Effects of Shelf-Life on Phytonutrient Composition in Stored Non-Alcoholic Beer

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ABSTRACT

The health benefits of beer phytonutrients are well known but there is no information on how they change under household refrigerated storage. The objective of the study is to determine the phytonutrient concentration in four commercial non-alcoholic beer beverages during 60-day storage and compare to an alcoholic beer beverage. Phytonutrient concentration was evaluated as the total polyphenol content and the flavonoid content [(+) - catechin and (-) - epicatechin]. The beverages were stored at 32° F and analyzed at day 0, 30 and 60. Statistical analysis of the data was carried out using one way analysis of variance (ANOVA) and Student t-test with a significance level of $p< 0.05$ (JMP 6.0, Statistical Analysis System (SAS®), Cary, North Carolina).

A significant increase ($p< 0.05$) in total polyphenol content occurred in all the beer beverages except for Beck's® non-alcoholic beer, suggesting that the beer beverages contained

more health benefits up to 60 days of refrigerated storage. (+) - Catechin generally decreased in the beer beverages up to 60 days and this was significant in Kaliber® and Leinenkugel`s® beverages. Overall, O'Doul's® non-alcoholic beer had the greatest total polyphenol and (+) - catechin content than all the non-alcoholic beer beverages. (-) - Epicatechin was quantified and detected only at day 0 indicating interaction of polyphenols of differing polarities as the beer aged may have occurred and the methods of analyses were not selective and sensitive to detect (-) - epicatechin at day 30 and 60. The study showed that beer total phytonutrients increase when the beer beverage is stored for 60 days at household refrigerated temperatures (32° F), and to obtain greater health benefits from the non-alcoholic beer beverages O'Doul's® may be consumed.

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Chapter I: Introduction

Beer is an alcoholic beverage produced from the brewing and fermentation of various cereals, particularly malted barley, and then flavored with hops. Hops are added to beer as a flavoring substance and they give beer its unique aroma and bitterness. The history of beer dates from Mesopotamia, an ancient region of Asia, included in modern Iraq. Beer was first made of naturally fermented bread that was baked using powdered malt (Kondo, 2004). This type of beer was rich in amino acids, vitamins, and minerals. Beer was an important food item in the daily diet of people and they attributed therapeutic effects to the beer. This ancient beer was flat and was not bitter like today's varieties since the hops were not used until the eighteenth-century in beer production (Kondo, 2004).

Beer is a widely consumed beverage in the world and may represent a vehicle for increasing the consumption of natural products having antioxidant and other health promoting properties (Stevens & Page, 2004). Two technologies that impart the conversion of the raw materials into beer are malting and fermentation (Robertson, 1993). Malting allows the ultimate production of a fermentable extract through the activities of enzymes formed during germination, while fermentation is the action of yeast on fermentable sugars and their conversion into alcohol and other by-products.

Beer is rich in several by-products including amino acids, peptides, B vitamins, and phenolic compounds that are derived from hops and malts; therefore, even non-alcoholic components of beer may have health benefits.

Vinson, Mandarano, Hisrt, Trevithick, and Bose (2003) stated that moderate consumption of alcoholic beverages has been associated with significant reductions in coronary heart disease (CHD) mortality. Additional findings indicate that the components contributing to the healthy

benefits of beer are called phytonutrients, which are found in the hops and malt. These phytonutrients are derived from the process of converting sunlight into energy by the plant from which hops and malt are used. Phytonutrients are certain organic components of plants and these components are thought to promote human health. They are defined by the United States (US) Food and Drug Administration (FDA) as phytochemicals or polyphenols, which are substances of plant origin that may be ingested by humans in gram quantities and which exhibit the potential for modulating metabolism such as to be favorable for cancer prevention and cardiovascular potential according to Lincoln-Lean (2003) (as cited in Bamforth, 2004). Phytonutrients are usually found in vegetables, fruits, and beverages. The California Cuisine Food Pyramid even includes phytonutrients in the base of the pyramid along with fruits and vegetables.

Polyphenols are a type of phytonutrient that exhibit antioxidant activity and are common substances in the plant kingdom. The polyphenols can be subdivided into three groups: phenolic acids, flavonoids, and stilbenes. Flavonoids are divided into different categories depending on the carbon skeleton and include anthocyanins, flavones, flavonones, flavanols, and flavonols. It is noted that flavonoids are very effective scavengers of free radicals (Rice-Evans, Miller, and Paganga, 1996) and they are the largest and best-studied group (Ferguson, 2000). These compounds are excellent candidates to explain the health benefits of diets rich in fruits, vegetables, and beverages such as wine or beer. The polyphenol antioxidant activity depends on structural features, such as the number and positions of the hydroxyl moieties on the ring system (Bravo, 1998). Rivero et al. (2005) stated that beer is reportedly a good source of polyphenols due to the significant amounts present in barley, malt, and hops.

The common polyphenols found in beer are flavonols, isoflavonoids, phenolic acids, chalcones and flavanols such as catechin, proanthocyanidins, and epicatechin (Rivero et al.,

2005). These have displayed antioxidant properties *in vivo* and *in vitro* and act as a defense against oxidative damage, especially from the free radicals generated in our bodies. Free radicals, if left unattended, attack DNA and eventually accelerate the aging process as well as the likelihood of cancer. Catechin is firmly accepted as an antioxidant through its ability to scavenge oxygen radicals and to inhibit the enzyme lipoxygenase, which promotes the initial breakdown of unsaturated fatty acids to carbonyls (Bamforth, 2004). Human exposure to these beneficial phytonutrients can be attained through beer consumption. For example a series of studies using animal models have shown that beer may prevent against carcinogenesis and osteoporosis since beer can provide plasma with significant protection from oxidative stress (Kondo, 2004). In addition, isohumulones, the bitter substances derived from hops, may decrease obesity and type-2 diabetes, and may prevent low density lipoprotein from oxidation and even suppress atherosclerosis (Kondo, 2004).

Although beer has myriad purported health benefits, consuming high quantities can lead to liver cirrhosis, inebriation, and other health complications. Heavy alcohol consumption is also blamed for approximately six per cent of all cancers in western countries, although moderate consumption reduces the risk of heart disease (*New Scientist*, 2005). While drinking excess alcohol increases the risk of cancer, it is believed that non-alcoholic beer may have a protective effect without complications from alcohol. Therefore, if the same amount of phytonutrients contained in alcoholic beer can be quantified in non-alcoholic beer, those people who prefer not to or are unable to consume alcoholic beer such as pregnant women (Garcia, Grande & Gandara, 2004) can obtain the same health benefits from the non-alcoholic beverage. In addition, non-alcoholic beer is much lower in calories than beer and is also beneficial for people who are concerned regarding caloric intake.

Non-alcoholic and alcoholic beers are made from the same raw materials and differ only in the alcohol content. Removal of alcohol is performed by techniques such as vacuum distillation, reverse osmosis, or by restricting the ability of the yeast to ferment wort, either by making a wort containing very low levels of fermentable sugars or by ensuring that the contact between the yeast and wort is at a very low temperature and brief time (Bamforth, 2004). Every brewery has its own methods and trade secrets on manufacturing beer.

The study of phytonutrient characterization in non-alcoholic beer during a 60-day refrigerated storage shelf-life will help educate people regarding the antioxidant and health benefits in this beer compared to alcoholic beer, which can then result in reducing the problems associated with high consumption of alcohol. This research study will evaluate the total polyphenol content and, flavonoid content as (+) - catechin and epicatechin concentration, in order to characterize the health benefits of various non-alcoholic beer types.

Statement of the Problem

There has been limited specific research conducted on non-alcoholic beer during refrigerated storage and therefore this study will determine if the same phytonutrients found in alcoholic beer are present and stable over a 60-day shelf-life in non-alcoholic beer. The study will be assimilating the storage of beers in a refrigerated home setting to determine if the phytonutrients decrease or remain constant over the 60-day storage.

A literature review was conducted in the fall semester of 2005. The five beers under study comprised of four non-alcoholic types; Kaliber® (Daigaes Great Britain Ltd, Rucorn, UK), O'Doul's® (Anheuser Busch, St. Louis, Missouri, USA), Clausthaler® (Germany, distributed by Binding Braunei, Norwalk, Cincinnati, USA), Beck`s® (Germany, distributed by Beck`s North America, Norwalk, Cincinnati, USA) and one alcoholic type; Leinenkugel`s® Creamy Dark (Jacob Leinenkugel Brewing Co., Chippewa Falls, Wisconsin, USA).

The preliminary experimental research was done the first week of January, 2006. The actual experimentation was started on February 6, 2006. All beers were analyzed at 0 day. The other analyses were done at 30 and 60 days to complete the shelf-life study. The experiments were completed in April, 2006. The report writing was completed throughout the study and finished in December, 2006. The chemical analyses were done in the Chemistry Department Laboratory Room 313 and Room 318. The instrumentation for the study was the Agilent 8453 UV-Vis Spectrophotometer and Waters High Performance Liquid Chromatograph with 1525 binary pump, 717 plus auto sampler, carousel, 2996 photodiode-array detector and computer analysis system
(Millennium® software).

Objectives

The overall objective for the research was to determine the phytonutrients in non-alcoholic beverages and to determine if they are stable over a 60 day shelf-life. The study analyzed the concentration of the phytonutrients as total polyphenol content, flavonoid content as (+) - catechin and (-) - epicatechin content in the non-alcoholic beer and determined the effect of shelf-life on the stability of these phytonutrients. The specific objectives were to:

1. Utilize a method of detecting the phytonutrients from the non-alcoholic beer;

2. Determine the concentration of the phytonutrients present in the non-alcoholic beer and compare to reference standards; and
3. Evaluate the phytonutrients` stability over 60 days of refrigerated storage.

Definition of Terms

The following definitions are core in understanding the purpose of this study.

Antioxidant "is a substance that inhibits oxidation or inhibits reactions promoted by oxygen or peroxides" (Merrian-Webster`s online dictionary).

Beer is an alcoholic beverage made by brewing and fermentation from cereals (usually malted barley) and flavored with hops to give a bitter taste (Robertson, 1993).

Non-alcoholic beers are beers containing up to 0.5 percent alcohol by volume in the US. Although they are called non-alcoholic, they still contain some alcohol and some states have laws which prohibit the sale to minors (Moll, 1991).

Nutrients are nourishing ingredients in food which contribute to growth and/or other functions (Merrian-Webster`s online dictionary).

Phytonutrients are nutrients derived from a plant source and which are not required for normal functioning of the body, but have beneficial effects on health. Many phytonutrients are antioxidants that impart bright colors to fruits and vegetables (Wikipedia, the free encyclopedia).

Assumptions of the Study

The assumptions to the study were that non-alcoholic beer has beneficial phytonutrients as does alcoholic beer, and the phytonutrients would increase over 60-day storage. The study documented the changes that occurred to the phytonutrient content as the beer was stored under household refrigerated temperatures (32° F) over a 60-day shelf-life.

Chapter II: Literature Review

Beer and Health

Coronary artery disease (CAD) is a serious disorder that accounts for one of every three deaths of both men and women (Gorinstein, Caspi, Zemser & Trakhtenberg, 2000). Atherosclerosis is the main basis of CAD and efforts to control this disease are continuing (Gorinstein et al., 1998a). Research has shown that one of the mechanisms resulting in the development of atherosclerosis is the oxidation of low density lipoprotein cholesterol (LDL-C) (Gorinstein et al., 2000). Several phenolic substances inhibit the oxidation of LDL-C and research has implied that only some polyphenols exhibit antioxidant properties and may therefore inhibit CAD.

Polyphenols have received increasing interest from consumers and food manufactures owing to their role as antioxidants, antimutagens, and scavengers of free radicals (Lugasi & Hovari, 2003). Several studies have linked polyphenols to prevention of pathologies such as cancer and cardiovascular disease. Alcoholic beverages are an integral part of most western culture diets and one example is beer. Studies have shown that beer contains polyphenolic compounds that may exhibit antioxidant properties. The presence of these compounds arises from the plants used in the production process, such as barley, malt, and hops (Jandera et al., 2005). Gorinstein et al. (1998a) using animals showed that moderate consumption of beer and wine (both alcoholic and non-alcoholic) may lead to some positive biochemical changes in lipid metabolism, antioxidant activity, and blood coagulation, which may result in improved prevention from atherosclerosis in consumers.

