g
Calcium	18mg
Sodium	28mg
Thiamine	0.01mg
Riboflavin	0.11mg
Niacin	2.2mg

(Rabin & Forget)

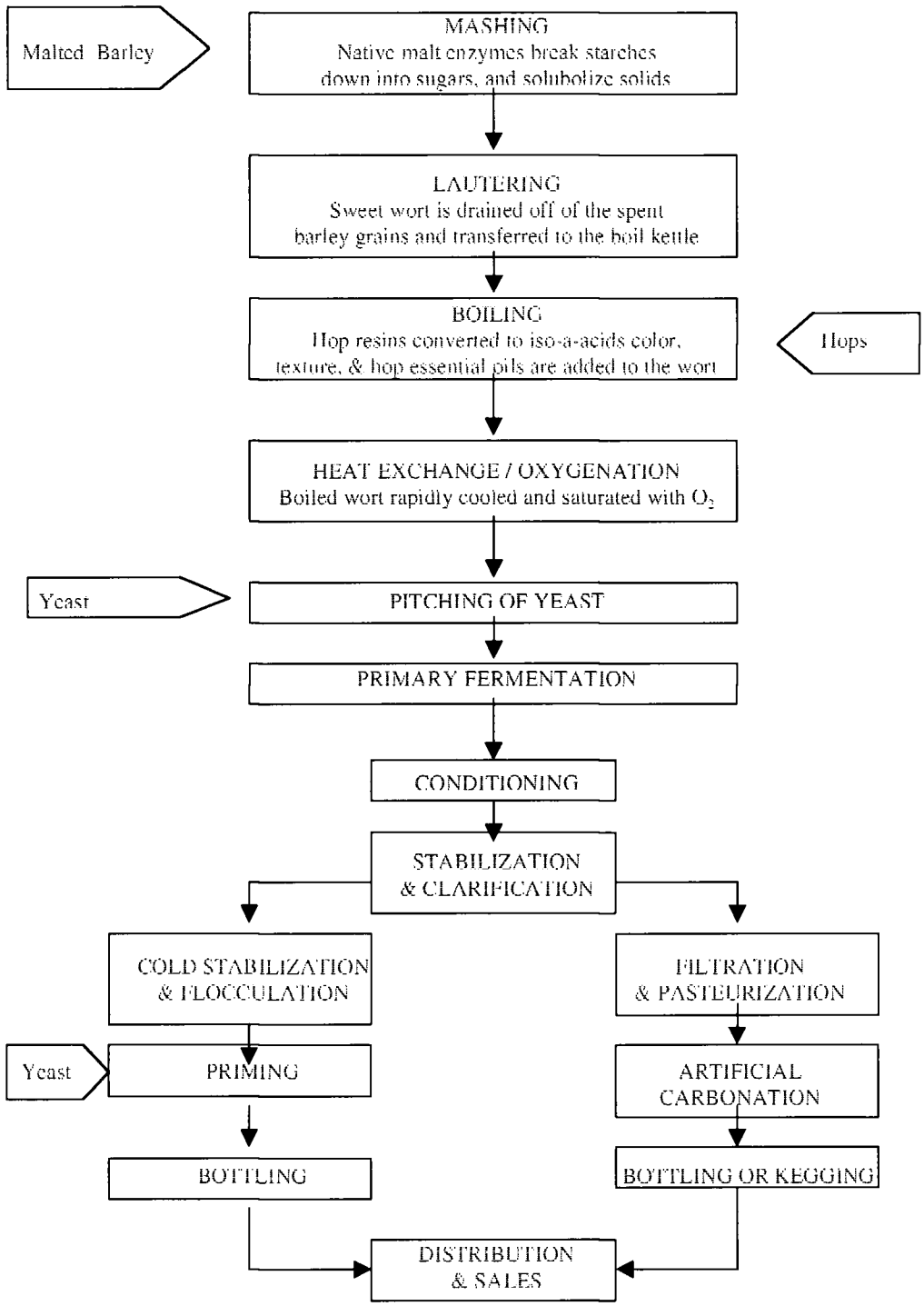


Figure 1. Overview of the Brewing Process
Boxed arrows indicate points of entry for antioxidant compounds

HEALTH BENEFITS OF ANTIOXIDANTS

The Oxygen Paradox coined by Kelvin Davies of the Albany Medical College in Albany New York, describes the constant battle in which higher eukaryotic organisms are engaged (6). Simply put, higher eukaryotes cannot live without oxygen, though oxygen is extremely toxic and dangerous to our systems. The reduction of oxygen to water through the mitochondrial electron transport chain is the process that fuels all cells and enables life. According to Davies, this reductive environment provides the opportunity for oxygen to undergo unscheduled and uncontrolled reductions, generating the superoxide anion radical, hydrogen peroxide, and the highly reactive hydroxyl radical; collectively known as free radicals or Reactive Oxygen Species (ROS). Free radicals are also generated as a result of normal oxidative reactions, or by components of tobacco smoke, diet, drugs, and other environmental pollutants (23).

Free radicals are highly reactive molecules that have one or more unpaired electrons. Free radicals react with and alter the function of cellular components such as cell membranes, lipoproteins, proteins, carbohydrates, RNA, and DNA, if they are not quenched by an antioxidant (23). Oxidative stress and cellular damage from free radicals has been implicated in a wide variety of degenerative processes, diseases, and syndromes, including: mutagenesis, cell transformation, and cancer; atherosclerosis, arteriosclerosis, heart attacks, strokes, and ischaemia/reperfusion injury; chronic inflammatory diseases, such as rheumatoid arthritis; acute

inflammatory problems, such as wound healing; photo-oxidative stresses to the eye, such as cataract; central-nervous-system disorders, such as Parkinson s disease and Alzheimer s dementia; and a wide variety of age related disorders (6). In fact, some of these oxidation-linked diseases and disorders are compounded, and sometimes even initiated, by reactions with free radicals generated by pro-oxidants absorbed environmentally or in drugs and foods (6).

Higher eukaryotic organisms possess enzymatic and non-enzymatic defense systems that prevent free-radical formation, remove radicals before damage can occur, repair oxidative damage, eliminate damaged molecules, and prevent mutations (13). Unfortunately, the antioxidant system is not perfect. Despite every enzymatic antioxidant or repair effort, inevitably oxidative stress and oxidative damage are inescapable (23). This is where some key non-enzymatic defense systems assist. One of those defense systems is the garnering of water- and lipid-soluble antioxidant compounds from their surroundings (6).

Antioxidant is a general term that defines a compound capable of scavenging oxygen radicals. Natural antioxidants prevent uncontrolled oxidative reactions by decreasing molecular oxygen levels, scavenging chain-initiating and chain-propagating free radicals, chelating metals, or decomposing peroxides (2). Our cells use a variety of antioxidant compounds to directly react with and disarm oxidizing agents (6).

Foods that contain high amounts of antioxidants such as wine, vegetables, fruit, and cereal grain products are beneficial to health (29). Evidence suggests that

antioxidants increase levels of HDL-cholesterol and protect the LDL-cholesterol complex from free radical attack and oxidation (15, 29). It has also been shown that the oxidation of LDL-cholesterol is the major building block of atherosclerotic plaques and the penetration of lipids into the arterial wall (14). Moreover, the same study proved that the development of atherosclerotic plaques and penetration of lipids only occurs after peroxidation of the LDL-cholesterol complex (14). It is therefore suggested by many researchers that a diet rich in antioxidants will prevent the development of atherosclerotic plaques and control lipid penetration of arterial walls (15). Further, increased intake of antioxidants has been linked to reduced DNA mutations and oxidative DNA base damage (8). Additionally, numerous other studies have pointed to the decrease in oxidative damage to the liver, and a reduction in cardio-vascular-disease risk factors in the blood, with increased antioxidants (15).

Another particular study of interest shows that a diet that is rich in high antioxidant foods may slow ageing and prevent the loss of long-term memory and learning ability (27). A similar study shows good evidence of a link between high antioxidant intake and the reduced risk of age-related diseases (32). That study concluded that antioxidant trace elements and antioxidant vitamin supplementation might be the best way to prevent accelerated ageing and age-related diseases (32).

However, it is not enough that a food contains antioxidants, but the antioxidants must also be easily assimilated or absorbed (29). Only some antioxidants are readily absorbed and remain for a significant time in the body (37).

For example, the fruits and vegetables most normally consumed in the human diet contain more than forty different types of carotenoids, as well as many other antioxidants, but only fourteen of the forty carotenoid molecules have been identified in human tissue or plasma (8). In fact, some of the antioxidant molecules are preferentially selected by binding proteins over other similar molecules even though the mechanisms of absorption are similar (3). One such example is α-tocopherol. While the process of absorption of all eight tocopherols is the same in humans, α-tocopherol predominates over the others in blood and tissues, even though some of the other tocopherols have higher antioxidant potential in chemical systems (8). The explanation for this phenomenon lies in the action of a 30kDa protein in the cytoplasm of the liver, which selects α-tocopherol and incorporates it into nascent very low-density lipoprotein (35). Similarly, α-tocopherol is selected preferentially over the other tocopherols for transportation intracellularly by a smaller binding protein 14.2kDa in size (9). This helps to illustrate why some antioxidant compounds have different antioxidant potentials *in vitro* compared to *in vivo* (8). Other hurdles that must be overcome are, often the antioxidant compounds are destroyed by gut flora or not extracted from the food matrix in the gut. They may be rapidly metabolized by the liver and excreted, or absorption through the intestinal wall may not take place (8).

ANTIOXIDANT ACTIVITY OF BEER

Beer is rich in antioxidants, derived primarily from malt and hops compounds, consisting mostly of flavanols and phenolic secondary plant metabolites (37). Some of the reduced proteins may also act as antioxidants (29). Yeast is also important in providing antioxidant activity during fermentation and conditioning (39). Beer itself contains many antioxidants of varying type and action.

Silicic acid is one such antioxidant compound found in beer. Human intake of Aluminum results in the average American consuming up to 20mg per day (37). It has been shown that at least 1% of all Aluminum we consume is accumulated in liver, muscle, bone, brain, and spleen tissue, and further studies point to a link between Aluminum content in tissues and stroke (10). Silicic acid and Aluminum form the stable and excretable hydroxyaluminosilicate, which is quickly eliminated by the kidneys, allowing for the depletion of Aluminum from body tissues and decreased incidence of Aluminum toxicity (37).

Significant amounts of niacin, riboflavin, and folate are found in beer (37). Folates are important for the metabolism of single carbon units, and therefore participate in the processes of DNA and protein synthesis, and subsequently cell division. Epedemiological studies show that folates can protect against neural tube defects, cardiovascular disease, colon cancer, and may have a role in protecting against Alzheimer s disease and cervical cancer (37).

Another very important source of antioxidants are the polyphenolic compounds known as tannins. Tannins are integral plant compounds with a relatively high molecular weight and an antioxidant activity more than twenty times that of vitamin E (14). Malt delivers many polyphenolic tannin compounds to beer during the mashing and sparging stages of the brewing process. Catechin, epicatechin, quercetin, and *p*-coumaric, caffeic, sinapic, ferulic, vanillic, and gallic acids, and the procyanidins and proanthocyanidins are all biologically active antioxidant tannins and polyphenols in beer and wine (14). It was initially thought that the more antioxidant rich the food source is, the more antioxidant compounds would be absorbed and utilized by the body. However, Gorenstein *et al* have shown that while some white wines were higher in total polyphenols, beer still had a higher antioxidant activity and in clinical trials was shown to have a significantly stronger ability to lower blood plasma lipid peroxidases and lipoxygenase activity (14, 34).

In addition to the malt compounds present in beer, the hops also provide significant antioxidant activity, and some hop components can transfer antimutagenic and anticarcinogenic compounds to the beer (34). It is important to first point out that on a weight-to-weight basis, the hops components have about ten-fold more tannin compounds than malt, except for the catechins and proanthocyanidins (34). Additionally, hops contain high amounts of other antioxidant compounds; flavone glycosides, chlorogenic acid derivatives, coumarins, and α-acids (34). Moreover, those compounds are more soluble at

higher temperatures and therefore more will be abstracted from the hops during the boil than from malt during the mash at lower temperatures (34).

Hydroxycinnamic acids from hops have been shown to have potent antioxidant and anticarcinogenic effects *in vitro* (34). Another hop compound, xanthohumol, has been shown to have antioxidant and anticarcinogenic abilities (18, 33, 38). Xanthohumol exhibited the ability to inhibit the cytochrome P450 systems responsible for the formation of carcinogens and even stimulating the detoxifying enzyme quinone reductase (37). The α-acids also exhibit anticarcinogenic activity via enhancing cell differentiation, and inhibiting tumor formation and/or growth (37). During the boil stage the α-acids undergo isomerization. Both high amounts of iso-α-acids and α-acids from aroma hops possessed very high free radical scavenging and lipid peroxidation inhibition activity (34). Perhaps most importantly, these compounds all show strong activity even at micro-molar levels (37).

Hops are also a source of phytoestrogens, plant derived compounds, which have been linked to protect against several chronic diseases and conditions (37). Phytoestrogens are present in many plant and cereal grains, and high in many legumes. In other studies they have shown protection against cardio-vascular disease, breast cancer, and prostate cancer (37).

Epidemiological studies have indicated the consumption of moderate quantities of alcoholic beverages, particularly wine and beer, reduce the mortality rate from coronary artery disease (15, 21). Other studies have shown that

polyphenolic and phenolic antioxidants in beer are bioavailable, easily assimilated, and remain in the body for periods in excess of 24 hours (29, 37). It appears that ethanol and antioxidants may work together to be beneficial to health. Ghiselli and others have shown that there seems to be a synergistic effect provided by the ethanol for the absorption of phenolic compounds in beer. They stated that ethanol plays an important role in phenolic compound absorption:

> Phenolics are aromat ic compounds hardly soluble in water, but easily soluble in ethanol. The increased solubility of these compounds in hydroalcoholic solutions may affect the rate and amount of their absorption. Beer, with a moderate antioxidant capability coupled with low ethanol content, is an alcoholic beverage that is able to improve plasma antioxidant capacity without the negative effects produced by high doses of ethanol. In fact, although the amount of ethanol present in 500mL of beer did not induce any appreciable change in the markers of metabolic control, it is able to facilitate the transfer of the antioxidant capacity from beer to body fluids.

In other words, while some foods may be rich in antioxidants, those antioxidants are never absorbed into the body and are merely excreted. However, due to the increased solubility of the highly antioxidative plant phenols and flavanols in ethanol, beer not only is high in antioxidants, but those antioxidants are able to be absorbed with the aid of ethanol and utilized by the body. Furthermore, one hospital study has shown that the absorption of at least one recognized dietary antioxidant from beer is actually more efficient than from vegetables (4). Another study, which shows the total antioxidant activity of beer is roughly the same as orange juice, suggests that since the typical serving of beer is larger than that of

orange juice, beer is a better source of antioxidants (37). In fact, my own research indicates that depending upon the style, beer ranges anywhere between tomatoes and blueberries in antioxidants on a per weight basis; higher than that of: peas, carrots, white cabbage, snap beans, white onion, cauliflower, red pepper, beet, broccoli, purple onion, spinach, green pepper, red and green Thompson seedless grapes, apple juice, whole milk, and Gatorade.

Further studies have shown that beer significantly increases the antioxidant activity of human plasma (12). A similar study confirmed that it is not just the ethanol or the antioxidants in a food product, but both together that significantly increases human blood plasma antioxidant activity (31). Perhaps most significant, a survey done at the Brewing Research Institute in England, has shown that the antioxidant activity of beer is higher than some fruit juices and similar to that of some wines (29). Meaning that not only is beer a superior vector for antioxidant absorption over some fruits and vegetables, but it also actually has more antioxidants to be absorbed.

The majority of research concerning the antioxidant activity of beer has been in relationship to flavor stability rather than health benefits. However, some of this research can be applied directly to health benefits as well. There is direct evidence that reactive oxygen species are responsible for the staling processes that occur with beer (29). Uchida and Ono have demonstrated the delay of free radicals and the reactive oxygen species' deleterious effects by antioxidants in beer (36). That is, there is a significant lag time during which all of the antioxidant capability

is used up; then, the radicals appear and staling of the beer begins (36). It may then be inferred that fresh beer will have negligible amounts of free radicals and will therefore be high in antioxidant compounds available for absorption after beer consumption. Similar results have been shown by another group whom has developed tests that correlate the beginning of perceived staling of beer to the appearance of free hydroxyl radicals (20). Furthermore, this protection from staling or oxidation is occurring even though molecular oxygen is constantly permeating into the bottle. In fact, one study showed that molecular oxygen uptake was 1.4mg/L of beer over a six month period, when using a standard crown cork barrier (26).

YEAST AND ANTIOXIDANT ACTIVITY

Yeast can also be classed as an antioxidant, due to the fact that yeast generates reducing power (29). Different strains are thought to vary in this ability (29). In order to maintain reactive oxygen radicals at unharmful levels, yeast cells possess both enzymatic and non-enzymatic primary antioxidant defenses (28).

Yeasts express levels of peroxisomal catalase A, which is important in the decomposition of high amounts of hydrogen peroxide resulting from fatty acid oxidation (28). Glutathione is one non-enzymatic defense against oxidants. Because of the redox-active sulfhydryl group, which acts directly with strong

oxidants, glutathione is an important free-radical scavenger (28). In addition, glutathione is one of the metallothiones; antioxidant compounds expressed by yeast to minimize the formation of hydroxyl radicals by ensuring the sequestration of transition metals, including copper and iron (28). The metallothiones have been linked to defense systems of metal-ion detoxification and homeostasis (28). Further, the metallothiones have been shown to protect against and prevent the formation of both the superoxide radical and hydrogen peroxide through magnesium, copper, and cadmium homeostasis (28).