Gorinstein, Zemser, Weisz, Haruenkit and Trakhtenberg, (1998b) also assessed the influence of alcoholic and non-alcoholic beer on lipids, proteins, and antioxidant activity using

animals. The study focused on investigating the influence of beer polyphenols would have on the lipids, proteins, and antioxidant activity on rats supplemented for 4 weeks with both alcoholic and non-alcoholic beer. After completion, it was noted that both beverages showed antioxidative effects by decreasing the level of lipid peroxides, total cholesterol, triglycerides, and elevating high density lipoproteins in the rats. Therefore, additional evidence has been provided to show that the antioxidative properties of beer may arise from the raw materials as a result of the effect of non-alcoholic beer (Gorinstein et al., 1998b). The corresponding topics will provide an overview of the beer brewing process, polyphenol composition, and purported health benefits.

Beer Brewing

Beer is a major alcoholic beverage of the world. The vast majority of beers comprise at least 90% water, along with ethanol and carbon dioxide (Bamforth, 2004). Malting and brewing are the processes designed to maximize the extraction and digestion of starch and protein, yielding a highly fermentable extract, the wort. The majority of beer nutrients are derived either directly from the malted barley, adjuncts, water and hops, or are produced during the fermentation process by the metabolism of yeast.

Raw Materials

The main raw materials used in the brewing process are hops, barley malt, water, and yeast. Adjuncts, corn grits or cornstarch, are sometimes used to provide a source of fermentable carbohydrates and are utilized in the corn cooker.

Hops

Hops are crucial as a source of bitterness (from hop resins) and aroma (from the essential oils). In addition, hops are the antioxidant-rich material found in beer (Bamforth, 2004).

Barley

Barley is grown in many parts of the world for both food and feed uses (Shahidi & Naczk, 1995). Various types of phenolic compounds are present in the barley and their content and concentration depends on the cultivar and growing location. Phenolic compounds present in barley include phenolic acids and anthocyanins responsible for the blue and red color of barley tissue.

Barley contains β glucan and arabinoxylan polysaccharides in the cell wall as the major carbohydrates. According to Ahluwalia and Fry (1986), arabinoxylan is covalently linked to ferulic acid in the cell wall (as cited in Bamforth, 2004). Ferulic acid, is a phenolic acid released during mashing and survives into beer and is contained in beer so that it may act as an antioxidant *in vivo*.

Barley grains also contain a range of flavonols (monomeric, dimeric, and trimeric flavonols) and higher molecular weight flavonoid tannins. Table 1 demonstrates the flavonoid content of barley varieties grown in Ireland (Bamforth, 2004).

Table 1

Flavonol Content (mg/kg) in Three Barley Varieties Grown in Ireland in 1981

Barley variety	(+) Catechin (mg/kg)	Procyanidin B3 (mg/kg)	Prodelphinidin B3 (Condensed Polyphenols) (mg/kg)	Trimers[a] (mg/kg)	Total[b] Polyphenols (mg/kg)
Ark royal	41	202	281	464	1460
Emma	29	130	218	388	1220
Triumph	25	138	186	413	1300

[a] Sum of four components separated by HPLC

[b] Measured with Folin-Ciocalteu reagent (Shahidi & Naczk, 1995).

Barley Malt

Specific malting varieties of barley are employed for beer production (Bamforth, 2004). Barley malt contributes phenolic and polyphenolic compounds to the early stages of the brewing process (Shahidi & Naczk, 1995).

Water

Most beers are comprised of 90-95% water and its mineral composition is critical as a determinant of beer quality since water varies from region to region and by source.

Yeast

Saccharomyces cerevesiae is normally used as the yeast strain in the brewing process at the fermentation stage. However, each brewery has its own strain. Normally, yeast is used up to the seventh generation after which it is discarded, a process known as yeast scrapping.

Overview of the Brewing Process

The brewing process is outlined in the following processes:

Malting

Malting produces barley malt that has the enzymes required for the brewing process. The malting process occurs in three stages: steeping, germination, and kilning.

The barley is steeped in water at 14-18° C for up to 48 hours, until it reaches a moisture content of 42-46% (Bamforth, 2004). The water in the steep tank is continually changed every six to eight hours and the grain is aerated to prevent the barley kernels from using up dissolved oxygen in the tank during respiration. The germination process is done for 4-6 days at 16-20° C. The barley grain is placed on a germination bed and sparged with water at 16-20° C until a moisture content of 43-46% is attained. The grain starts to germinate, the enzymes break down the cell

walls and some protein in the starchy endosperm, rendering the grain easily crumbled (Bamforth, 2004). The controlled germination softens the grain, and makes it more readily milled.

The germination process is stopped and moisture content of malt is lowered during kilning. Kilning is done for up to 4 hours at a temperature range of 80 and 100° C to allow drying to < 5% moisture, while preserving those enzymes that are sensitive to heat. The temperature used depends on the color of the malt to be attained. Dark colored malts usually reach a maximum of 105 to 250° C for 3 hours.

Brewing

Brewing starts in the brew house where raw materials are blended together to produce wort, unfermented beer. The wort is subsequently pitched with yeast (yeast is added to wort), in fermentation to produce alcohol, carbon dioxide, and other products. The malt is wet milled to generate fine particles and mixed with hot water in a process called mashing.

Corn Cooking

Starch gelatinization and liquefaction occurs in the corn cooker. Malt (10%) and corn grits/cornstarch are mixed with water and the temperature is raised to 56° C. Beta glucanase activity occurs so that a break down of glucans to soluble glucose chains results. The resulting glucose chains serve as a substrate for the yeast to produce CO_2 and ethanol.

Mashing

Conversion of starch into simple fermentable sugars such as glucose occurs during mashing. Malt is mixed with water and combined with the cooked starch at a temperature of 62° C and then the temperature of the mixture is further raised to 76° C. Extractions of enzymes (β- and α-amylase) occur, which act on the starch to produce maltose, oligosaccharides, dextrins, and glucose that are used by yeast during the fermentation to produce CO_2 and ethanol.

Lautering

Lautering is essential for separating solids from the clear wort. A filter bed is created by allowing the mash to settle on a false slotted bottom. The filter bed is washed with water at 76° C to exhaust the bed of residual sugars.

Wort Boiling

The wort is boiled and the importance is to sterilize the wort, precipitate tannins and proteins, and drive away any unpleasant grainy notes from the malt (Bamforth, 2004). Wort boiling serves many purposes and all the actions occur simultaneously in the hop kettle (Moll, 1991), which include: inactivation of enzymes, sterilization of wort, concentration of wort, and removal of any volatiles that could spoil the beer flavor. The brewing operation is finished in the whirlpool. The wort whirls the precipitated proteins, polyphenols, and sediment to the bottom resulting in clear wort that is used in fermentation.

Fermentation

The clear wort is cooled and inoculated with yeast (*Saccharomyces cerevisae*). Ale fermentations usually complete within a few days at temperatures as high as 20° C, whereas larger fermentations can be as low as 6° C for several weeks. Fermentation is complete when the desired alcohol content has been reached and when an unpleasant butterscotch flavor, which develops during all fermentations, has been removed by yeast (Bamforth, 2004).

Storage Tanks

Beer is transferred from the fermentation vessels where it undergoes four days of maturation at 0° C, so that volatiles can be produced, carbon dioxide concentration increases, yeast will flocculate to the bottom of tank, proteins precipitate, and esters begin to form.

Filtration and Packaging

The beer passes through the filter and impurities are removed to produce clear beer. The alcohol concentration is adjusted as the beer moves from the filter to the bright beer tanks. The beer is transferred to bright beer tanks which hold filtered beer prior to bottling. Beer stabilization and homogenization occur, as well as final quality control checks are conducted prior to bottling. The filtered beer is adjusted to the required carbonation and packaged into cans, kegs, glass or plastic bottles.

Methods of Producing Non-Alcoholic Beer

Theoretically an alcohol-free beer should not contain any alcohol and this is perfectly achievable by distillation (Moll, 1991). However, analytical methods using distillation to measure the alcohol content are less sensitive than enzymatic methods and international regulations have directed towards a maximum alcohol concentration of 0.5% by volume.

Restricted fermentation (stopped fermentation). One technique to produce non-alcoholic beer is through restricted fermentation, which requires great care in the selection of flocculent yeast, as well as the use of lower amounts of yeast to allow tighter control of the fermentation process (Moll, 1991). The fermentation can be stopped by use of plate heat exchangers using High Temperature Short Time (HTST) centrifugation, filtration, a cold thermal shock, or high carbon dioxide pressure (Moll, 1991). The beer produced has an alcohol concentration of 0.5% v/v.

Special yeast or other microorganisms. The yeast *Saccharomycoides ludwiggi* ferments only glucose, fructose, and sucrose (Moll, 1991), so that by proper selection of the starting raw materials and the mashing method, it is possible to adjust the carbohydrate spectrum to obtain a low ethanol (< 1%) concentration in the beer.

Spent grains usage. Another technique to obtain low alcohol beer is allowing the spent grains to be extracted with water to obtain wort with low extract content (Moll, 1991). The wort is boiled with hop extracts for 90 minutes to provide 6-10 mg/L of isohumulones in the beer. Fermentation of the spent grain extract produces a beer containing 1% alcohol by volume. The beer is matured for 14 days after fermentation.

Distillation. A fourth method to obtain non-alcoholic beer is by removal of alcohol using distillation, in which 30% of hot water is added to normal beer in the wort kettle. Boiling is continued until the volume has decreased to its original volume again. The boiling stage distills off most of the alcohol, resulting in an alcohol content of 0.5% v/v (Moll, 1991).

Vacuum distillation. A fifth technique to obtain non-alcoholic beer is by passing alcoholic beer through a plate heat exchanger at 50° C and deesterifing under high vacuum to produce beer, carbon dioxide, and volatiles (Moll, 1991). The beer is then distilled under vacuum to allow the alcohol to be removed. Then this beer is remixed with the carbon dioxide and volatiles and further passed through a plate heat exchanger to produce beer with 0.5% alcohol content.

Dialysis. The sixth technique to produce non-alcoholic beer is through dialysis so that the alcohol in the beer can be removed through membranes into the dialysate as shown in the Figure 1 (Moll, 1991). Dialysis operates as a result of concentration differences and the presence of small pressure differences.

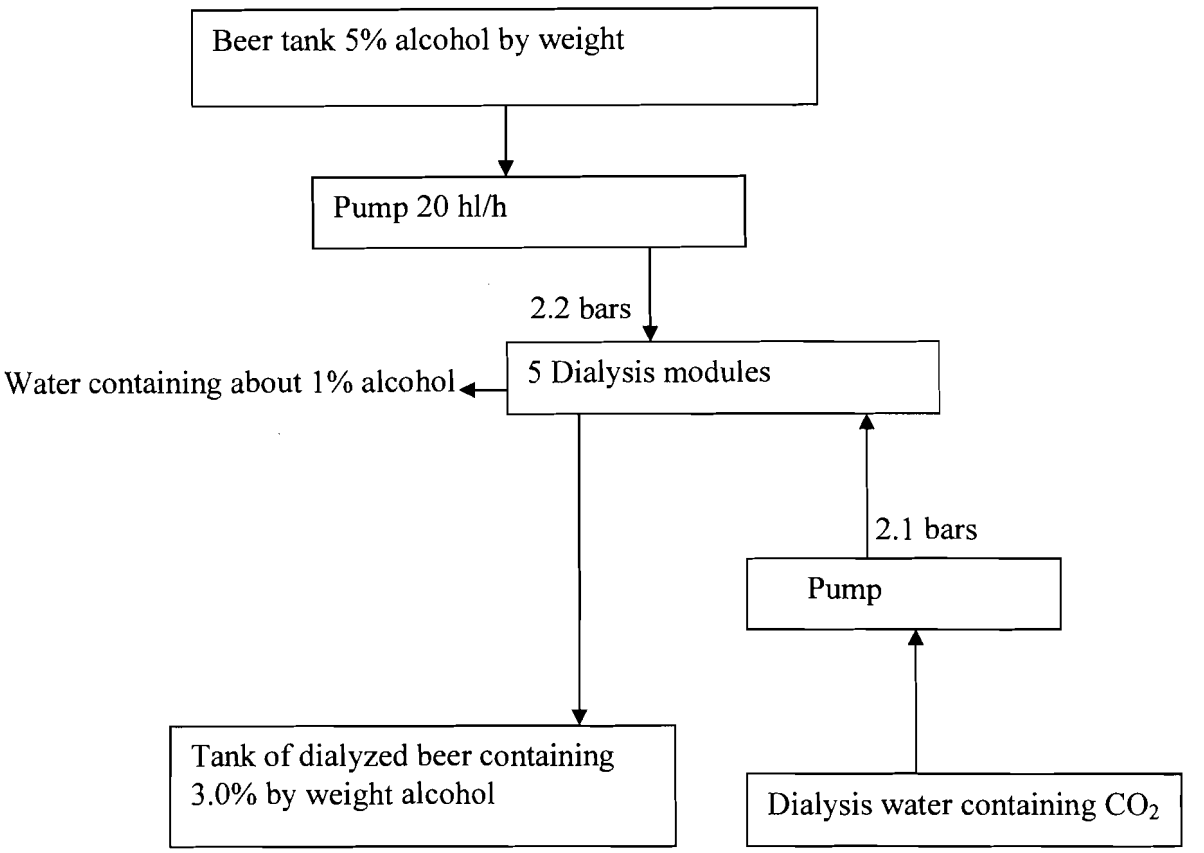


Figure 1. Diagram of removal of alcohol from beer by dialysis (Moll, 1991)

Alcohol removal through dialysis may be performed to a greater extent to obtain a beer with low alcohol content and a water fraction containing more alcohol. The alcohol-free fraction is reincorporated into the low alcohol beer.

Reverse osmosis. The final technique of obtaining non-alcoholic beer is through the process of reverse osmosis which employs filtration at high pressure (30-60 bars) through a semi permeable membrane (Moll, 1991). The semi permeable membrane allows water and ethanol to pass through. The mixture of water and alcohol may be processed using a distillation column and the alcohol–free fraction containing the volatiles may be added to the dealcoholized beer.

Overview of Phytonutrients in Beer

Brewing materials such as barley, malt, husks, and hops contribute phenolic and polyphenolic compounds to the early stages of the brewing process (Shahidi & Naczk, 1995). Phenolic compounds constitute a large group of secondary plant products, which differ in structural properties (Shahidi & Naczk, 1995). The chemical structure of polyphenols may range from simple compounds like phenolic acids to highly polymerized, tannins such as epicatechin, which is characterized in the present study.

Plant phenols play an important role in determining the organoleptic properties of food products of plant origins as well as participating in the formation of haze during processing of beer and wine (Shahidi & Naczk, 1995). Beers are known to contain a wide variety of phenolic compounds, which mainly result from raw materials of brewing (Madigan, McMurrough & Smyth, 1994). It is estimated that beer contains proanthocyanidins: up to 70- 80% are from barley while 20-30% originate from the hops (Stevens et al., 2002). The proanthocyanidins from hops are structurally very similar to those found in barley, the main difference being the higher proportion of gallocatechin (flavan-3-ols) units in the barley oligomers. Therefore, malt and hops play an integral role in the composition of beer polyphenols and since they are the starting material for beer production, they may explain the presence of such polyphenols in non-alcoholic beers.

Role of Phytonutrients

Reactive oxygen species can attack some cellular structures and molecules, such as lipids, proteins or DNA. DNA oxidative damage can lead to base mutation, single- and double–strand breaks, DNA cross-linking, and chromosomal breakage with rearrangement (Ames, Shigenga, & Gold, 1993). According to Halliwell and Gutteridge (1996), the damage to cellular structures in

the human body is associated with the aging process, and chronic diseases such as cancer and coronary heart disease (as cited in Rivero et al., 2005). Antioxidants, such as phytonutrients, are effective in lowering the risk of toxic effects of reactive oxygen species. Antioxidants act by reacting with free radicals produced and terminating the chain reaction before any vital molecules are damaged.

Beer is a low-alcoholic beverage that is brewed from natural ingredients, which may be involved in the prevention of cardiovascular and carcinogenesis diseases (Rivero et al., 2005). Beer contributes positively to health effects due to its antioxidant components. Ghiselli et al. (2000) suggested that the positive association between moderate intake of alcoholic beverages and reduced risk of cardiovascular disease may be linked to total polyphenol content of the beer beverages. In their human study involving 14 healthy participants, an increase in plasma antioxidant capacity was reported to occur following consumption of alcoholic beer (500 mL) but dealcoholized beer resulted in a lower increase in plasma antioxidant capacity and ethanol alone (4.5%) did not affect plasma antioxidant capacity or any of the parameters measured. There was a low absorption of polyphenol compounds to body fluids from dealcoholized beer and that accounts for the lower increase in plasma antioxidant capacity. Ethanol removal impaired the absorption of the polyphenol compounds. Therefore, ethanol (at low doses) can influence polyphenol absorption and its role in protection against CHD.

Gasowski et al. (2004) assessed the influence of alcoholic beer with different antioxidant potentials on plasma lipid metabolism, plasma antioxidant capacity, and bile excretion of rats (n=60) fed cholesterol-containing and cholesterol-free diets. The rats were given on average 2 mL/day of beer for the four week study. The study aimed at assessing the effect of beer with the highest and lowest contents of total polyphenols, total flavonoids, and antioxidant potential on

experiments *in vivo.* The results indicated that the supplemented diets of rats containing beer with the greatest and to lesser extent beer with lowest of the above mentioned components significantly hindered a rise in plasma lipids (total cholesterol, low density lipoprotein cholesterol, triglycerides, total phospholipids), and increased bile excretion but high density lipoprotein cholesterol was not changed. It was shown that the polyphenols are the main components of alcoholic beverages that contribute significantly to the antioxidant potential of these beverages.

The *in vitro* results, total polyphenol content and total antioxidant potential, of the beer samples were directly proportional to the effect of the beer polyphenols on experiments *in vivo*. Therefore, the positive influence of beer is directly connected to the bioactive compounds, polyphenol compounds in the beer and in order to achieve the best results in terms of health benefits, beer with the greatest content of polyphenols and greater antioxidant potential may be consumed.

In another study (Honma, Tobe, Makishima, Yokoyama & Okabe-Kado, 1998), it was found that humulone, a bitter substance from hops, effectively inhibited bone resportion *in vitro* with an IC_{50} (concentration required 50% inhibition) of 3.4 μM. Additionally, the effect of humulone on the differentiation of human myelogenous leukemia cells was studied and it was found to inhibit the growth of monoblastic leukemia U937 cells (Honma et al., 1998). These results showed that humulone may help in differentiation therapy of monocytic leukemia. Therefore, additional evidence has shown the antioxidative properties of beer is probably derived from the raw materials used in the brewing process. These observations may explain the presence of these polyphenols in non-alcoholic beer and that they may perform the same functionality when non-alcoholic beer is consumed.

The polyphenols in beer may have an influence on beer flavor stability during storage. For example, (+) - Catechin suppressed the formation of some carbonyl compounds in forced aged beers (Walters, Heasman & Hughes, 1997a). The beers were aged by heating to 60° C and keeping them at that temperature for 24 hours. The subsequent formation of carbonyl compounds as beer ages has a negative influence on beer flavor stability (Walters et al., 1997a). Storage studies on (+) - catechin are also mentioned by (Walters, Heasman & Hughes, 1997b) and are explained in detail in chapter 4. One of the major changes in aged beer is the development of a characteristic "cardboard" flavor. The purpose of adding antioxidants into beer is to delay or prevent this detrimental flavor changes. Therefore, polyphenol compounds could delay beer detrimental flavor changes and thus avoiding the use of exogenous antioxidant compounds (for example, ascorbate and bisulfate), that are generally incorporated to make beer stable over time.

Polyphenol Compounds as Antioxidants

Polyphenol antioxidants function as terminators of free radials and chelators of metal ions that are capable of catalyzing lipid peroxidation (Bravo, 1998), which is thought to play a role in atherosclerosis. Phenolic antioxidants interfere with the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals. The hydrogen atom is obtained from the hydroxyl group on the phenolic ring of the phenolic compounds.

Polyphenols are multifunctional and can act as reducing agents, hydrogen donating antioxidants and singlet oxygen quenchers (Rice-Evans, Miller, & Paganga, 1996). Polyphenols also exhibit antioxidant activity when present in low concentration relative to the substrate to be oxidized; they can prevent auto oxidation and form a stable radical after scavenging through intramolecular hydrogen bonding so that additional free radicals do not form which cause damage to cellular tissues *in vivo.* Polyphenol compounds are generally found in limited

quantities but even the low levels are effective in acting as antioxidants and thereby contributing to the health benefits received by consumers from ingesting polyphenol containing food and beverages. Therefore, the small concentrations of (+) - catechin and (-) - epicatechin found in the beers under the present study may be effective in acting as antioxidants.

Bamforth (2004) evaluated the different polyphenols found in lager beer (Table 2). The polyphenols ranged from < 1mg/L (catechin) to 80 mg/L (leucocyanidin) in concentrations. Epicatechin was also in the lower range of 1-10mg/L indicating that the compound is generally present in lower concentrations in beer. Greater concentrations of other compounds suggest beer is a composition of different polyphenolics.

Table 2

Polyphenol Compounds and Range of Concentrations Found in Lager Beer

Compound	Example	Levels (mg/L)
Phenolic alcohols	Tyrosol	3-40
Phenolic acids	Ferulic acid, p-coumaric acid, vanillic acid, caffeic acid, gallic acid	10-30
Phenolic amines and amino acids	Hordenine, tyramine, tyrosine	10-20
Flavan-3-ols	Catechin Epicatechin Proanthocyanidins	0.5-1.3 1-10 20-60
Flavan-3, 4-diols	Leucocyanidin	4-80
Flavonols	Quercetin, myrecetin, rutin	<10
Condensed polyphenols	Dimeric catechins Polymeric catechins Proanthocyanidins Prodelphinidins	5-8 <1 20-60 3-10

(Bamforth, 2004, p. 114)

According to Walker et al. (2001a), the quality of antioxidants is more relevant than their absolute quantity (as cited in Bamforth, 2004). In the researcher's study, they divided the antioxidants into ascorbic acid and its related compounds, polyphenolic flavonoids, catechin and its related compounds, and epicatechin and its related compounds. The antioxidant concentration of each alcoholic beverage was measured (red wine, cider and beer) using high performance liquid chromatography. The test revealed that cider had a greater antioxidant concentration among the beverages tested. When the antioxidant activity was measured of various foodstuffs in a laboratory assay, beer had slightly lower antioxidant activity than cider and red wine. However, more studies are needed *in vivo* to determine if the body utilizes the antioxidants found from beer and other sources in the same way as they act *in vitro*. It is important to note how the ingested polyphenols behave in the digestive tract as their nutritional significance to consumers and potential health effects depend on the bioavailability of polyphenols.

Gorinstein et al. (1998a) using laboratory animals and clinical investigations showed that although the content of total polyphenols in white wine was greater than beer, beer possessed a greater antioxidant activity. The experiments included lipid peroxides (LP) as an indicator of the status of antioxidant activity. The researchers found that a decrease in the level of LP was significantly greater in diets supplemented with beer than white wine. This is ascribed to the levels of procyanidins, epicatechin, and ferulic acid that are significantly greater in beer than in white wine (Gorinstein et al., 2000). Epicatechin (flavan-3-ol) is high molecular weight tannin and tannins have been shown to be 20 times more active than vitamin E as antioxidants (Gorinstein et al., 1998a). Therefore, not only the quantitative content of polyphenols must be taken into consideration but also it is important to study the content of various phenolics in beverages. Proanthocyanidins, epicatechin, catechin, quercetin, p-coumaric, and gallic acids are

several of the important constituents of polyphenols found in beer that may possess important health benefits when ingested. Additional information on total polyphenols of lager and low alcohol beer beverages are published in (Denke, 2000) and methods for analyzing total polyphenols of beverages (Singleton & Rossi, 1965, & Singleton, Orthofer & Lamuela-Raventos, 1999). Vinson, Proch and Bose, (2001) describes a faster Folin-Ciocalteu procedure for measuring total polyphenols in beverages.

Flavonoids

The polyphenolic flavonoids have the diphenylpropane ($C_6C_3C_6$) skeleton (Rice-Evans et al., 1996) and consist of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (Bravo, 1998). The family includes flavonols (e.g. quercetin, myricetin), flavanones (e.g.naringenin, hesperidin), flavones (e.g. apigenin, luteolin), flavan-3-ols (e.g. (+) - catechin, (-) - epicatechin, proanthocyanidins), anthocyanidins (e.g. cyanidin, pelargonidin), chalcones (e.g. xanthahumol), and isoflavones (e.g. genistein, diadzein) (Miranda et al. 1999, Ross & Kasum, 2002). Flavonoids constitute a large class of compounds in plants that contain a number of phenolic hydroxyl groups attached to the ring structures, which confer antioxidant activity (Bravo, 1998). They are a particular interest to the brewer as they have been associated with the development of haze in beers (Madigan, McMurrough & Smyth, 1994). During beer storage proteins and polyphenols slowly react to form a colloidal complex which becomes insoluble at cold temperatures and beer becomes cloudy. This is known as chill haze. In addition, McMurrough, Madigan, Kelly and Smyth (1996) found that (+) - catechin and procyanidin B3 may prevent staling of beer by protecting susceptible substrates from oxidation and therefore these compounds may decrease in stored beer.