If the yeast s primary antioxidant defenses do not overcome all the oxidants thrown at them, and the cells experience some degree of oxidative stress, they produce secondary defense compounds (28). Superoxide dismutase and thioredoxin peroxidase are two such proteins. Antioxidative properties of superoxide dismutase from purified yeast extracts have been shown to inhibit the oxidation of emulsified linoleic acid, emulsified cholesterol, and ascorbic acid (25). Superoxide dismutase has likewise been shown to eliminate the superoxide radical (28). Use of reducing equivalents from yeast thioredoxin has been shown to reduce hydrogen peroxide and hydroperoxides (19). Another antioxidant yeast protein, catalase T, is transcribed and produced after oxidative stress occurs from the formation of excess hydrogen peroxide and acts directly to control it s toxic effect (28).

Apart from the yeast s innate antioxidant defenses, during fermentation the yeast produce secondary metabolites, some of which exhibit strong antioxidant

activity. As shown previously, ethanol is essential in the aid of poly- and mono-phenolic absorption in the human gut. All of the ethanol present in beer is the result of yeast fermentation and metabolism. The yeast converts fermentable sugars to ethanol and carbon dioxide during fermentation. Additionally, the yeast produces smaller quantities of secondary metabolites, one of which is sulfur dioxide (SO_2); a strong antioxidant utilized itself as an additive in many food systems. Many studies have shown a linear correlation between the level of sulfite in beer and the length of the shelf-stability of the beer (1, 36). The higher the amount of sulfite directly relates to longer shelf life and greater flavor stability. Therefore, the same can be inferred that greater amounts of sulfite in the finished beer will equal greater protection against oxidation, and therefore longer protection of poly- and mono- phenolic compounds that are bioavailable during consumption. Glucose addition to beer has also been shown to increase sulfite levels (22). This can be viewed as strong evidence that during a secondary fermentation or bottle conditioning, more sulfite will naturally be produced. This being true, any bottle-conditioned product will have greater protection against oxidative staling, and subsequently will deliver more antioxidant compounds to the body when the beer is imbibed.

MEASUREMENT OF ANTIOXIDANT ACTIVITY

As stated previously, antioxidant compounds encompass a wide range of molecules, each with varying mechanisms of action on a wide range of molecules with oxidative and pro-oxidative activities. Common methods or assays employed in the measurement of antioxidants are: Ferric Reducing Antioxidant Potential (FRAP), Trolox Equivalent Antioxidant Capacity (TEAC), Oxygen Radical Absorbance Capacity (ORAC), Total Radical Absorption Potential (TRAP), Total Antioxidant Activity (TAA), High Performance Liquid Chromatography (HPLC), Reverse-Phase High Performance Liquid Chromatography (RP-HPLC), Electron Spin Resonance spectroscopy (ESR), Chemiluminescence-flow injection analysis, and Gas Chromatography —Mass Spectrometry (GC-MS), as well as many other methods of generating radical or oxidative species and observing the period in which they are absorbed or quenched by a given sample or standard. The most common methods rely on a sample s ability to absorb the oxidative species either being generated by - or added to the reaction mixture, compared to a standard curve based on the ability of a known antioxidant to do the same.

Presently there is no standard assay for the analysis of antioxidant activity. The most commonly employed methods are the ORAC and FRAP assays. The FRAP assay is a rapid and economical assay best utilized for measuring many samples at one time.

YEAST IN BREWING

Brewing yeasts are classed into two categories: bottom fermenting yeasts (*Saccharomyces carlsbergensis,* reclassified *Saccharomyces uvarum*) and top-fermenting yeasts (*Saccharomyces cerevisiae*); there are also wild yeasts that have many species. Each of these categories is further subdivided into strains. More than 500 types of yeasts have been isolated, not including the numerous wild strains (7). The yeasts play a very important part in the fermentation process, as they convert the natural malt sugars into alcohol and CO_2. The different yeast strains also impart particular flavor and aroma characteristics in the finished beer. Desirable attributes of brewing yeasts are a rapid fermentation rate, efficient conversion of sugars to ethanol, CO_2, and not other metabolites, ability to withstand alcohol, reproducible and consistent flavor compound production, and good flocculation character (30). The yeast cells quickly absorb all available oxygen species to synthesize sterols and unsaturated fatty acids for their cell membranes and cell walls (11). Yeasts metabolize D-glucose, D-fructose, maltose, and maltotriose sugars into ATP. Some lager yeasts can also metabolize maltotetrose, resulting in a drier, less sweet beer.

Other than for fermentation of sugars to ethanol and CO_2, yeast plays vital roles in flavor, mouthfeel, and conditioning of beer. Conventional beers are sterile filtered to remove all yeast and bacterial cells (live or dead) before being packaged.

However, the bottle-conditioned craft beers contain yeast as part of the finished, packaged product.

Bottle conditioning is the process of adding sugar and yeast, called priming, to a completely fermented beer just prior to bottling (30). This has the effect of carrying out a secondary fermentation in the bottle producing 2.5 atmospheres of dissolved CO_2, and another 0.511% ethanol. Bottle conditioned beers contain yeast cells, live or dead. Conventional beers do not contain any yeast cells at all, due to their sterile filtration. Since yeasts inherently possess antioxidant capability and reducing power, bottle conditioned beers will have a higher antioxidant capacity than conventional (sterile filtered) beers.

MATERIALS AND METHODS

YEAST AND MEDIA

Isolation and Enrichment

Saccharomyces cerevisiae ssp. California Ale Yeast (#WLP001), *Saccharomyces uvarum* ssp. San Francisco Lager Yeast (#WLP810), and *Saccharomyces cerevisiae* ssp. Belgian Wit Ale Yeast (#WLP400) were obtained from White Labs, Inc., San Diego, CA., U.S.A. Yeasts were plated on malt extract broth media mixed with plate count agar from Difco Media, Voigt Global Distribution, LLC., Kansas City, MO., U.S.A. This was followed by purification via selection of a single colony and propagation on malt broth agar. For separate trials and treatments, individual colonies were selected and enriched on malt extract broth agar as above, or in malt extract broth with specific gravity 1.040 from John Bull-Bulldog Blend light un-hopped liquid malt extract, Paine s Malt LTD., Newark, England. Yeast cells were enumerated by American Society of Brewing Chemists (ASBC) methods using a Thoma type Hemacytometer counting chamber at 400x magnification.

Yeast Extracts

Isolated colonies of yeast cells were taken from the stock culture of yeast for each strain used in the bottle-conditioning experiments and for the addition to the artificially carbonated beers employed in the other experiments of this research. The yeast was cultivated in 500mL malt extract broth for 6 days at room temperature, during which time primary fermentation and yeast cell flocculation had occurred. The cells were then cultivated, centrifuged, washed in sterile diluent, vortexed, and centrifuged again. This process of washing the cells was repeated 10 times in sterile diluent. The cells were again centrifuged and the aqueous layer discarded. The cleaned cells were observed under the microscope at 400X magnification to confirm morphology and viability. The cells were then crushed in a mortar and pestle with glass beads, observed microscopically to confirm cell disruption, and centrifuged once more. This time the aqueous phase was reserved for analysis as a crude yeast extract consisting of yeast intracellular contents and soluble membrane proteins. This process was repeated for each of the three strains.

Yeast Fractions

Fractions of the crude yeast extracts and treatments were divided above 10,000MW, between 5,000MW and 10,000MW, and below 5,000MW. This was

accomplished employing Centricon Plus-20 centrifugal filter units at 10,000 and 5,000 molecular weight cut-offs from Amicon, of Millipore Corporation, Bedford MA. Crude yeast extracts were prepared as above, then 1 mL reserved for the unfiltered control, and 4mL were spun through the 10,000MW Amicon centrifuge filters at 4000 x gravity for 15 minutes. Next, the 1mL of filtrate was reserved for the below 10,000MW sample, while the other 3mL were spun through the 5,000MW at 4000 x gravity for 15 minutes. Both filters were spun until there was no visible liquid above the filters. The filters were then inverted into the centrifuge and the retentate recovered from the filters by gently spinning at 1,000 x gravity for 1 minute. The retentates were brought back to the original volume and reserved for analysis.

Yeast Lipid Extraction

The crude yeast extracts were also analyzed for antioxidant activity after having the lipid portion extracted. The lipid portion was also resuspended and analyzed. The crude yeast extracts were prepared as above. Following preparation, 3mL crude yeast extract was added to 3mL Chloroform and vortexed. The mixture was left to stand for 20 minutes followed by centrifuge separation. The two layers were separated, the aqueous phase reserved for the lipid extracted sample, and the

chloroform or solvent phase was evaporated under nitrogen gas until dry and then resuspended to the original volume in sterile buffer until analysis.

BREWING FORMULATION AND TREATMENTS

For consistency, the conventional beer used for the standard and the bottle-conditioned beers were made using an unhopped malt extract. The use of unhopped malt extract allowed for the evaluation of antioxidant contribution specifically from the yeast and malt, essentially removing the antioxidant activity provided by the hop components. The beers were made by adding the malt extract to 75.7 Liters of water, until the specific gravity reached 1.040. The total volume was brought up to 151.5 Liters and boiled for 90-minutes. The actual specific gravity of the sweet wort was 1.0415 at a volume of 79.5 Liters.

After the boil, 4.3 liters of the sweet wort were bottled into 355ml brown glass beer bottles, capped, and stored at 4°C until analysis. The remaining sweet wort was inoculated with White Labs English Ale Yeast (#WLP002) and allowed to complete primary fermentation at 21°C for 14 days. Of the now fermented green beer, 4.3 liters were bottled into 355ml brown glass beer bottles, capped, and stored at room temperature until analysis. The remaining green beer was then divided into 7 lots of 4.3 liters each. Three of the lots were bottled into 355ml brown glass beer bottles, inoculated with 1mL of yeast slurry containing a second yeast strain

standardized to 3.8 x 10^8 cells per mL, 0.355g. of glucose added, capped, and stored at room temperature until analysis. The three yeast strains used were White Labs California Ale Yeast (#WLP001), White Labs San Francisco Lager Yeast (#WLP810), and White Labs Belgian Wit Ale Yeast (#WLP400). The remaining 4 batches of green beer were transferred to Cornelius type kegs and chilled to 4.5°C for artificial carbonation. The kegs were pressurized to 12.3 psi with food grade carbon dioxide gas to obtain 2.5 atmospheres of dissolved CO_2.

After six days of bottle conditioning, one bottle from each treatment was analyzed for CO_2 and total yeast concentration. As the control, one of the four batches of artificially carbonated beer was bottled into 355ml brown glass beer bottles, capped, and stored at room temperature until analysis. The last three artificially carbonated treatments, were bottled into 355ml brown glass beer bottles for each strain, inoculated with 3.8 x 10^8 live yeast cells (one strain each for the three strains used for bottle-conditioning), capped, and stored at room temperature until analysis.

ANALYSIS

At 1 month from completion of bottle conditioning, bottles from each treatment, as well as the unfermented sweet wort and the non-carbonated green

beer, were selected and analyzed for dissolved oxygen, yeast viability, free and total sulfur dioxide, and antioxidant capacity. This was repeated at 2, 3, 4, 5, and 6 months. The industry standard for the typical shelf life of beer is generally four months.

Dissolved Oxygen

Dissolved Oxygen was determined using an YSI model 95 dissolved oxygen meter and expressed in mg/L (parts per million). Individual treatments were opened, half of the contents removed, and the remaining half reserved and resealed for antioxidant analysis. Of the portion removed two 1mL aliquots were taken for yeast viability analysis and the remainder poured into a 250mL beaker. The dissolved oxygen meter was submerged into the sample and swirled steadily until the reading was stabilized.

Viable Yeast

Viable yeast was determined by plating 1mL beer from each treatment containing yeast on malt agar, dried, and incubated for 3 days at 37°C in duplicate.

After incubation, the plates were analyzed for viable yeast indicated by the presence of formed colonies.

Sulfur Dioxide

Sulfur dioxide was determined using the pararosanaline method for free and total sulfite. Sample preparation and analysis consisted of adding 100µL mercurichloride reagent to a test tube, followed by 100µL 0.1N H_2SO_4, and 200µL beer sample or standard solution. The standard solution consisted of 0-25 mg/L sulfur dioxide. For free sulfite determination, 1mL distilled water, 400µL color reagent, 400µL 0.2% formaldehyde, and 1.3mL distilled water were added to each test tube, with gentle mixing after each addition. For total sulfite determination, 300µL 0.1N NaOH was added and allowed to react for 1 minute. This was followed by addition of 200µL 0.1N H_2SO_4, 1mL distilled water, 400µL color reagent, 400µL 0.2% formaldehyde, and 0.8mL distilled water, again with gentle mixing after each addition. All samples were incubated at room temperature for 30 minutes, followed by absorbance measurement at 580nm.

Antioxidant Capacity

Antioxidant capacity was determined by employing the Ferric Reducing Antioxidant Potential (FRAP) assay, using a Molecular Devices SpectraMax 190 microplate reader and SoftMAX Pro software from Molecular Devices, Sunnyvale, CA. This assay is based on the ability of the assayed sample to reduce the ferric ion to ferrous followed by the formation of a colored ferrous-tripyridylpyrazine complex and comparison to a standard curve containing ferrous ions of known concentration.

Sample preparation and analysis consisted of adding 80μL of each sample in duplicate to the first 2 wells in row A of a 96 well flat bottom microplate. This was repeated for samples until the first row (A) of the plate was complete (6 samples total per plate). 40μL was removed and added to the second row (B) of the plate containing 40μL of de-ionized, distilled water treated with a metal chelator and mixed, removing 40μL after mixing. This results in a volume of 40μL in each of the first two rows of the plate, the first row undiluted and the second row at dilution factor 2. The 40μL removed from row two was added to 40μL of water in row 3 (C) and mixed, removing 40μL again. This results in a dilution factor of 4 in row three. This was continued through row seven (H) resulting in a dilution factor of 64. Therefore, each sample in duplicate was also at concentrations from undiluted through dilution factors of 2, 4, 8, 16, 32, and 64. To the eighth and final row, 40μL of de-ionized, distilled water treated with a metal chelator was added. To the

first two wells, 40μL of a one molar concentration of Trolox was added and mixed, removing 40μL and adding them to the third and fourth wells and mixing. This was repeated in the fifth and sixth wells and again in the seventh and eighth wells. This last row represents the standard curve of Trolox at 500μM, 250μM, 125μM, 62.5μM and 0μM Trolox, respectively. To each well of the 96-well plate, 300μL of warmed FRAP reagent were added and incubated for 15 minutes at 37°C. After 15 minutes, absorbance is read at 550nm. Antioxidant activity is calculated against the Trolox standard curve and calibrated using linear approximation with corresponding activity for each sample, expressed in μg/mL Trolox equivalents using SoftMax software.

RESULTS AND DISCUSSION

The experimental portion of this research took place in three phases (Figure2). The first phase was a set of initial experiments designed to establish background knowledge of the ranges of antioxidant values for a variety of foods and drinks, and compare that to the values for different styles of beer made using different processes. Next, being interested in the antioxidant contribution specifically from yeast, live and dead yeast was added to commercial beer at different levels. The initial set of experiments was concluded with the first set of beer trials. In which, wort was prepared, inoculated with brewers yeast, and allowed to complete primary fermentation. Treatments with the green beer consisted of bottle-conditioning with either a high (10g/L) or low (5g/L) level of priming sugar and one of three yeast strains. The control consisted of artificially carbonating the green beer. The two levels of sugar resulted in high (~1.1 x 10^6 cells/mL) and low (~5.66 x 10^5 cells/mL) levels of live yeast cells upon completion of bottle-conditioning. Throughout the 3-month experiment, increases in antioxidant activity were detected in the bottle-conditioned beers, regardless of high or low level of yeast, compared to the artificially carbonated control.

The second phase of experiments consisted of a second set of beer trials. During this experiment, wort was prepared, inoculated with brewers yeast, and allowed to complete primary fermentation, as in the first set of beer trials.

Treatments consisted of bottle-conditioning the beer with the addition of yeast and priming sugar, or artificially carbonating the beer followed by the addition of yeast, but no sugar. The control consisted of artificially carbonating the beer, with no yeast present. Dissolved oxygen, sulfur dioxide, yeast viability, and antioxidant potential were all analyzed throughout the 6-month trial. The results showed the highest levels of SO_2 and antioxidant activity among the bottle-conditioned beers, where the yeast underwent a secondary metabolism in the bottle. Furthermore, the presence of yeast alone, without having undergone secondary fermentation through bottle-conditioning, still provided elevated levels of SO_2 and antioxidant activity compared to the control.

The third and final phase of experiments was centered on specific contributions from the yeast that could provide antioxidant activity. Isolated, purified yeast cells were cultivated, washed, and disrupted, followed by centrifugation and removal of the aqueous layer as a crude yeast extract. The yeast extracts were then heat-treated, fractionated and heat treated or treated with protease (10mg/mL), or lipid extracted, and subjected to antioxidant analysis. Glutathione concentration and activity was also determined. Results did not point to any one smoking gun antioxidant compound, but rather a complex interaction between at least several antioxidant components.