Flavonoids occur in plants as aglycones although commonly found as glycoside derivatives. Figure 2 shows the basic structure of flavonoids and the system used for carbon numbering of the flavonoid nucleus.

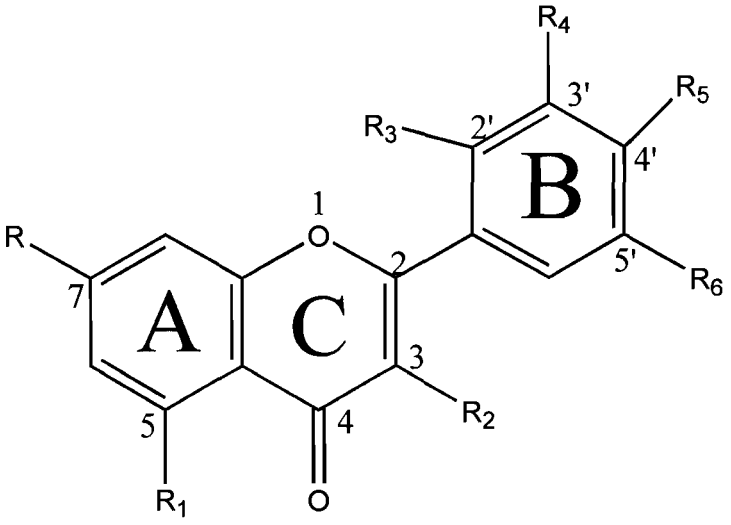


Figure 2. Basic flavonoid structure having A and B rings. An R group represents the OH group.

Flavonoids as Antioxidants

Flavonoids are among the most potent plant antioxidants because they possess one or more of the following structural elements involved in antiradical activity (Figure 3). They have an *o*-diphenolic group in ring B (2 hydroxyl groups attached to carbon 1 and 2 on the benzene ring), a 2-3 double bond conjugated with the 4-oxo function and hydroxyl groups in positions 3 and 5 (Bravo, 1998). Flavonoids donate an electron accompanied by an H^+ nucleus from the OH group to a free radical. The 4-oxo function in ring C allows delocalization between A and B rings which stabilizes the aroxyl radical formed after donation of an H^+ atom to a free radical during the free radical termination process. The flavonoids become an aroxyl radical which is more stable than a free radical and the free radical becomes inactivated. Quercetin, a flavanol (Figure 3) combines all of these characteristics and is one of the most potent natural antioxidants (Bravo,

1998). Quercetin has been shown to be more effective in protecting lipoproteins from oxidation in copper-mediated peroxidation systems than catechin and epicatechin.

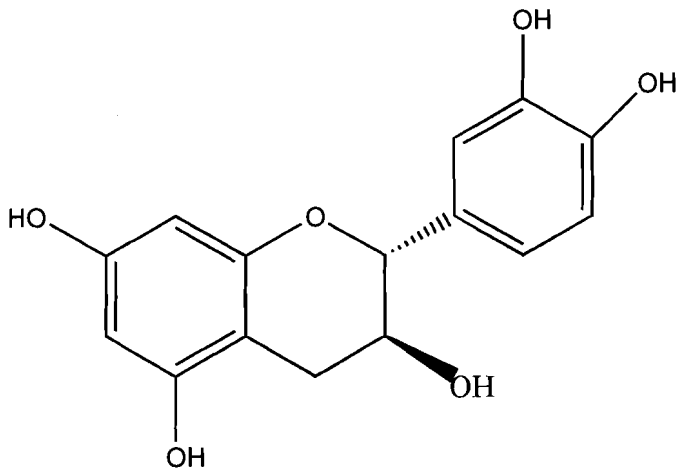


Figure 3. Structure of quercetin showing the structural characteristics related to its antioxidant capacity.

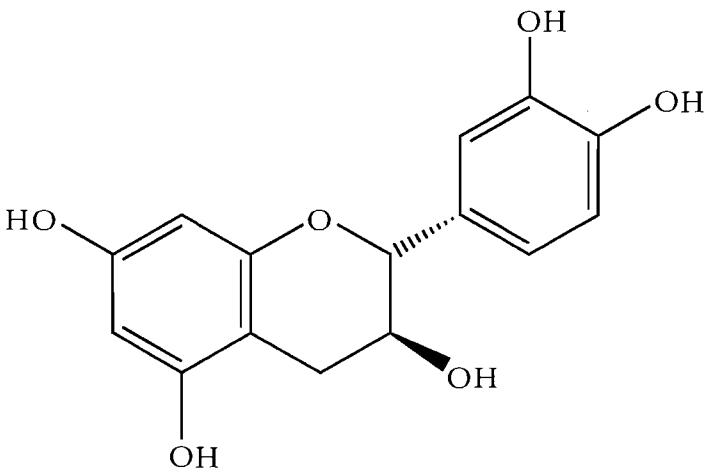
The antioxidative effect of flavonoids has been of interest for a considerable time (Bors, Heller, Michel, & Saran, 1990), because of their role in free radical scavenging, modulation of enzyme activity and inhibition of cellular proliferation (Bravo, 1998). Flavonoids (e.g. catechin, epicatechin, gallocatechin) are monomeric constituents of the condensed tannins. Tannins are a unique group of phenolic metabolites and the most biologically active is epicatechin (Gorinstein et al., 2000). Different classes of flavonoids differ in their antioxidant potential.

The polyphenol compounds mentioned in Figure 3 and Figure 4 have identical five hydroxyl groups; quercetin has an identical number of hydroxyl groups in the same positions as catechin but also contains the 2, 3-double bond in the C-ring and the 4-oxo-function. This structural arrangement allows quercetin to have a greater antioxidant activity value than catechin and epicatechin. Catechin and epicatechin have a saturated heterocyclic ring and have half the

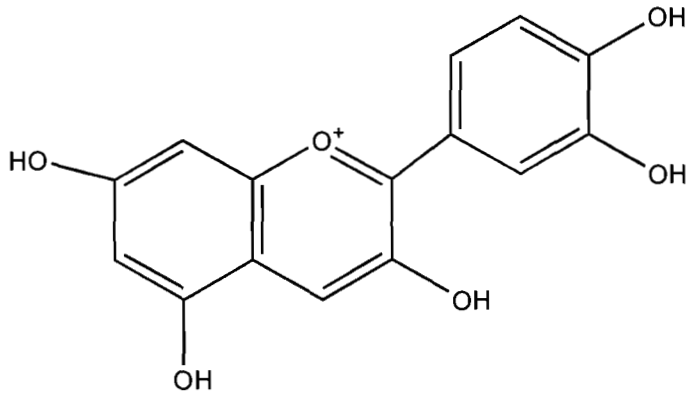
antioxidant activity as quercetin. Cyanidin has a central C ring allowing conjugation and has a slightly lower antioxidant potential than quercetin (Rice-Evans et al., 1996).

Therefore, the criteria for effective radial scavenging are: the *o*-dihydroxy structure in the B ring, which confers greater stability to the radical form and participates in electron delocalization; electron delocalization as a result of 2, 3 double bond conjugation with a 4-oxofunction in the C-ring and; the 3- and 5-OH groups with 4-oxo function in the A and C rings which are required for maximum radical scavenging. Figure 4 shows the structures of catechin and epicatechin (flavan-3-ols) and cyanidins (anthocyanidin).

Catechin



Cyanidin



Epicatechin

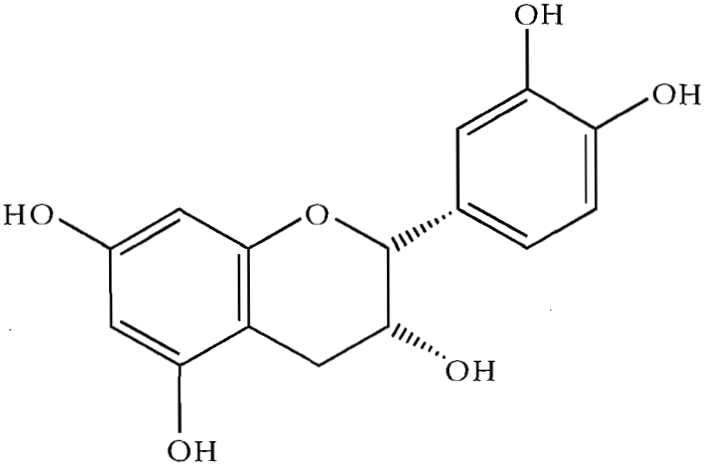


Figure 4. Catechin, cyanidin, and epicatechin structures.

Bors et al. (1990) determined the radical scavenging efficiencies of flavonoids and the researcher's findings indicate that the three structural groups of flavonoids mentioned above were important determinants for radical scavenging and/or antioxidative potential. The flavan-3-ols [(+) - catechin, (-) - epicatechin] and flavonols (kaempferol, quercetin) were effective as antioxidants in free radical scavenging. Therefore, the presence of such compounds in beer further indicates a basis for possible functional properties of beer.

Flavanols: Proanthocyanidins

Oligomeric proanthocyanidins constitute a group of water-soluble polyphenolic tannins that are present in the hop plant (0.5 to 5% dry weight) and are built up from polyhydroxy flavan-3-ols units such as catechin, epicatechin, gallocatechin, and their epimers (Stevens et al., 2002). Human exposure to hop proanthocyanidins is obtained generally through consumption of beer.

A study conducted by Stevens et al. (2002) identified and characterized the chemical structure and *in vitro* functions of proanthocyanidins from hops used in beer brewing. The study showed hop oligomeric proanthocyanidins at concentration of 25 μg/mL were potent inhibitors of rat neurons nitric oxide synthase activity which is an enzyme used for the production of nitric oxide (NO) in the nervous system. Excess amounts of NO may be involved in induced toxicity in the brain. Products from reactions of NO with super oxide anions may result in the oxidation of low-density lipoprotein (LDL), and may be involved in neurological disorders such as stroke, Parkinson disease, Huntington disease, and atherosclerosis. Therefore, after identification and characterization of proanthocyanidins in hops, it was revealed that they mainly contain subunits of catechin and epicatechin. The study showed that some components of proanthocyanidins from hops inhibit rat neurons NO synthase activity and LDL even stronger than several well known antioxidants (i.e α-tocopherols or ascorbic acid). They observed *in vitro* results that suggested a health promoting impact of dietary proanthocyanidins on NO related disorders. Therefore, hop oligomeric proanthocyanidins found in beer may prevent diseases associated with reactive nitrogen or oxygen species, which is additional to the functional properties of beer. However, more studies *in vitro* are needed to determine the utilization and absorption of proanthocyanidins obtained from beer.

Chalcones: Xanthohumol

Xanthohumol is a major prenylated chalcone that occurs in the hop plant *Humulus lupus* L. (Cannabaceae), and in beer (Stevens & Page, 2004). Hops, which are added for bitterness and flavor of beer, are a main dietary source of xanthohumol and related prenylflavonoids in beer.

Xanthohumol and other prenylated chalcones have been characterized as a cancer chemopreventive agent and related prenylflavonoid such as 8-prenylnaringenin and isoxanthohumol, have been suggested as one of the most potent phytoestrogens isolated currently (Stevens & Page, 2004).

Miranda et al. (2000) showed that xanthohumol exhibited a greater antioxidant activity at a concentration of 5μM by inhibiting LDL oxidation, which was higher than α-tocopherol but lower than the flavonol quercetin. Xanthohumol and other prenylated chalcones are lost during the wort boiling step of beer brewing however, 22-30% ends up in beer (Stevens & Page, 2004). Based on the health promoting properties of xanthohumol, increasing intake of this compound can be achieved by consuming non-alcoholic beer. Greater consumption of alcoholic beer to increase the intake of xanthohumol may result in health problems due to alcohol-related disorders.

Dietary Intake of Polyphenols

Hertog et al. (1995) reported that the intake of flavonoids ranged from 2.6 mg/day in West Finland to 68.2 mg/day in Japan using 16 cohorts from a study in seven countries. The study reported flavonoid intake from several food sources and beverages.

The proportion of polyphenols depends largely on the type of food source being consumed. For persons including beer in their diet (moderate intake), the most abundant polyphenols they will consume would be flavanols (mainly catechin, epicatechin and proanthocyanidins) and these account for more than two-thirds of the total polyphenol content (Scalbert & Williamson, 2000). The Japanese flavonoid intake was greater because of their consumption of beverages such as green tea which have large amounts of polyphenols.

Bioavailability of Polyphenols

In a study conducted by Bourne, Paganga, Baxter, Hughes and Rice-Evans (2000) using five healthy males, it was shown that ferulic acid (5.8 ± 3.2 mg) could be detected in urine after consumption of beer. The volunteers for the study consumed 4 liters of beer over 4 hours. The study investigated uptake of ferulic acid from low alcohol beer (approximately 1% alcohol by volume) as measured by urinary excretion. The findings were comparable with the uptake of ferulic acid from other dietary sources, such as tomatoes. For a material to reach the kidney, it must first be absorbed by the digestive system. This study does not show that the antioxidants reached all tissues in the body but it does appear to demonstrate that all the ferulic acid was absorbed by the body suggesting the availability of this substance from beer (Bamforth, 2004) and even from low alcohol beer.

The study also indicates the importance of understanding the absorption, distribution, metabolism, and excretion of polyphenol compounds. Vinson et al. (2003) stated that assuming that lager beer is consumed, the average phenol intake as catechin equivalents was 42 mg/day.

The Impact of Polyphenols in Beer on Health

Role of Polyphenols on Heart Diseases. According to the American Heart Association (2003), coronary heart disease (CHD) and related cardiovascular diseases are responsible for one

in two deaths among Americans, and more deaths than caused by cancer. Atherosclerosis is the term used for the hardening of the arteries and is responsible for CHD, stroke, and diseases of the circulatory system (Bamforth, 2004).

It is believed that polyphenols may combat coronary heart diseases. Gorinstein et al. (1997) evaluated the influence of a short period of moderate beer consumption on the status of thrombolitic activity in patients with coronary artery disease (CAD). The study involved twenty-eight males aged between 51 and 73 years with well documented CAD (two to three vessels CAD) and all having undergone coronary bypass surgery. The patients in the experimental group ($n = 22$) consumed 330 mL of beer (about 20 g of alcohol) for 30 days. Patients in the control group did not consume alcoholic beverages. Several blood indicators of thrombolitic activity (fibrinogen, prothrombin time (PT), coagulant activity of Factor VII, Factor VII antigen and plasminogen activator (PAI) levels) were measured. The results indicated that even a short period of moderate beer consumption decreased thrombogenic activity in the experimental group. The decrease in thrombogenic activity may be the main cause of decreased mortality in patients with CAD who consume moderate quantities of alcoholic beverages (Gorinstein et al., 1997).

Gorinstein et al. (1998b) conducted experiments on 60 male rats in an attempt to answer the differing opinions regarding biologically-active compounds in beer that cause significant reductions in coronary heart disease mortality. Tests included measurement of total cholesterol, low-density lipoprotein and high-density lipoprotein cholesterol, triglycerides, and lipid peroxides. The rats were divided into five groups and supplemented daily with alcoholic dry red wine (2 mL), alcoholic beer (6 mL), non-alcoholic dry red wine or non-alcoholic beer. The non-alcoholic wine and beer were at concentrations corresponding to an intake of 2 mL of alcoholic

wine and 6 mL of alcoholic beer. There were no statistically significant differences ($p < 0.05$) in the results between groups supplemented with alcoholic and non-alcoholic beverages. Additionally, the alcoholic and non-alcoholic beverages both exerted beneficial lipidemic and antioxidant properties by reducing total cholesterol, low-density lipoprotein cholesterol, triglycerides and lipid peroxides in the rats. Therefore, it was suggested that the biologically active compounds of the beverages responsible for the healthy effects in rats were attributed to the polyphenols contained in raw materials such as hops and barley malt.

Vinson et al. (2003) studied beer and heart disease using an animal model of atherosclerosis. The quantity of phenols (total polyphenols) were also studied, and ranged from 187-440 μM catechins in non-alcoholic and 165-1038 μM catechins in alcoholic lager beers. These values were equivalent to total polyphenols measured in mg/L gallic acid equivalents. When the quality of antioxidants in the beers was measured, epicatechin showed that it has a greater antioxidant potential. The lager beer inhibited atherosclerosis at 225 mL/day (equivalent to 42 mg/day of catechin equivalents) in the animal model of atherosclerosis by decreasing total cholesterol, triglycerides and oxidability of LDL *in vivo.* Therefore, polyphenols in the beer beverages appear to be responsible for the benefits in this model.

Role of Polyphenols on Cancer. There is substantial evidence that components found in beer may prevent cancer. These compounds act as therauptic agents; they kill the cancer cells or stop their growth (Miranda et al., 1999). Some unique flavonoids have been isolated from beer that may inhibit cytochrome P450 enzymes responsible for activating carcinogenesis. These components include xanthohumol, 8-prenylnaringenin and isoxanthohumol (Bamforth, 2004). Miranda et al. (1999) showed that *in vitro* proliferation of human breast cancer (MCF-7), and ovarian cancer (A-2780) cells were inhibited by prenylated flavonoids derived from hops, with

xanthohumol highly antiproliferative. Normal cells were not affected. These molecules may inhibit the activation of the cytochrome P450 enzymes that catalyze the conversion of procarcinogenesis into carcinogens which have an indicative role in cancer formation. In addition, xanthohumol may have potential chemopreventive activity against breast and ovarian cancer in humans. However, xanthohumol is isomerized to isoxanthohumol during wort boiling, a compound which has reduced antiproliferative activity in human cancer and ovarian cancer cells *in vitro*. Therefore, beer may not have a greater concentration of xanthohumol, although if isomerized to isoxanthohumol, antiproliferative effects may be exhibited only at a lower rate.

The antioxidant activity of polyphenols is generally evaluated on lipid systems. Plumb, Pascual-Teresa, Santos-Buelga, Cheynier and Williamson (1998) states that the antioxidant properties of catechins in lipid phase systems (erythrocyte membranes, rat liver microsomes and phospholipids bilayers) is equivalent to that of quercetin. This implies that catechin and epicatechin may be as potent as quercetin in preventing LDL oxidation. However, several studies have shown that quercetin is a potent natural antioxidant than catechin or epicatechin. For example, quercetin (100 μM) delayed cell proliferation on gastrointestinal tumor cells, which cause human colon cancer *in vivo* (Hosokawa et al., 1990). Also, Uddin and Choudhry (1995) stated that quercetin may have the potential of being an anticancer agent, as it inhibited the synthesis of DNA in human leukemia cells (HL-60) in culture at a concentration of 10 μM. The inhibitory effect was evident 24 hours after incubation. In addition, the polyphenol quercetin inhibited propagation of gastric cancer cells in humans (Yoshida et al., 1990). Therefore, in order to evaluate the content and shelf-life stability of polyphenols in non-alcoholic beer beverages, the present study was conducted. There have been previously limited studies to evaluate the stability

of these polyphenols in beer samples especially at refrigerated household temperatures of 32° F, and to determine if over storage consumers can still obtain health benefits.

Chapter III: Methodology

The objective of the research study was to determine the phenolic compounds in non-alcoholic beer. Two different families of phenolic compounds were measured; total polyphenol (TPP) content was evaluated by the Folin-Ciocalteu reaction and quantified as the mg GAE/L (milligram gallic acid equivalents per liter), and the flavonoid content measured as (+) - catechin and (-) - epicatechin content and expressed in mg/L of (+) -catechin and (-) - epicatechin, respectively using the High Performance Liquid Chromatograph (HPLC) method. The analyses were done at day 0, 30 and 60 for the shelf-life study.

Five types of beer beverages were studied (Figure 5); four were non-alcoholic lager beers (alcohol levels lower than 0.5%) and one alcoholic dark lager beer. The beers were purchased from Market Place Grocery Store [Menomonie, Wisconsin, United States of America (USA)] and transported to the laboratory UW-Stout Home Economics building Room 242. The four non-alcoholic types were from; Kaliber® (Daigaes Great Britain Ltd, Rucorn, United Kingdom), O'Doul's® (Anheuser Busch Inc, St Louis, Missouri, USA), Clausthaler® (Germany, distributed by Binding Braunei, Norwalk, Connecticut, USA), and Beck`s® (Germany, distributed by Beck`s North America, Norwalk, Connecticut, USA). The alcoholic type was Leinenkugel`s® Creamy Dark Lager (Jacob Leinenkugel`s Brewing Co., Chippewa Falls, Wisconsin, USA).

Figure 5. Beer beverages used in the study.

Materials and Method

Chemicals and reagants. The reagents, (+) - catechin, (-) - epicatechin, Folin–Ciocalteu reagent, and gallic acid were purchased from Sigma Chemical Co. (St Louis, Missouri). Glacial acetic acid, methanol, and sodium carbonate were purchased from Fisher Scientific (Fairlawn, New Jersey), acetonitrile was purchased from EM Science (Gibbstown, New Jersey), and ethanol was obtained from AAPER Alcohol and Chemical (Shelbyville, Kentucky).

Sep-Pak® cartridges Plus L-C18 were obtained from Waters Corporation (Milford, Massachusetts). Ultra pure water was purified in a Milli-Q® water purification system manufactured by Millipore® Corporation (Bedford, Massachusetts).

Beer samples. The beer beverages were stored at a refrigerated temperature (32° F) and the temperature was recorded each morning on a chart. The beer samples were coded with five different letters for analysis purposes. The analyses were carried out immediately upon opening the bottles to prevent loss of phenols by oxidation. The beers were degassed and equilibrated to 25° C, using a water bath prior to analysis of total polyphenols and flavonoid content.

Total Polyphenol Content: Determination of Total Polyphenol Content

The total polyphenols were determined spectrophotometrically using the Folin-Ciocalteu method (Singleton & Rossi, 1965, & (Singleton et al., 1999). The Folin method is an excellent colorimetric method as it operates by an oxidation-reduction mechanism measuring polyphenols, which are antioxidants (Vinson, et al., 2001).

Spectroscopy instrumentation. The Agilent 8453 UV-Visible photodiode-array spectrophotometer (Palo Alto, CA) was used for analyzing total polyphenols. The instrument has a wavelength range of 190 to 1100 nm, a 1-nm slit width, less than 0.03 % stray light, and requires Agilent Chemstation software and Microsoft® Windows® 2000 Professional built on NT technology operating system running on a PC. The Agilent 8453 is a single beam diode array spectrophotometer and is equipped with deuterium (UV) and tungsten halogen (visible) light sources.

Gallic acid stock preparation. Gallic acid stock solution was prepared by diluting 0.500 g of gallic acid, weighed using on a Mettler Toledo Analytical balance AG135, with 10 mL ethanol in a small beaker. The mixture was transferred quantitatively to a 100-mL volumetric flask and diluted to the mark with Milli-Q® water. The mixture contained 5000 mg/L gallic acid in order to prepare standard dilutions for the standard curve.

Gallic acid standards preparation. The standards were prepared by pipetting 2 mL, 5 mL, 8 mL, 11 mL, 14 mL, 17 mL, and 20 mL of the gallic acid stock solution (5000 mg/L) into 100-mL separate volumetric flasks, and then diluting to volume with Milli-Q® water. These standard solutions had phenol concentrations of 100 mg/L, 150 mg/L, 250 mg/L, 400 mg/L, 550 mg/L, 700 mg/L, 850 mg/L, and 1000 mg/L Gallic acid. The calibration curve was prepared from the gallic acid standards.

Colometric measurements. The beer samples were filtered through a 0.45-μm filter, Puradisc™ 25 PP (0.45μm polypropylene medium with polypropylene housing) (Whatman® Inc, Clifton, New Jersey) prior to analysis. Analysis of beer samples was done in triplicate for each beer type.

Samples having a volume of 100 μL of beer, standard, and blank (Milli-Q® water) were mixed with 900 μL of Milli-Q® water in 10 mL volumetric flasks. Five milliliters of 0.2 N Folin-Ciocalteu reagent was added and the mixture shaken. After 30 seconds and before 8 minutes, 4 mL of saturated sodium carbonate (75 g/L) was added to the mixture. The resultant mixture was 10 mL and was shaken to allow contents to mix. The samples were placed in an isotemp water bath (Fisher Scientific) at 23 °C for 2 hours. The samples were taken from the water bath after 2 hours and transferred to a 10 mm cuvette and absorbance was read at 765 nm with an Agilent 8453 UV-Visible spectrophotometer against the blank (Singleton et al., 1999). The blank was measured first prior to measurement of sample spectra and was done once at the beginning since all samples were measured within an hour.