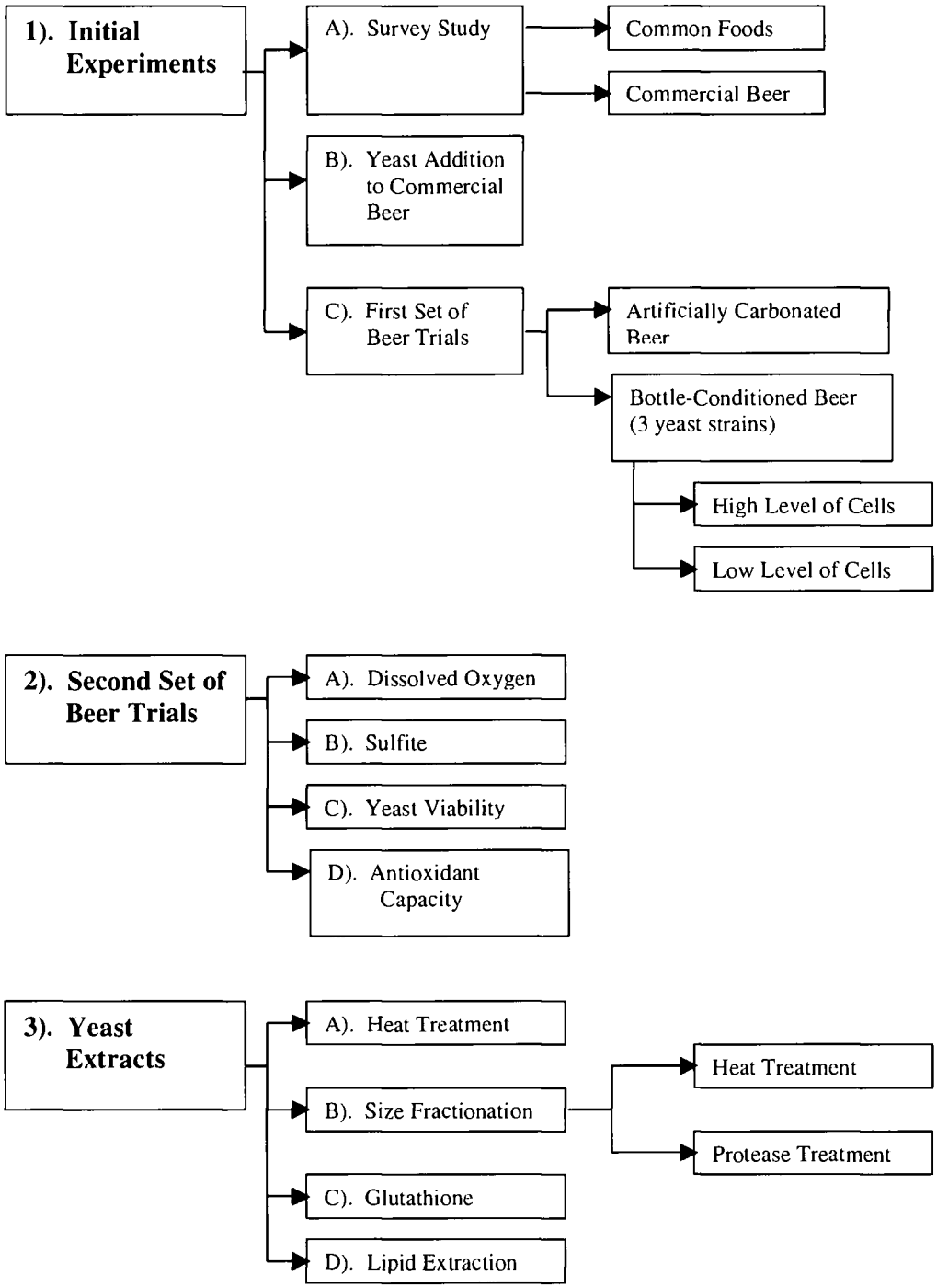


Figure 2. Overview of All Experiments

INITIAL EXPERIMENTS

Survey Studies

Before comparing bottle-conditioned and live beer to force-carbonated beer, it was important to establish a benchmark of the antioxidant activity of various commercial beers, and how they compare to other common foods (figure 3). The beer samples shown reflect the average antioxidant activity of two commercially available force-carbonated beers analyzed in duplicate, and two commercially available bottle-conditioned beers also analyzed in duplicate. Other drinks and foods represent the average antioxidant activity of four samples. It was interesting to observe values that beer has, depending upon style and process. It should also be noted that the values of beer lie in what is considered the high antioxidant food range, along with apple juice and tomatoes, and above Thompson seedless grapes.

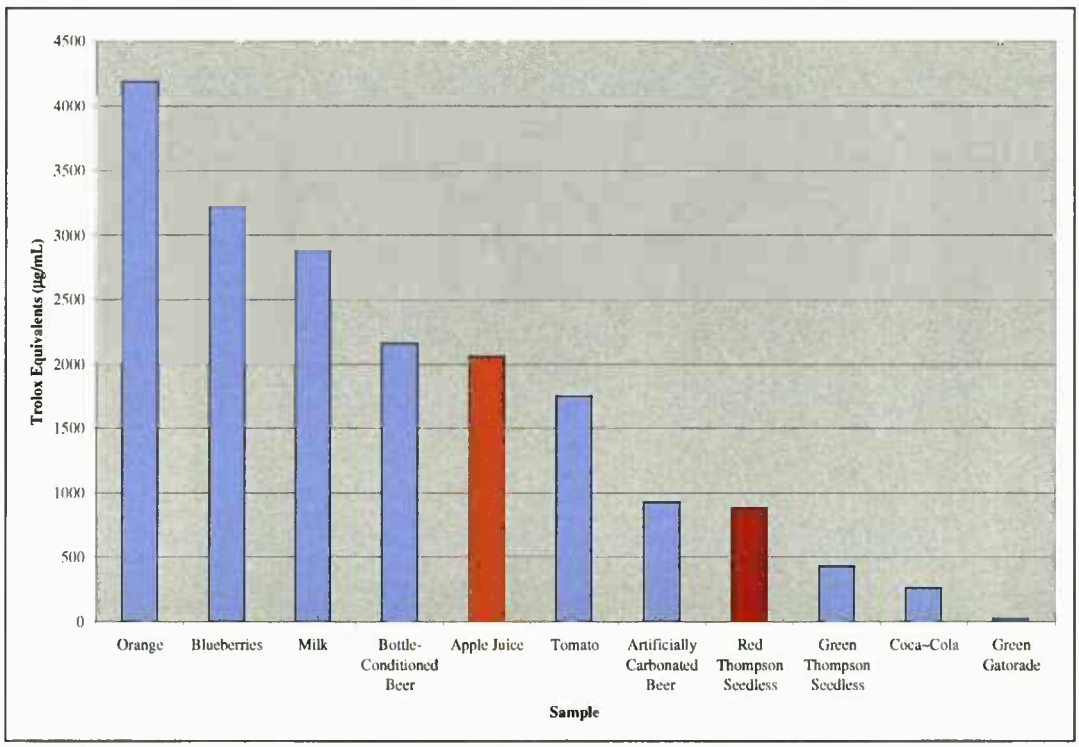


Figure 3. Antioxidant Activity of Some Common Foods and Drinks

It is well known, in commercial breweries and among home brewers, that bottle conditioned beer has a longer shelf life in terms of freshness and flavor than its artificially carbonated or force-carbonated counterpart. To analyze this phenomenon in terms of antioxidant potential, group of commercially sold beers, consisting of force-carbonated, bottle-conditioned, unfiltered, and fruit flavored beers was selected and tested for antioxidant capacity. The results indicated that the bottle-conditioned, unfiltered, and live beers clearly had the highest antioxidant capacity (figure 4). The live fruit beer was next, followed by the force-carbonated fruit flavored beers, and lastly the force-carbonated beers (figure 4).

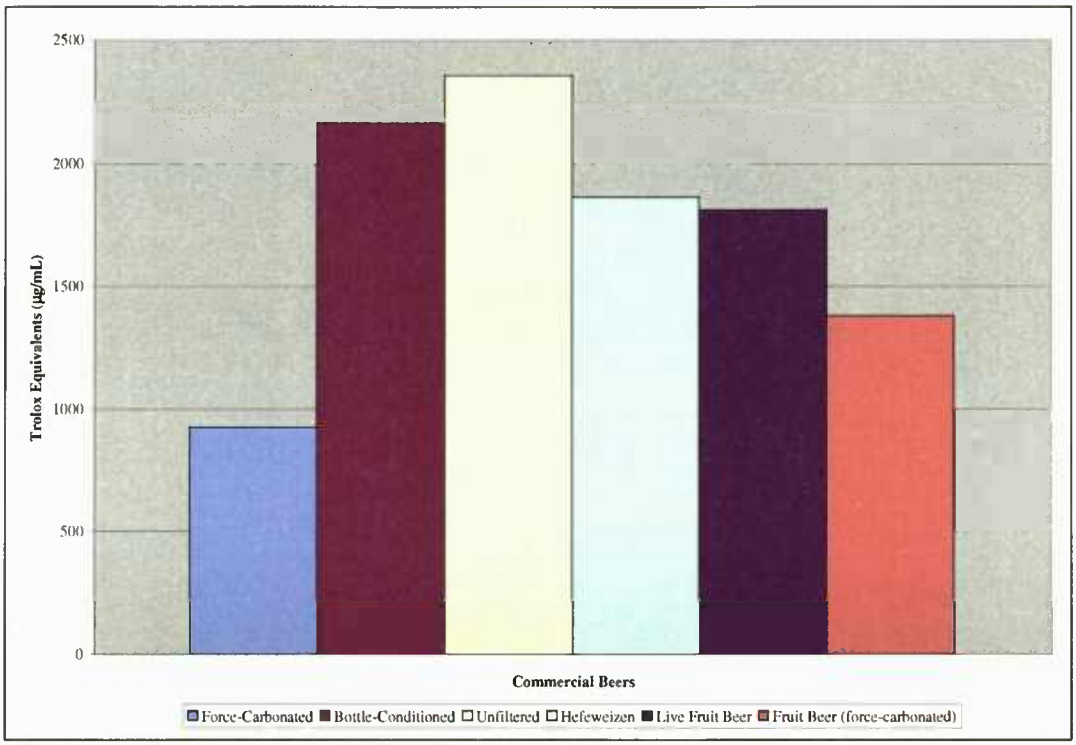


Figure 4. Antioxidant Activity of Various Types of Commercial Beers

Yeast Addition to Commercial Beer

Obviously, the beers that had undergone secondary fermentation in the bottle, or beers with live yeast present had higher antioxidant potential. Clearly, style of beer has a lot to do with antioxidant activity, considering the majority of antioxidant activity is contributed by the malt and hops components. However, our interest was in the contribution of antioxidant activity specifically from the yeast. With this in mind, different levels of yeast, both live and dead, were added to a force-carbonated commercial beer and analyzed for antioxidant activity (figure 5).

This figure illustrates that while there was yeast present, there was little or no change in antioxidant activity, whether or not the yeast was live or dead. The yeast play a significant role in the raised levels of antioxidants in the bottle-conditioned beer, as further discussed in detail later, although it seemed apparent that based on this experiment, the presence of yeast did not impact the antioxidant capacity. It is important to distinguish that this data represents yeast added the day of the FRAP analysis, whereas in the second set of beer trials, the yeast was added at the time of bottling.

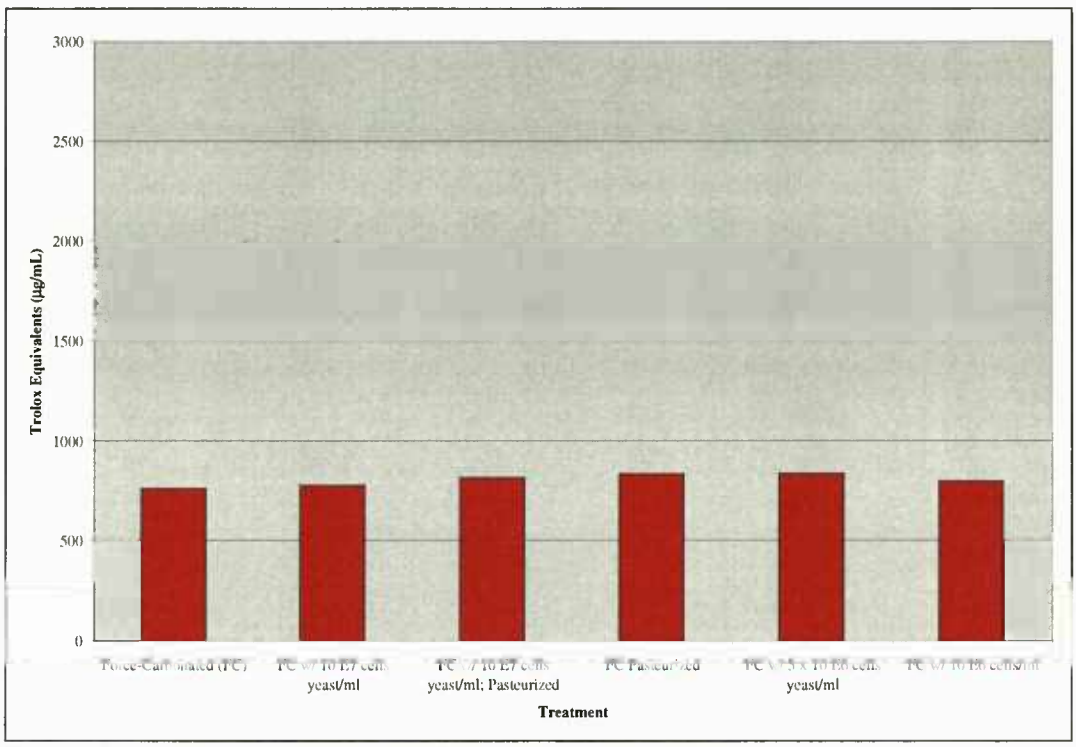


Figure 5. Artificially Carbonated Beer with Yeast Added

First Set of Beer Trials

It was concluded that the yeast must undergo metabolism through completion of a secondary fermentation in the bottle, essentially the process of bottle-conditioning, in order to contribute the additional antioxidant capacity. To further investigate this theory, an experiment was designed to observe whether or not a significant difference in antioxidant capacity would exist between beers from the same original batch that had undergone bottle-conditioning or artificial carbonation (Figure 6). Further, three different strains were used at two different levels of total yeast cells present. This was accomplished by adding 10g/L priming sugar for the High level of yeast, and 5g/L priming sugar for the Low level of yeast. The number of total cells present at the end of secondary fermentation (bottle-conditioning) is proportionate to the amount of priming sugar added at the time of bottle-conditioning. The two levels of sugar resulted in high (~1.1×10^6 cells/mL) and low (~5.66×10^5 cells/mL) levels of live yeast cells upon completion of bottle-conditioning. The results indicate that the antioxidant capacity was increased in all the beers with live yeast present, regardless of the final number of yeast cells in the beer, when compared to the artificially carbonated beer. This helped to confirm that it was indeed some action taken by the yeast, or the production of some metabolite during fermentation, that lead to the increase in total antioxidant activity. It is interesting to note that the antioxidant capacities of the beers with high levels of yeast were not necessarily more elevated than those with

the low levels of yeast. It should also be noted that while the difference between the artificially carbonated beer and the beers with live yeast does not look significant, when compared to the Trolox level of a standard curve of sulfur dioxide, the differences range between 22 and 35 mg/L SO_2; a significant level of antioxidant potential.