Quantification was based on a standard curve of absorbance as a function of concentration for 100 mg/L, 250 mg/L, 400 mg/L, 550 mg/L, 700 mg/L, 850 mg/L, and 1000 mg/L gallic acid standards prepared simultaneously. Gallic acid standard curves are shown in

Appendix B through Appendix D. The standard curves were made by plotting the concentration of gallic acid (mg/L) on the x-axis and absorbance (AU) reading on the y-axis. The total polyphenols were obtained by interpolating the absorbance values of beer samples on the standard curves and determining the concentration in the beer beverages. The results were reported as milligram gallic acid equivalents (GAE) per liter. The data of the absorbance values for standard concentrations at day 0, 30 and 60 are shown in Appendix A, standard curves for gallic acid at day 0, 30 and 60 are shown in Appendix B through Appendix D, and data for the total polyphenol measurements are shown in Appendix E.

Flavonoid Content: Determination of (+) - Catechin and (-) - Epicatechin

Stock and standard preparation. Stock solutions for (+) - catechin and (-) - epicatechin (1000 mg/L) were prepared by weighing 0.100g of each phenolic compound, and transferring to 100-mL volumetric flask. The phenolic compounds were diluted to volume with methanol. (-) - Epicatechin was crushed with a mortal and pestle prior to weighing and preparing the stock solution. The stock solutions were stored in the freezer, Chemistry Room 320 for three months with foil placed around the flasks.

A mixture of standard solutions of 10 mg/L, 20 mg/L, 30 mg/L, 40 mg/L and 50 mg/L of (+) - catechin and (-) - epicatechin were prepared by placing 1 mL, 2 mL, 3 mL, 4 mL and 5 mL of each stock solution into 100-mL volumetric flasks. The flasks were diluted to the volume with methanol. The standard solutions were foil wrapped and stored in the refrigerator at 4 °C in Chemistry Room 313A for three months.

Polyphenol extraction. The beers were degassed, equilibrated to 25° C and concentrated 25 fold prior to injection into the HPLC system. A 500-mg C18 Sep-Pak® cartridge was conditioned with 4 mL of methanol followed by 4 mL of Milli-Q® water without allowing the

cartridge to dry out. An aliquot of beer sample (100 mL) was passed through the cartridge. The phenolic compounds were eluted using 4 mL of methanol and the organic eluate was homogenized by shaking. The eluate was then filtered through a 0.45-μm filter Puradisc™ 25 PP (0.45 μm polypropylene medium with polypropylene housing) (Whatman® Inc, Clifton, New Jersey) and transferred to a Waters auto sampler vial (1 mL) (Waters Corporation, Milford, MA). The vial was placed in a carousel and placed in the HPLC autosampler for chromatographic analysis. Polyphenol extraction was conducted in triplicate for each beer type.

Mobile phases. The HPLC required a solvent gradient using A and B where Solvent A (0.5% glacial acetic acid, 99.5% Milli-Q® water) was prepared by diluting 5 mL reagent grade acetic acid to volume in a 1000 mL volumetric flask with Milli-Q® water. Solvent B (40% acetonitrile, 59.5% Milli-Q® water and 0.5% acetic acid) was prepared by mixing 400 mL acetonitrile, 595 mL Milli-Q® water and 5 mL reagent grade acetic acid in a 1000 mL volumetric flask.

Chromatographic system and operating conditions. (+) - Catechin and (-) - Epicatechin compounds were isolated from their fractions by a Waters high performance liquid chromatography (HPLC) system equipped with 1525 binary pump, 717 plus auto sampler, carousel, 2996 photodiode-array detector linked to a computer running the Millennium® chromatography software. The analytical column (Waters Radial™ Compression) used was a 10 cm x 8 mm ID Novapak C_{18} column. The guard column, NovaPak GuardPak, was packed in an RCM-100 radial™ compression module.

For the HPLC analysis an aliquot (25 μL) was injected into the columns and eluted at room temperature with a constant flow of 2.0 mL/min and the run time was 35 minutes. Solvent A and B were used to generate a solvent gradient. The following gradient separating conditions were used and are shown in Table 3, for the mobile phases- A and B: A: B (100:0) for 30 minutes, changed to A: B (0:100) for 1 minute, changed to A: B (100:0) for 4 minutes. The mobile phase was acidified to allow total protonation of the compounds being studied.

Table 3

Mobile Phase Gradient Separation Conditions

Time (min)	Flow (mL/min)	% A	% B
	2.00	100.0	0.0
30.00	2.00	0.0	100.0
31.00	2.00	100.0	0.0
35.00	2.00	100.0	0.0

Quantification of (+) - catechin and (-) - epicatechin. The identification of the peaks was carried out by their retention times in comparison with standards retention times at 278 nm, 290 nm and 300 nm for both (+) - catechin and (-) - epicatechin. Results from the 278 nm chromatogram are reported in this research because the wavelength is most nearly λ_{max} for the analytes investigated.

Quantification of the (+)-catechin and (-) - epicatechin concentrations in the non-alcoholic and alcoholic beer beverages were obtained from their peak areas. The peak areas were interpolated on the individual standard curves of (+) - catechin and (-) - epicatechin and the concentration of the compounds were obtained. The concentrations were divided by 25, which was the concentration factor used in the analyses. The data used for the standard curves of (+) - catechin and (-) - epicatechin for the shelf-life study is shown in Appendix F through Appendix G. Sample chromatograms at day 0 for the standards (+) - catechin and (-) - epicatechin, O'Doul's® non-alcoholic beer and Leinenkugel`s® alcoholic beer are shown in Appendix I through Appendix K. The standard curves for the shelf-life study are shown in Appendix L through Appendix O. The standard curves were made by plotting the concentration of (+) - catechin and (-) - epicatechin (mg/L) on the x-axis and peak area (μV*Sec) on the y- axis at 278 nm and 290 nm.

Statistical Analysis

Statistical analysis of the data was carried out using one way analysis of variance (ANOVA) and Student t-test to determine significance with beer type (alcoholic versus non-alcoholic) and time of storage (0, 30 and 60 days) as the independent variables, total polyphenol and flavonoid content as dependent variables. A significance level of $p < 0.05$ was considered. Statistical analyses were conducted using JMP 6.0 Statistical Analysis System (SAS®), 2005, Cary, North Carolina.

Chapter IV: Results and Discussion

Non-alcoholic beers are often selected as an alternative to alcoholic beverages by many people such as pregnant women, sporting professionals, and people with cardiovascular diseases (Garcia et al., 2004) since these groups of people have restrictions on alcohol intake. These beverages contain a mixture of polyphenol compounds extracted from malt and hops that have useful antioxidant properties. Flavan-3-ols, mainly monomers such as (+) - catechin, and (-) - epicatechin, dimers (prodelphinidin B3 and procyanidin B3), and trimers (procyanidin C2) in beer are obtained from malt and hops (Garcia et al., 2004). The final content of the polyphenol compounds in beer depends on both the raw materials and the brewing process. In the current study, the analyses were carried out in triplicate for each beer beverage type. The concentrations for total polyphenols and flavonoid content were expressed in mg/L and then in mg/340 mL in order to determine the amount of phytonutrients contained in a beer serving by multiplying the initial concentration in mg/L by a factor of 0.34 as shown in the formula below:

concentration [mg/340 mL (beer serving size)] = concentration in mg/L × 0.34

Total Polyphenol Content

The total polyphenol content of the beer beverages was measured using the Folin-Ciocalteu method and gallic acid as the standard (Singleton et al., 1999). Table 4 indicates the total polyphenol content in the beer beverages expressed as milligram gallic acid equivalents (GAE) per liter (mg/L) over the 60-day shelf-life. One way analysis of variance (ANOVA) and comparisons of means using the Student t-test (day 0 and 30) showed that up to 30 days there was no significant ($p<0.05$) change in the total polyphenols in any of the non-alcoholic beer beverages. However, at day 60, there was a significant increase in the total polyphenol content of all the non-alcoholic beverages except for Beck`s®, which did slightly increase from day 30 (233

mg/L) to day 60 (249 mg/L) although not statistically different. In the alcoholic beverage, Leinenkugel`s®, a significant increase ($p< 0.05$) in total polyphenols was noted at 30 days (669 mg/L) compared to day 0 (622 mg/L). These results indicate that an increase in polyphenol compounds occurred in the beer beverages over the 60-day storage except for Leinenkugel`s®, which did not change from 30 days to 60 days of storage.

Table 4 also shows the wide range in total polyphenol content among all non-alcoholic beer beverages. For example at day 0 the total polyphenol content ranged from 229 mg/L in Beck`s® and 485 mg/L in O'Doul's®. At day 30, the range was from 233 to 480 mg/L and at day 60, the range was from 249 to 505 mg/L. The results indicate that additional beer polyphenols in the non-alcoholic beer may have continued to form during the 60-day refrigerated storage, thus implying greater health benefits may be obtained from consuming stored beer (Shahidi & Naczk, 1995). For example, total polyphenol content of O'Doul's® non-alcoholic beer increased from 485 mg/L to 505 mg/L up to 60 days. Therefore, consuming O'Doul's® beer at 60 days may provide more health benefits than consuming the beer at day 0 according to the present study. Leinenkugel`s® alcoholic beer beverage total polyphenol content increased from 622 to 669 mg/L up to 30 days of storage and remained unchanged (668 mg/L) up to 60 days. This is postulated that the beer phytonutrients may have stopped forming in the alcoholic beer beverage when the time of storage was continued up to 60 days or may be that the alcohol content had an influence on the phytonutrient polymerization. The total polyphenol content of the beer beverages were related to their beer color level. The darker beer beverages had a greater total polyphenol content compared to the light colored beer. Figure 5 shows the beer beverages used in the current study. Leinenkugel`s® alcoholic beer was the darkest in color level and had the greatest total polyphenol content of 622 mg/L at day 0. Beck`s® non-alcoholic beer had the

lightest beer color level and its total polyphenol content was lowest, 229 mg/L at day 0. Lugasi and Hovari (2003) reported the total polyphenol content of dark beers and lager beers as ranging from 380 to 600mg/L and 270 to 470 mg/L respectively. The results indicate that beer with a darker color level has more total phytonutrients compared to beer which is lighter in color (for instance, the lager beer in Lugasi & Hovari, 2003 study) as demonstrated in the current study.

Table 4

Total Polyphenol (TPP)[1] Content of Four Non-Alcoholic and One Alcoholic Beer Beverages[2] Measured at Wavelength 765 nm

Beer beverage type	TPP at day 0 (mg GAE/L)	TPP at day 30 (mg GAE/L)	TPP at day 60 (mg GAE/L)
Beck`s®[3]	229 ± 13^{A}	233 ± 1^{A}	249 ± 4^{A}
Clausthaler®[3]	271 ± 4^{B}	275 ± 1^{B}	281 ± 3^{A}
Kaliber®[3]	265 ± 10^{B}	264 ± 4^{B}	283 ± 4^{A}
O'Doul's®[3]	485 ± 8^{B}	480 ± 2^{B}	505 ± 4^{A}
Leinenkugel`s®[4]	622 ± 9^{B}	669 ± 5^{A}	668 ± 9^{A}
Average	374 ± 159	384 ± 173	397 ± 169

Values followed by different upper case letters in each row are significantly different, ($p < 0.05$),

[1]TPP content expressed as milligram gallic acid equivalents (GAE) per liter (mg/L),

[2]Mean of three trials ± standard deviation (SD),

[3]Non-alcoholic beer

[4]Alcoholic beer.

The levels of total polyphenols illustrated in Table 4 were noted to agree with literature concentrations (e.g. Lugasi, 2003). For instance, 622 mg/L of total polyphenols was contained in Leinenkugel`s® alcoholic beer at day 0 which corresponds to 600 mg/L in the alcoholic beer as shown in Lugasi, 2003 study. The beer beverages reported in the literature (alcoholic and non-alcoholic) were also studied for their antioxidant activity, this suggests, the beer beverages in the present study may possess the same antioxidant properties *in vivo* if consumed although most research show *in vitro* studies. For instance, Lugasi (2003) investigated the total polyphenol content and *in vitro* antioxidant properties in five lager and dark beers. The total polyphenol content in the Germany Hungarian alcoholic dark beers was reported as 380 mg/L and 600 mg/L, respectively and in the present study the dark alcoholic beer was 622 mg/L at day 0. In one study (Gasowski et al., 2004), researchers found that the beer with greatest antioxidant potential had 668 mg/L and beer with lowest antioxidant potential had 442 mg/L total polyphenols expressed as milligram gallic acid equivalents per liter. In comparison the findings of the current study show that the total polyphenol content was greatest in Leinenkugel`s® (622 mg/L) alcoholic beer and lowest in Beck`s® (229 mg/L) non-alcoholic beer at day 0. In Gasowski et al. (2004), beer with greatest content of total polyphenols had more influence on improving plasma lipid levels, increasing plasma antioxidant potential and bile excretion rates on experiments with laboratory animals. Therefore, health benefits from consuming Leinenkugel`s® beer may be greater than Beck`s® beer since beer with the greatest total polyphenol content has the greatest antioxidant potential.

Table 5 shows the total polyphenol values in the beer beverages expressed as milligram gallic acid equivalents (GAE) per 340 mL (mg/340 mL), which a typical beer is serving size from one standard-sized bottle. Table 5 illustrates the total polyphenols that may be present in a

beer serving and therefore educates consumers on the quantity of polyphenol compounds found in a one beer serving size. The quantity of total polyphenols in a beer serving is low as compared to values in Table 4, suggesting that to obtain greater health benefits, at least two beer beverages may be consumed. It would therefore be advantageous to consume non-alcoholic beer than alcoholic beer because of the deleterious effects of increased alcohol intake if a consumer chooses to get these beneficial phytonutrients from beer. In addition, O'Doul's® non-alcoholic beer contains a greater amount of the total polyphenols per serving as compared to the other non-alcoholic beers.

Table 5

Total Polyphenol (TPP) [1] *Content of Four Non-Alcoholic and One Alcoholic Beer Beverages Measured at Wavelength 765 nm*

Beer beverage type	TPP at day 0 (mg GAE/340 mL)[2]	TPP at day30 (mg GAE/340 mL)	TPP at day 60 (mg GAE/340 mL)
Beck`s®[3]	78 ± 4^{B}	79 ± 0^{B}	85 ± 1^{A}
Clausthaler®[3]	92 ± 1^{B}	94 ± 0^{B}	96 ± 1^{A}
Kaliber®[3]	90 ± 3^{B}	90 ± 1^{B}	96 ± 1^{A}
O'Doul's®[3]	165 ± 3^{B}	163 ± 1^{B}	171 ± 1^{A}
Leinenkugel`s®[4]	211 ± 3^{B}	226 ± 2^{A}	227 ± 3^{A}
Average	127 ± 58	130 ± 63	135 ± 56

Values followed by different upper case letters in each row are significantly different, ($p < 0.05$),
[1]TPP content expressed as milligram gallic acid equivalents (GAE) per 340 mL (beer serving size),
[2]Mean of three trials ± standard deviation (SD)
[3]Non-alcoholic beer
[4]Alcoholic beer.

Denke (2000) reported that the total polyphenol content of lager beer was 112.3 mg/340 mL and low-alcohol beer was 119.9 mg/340 mL and has stated that the beneficial effects of beer could be caused by the non-alcoholic components of beer. The values per 340 mL in the present study for the non-alcoholic beer are even greater at day 0 in O'Doul's® (165 mg/340 mL) than found by Denke (2000) which suggests that health benefits can be obtained from consuming O'Doul's®. Gorinstein et al. (1998a) showed that beer (total polyphenol content = 345 mg/L) exhibited a greater antioxidant potential than white wine (total polyphenol content = 436 mg/L) even though white wine had greater total polyphenol content. This implies the importance to study individual beer polyphenols, such as catechin and epicatechin, which may contribute to the antioxidant potential of both alcoholic and non-alcoholic beverages. Therefore, it is generally less advantageous to rely on total polyphenols alone but more beneficial to also quantify individual beer polyphenols that could contribute to the antioxidant properties of beer. Bartolome et al. (2000) (as cited in Garcia et al., 2004) compared the polyphenol compounds of several commercial non-alcoholic and alcoholic beers and found that the level of total polyphenols in non-alcoholic beer beverages were lower than for alcoholic beer beverages. This may have been attributed to the duration of fermentation and the yeast strains employed in brewing non-alcoholic beers as well as to a possible loss of polyphenols due to the dealcoholization process. Therefore, generally non-alcoholic beer beverages may have a lower total polyphenol content, which were found in the current study since Leinenkugel's® had a greater total polyphenol content than non-alcoholic beers and the antioxidant potential may be somewhat lower in non-alcoholic beers as opposed to alcoholic beer beverages. However, even if the total polyphenol content may be reduced, non-alcoholic beer beverages are still beneficial to the health of consumers.

The means of the total polyphenol content of the beer beverages were compared using a Student t-test at day 0 and Figure 6 indicates the total polyphenol content of each beer beverage at day 0. The total polyphenol content of Leinenkugel`s® beer (622 mg/L) was significantly greater than all the non-alcoholic beer beverages. In addition, the total polyphenol content of non-alcoholic beer beverages was also significantly different. For instance, O'Doul's® (485 mg/L) was significantly greater than Beck`s® (229 mg/L), Kaliber® (265 mg/L), and Clausthaler® (271 mg/L); whereas Clausthaler® and Kaliber® were significantly greater than Beck`s® beer. The beer beverage with the greatest total polyphenol content indicates that the beverage may have greater health benefits in comparison with the other beer beverages.

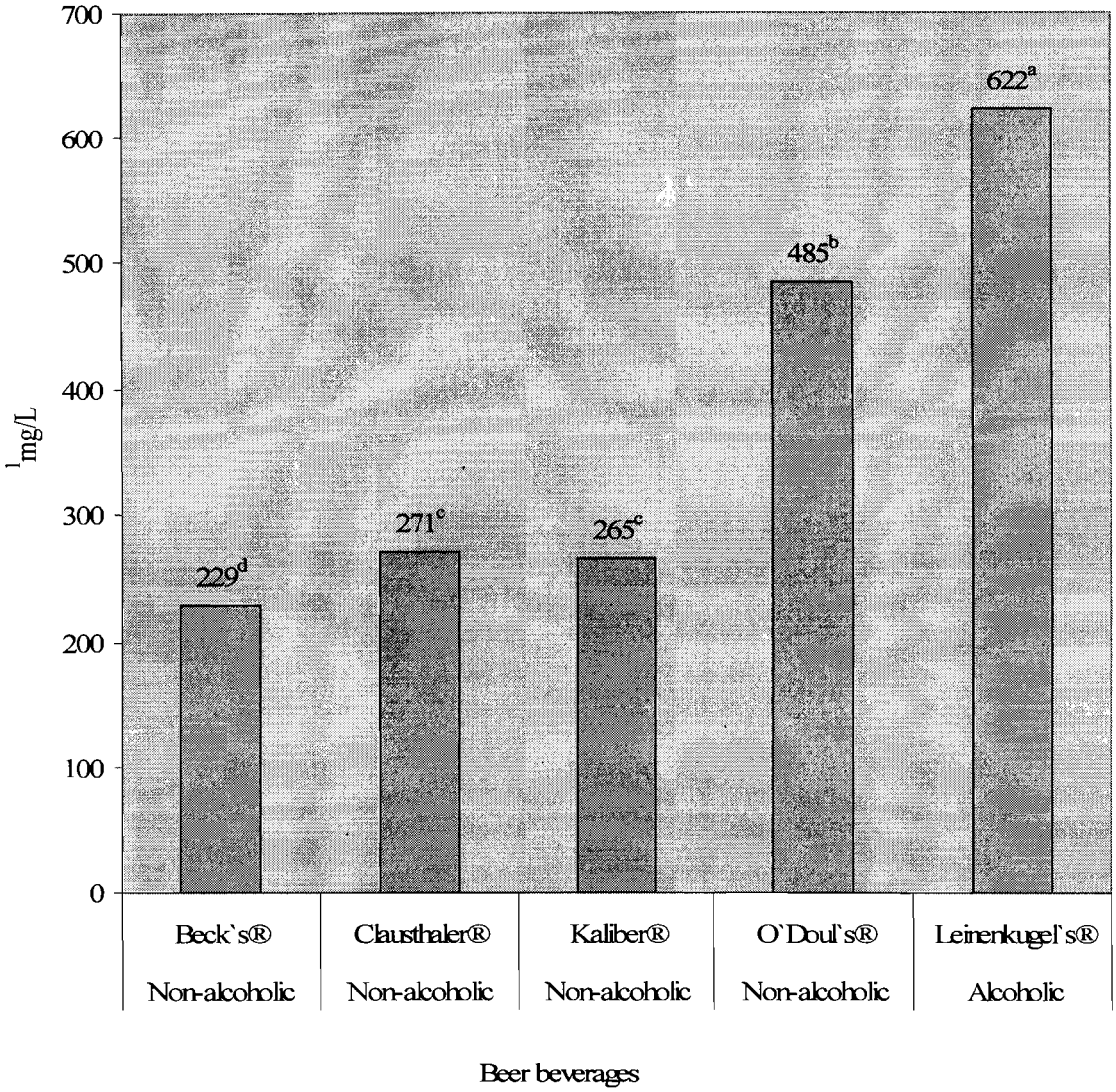


Figure 6. Average total polyphenol content (mg/L) of the beer beverages at day 0. Values followed by different lower case letters are significantly different, ($p<0.05$). [1]milligram gallic acid equivalents per liter.

At day 30, the means of the total polyphenol content of the beer beverages were compared using a Student t-test. Figure 7 indicates the total polyphenol content of each beer beverage at day 30. The total polyphenol content of Leinenkugel`s® beer (669 mg/L) was significantly greater ($p<0.05$) than all the non-alcoholic beer beverages.

The total polyphenol content of non-alcoholic beer beverages was also significantly different. It was noted that O'Doul's® (480 mg/L) was significantly greater than Beck`s® (233 mg/L), Kaliber® (264 mg/L), and Clausthaler® (275 mg/L); whereas Clausthaler® was significantly greater than Kaliber® and Kaliber® was found to be significantly greater than Beck`s® beer. This indicates that O'Doul's® would be the best choice to consume in terms of the beneficial phytonutrients when comparing the beer beverage to the other non-alcoholics which were studied in the current research.

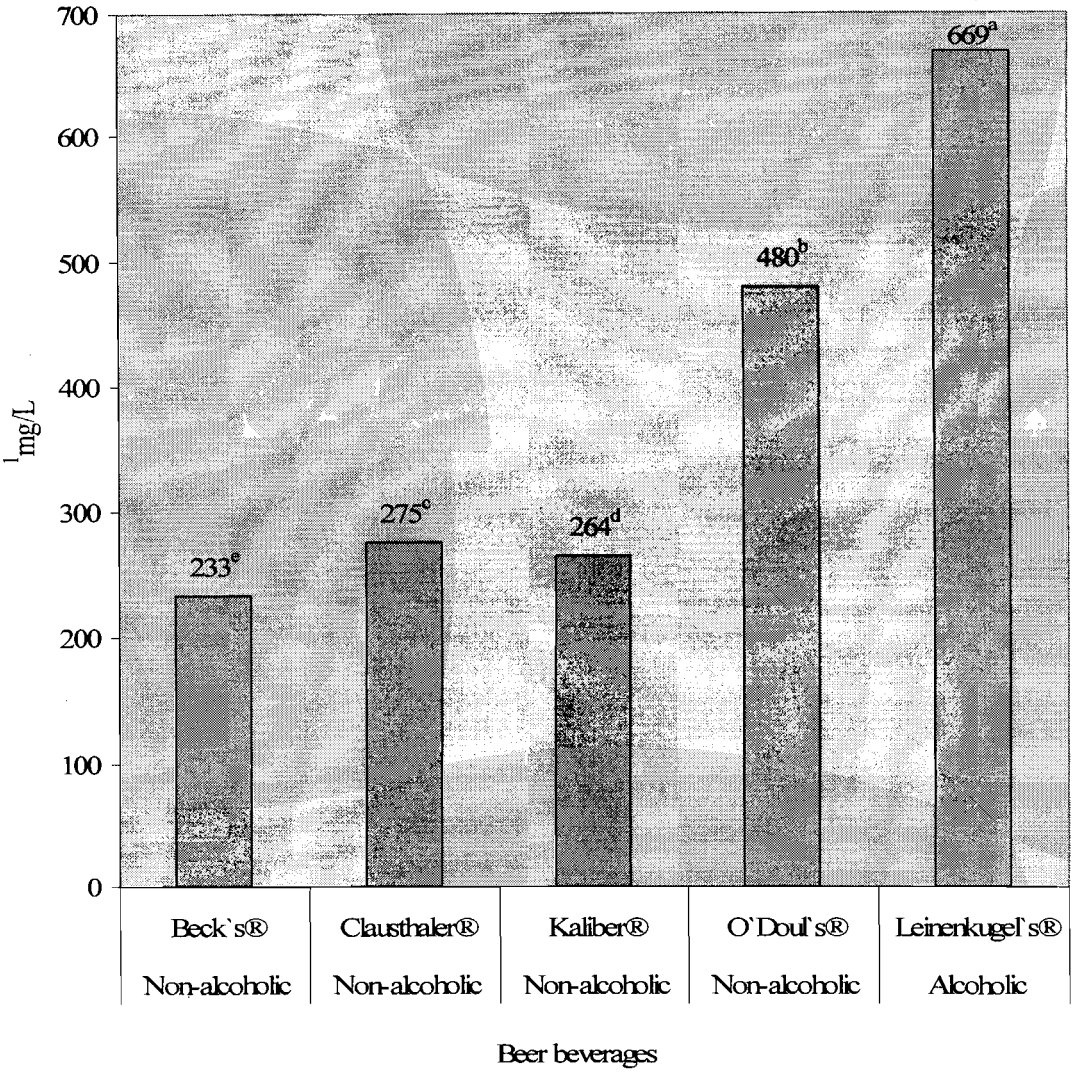


Figure 7. Average total polyphenol content (mg/L) of the beer beverages at day 30. Values followed by different lower case letters are significantly different, ($p<0.05$). [1]milligram gallic acid equivalents per liter.