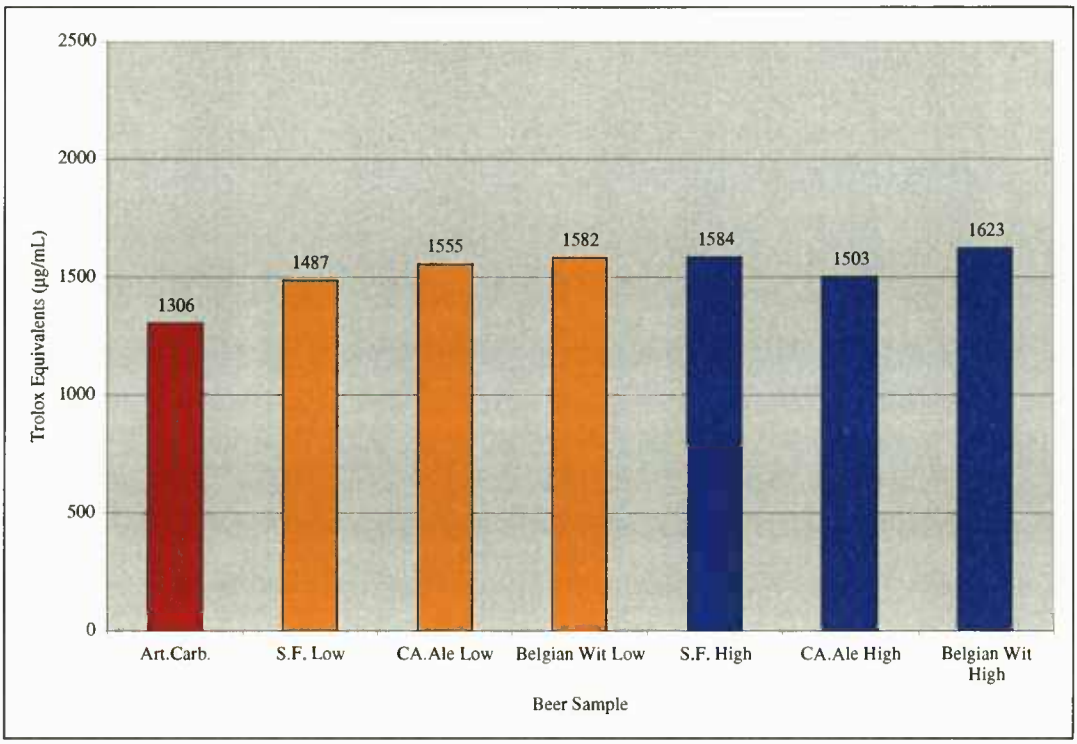


Figure 6. Antioxidant Capacity of Artificially Carbonated and Bottle-Conditioned Beers at High (~1.1 x 10^6 cells/mL) and Low (~5.66 x 10^5 cells/mL) Levels of Live Yeast Cells

SECOND SET OF BEER TRIALS

Dissolved Oxygen

Determining the yeast s inherent ability to scavenge and metabolize any free oxygen species was one objective of this research. Upon bottling, any available oxygen would be assimilated and metabolized by the yeast for cell growth. Subsequently resulting in fewer oxygen species available to undergo possible transformation to reactive oxygen species. Evidence of this is pointed to by the lower levels of dissolved oxygen in the bottle-conditioned beers compared to the artificially carbonated beers in the first two months (figure 7). Interestingly, this trend was not evident in the observed levels of dissolved oxygen in the third month, during which the control beer-with no yeast present-had the lowest level of dissolved oxygen. This phenomenon might be explained by the breakdown of molecular oxygen to reactive species in the artificially carbonated beer, while in the beers with live yeast present, the molecular oxygen was protected. In fact, when compared to viable yeast (Table 3), it is evident that the molecular oxygen was both protected and steadily absorbed while the yeast remained viable.

It was interesting to note that the difference in the levels of dissolved oxygen between the treatments is not as great as anticipated. As discussed later, there is an increase in the antioxidant potential in the trials with live yeast present. The increase in antioxidant potential can t be explained by the decrease in

molecular oxygen alone. This is suggestive of the yeast playing a more active role than simply scavenging free oxygen.

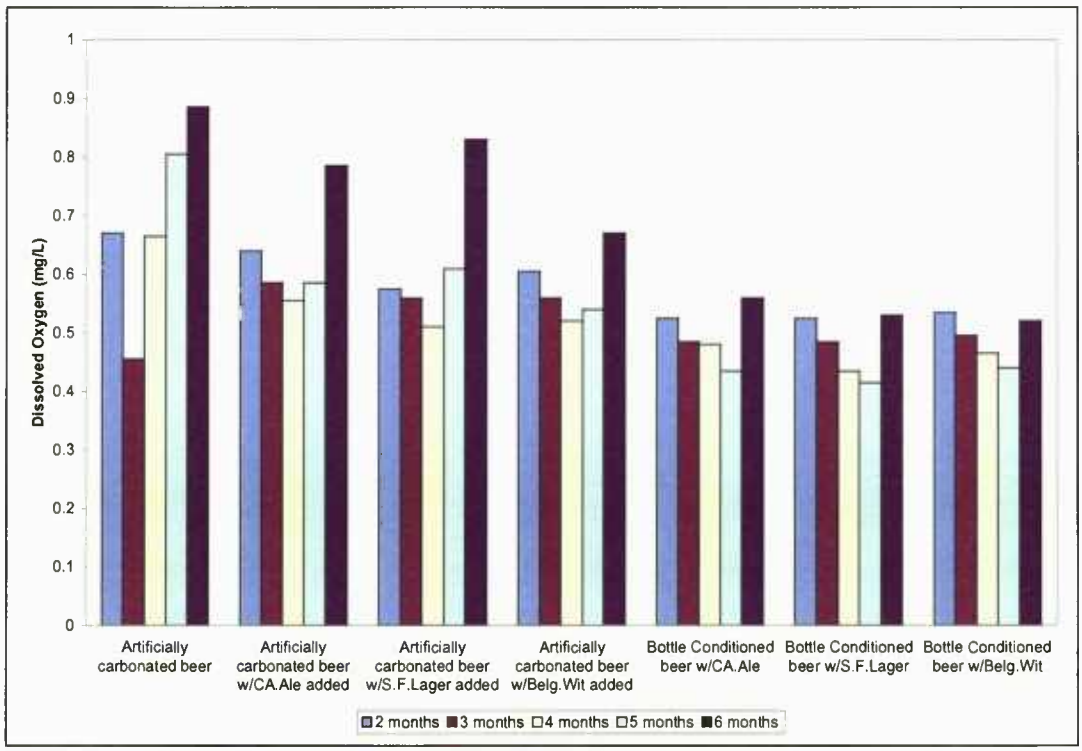


Figure 7. Dissolved Oxygen for second set of beer trials at 2, 3, 4, 5, and 6 months

Yeast Viability

The yeast remained live and viable for almost the duration of the 6-month experiment (Table 3). It was not surprising that the yeast was viable in both the force-carbonated beers with live yeast added, as well as the bottle-conditioned beers. It is interesting to note, that when compared to the dissolved oxygen (figure 7), a downward trend in dissolved oxygen is observed as long as viable yeast

remain. However, as the yeast begins to die off, the dissolved oxygen levels start to increase. While it has been shown there is a constant migration of oxygen into the bottle, the dissolved oxygen levels descend as long as yeast is viable. Only after the yeast begin to die off, does the dissolved oxygen level start to increase, even though oxygen has been crossing the barrier at the same rate since the time of bottling. It appears that, in addition to slowly being absorbed and utilized by the yeast, the molecular oxygen present was also being protected from breakdown to radical oxygen species.

	Yeast Viability											
	1 Month		2 Months		3 Months		4 Months		5 Months		6 Months	
FC + CA.Ale	++	++	++	++	++	++	++	++	-	-	-	-
FC + SF.Lager	++	++	++	++	+	+	++	++	-	-	-	-
FC + Belg.Wit	++	++	++	++	+	+	++	++	+	+	-	-
BC w/ CA.Ale	++	++	++	+	++	++	++	++	++	+	+	-
BC w/SF.Lager	++	++	++	++	+	+	++	++	++	+	-	-
BC w/Belg.Wit	++	++	++	+	+	+	++	+	++	++	+	-

Table 3. Yeast Viability. Abundant Growth (++), Visible Growth (+), No Visible Growth (-).

Sulfur Dioxide

Since sulfur dioxide, or sulfite, is a natural metabolite of yeast fermentation, and bottle-conditioned beer undergoes a secondary fermentation, it is reasonable that bottle-conditioned beer has higher levels of sulfite than artificially carbonated

beer. In fact, free and total sulfite levels in the bottle-conditioned beers were higher than the artificially carbonated beer, and the artificially carbonated beers with yeast added throughout the duration of the experiment (figures 8.1-8.6). However, even though the artificially carbonated beers with live yeast added didn t undergo secondary fermentation, elevated levels of sulfite compared to the control were still observed. Another observation is that the free sulfite is bound up rather quickly in the artificially carbonated beer with no yeast present. While in the beers with live yeast, whether bottle-conditioned or artificially carbonated, it appears the sulfite is remaining free for a longer period of time, regardless of the initial level. This suggests that in the beers with live yeast present, the sulfite is being protected by some other source of antioxidant. As that other antioxidant starts to become depleted, the free sulfite present starts to bind with and reduce the oxidants involved. This loss of antioxidant capacity and subsequent depletion of free sulfite happens early on in the artificially carbonated beer with no yeast present. Then, in the later months of the experiment, the process begins in the artificially carbonated beer with live yeast added, followed lastly by the bottle-conditioned beer. By the sixth month there were no detectable levels of free or total sulfite in any of the beer samples.

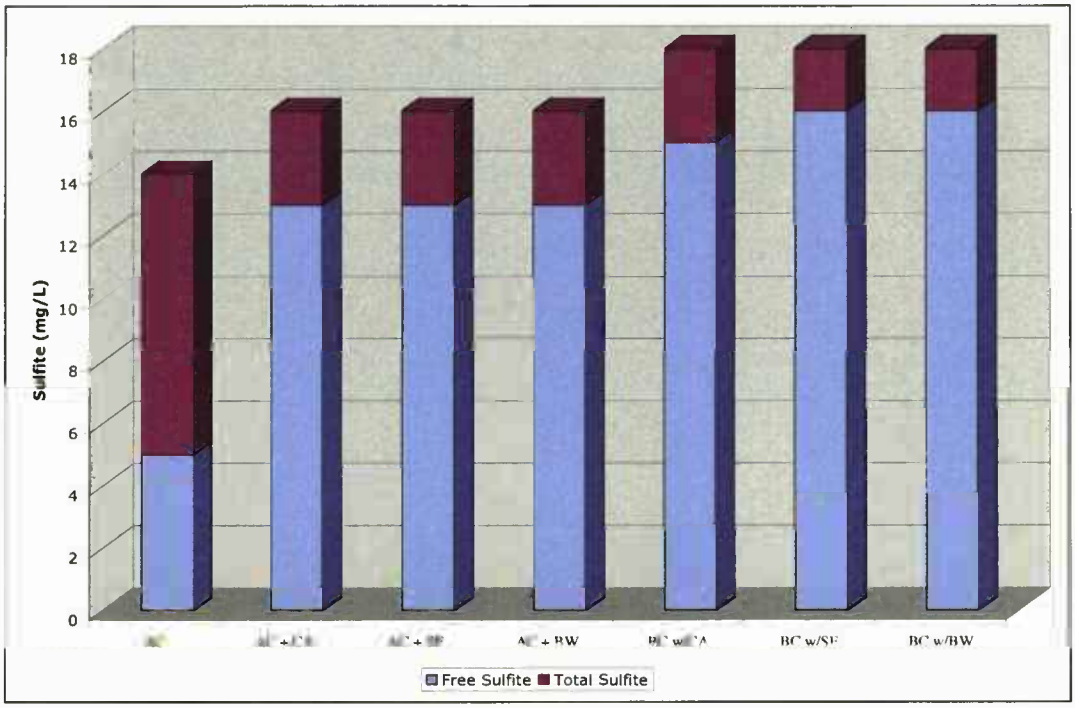


Figure 8.1. Free and Total Sulfite at 1 Month
AC=Artificially Carbonated Beer (Control)
AC+CA=Artificially Carbonated Beer + California Ale Yeast
AC+SF= Artificially Carbonated Beer + San Francisco Lager Yeast
AC+BW= Artificially Carbonated Beer + Belgian Wit Yeast
BC w/CA= Bottle-conditioned Beer with California Ale Yeast
BC w/SF= Bottle-conditioned Beer with San Francisco Lager Yeast
BC w/BW= Bottle-conditioned Beer with Belgian Wit Yeast

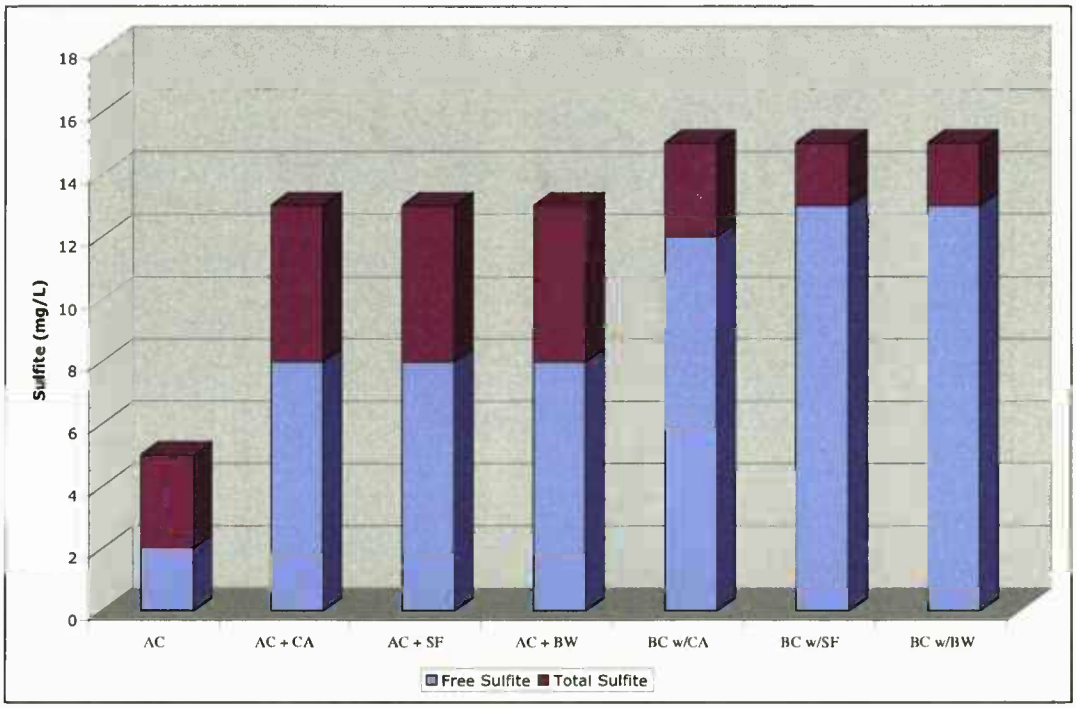


Figure 8.2. Free and Total Sulfite at 2 Months

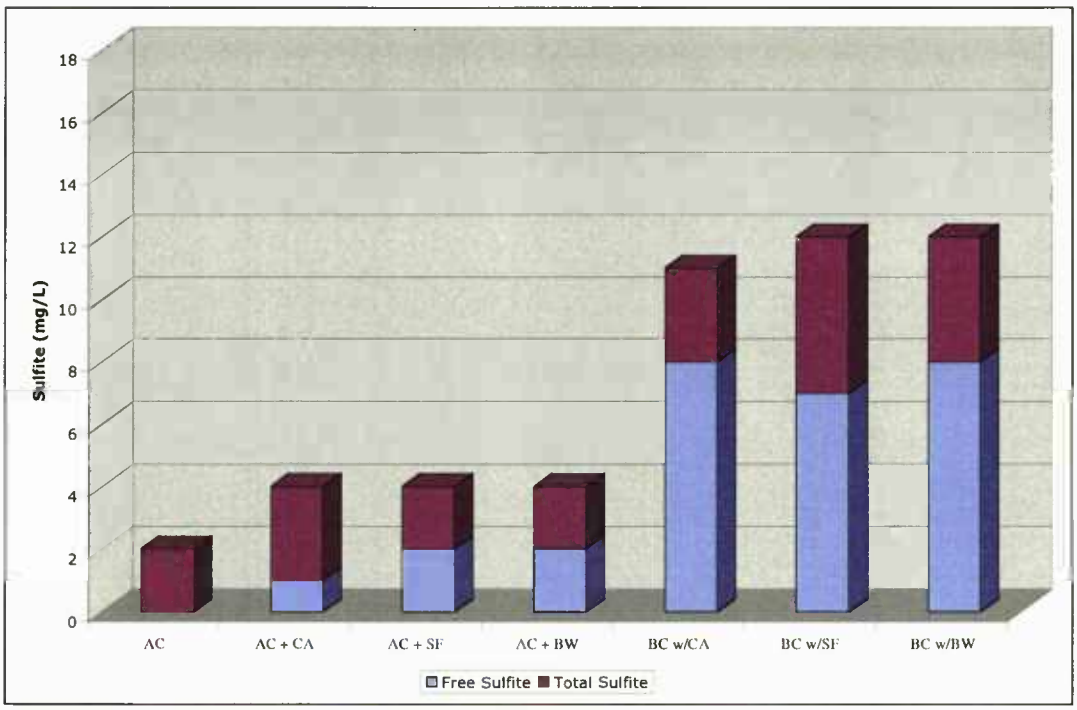


Figure 8.3. Free and Total Sulfite at 3 Months

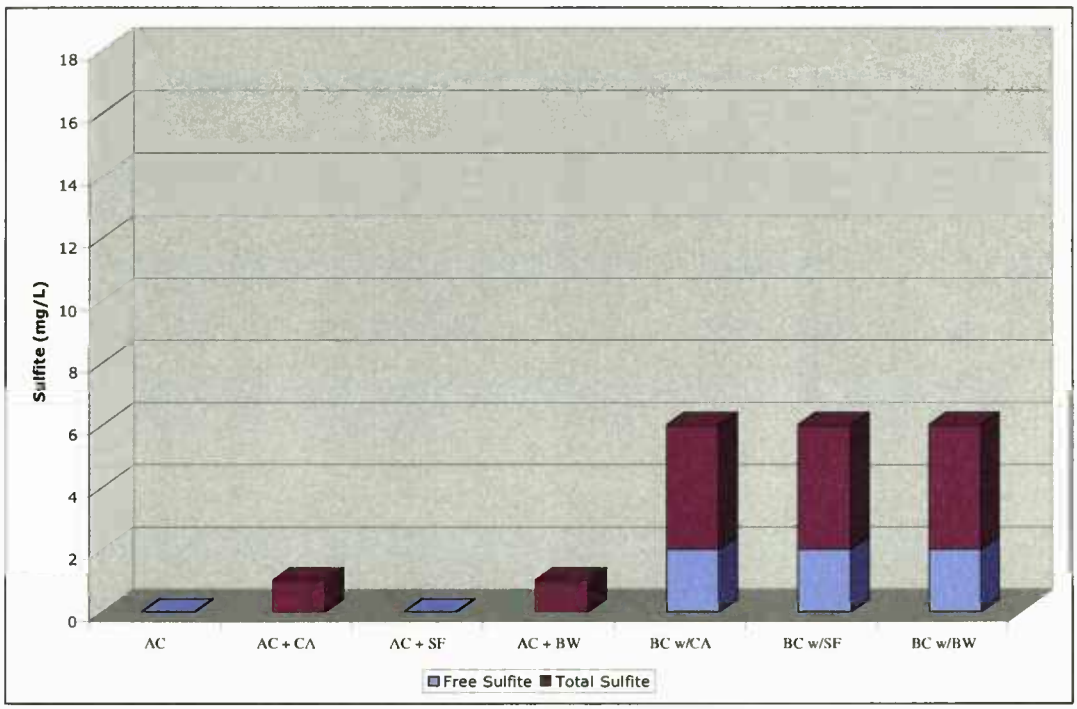


Figure 8.4. Free and Total Sulfite at 4 Months

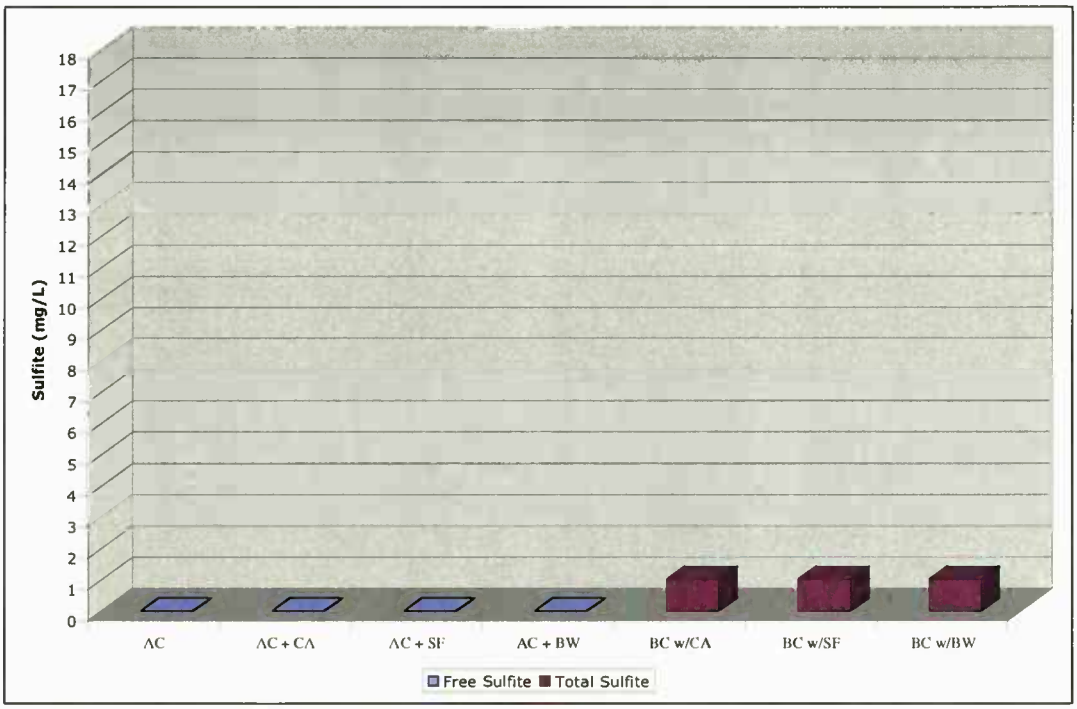


Figure 8.5. Free and Total Sulfite at 5 Months

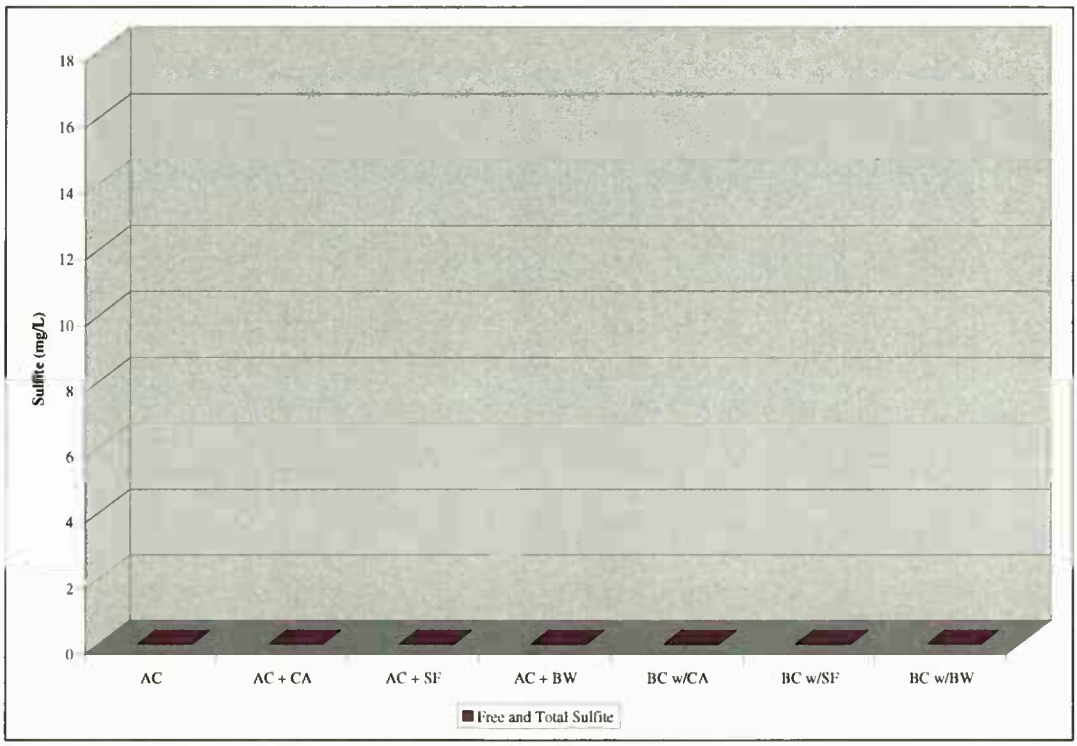


Figure 8.6. Free and Total Sulfite at 6 Months

ANTIOXIDANT POTENTIAL

In contrast to the artificially carbonated beer, both the bottle-conditioned beers and the artificially carbonated beers with live yeast added had a significantly elevated antioxidant capacity (Figure 9). Both treatments consistently showed the highest levels of antioxidant capacity compared to the artificially carbonated control beer with no yeast present. As expected, when compared to the artificially carbonated beers with live yeast added, the bottle-conditioned beers had even

higher levels of antioxidant capacity beginning in the third and fourth months, and becoming even more evident in the fifth and sixth months.

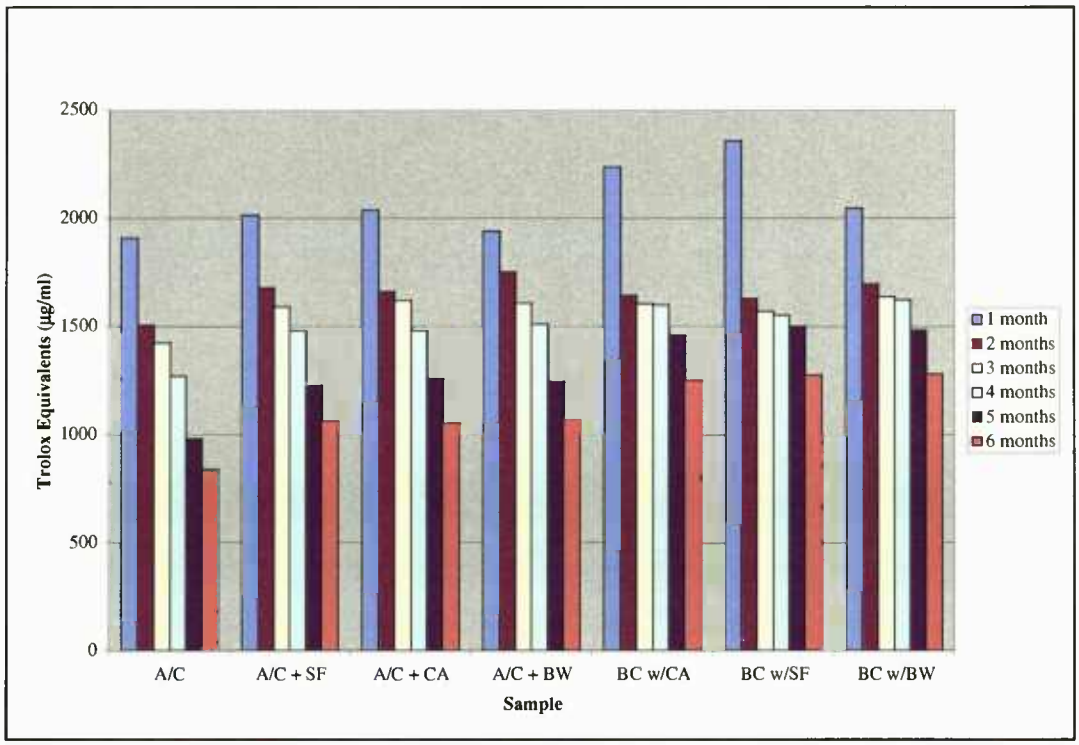


Figure 9. Antioxidant Capacity of Artificially Carbonated and Bottle-Conditioned Beers

The bottle-conditioned beers had the greatest overall antioxidant activity as expected. The trend for all three strains is uniform and consistent. Figures 10.1, 10.2, and 10.3, depict the antioxidant capacities for the specific strain, comparing bottle-conditioned beer to artificially carbonated beer with live yeast added, and artificially carbonated beer for California Ale yeast, San Francisco Lager yeast, and Belgian Wit yeast, respectively. Each figure shows similar levels in antioxidant capacity in the beginning of the experiment. However, around the second month,

the bottle-conditioned beers level off, while the antioxidant capacities of the two artificially carbonated treatments for each strain continue to slowly decrease. The protection observed, or the slowed decrease, in antioxidant activity is observed in the bottle-conditioned beers past the fourth month, before the drop in antioxidant activity resumes in the bottle-conditioned treatments. Another important aspect of the figures is the consistently higher antioxidant levels in the artificially carbonated beer treatment with live yeast added compared to the control - or artificially carbonated — beer. More simply, just having live and viable yeast increases antioxidant activity of the beer. This is in part due to the oxygen scavenging abilities, but also involves some production of sulfite, and possibly the action of some yeast oxygen defense enzymes.

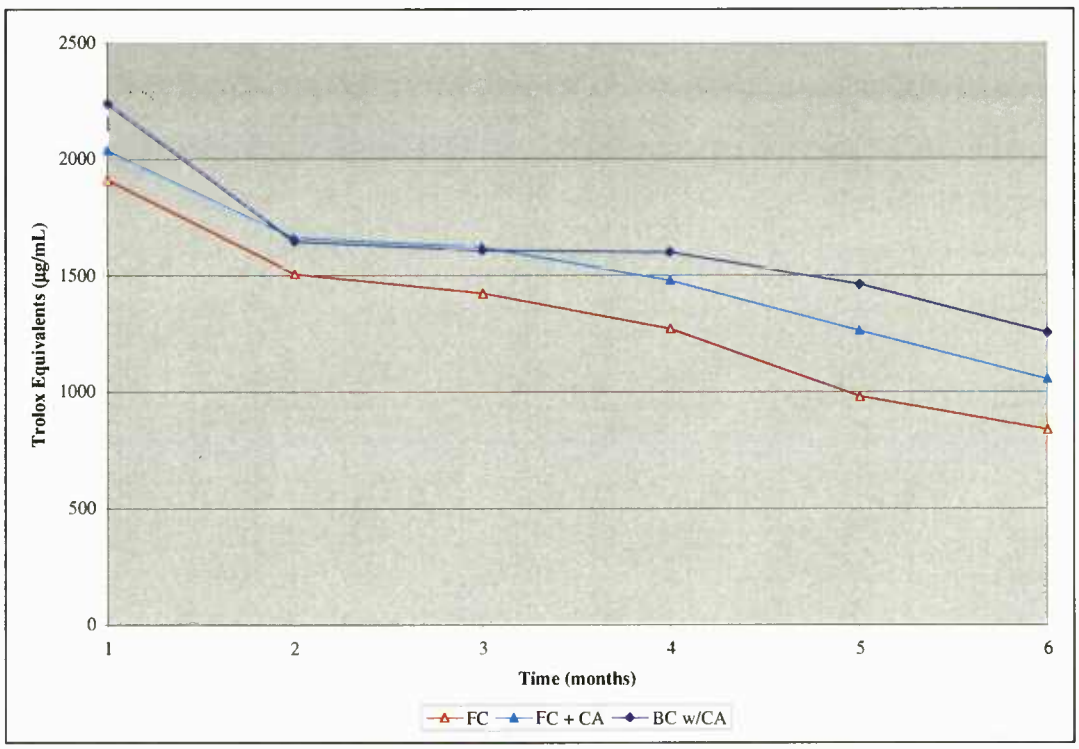


Figure 10.1. Antioxidant Capacity of California Ale Yeast Treatments

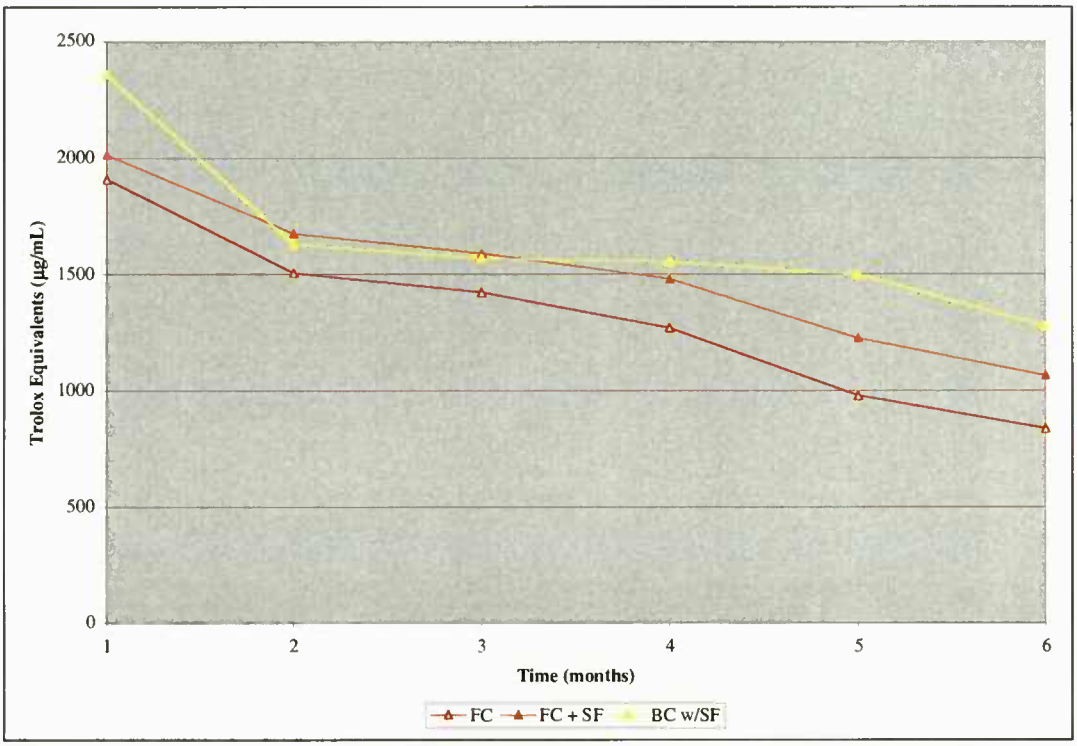


Figure 10.2. Antioxidant Capacity of San Francisco Lager Yeast Treatments

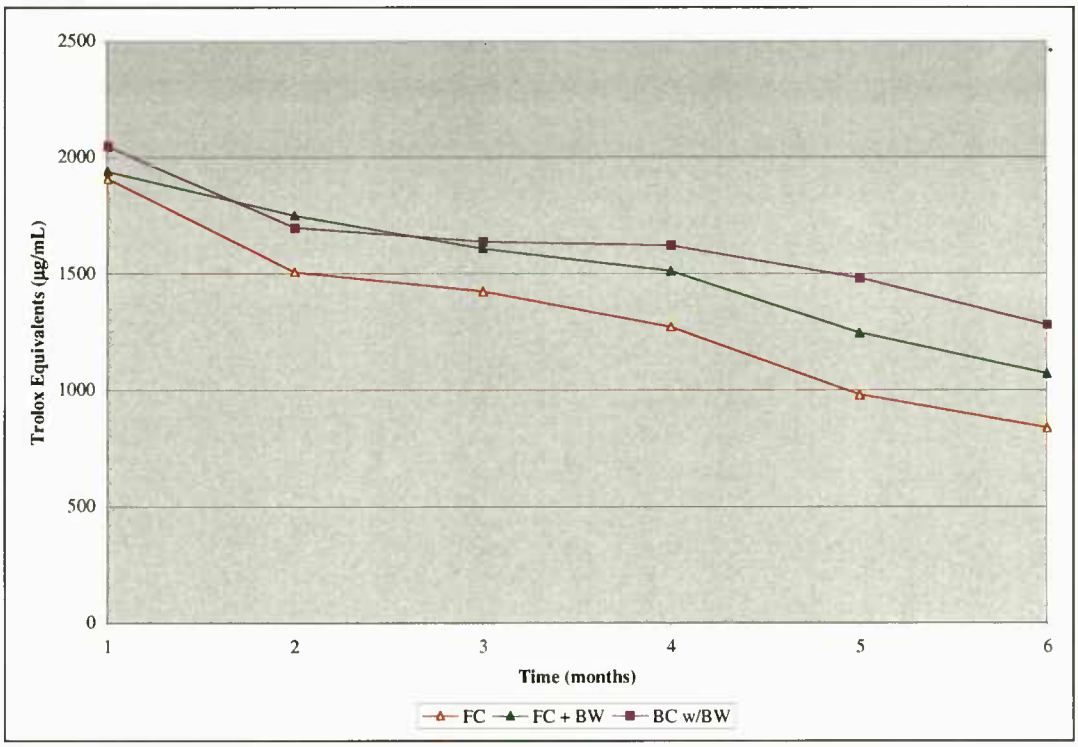


Figure 10.3. Antioxidant Capacity of Belgian Wit Yeast Treatments

YEAST EXTRACTS

The main objective of this research was to determine whether the yeast itself, through bottle-conditioning (or secondary fermentation), has an effect on the antioxidant activity of the beer. Assuming so, aside from producing SO_2 and scavenging and protecting oxygen, what else was the yeast contributing? With this in mind, and after considering research on yeast metabolism and fermentation behavior, antioxidant analysis was conducted on yeast extracts made from the 3 strains of yeast used for bottle-conditioning and adding to the artificially carbonated beer in the beer trials. Speculating that there could indeed be extracellular yeast enzymes involved in antioxidant activity, the extracts were subjected to heat treatment, protease treatment, and lipid extraction. Furthermore, the heat-treated and protease treated samples were further subjected to fractionation above 10,000 MW, between 5,000 MW and 10,000 MW, and below 5,000 MW.

Heat Treatment

The results in the heat-treated experiment show differences in overall antioxidant capacity for each treatment between the different strains (Figure 11). This may be attributed to human error during the heat treatment itself, or during extraction of the yeast sample. However, while there are unexplainable

discrepancies between the different strains of yeast when comparing overall antioxidant activity, the important thing to note in this graph is the trend observed for all the strains when subjected to heat treatment. Antioxidant activity in general slowly goes down for all strains. Though this data shows averages, the same trend was observed for three separate batches of extracts and duplicate analysis for each sample on three separate occasions.

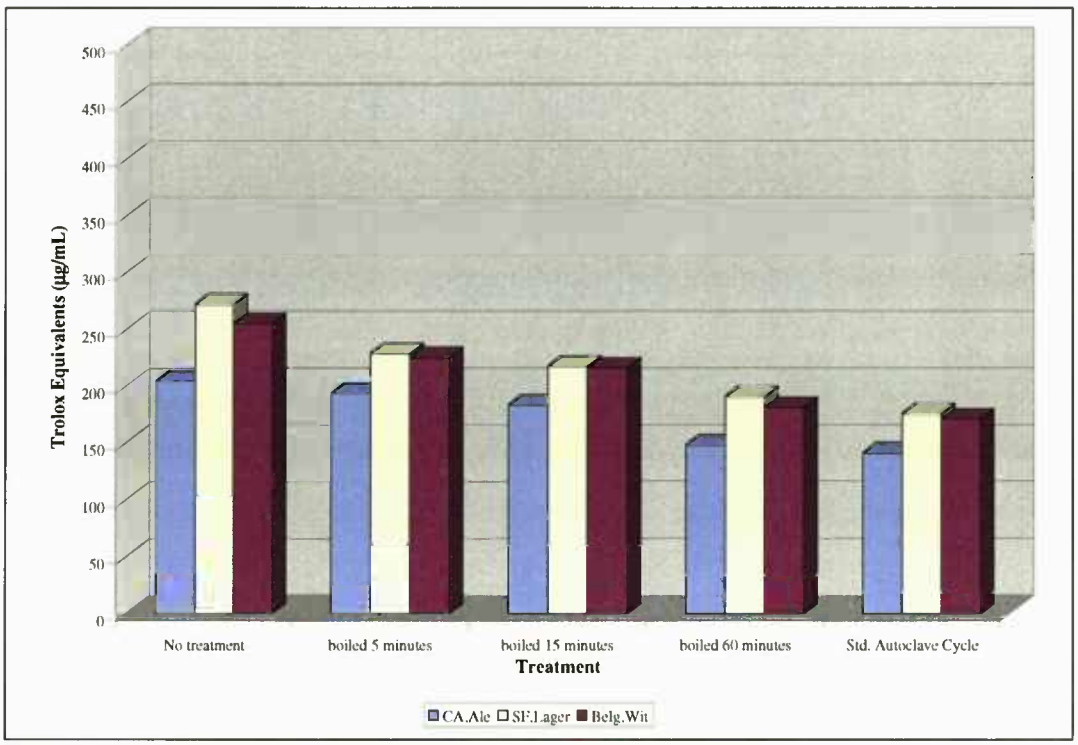


Figure 11. Antioxidant Capacity of Crude Yeast Extracts-Heat Treated

Size Fractionation

Based on these results, the theory that an enzyme could be contributing still seemed pliable. To further investigate this theory, yeast extracts were again prepared and heat-treated, as well as treated with protease enzyme at 10mg/mL and then subjected to fractionation (Figures 12.1, 12.2, & 12.3). The data in figure 12.1 represents the control, and shows the general breakdown of antioxidant activity in the crude yeast extracts from the three different yeast strains used for the beer trials. Without a doubt, the majority of the antioxidant activity present lies in the fraction above 10,000MW. Of the fractions below 10,000MW, the fraction below 5,000MW has the highest antioxidant capacity.

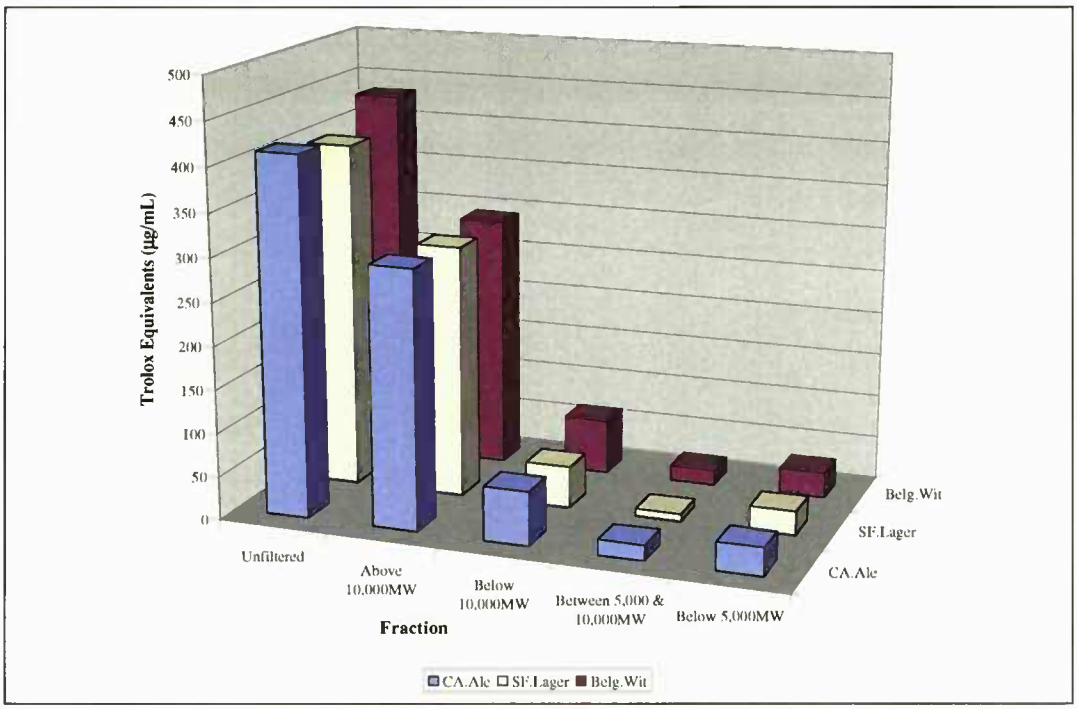


Figure 12.1. Antioxidant Capacity from Different Molecular Weight Fractions of Crude Yeast Extracts-Control

While the general trend is the same for all three strains, with the majority of antioxidant activity above 10,000MW, followed by below 5,000MW, when heat treated, both fractions below 10,000MW saw elevated levels in antioxidant activity when compared to the control (Figure 12.2). This might be explained by the liberation of small molecular weight compounds such as bound SO_2.

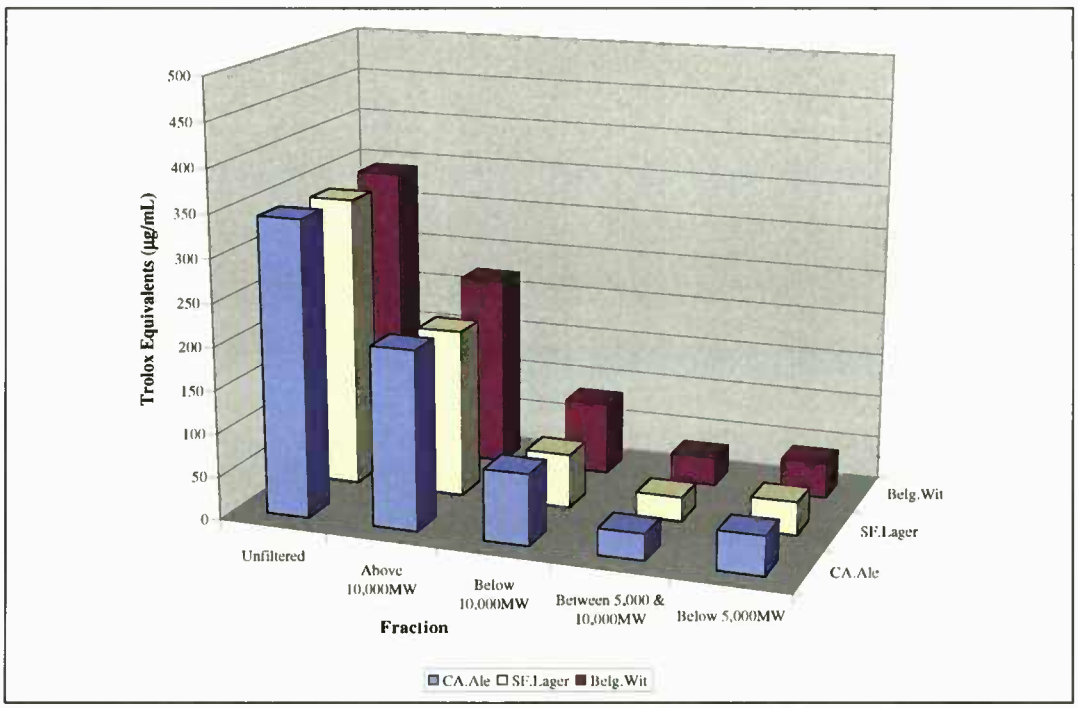


Figure 12.2. Antioxidant Capacity from Different Molecular Weight Fractions of Crude Yeast Extracts -Boiled 60 Minutes

When comparing the results of Figure 12.3 to the control (Figure 12.1) one observes the total loss of antioxidant activity in the fraction between 5,000MW and 10,000MW. This too lends to the possibility of a low molecular weight enzyme,

designed for the yeast cell to combat oxidative damage, which could be contributing to the antioxidant activity.

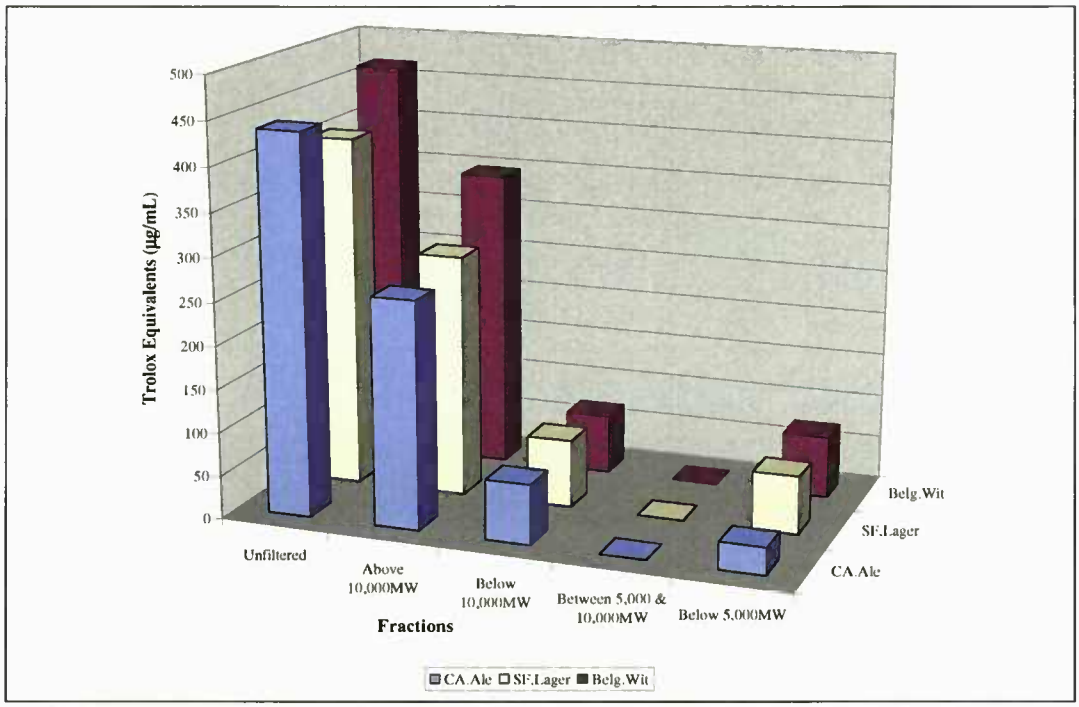


Figure 12.3. Antioxidant Capacity from Different Molecular Weight Fractions of Crude Yeast Extracts -Treated with 10mg/mL Protease

Glutathione Concentration

Throughout the research phase of this thesis, glutathione continued to stand out as a possible contender for our low molecular weight enzyme. Glutathione is a small molecular weight enzyme responsible for reduction of disulfides and scavenging of free radicals. It is 600kDa when reduced, and 1200kDa when it has

become oxidized through the reduction of a radical or other electron donor. When combined with another enzyme, glutathione reductase and NADPH as a co-factor, glutathione in the oxidized form can be reduced again for continual scavenging of radical species until the NADPH is used up. The next experiment was designed to amplify any glutathione present in the crude yeast extracts by supplying NADPH and glutathione reductase (Figure 13). The results were quite surprising. While a noticeable increase is observed in the antioxidant activity of the extract and the NADPH, it is not so elevated in the treatment with glutathione reductase. Further, it is a little mysterious to note the low antioxidant level of the extract alone, and the very uniform and unusually high level of antioxidant activity for the glutathione reductase and buffer. This data does suggest that with NADPH supplied, one of the many other possible enzymes to use it as a cofactor could still be working as an antioxidant.

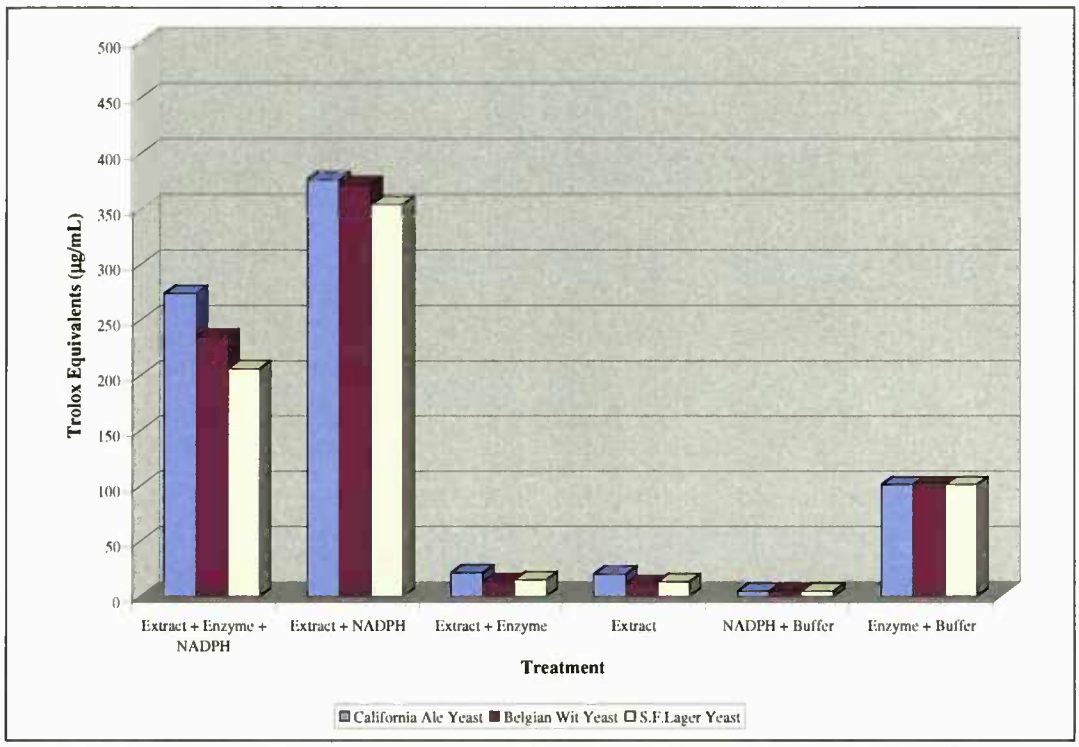


Figure 13. Antioxidant Capacity of Crude Yeast Extracts with Glutathione Reductase (Enzyme) and NADPH

Lipid Extraction

A lipid protein from the yeast cell membrane involved in the uptake of molecular oxygen, or some other lipid protein, might be a source of yeast antioxidant activity. Lipid extraction was performed on the crude yeast extracts and then assayed for antioxidant activity (Figure 14). While slight, there is an observable increase in the antioxidant activity provided by the lipid component of the crude extracts. The important aspect of this figure once again, is the trend observed for all three strains throughout the treatments.

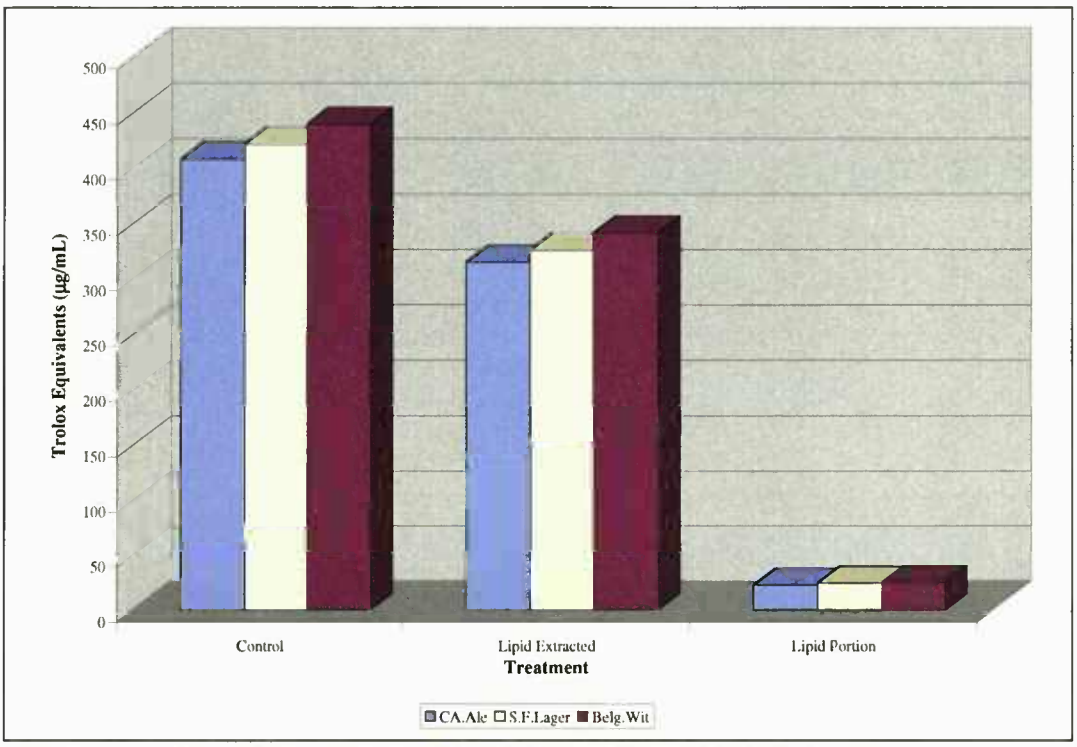


Figure 14. Antioxidant capacity of lipid extracted crude yeast extracts

CONCLUSION

Because bottle-conditioned beers and beers containing live yeast have greater antioxidant activity and are more protected from oxidative reactions and the generation of free radicals, it can be concluded that these beers will contribute higher amounts of antioxidant compounds for absorption when consumed. From this, it can also be inferred that because of the increased antioxidants available and the reducing power offered by live yeast, perhaps it would be more beneficial to drink beer containing live yeast. In fact, this and other research has shown that beer in general is a better source of dietary antioxidants than most vegetables and fruit due to the increased solubility and absorption facilitated by the ethanol in beer. Furthermore, it also may be concluded that the extracellular activity of some of the yeast s enzymatic defenses against reactive oxygen species and free radicals add protection against oxidation in beers with live yeast present. This would help describe the increased antioxidant activity of all beers containing live yeast.

Bottle conditioned beer and live beer have greater antioxidant capacity than artificially carbonated beer; equivalent to 20-35ppm SO_2 in some cases. Simply the presence of live yeast in the finished product has a greater antioxidant capacity than the artificially carbonated beer. When the live yeast is allowed to undergo a secondary fermentation in the bottle, the antioxidant capacity is greater still.

This might be explained by any number of actions. First of which is the added protection provided by the increase of secondary yeast metabolites, primarily -- but not limited to-- sulfite. Also, the elevated level of antioxidant capacity in the beers containing live yeast, either added or used in bottle conditioning, can partially be explained by the yeast s inherent ability to scavenge and metabolize any free oxygen species, and protect the oxygen species present while the yeast remains viable. Further, there is likely some extracellular yeast oxidative defense enzymes providing some level of antioxidant activity. The antioxidant activity not explained by the SO_2, is likely a complex relationship between the yeasts inherent ability to scavenge and metabolize or protect any free oxygen species present, produce antioxidative secondary metabolites, chelate transition metals and most likely also involves some other enzymatic and non-enzymatic yeast defenses against molecular oxygen.

This research has shown that bottle-conditioned beer and beer with live yeast present is higher in antioxidant activity than artificially carbonated beer. Further, the data suggests that there are several different specific yeast components and reactions that combined are likely to provide a cumulative antioxidant effect.

BIBLIOGRAPHY

1. Andersen, Mogens L., Henrik Outtrup, & Leif H. Skibsted (2000). Potential Antioxidants in Beer Assessed by ESR Spin Trapping. *J.Agric.Food Chem.* **48**: 3106-3111.

2. Araki, S., Kimura, T., Shimizu, C., Furusho, S., Takashio, M., & Shinotsuka, K. (1999). Estimation of Antioxidative Activity and its Relationship to Beer Flavor Stability. *J.Am.Soc.Brew.Chem.* **57(1)**: 34-37.

3. Beecher G.R., & F. Khachik (1992). Qualatative Relationship of dietary and plasma carotenoids in human beings. *Ann. N.Y.Acad. Sci.* **669**: 320-321.

4. Bourne, L. (2000). Bio-availability of ferulic acid from beer. *Free Radical Res.* **32**: 273-280.

5. Bright, David, Graham G. Stewart, & Hugo Patino (1999). A Novel Assay for Antioxidant Potential of Specialty Malts. *J.Am.Soc.Brew.Chem.* **57(4)**: 133-137.

6. Davies, Kelvin J.A. (1994). Oxidative Stress: the paradox of aerobic life. *Biochem. Soc. Symp.* **61**: 1-31.

7. DeKeersmaecker, J. (1996). The Mystery of Lambic Beer. *Scientific American.* **August**: 74-80.

8. Duthie, Garry G. (1999). Determination of activity of antioxidants in human subjects. *Proc. Nutr. Soc.* **58**: 1015-1024.

9. Dutta-Roy, A.K., M.J. Gordon, F.M. Campbell, G.G. Duthie, & W.P.T. James (1994). Vitamin E requirements, transport, and metabolism: role of α-tocopherol-binding protein. *J. Nutr. Biochem.* **5**:562-570.

10. Fahal, I.H., R. Ahmad, G. Bell, J.D. Birchall, & N.B. Roberts (1993). Profile of serum silicon in aluminum-overloaded patients on haemodialysis treatment. *J. Anal. At. Spectrom.* **8**: 911-913.

11. Fix, George. (1989) Principles of Brewing Science. Brewers Publications.

12. Ghiselli, Andrea, Fausta Natella, Alessia Guidi, Luigi Montanari, Paolo Fontozzi, & Cristina Scaccini (2000). Beer increases plasma antioxidant capacity in humans. *J. Nutr. Biochem.* **11**: 76-80.

13. Ghiselli, A., M. Serafini, G. Maiani, E. Azzini, & A. Ferro-Luzzi (1995). A fluorescence-based method for measuring total plasma antioxidant capability. *Free Radic. Biol. Med.* **18**: 29-36.

14. Gorinstein, Shela, Abraham Caspi, Marina Zemser, & Simon Trakhtenberg (2000). Comparative contents of some phenolics in beer, red and white wines. *Nutr. Res.* **20 (1)**: 131-139.

15. Gorinstein, S., M. Zemser, M. Berliner, R. Goldstein, I. Libman, S. Trakhtenberg, & A. Caspi (1997). Moderate beer consumption and positive biochemical changes in patients with coronary atherosclerosis. *J. Int. Med.* **242**: 219-224

16. Harayama, K., Hayase, F., & Kato, H. (1994). New Method for Analyzing the Volatiles in Beer. *Biosci. Biotech. Biochem.* **58**: 2246-2247.

17. Hickenbottom, J.W. (1996). Processing, Types, and Uses of Barley Malt, Extracts, and Syrups. *Cereal Foods World.* **41**: 788-790.

18. Honma, Y., H. Tobe, M. Makishima, A. Yokoyama, & J. Okabe-Kado (1998). Induction of differentiation of myelogenous leukemia cells by humulone, a bitter in the hop. *Leuk. Res.* **22**:605-610.

19. Jeong, Jin Sook, Soo Jin Kwon, Sang Won Kang, Sue Goo Rhee, & Kanghwa Kim (1998). Purification and Characterization of a Second Type Thioredoxin Peroxidase (Type II TPx) from *Saccharomyces cerevisiae*. *Biochemistry.* **38**: 776-783

20. Kaneda, H., Y. Kano, T. Osawa, N. Ramarathnam, S. Kawakishi, & K. Kamada (1988). Detection of free radicals in beer oxidation. *J. Food. Sci.* **53**: 885-888.

21. Klatsky, Arthur, and Mary Anne Armstrong (1993). Alcoholic Beverage Choice and Risk of Coronary Artery Disease Mortality: Do Red Wine Drinkers Fare Best? *Am. Journ. Cardiol.* **71**: 467-469.

22. Korch, Christopher, Harry A. Mountain, Hans Gyllang, Michael Winge, & Patrick Brehmer (1991). A Mechanism for sulfite production in beer and how to increase sulfite levels by recombinant genetics. *European Brewery Convention Congress 1991.* 201-208.

23. Lampe, Johanna W. (1999). Health effects of vegetables and fruit: assessing mechanisms of action in human experimental srudies1-3. *Am. J. Clin. Nutr.* **70(suppl)**: 475S-490S.

24. Lewis, M.J., & Young, T.W. (1995). Brewing. Chapman and Hall.

25. Lingnert, Hans, Goran Akesson, & Caj E.Eriksson (1989). Antioxidative Effect of Superoxide Dismutase from *Saccharomyces cerevisiae* in Model Systems. *J.Agric.Food.Chem.* **37**: 23-28

26. Meuller, K., H. Weissberg, & Fresing-Weithenstephan (2002). Gas Permeability of bottle closures. *Brauwelt International.* **20(4)**: 211-214

27. McBride, Judy. High-ORAC Foods May Slow Aging. *Agricultural Research News* www.usda.gov/is/pr/1999/99082.htm

28. Moradas-Ferreira, P., V.Costa, P.Piper, & W.Mager (1996). The Molecular Defences Agains Reactive Oxygen Species in Yeast. *Mol. Microbiol.* **19(4)**: 651-658.

29. Muller, R. (2000). A win-win proposition. *Brewers' Guardian.* **129(10)**: 26-28.

30. Rabin, Dan & Carl Forget (1998). Dictionary of Beer & Brewing 2nd Edition. Brewers Publications.

31. Rice-Evans, C. & N.J. Miller (1994). Total antioxidant status in plasma and body fluids. *Methods Enzymol.* **234**: 279-293.

32. Richard, Marie-Jeanne, and Anne-Marie Roussel (1999). Micronutrients and ageing: intakes and requirements. *Proc. Nutr. Soc.* **58**: 573-578.

33. Stevens, J.F., C.L. Miranda, D.R. Buhler, & M.L. Deinzer (1998). Chemistry and biology of hop flavanoids. *J. Am. Soc. Brew. Chem.* **56**: 136-145.

34. Swajgier, Dominik, & Zdzislaw Targonski (2000). Comparison of *in vitro* antioxidant activities of malt, hops, worts, and lager type beer. *Pol. J. Food Nutr. Sci.* **9/50(4)**:53-59.

35. Traber, M.G. & H.J. Kayden (1993). α-tocopherol as compared with γ-tocopherol is preferentially secreted in human lipoproteins. *Ann. N.Y. Acad. Sci* **570**: 95-108.

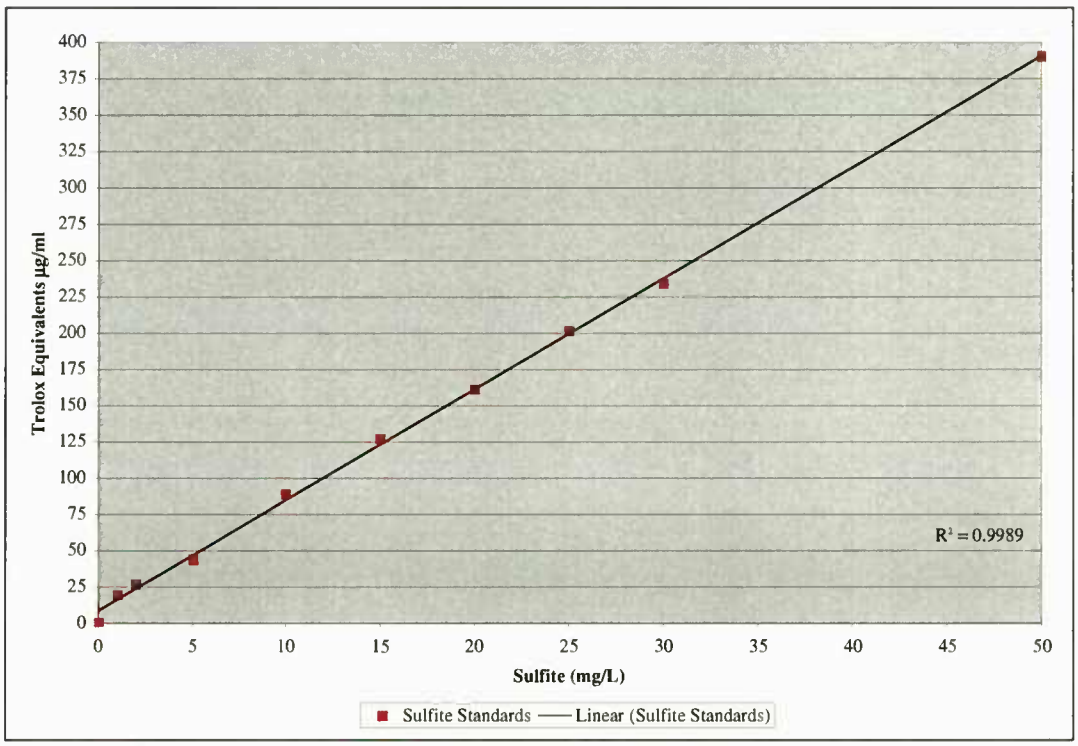
36. Uchida, M. & M.Ono (1996). Improvement for Oxidative Flavor Stability of Beer--Role of OH-Radical in Beer Oxidation. *J.Am.Soc.Brew.Chem.* **54(4)**: 198-204.

37. Walker, Caroline & E.D. Baxter (2000). Health Promoting Ingredients in Beer. *Technical Quarterly-Master Brewers' Association of America.* **37(2)**: 301-305.

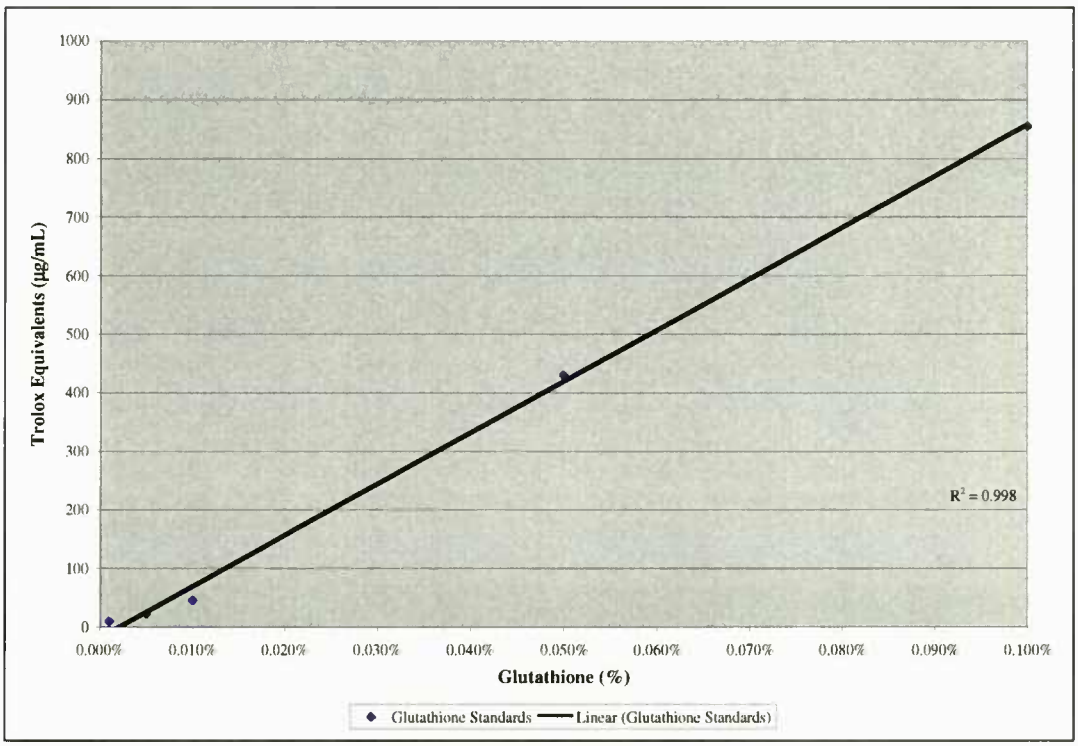
38. Yasukawa, K., M. Takeuchi, & M. Takido (1995). Humulon, a bitter in the hop, inhibits tumor promotion by 12-o-tetrade-canoylphorbol-13-acetate in two stage carcinogenesis in mouse skin. *Oncology.* **52**:156-158.

39. Young, T.W. (1987). The Biochemistry and physiology of yeast growth. Brewing Microbiology, 2nd Edition. Chapman & Hall, London. 1987.

APPENDICES



Appendix I. Trolox Equivalents for Standard Curve of Sulfite



Appendix II. Trolox Equivalents for Standard Curve of Glutathione

Appendix III. Table of Raw Data for Figure 3

Sample	Value 1	Value 2	Mean Result	Std.Dev. (+/-)
Orange	4125	4257	4191	93
Red Thompson Seedless	897	874	885	16
Green Thompson Seedless	428	428	428	0
Blueberries	3201	3241	3221	29
Tomato	1773	1737	1755	26
Apple Juice	2074	2051	2062	16
Green Tea	7358	6801	7080	394
Milk	2880	2880	2880	1
Coca~Cola	253	267	260	10
Green Gatorade	24	25	25	1

Appendix IV. Table of Raw Data for Figure 4

Type of Commercial beer	Value 1	Value 2	Mean Result	Std.Dev. (+/-)
Force-Carbonated	819.4	1032	926	150
Bottle-Conditioned	2136	2192	2164	40
Unfiltered	2153	2562	2358	289
Hefeweizen	1864	1864	1864	0
Live Fruit Beer	1813	1813	1813	0
Fruit Beer (force-carbonated)	1284	1478	1381	137

Appendix V. Table of Raw Data for Figure 5

Treatment	Value 1	Value 2	Mean Result	Std.Dev. (+/-)
Force-Carbonated (FC)	753.8	762.1	758.0	5.9
FC w/ 10 E7 cells yeast/ml	798.6	751.6	775.1	33.2
FC w/ 10 E7 cells yeast/ml; Pasteurized	790.6	837.2	813.9	33.0
FC Pasteurized	794.8	870.2	832.5	53.3
FC w/ 5 x 10 E6 cells yeast/ml	808.5	865	836.8	40.0
FC w/ 10 E6 cells/ml	754.2	840.3	797.3	60.9

Appendix VI. Table of Raw Data for Figure 6

Treatment	Value 1	Value 2	Mean Result	Std.Dev. (+/-)
Art.Carb.	1263	1348	1306	60.1
S.F. Low	1467	1507	1487	28.3
CA.Ale Low	1542	1570	1556	19.8
Belgian Wit Low	1558	1605	1582	33.2
S.F. High	1568	1600	1584	22.6
CA.Ale High	1497	1509	1503	8.5
Belgian Wit High	1618	1628	1623	7.1

Appendix VII. Table of Raw Data for Figure 7

Treatment	2 months	3 months	4 months	5 months	6 months
Artificially carbonated beer	0.67	0.455	0.665	0.805	0.885
Artificially carbonated beer w/CA.Ale added	0.64	0.585	0.555	0.585	0.785
Artificially carbonated beer w/S.F.Lager added	0.575	0.56	0.51	0.61	0.83
Artificially carbonated beer w/Belg.Wit added	0.605	0.56	0.52	0.54	0.67
Bottle Conditioned beer w/CA.Ale	0.525	0.485	0.48	0.435	0.56
Bottle Conditioned beer w/S.F.Lager	0.525	0.485	0.435	0.415	0.53
Bottle Conditioned beer w/Belg.Wit	0.535	0.495	0.465	0.44	0.52

Appendix VIII. Table of Raw Data for Figure 8

°	Free SO2 (A580)					
Treatment	1 month	2 months	3 months	4 months	5 months	6 months
AC	0.072	0.041	0.011	0	0	0
AC + CA	0.161	0.103	0.038	0.009	0	0
AC + SF	0.157	0.111	0.041	0.007	0	0
AC + BW	0.158	0.109	0.043	0.008	0	0
BC w/CA	0.183	0.155	0.104	0.042	0.004	0
BC w/SF	0.190	0.159	0.098	0.041	0.006	0
BC w/BW	0.189	0.161	0.101	0.04	0.006	0
°	°	°	°	°	°	°
°	Free SO2 (mg/L)					
Treatment	1 month	2 months	3 months	4 months	5 months	6 months
AC	5	2	0	0	0	0
AC + CA	13	8	1	0	0	0
AC + SF	13	8	2	0	0	0
AC + BW	13	8	2	0	0	0
BC w/CA	15	12	8	2	0	0
BC w/SF	16	13	7	2	0	0
BC w/BW	16	13	8	2	0	0

°	Total SO2 (A580)					
Treatment	1 month	2 months	3 months	4 months	5 months	6 months
AC	0.127	0.073	0.041	0.01	0	0
AC + CA	0.189	0.156	0.063	0.029	0	0
AC + SF	0.187	0.153	0.058	0.012	0	0
AC + BW	0.191	0.159	0.069	0.03	0	0
BC w/CA	0.210	0.181	0.143	0.086	0.032	0
BC w/SF	0.208	0.179	0.142	0.084	0.029	0
BC w/BW	0.211	0.184	0.146	0.087	0.033	0
°	°	°	°	°	°	°
°	Total SO2 (mg/L)					
Treatment	1 month	2 months	3 months	4 months	5 months	6 months
AC	14	5	2	0	0	0
AC + CA	16	13	4	1	0	0
AC + SF	16	13	4	0	0	0
AC + BW	16	13	4	1	0	0
BC w/CA	18	15	11	6	1	0
BC w/SF	18	15	12	6	1	0
BC w/BW	18	15	12	6	1	0

Appendix IX. Table of Raw Data for Figures 9 & 10

Month 1

Treatment	Value 1	Value 2	Mean Result	Std.Dev. (+/-)
AC	1964.9	1852.6	1908.8	56.2
AC + CA	1949.7	2077.6	2013.7	64.0
AC + SF	1967.1	2107.9	2037.5	70.4
AC + BW	2012.1	1869.2	1940.7	71.5
BC w/CA	2315.4	2398.7	2357.1	41.6
BC w/SF	2331.5	2146.1	2238.8	92.7
BC w/BW	2140.9	1954.3	2047.6	93.3

Month 2

Treatment	Value 1	Value 2	Value 3	Value 4	Mean Result	Std.Dev. (+/-)
AC	1512.4	1505.8	1481.1	1529.8	1507.3	17.5
AC + CA	1656.3	1663.8	1654.5	1670.2	1661.2	6.3
AC + SF	1696.5	1695.3	1703.7	1619.5	1678.7	34.4
AC + BW	1732.4	1772.3	1741.3	1760.1	1751.5	15.6
BC w/CA	1650.5	1645.4	1664.2	1616.1	1644.0	17.5
BC w/SF	1637.6	1644.5	1632.2	1605.2	1629.8	14.9
BC w/BW	1699.2	1724.5	1696.5	1672.7	1698.2	18.3

Month 3

Treatment	Value 1	Value 2	Value 3	Value 4	Mean Result	Std.Dev. (+/-)
AC	1398.0	1421.6	1408.6	1468.7	1424.2	27.0
AC + CA	1609.2	1659.5	1588.4	1625.8	1620.8	26.0
AC + SF	1659.0	1522.0	1668.1	1514.0	1590.8	72.9
AC + BW	1663.8	1608.5	1565.9	1592.8	1607.7	35.8
BC w/CA	1663.2	1656.6	1662.7	1645.7	1657.1	7.1
BC w/SF	1609.3	1619.5	1518.3	1530.9	1569.5	45.3
BC w/BW	1680.3	1584.6	1685.8	1599.8	1637.6	45.8

Month 4

Treatment	Value 1	Value 2	Value 3	Value 4	Mean Result	Std.Dev. (+/-)
AC	1381.9	1193.7	1367.4	1142.8	1271.5	104.9
AC + CA	1470.0	1462.1	1548.5	1441.6	1480.6	40.6
AC + SF	1405.8	1564.4	1515.8	1437.9	1481.0	62.6
AC + BW	1467.1	1568.0	1534.0	1477.7	1511.7	41.3
BC w/CA	1589.4	1592.3	1572.9	1642.9	1599.4	26.2
BC w/SF	1517.0	1594.0	1578.1	1526.3	1553.8	32.8
BC w/BW	1638.0	1628.3	1631.7	1594.8	1623.2	16.8

Month 5

Treatment	Value 1	Value 2	Value 3	Value 4	Mean Result	Std.Dev. (+/-)
AC	969.817	979.57	989.083	986.252	981.1805	7.4
AC + CA	1236.308	1288.17	1272.903	1254.543	1262.981	19.5

AC + SF	1184.781	1236.057	1242.539	1247.935	1227.828	25.2
AC + BW	1281.487	1214.141	1269.6	1220.666	1246.4735	29.5
BC w/CA	1494.946	1433.133	1446.413	1475.146	1462.4095	24.2
BC w/SF	1484.26	1495.268	1516.033	1505.73	1500.32275	11.8
BC w/BW	1494.44	1478.726	1486.675	1467.337	1481.7945	10.0
°						°
Month 6						°
Treatment	Value 1	Value 2	Value 3	Value 4	Mean Result	Std.Dev. (+/-)
AC	853.945	859.011	810.007	830.103	838.2665	19.6
AC + CA	1057.149	1058.556	1081.279	1069.708	1066.673	9.7
AC + SF	1065.586	1062.445	1047.571	1047.429	1055.75775	8.3
AC + BW	1067.41	1071.95	1072.031	1073.551	1071.2355	2.3
BC w/CA	1247.532	1252.264	1256.729	1257.037	1253.3905	3.9
BC w/SF	1268.696	1293.544	1274.528	1269.919	1276.67175	10.0
BC w/BW	1282.226	1273.995	1274.074	1289.629	1279.981	6.5

Appendix X. Table of Raw Data for Figure 11

Figure 11	CA.Ale		
°	Value 1	Value 2	Average
No treatment	182.62	226.19	204.40
boiled 5 minutes	169.02	218.45	193.74
boiled 15 minutes	165.26	201.12	183.19
boiled 60 minutes	133.82	162.18	148.00
Std. Autoclave Cycle	117.68	163.93	140.80
°	SF.Lager		
°	Value 1	Value 2	Average
No treatment	240.68	302.95	271.81
boiled 5 minutes	222.34	234.72	228.53
boiled 15 minutes	215.57	219.33	217.45
boiled 60 minutes	200.25	180.44	190.34
Std. Autoclave Cycle	177.31	173.33	175.32
°	Belg.Wit		
°	Value 1	Value 2	Average
No treatment	227.13	282.32	254.72
boiled 5 minutes	204.91	244.68	224.80
boiled 15 minutes	200.84	230.78	215.81
boiled 60 minutes	183.19	178.91	181.05
Std. Autoclave Cycle	168.67	173.23	170.95

Appendix XI. Table of Raw Data for Figure 12

Strain	Treatment	Fraetion	Value 1	Value 2	Average	STD.DEV.
CA	Boiled 60 min.	unfiltered	340.143	345.893	343.018	4.07
CA	Boiled 60 min.	above 10.000MW	201.154	215.227	208.1905	9.95
CA	Boiled 60 min.	below 10.000MW	83.724	80.545	82.1345	2.25
CA	Boiled 60 min.	between 5.000 & 10.000MW	31.177	29.462	30.3195	1.21
CA	Boiled 60 min.	below 5.000 MW	44.957	45.27	45.1135	0.22
CA	Control	unfiltered	413.349	423.804	418.5765	7.39
CA	Control	above 10.000MW	269.844	272.039	270.9415	1.55
CA	Control	below 10.000MW	70.78	72.077	71.4285	0.92
CA	Control	between 5.000 & 10.000MW	20.345	20.178	20.2615	0.12
CA	Control	below 5.000 MW	38.83	39.771	39.3005	0.67
CA	Protease @10mg/mL	unfiltered	490.758	498.632	494.695	5.57
CA	Protease @10mg/mL	above 10.000MW	227.731	231.964	229.8475	2.99
CA	Protease @10mg/mL	below 10.000MW	97.399	94.66	96.0295	1.94
CA	Protease @10mg/mL	between 5.000 & 10.000MW	19.718	19.028	19.373	0.49
CA	Protease @10mg/mL	below 5.000 MW	80.566	79.374	79.97	0.84
SF	Boiled 60 min.	unfiltered	332.986	341.777	337.3815	6.22
SF	Boiled 60 min.	above 10.000MW	187.565	201.018	194.2915	9.51
SF	Boiled 60 min.	below 10.000MW	62.25	62.885	62.5675	0.45
SF	Boiled 60 min.	between 5.000 & 10.000MW	27.907	27.335	27.621	0.40
SF	Boiled 60 min.	below 5.000 MW	37.78	38.204	37.992	0.30
SF	Control	unfiltered	418.386	423.997	421.1915	3.97
SF	Control	above 10.000MW	236.886	238.114	237.5	0.87
SF	Control	below 10.000MW	57.398	56.847	57.1225	0.39
SF	Control	between 5.000 & 10.000MW	9.899	9.899	9.899	0.00
SF	Control	below 5.000 MW	30.217	30.746	30.4815	0.37
SF	Protease @10mg/mL	unfiltered	464.613	469.91	467.2615	3.75
SF	Protease @10mg/mL	above 10.000MW	233.178	233.644	233.411	0.33
SF	Protease @10mg/mL	below 10.000MW	83.097	83.499	83.298	0.28
SF	Protease @10mg/mL	between 5.000 & 10.000MW	13.946	13.628	13.787	0.22
SF	Protease @10mg/mL	below 5.000 MW	82.906	83.394	83.15	0.35
BW	Boiled 60 min.	unfiltered	339.192	344.115	341.6535	3.48
BW	Boiled 60 min.	above 10.000MW	224.118	219.63	221.874	3.17
BW	Boiled 60 min.	below 10.000MW	84.019	83.333	83.676	0.49
BW	Boiled 60 min.	between 5.000 & 10.000MW	33.122	32.309	32.7155	0.57
BW	Boiled 60 min.	below 5.000 MW	41.12	41.159	41.1395	0.03
BW	Control	unfiltered	430.405	435.061	432.733	3.29
BW	Control	above 10.000MW	293.633	292.302	292.9675	0.94
BW	Control	below 10.000MW	63.503	62.114	62.8085	0.98
BW	Control	between 5.000 & 10.000MW	17.05	16.911	16.9805	0.10
BW	Control	below 5.000 MW	29.61	29.413	29.5115	0.14
BW	Protease @10mg/mL	unfiltered	467.545	472.249	469.897	3.33
BW	Protease @10mg/mL	above 10.000MW	284.96	274.758	279.859	7.21
BW	Protease @10mg/mL	below 10.000MW	93.947	92.737	93.342	0.86
BW	Protease @10mg/mL	between 5.000 & 10.000MW	10.505	11.167	10.836	0.47
BW	Protease @10mg/mL	below 5.000 MW	91.366	93.033	92.1995	1.18

Appendix XII. Table of Raw Data for Figure 13

°	California Ale Yeast	Belgian Wit Yeast	S.F.Lager Yeast
Extract + Enzyme + NADPH	273.964	234.382	205.736
Extract + NADPH	376.357	371.584	354.113
Extract + Enzyme	21.004	12.91	14.958
Extract	19.978	11.267	12.995
NADPH + Buffer	4.815	4.815	4.815
Enzyme + Buffer	100.917	100.917	100.917

Appendix XIII. Table of Raw Data for Figure 14

°	Value 1	Value 2	Value 3	Value 4	Average	STD.DEV.
CA.Ale-Control	396.663	391.031	426.342	417.612	407.9	16.8
SF.Lager-Control	407.621	411.462	435.895	430.356	421.3	13.9
Belg.Wit-Control	415.95	413.199	460.033	468.02	439.3	28.8
CA.Ale-Lipid Extracted	301.069	298.897	326.178	333.818	315.0	17.6
SF.Lager-Lipid Extracted	312.566	307.137	341.458	341.696	325.7	18.5
Belg.Wit-Lipid Extracted	318.147	312.914	354.499	367.302	338.2	26.8
CA.Ale Lipids	22.427	23.325	0	0	11.4	0.6
SF.Lager Lipids	22.275	26.435	0	0	12.2	2.9
Belg.Wit Lipids	24.554	25.566	0	0	12.5	0.7