Figure 8 indicates the total polyphenol content of each beer beverage at day 60. The means of the total polyphenol content of the beer beverages were compared up to 60 days using a Student t-test. The total polyphenol content of Leinenkugel`s® beer

(668 mg/L) was significantly greater ($p<0.05$) than for the non-alcoholic beer beverages and this indicates that even after 60 days of storage, the alcoholic beverage still contained a greater quantity of the beneficial phytonutrients that the other beer beverages The alcohol content, probably did not have an effect on the total polyphenol content during storage. The total polyphenol content of non-alcoholic beer beverages was also significantly different. It was found that O'Doul's® (505 mg/L) was significantly greater than Beck`s® (249 mg/L), Kaliber® (283 mg/L), and Clausthaler® (281 mg/L); whereas Clausthaler® and Kaliber® were significantly greater ($p<0.05$) than Beck`s® beer. The quantities of the total polyphenol content up to 60 days in the non-alcoholic beer differed because of the color of the beer. The beer color is an indication of the quantity of total polyphenols which may be present in the beer. For instance, O'Doul's® was darker in color as indicated in Figure 5 than the other non-alcoholics and that may explain why it remained significantly greater during shelf life.

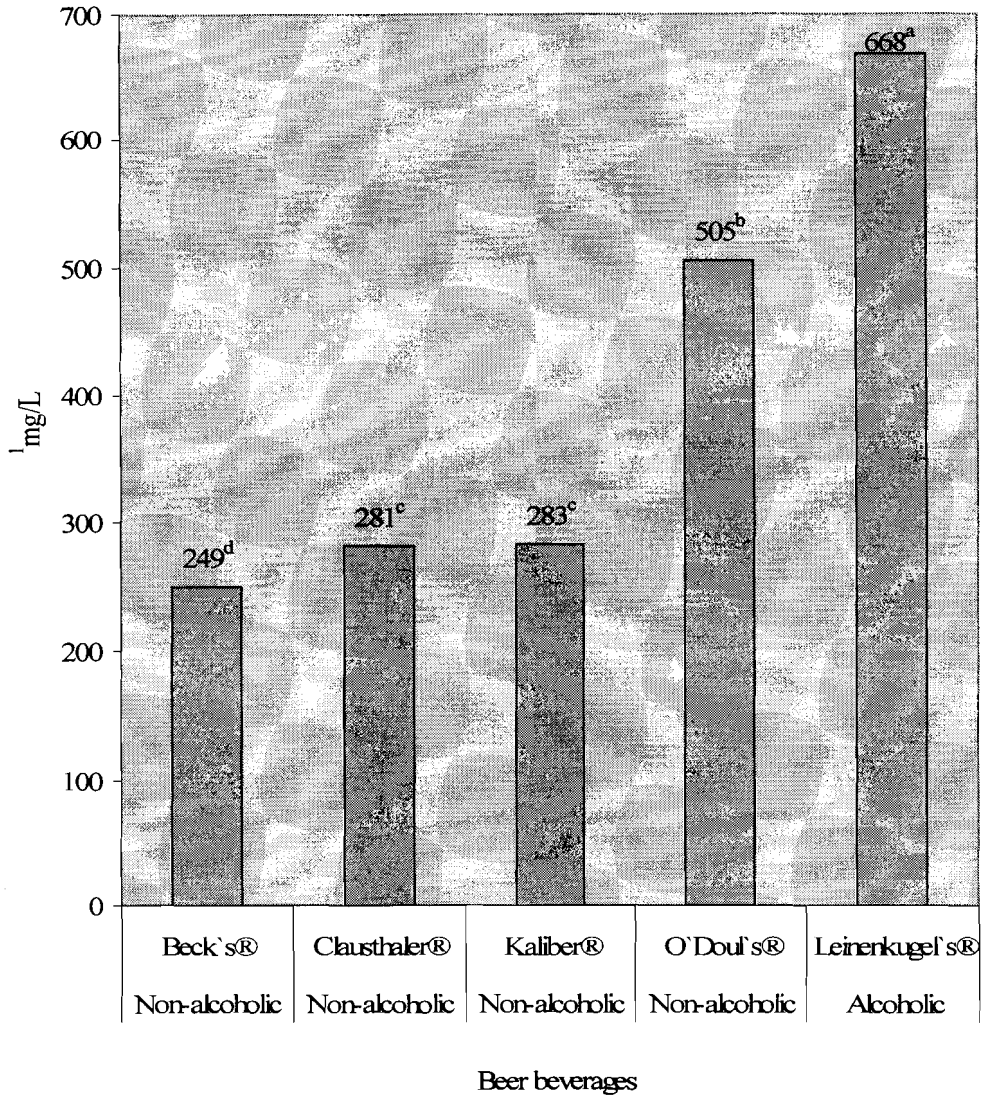


Figure 8. Average total polyphenol content (mg/L) of the beer beverages at day 60. Values followed by different lower case letters are significantly different, ($p<0.05$). [1]milligram gallic acid equivalents per liter.

Determination of (+) - Catechin and (-) - Epicatechin Content

The compounds, (+) - catechin and (-) - epicatechin, were evaluated for their content in commercial non-alcoholic and alcoholic beer for their stability over a 60-day shelf-life in order to individualize those components in the total polyphenols found previously.

Table 6 shows the average retention times and peak area ratios at 278 and 290 nm for (+) - catechin and (-) - epicatechin. The results show retention times (time at which each component was eluted) decreasing at day 30 and 60. However, the peak area ratios were relatively constant during the shelf-life study.

Table 6

Peak Area Ratio at 278 and 290 nm for (+) - Catechin and (-) - Epicatechin[1]

Compound	Day	Retention Time	Peak Area Ratio (278/290 nm)
(+) - Catechin	0	18.416 ± 0.03	2.790 ± 0.04
	30	18.130 ± 0.02	2.750 ± 0.01
	60	17.992 ± 0.02	2.780 ± 0.02
(-) - Epicatechin	0	20.953 ± 0.03	2.940 ± 0.02
	30	20.606 ± 0.01	2.930 ± 0.01
	60	20.585 ± 0.02	2.930 ± 0.02

[1]Average at day 0, 30 and 60 trials ± standard deviation (SD).

Table 7 shows the average values ± SD of (+) - catechin content (expressed in mg/L) over the 60-day shelf-life and (-) - epicatechin at only day 0. It was found that from day 0 to day 30 there was no significant change in the (+) - catechin content of all beer beverages. Therefore, the (+) - catechin content did not decrease significantly up to 30 days of refrigerated storage implying that the antioxidant was still present in the beer beverages after 1 month of storage. Comparing means of beer stored at day 0 and day 60, it was found that a significant decrease in the (+) - catechin was observed in non-alcoholic beers, Kaliber®, Clausthaler®, and in the alcoholic beer, Leinenkugel's®. This indicates that the (+) - catechin decreased at the same rate

as in alcoholic beer and may suggest that the presence of alcohol did not have an effect on the storage stability of (+) - catechin. Interestingly, Beck`s®, a non-alcoholic beer, had no detectable catechin at day 60. The (+) - catechin content in Beck`s® was initially low compared to the other beer beverages at day 0 and may have depleted during storage such that there was no (+) - catechin present after 60 days. O'Doul's® and Clausthaler®, both non-alcoholic beer beverages, showed no significant decrease. Overall, (+) - catechin content was generally lower in all the beer beverages at 60 days storage. This decrease after 60 days of refrigerated storage might be as a result of the flavonoid acting as antioxidants in beer flavor stability during beer storage (Walters et al., 1997b). During beer storage, staling of beer may occur as a result of oxidation. This is a free radical process in which molecular oxygen is converted to reactive oxygen species which oxidize beer components into free radicals. The free radicals in beer react further, resulting in the formation of carbonyl compounds such as aldehydes and ketones. The carbonyl compounds give rise to the cardboard-like flavor of stale beer. Therefore, antioxidants, such as catechins, in beer may help prevent the formation of free radicals and increase beer flavor stability and thereby decrease in quantity during subsequent beer storage. The results also suggest that (+) - catechin may not be present in substantial amounts as beer is stored but that there are probably other polyphenols present in beer which should be investigated further over shelf-life. A noticeable trend occurred in the beer beverages at day 30 and 60 was that (-) - Epicatechin, detectable at day 0, was not identifiable at later days of storage. This may have been due to an interaction with other polyphenols of differing polarities as the beer aged and possibly also a result of the methods of analyses that may have not been sensitive and highly selective in detecting the (-) - epicatechin content at day 30 and 60.

Table 7

Flavonoid Content [(+) - Catechin and (-) - Epicatechin Concentration] of the Four Non-Alcoholic and One Alcoholic Beer Beverages expressed in mg/L[1]

Beer Samples	(+) - Catechin (mg/L)			(-) - Epicatechin (mg/L)		
	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60
Beck`s®[2]	0.04 ± 0.00[A]	0.05 ± 0.02[A]	nd[4]	0.43 ± 0.32	nd	nd
Clausthaler®[2]	0.16 ± 0.00[A]	0.17 ± 0.02[A]	0.08 ± 0.00[A]	0.33 ± 0.02	nd	nd
Kaliber®[2]	0.27 ± 0.02[A]	0.20 ± 0.04[A]	0.12 ± 0.00[B]	0.77 ± 0.02	nd	nd
O'Doul's®[2]	0.58 ± 0.02[A]	0.53 ± 0.05[A]	0.52 ± 0.11[A]	0.88 ± 0.04	nd	nd
Leinenkugel`s®[3]	0.12 ± 0.00[A]	0.08 ± 0.00[A]	0.04 ± 0.00[B]	0.69 ± 0.02	nd	nd
Average	0.23 ± 0.20	0.20 ± 0.17	-	0.62 ± 0.25	-	-

Values followed by different upper case letters in each row are significantly different ($p < 0.05$), [1] Mean of three trials ± standard deviation (SD), [2] Non-alcoholic beer, [3] Alcoholic beer, [4]nd Concentration below the detection limit.

Walters et al. (1997b) compared (+) - catechin and ferulic acid as natural antioxidants and their impact as beer flavor stabilizers in extended storage trials (five months). The antioxidants (1 mM in 25 L of beer) were added to beer before filtration and packaging and the beers were stored at 25° C and 40° C. Both (+) - catechin and ferulic acid were separated and detected using HPLC with ultraviolet detection at 280 nm. Results indicated that (+) - catechin and ferulic acid decreased during storage and this was temperature dependent. The greatest loss rate occurred at 25° C and 40° C for (+) - catechin, thus suggesting that the temperature used in the current study (0° C) may have showed minimal losses of (+) - catechin in the beer beverages. The decrease of (+) - catechin could have been larger if elevated temperatures were used in the current study.

The levels of (+) - catechin content of the beer beverages (at day 0) in the current study agree with phenolic concentrations by other researchers in the literature. For instance, Garcia et al. (2004) determined the content of (+) - catechin in nine commercial non-alcoholic beer beverages from Spain and found that (+) - catechin ranged from 0.31 to 4.5 mg/L. In the present study, (+)-catechin ranged from 0.04 to 0.58 mg/L at day 0 in the non-alcoholic beer beverages, which suggests the validity of concentrations found in the current research as well as available methodology for this compound. Additionally, Madigan et al. (1994) showed that (+) - catechin concentration in lager beer was 2.7 mg/L; however, this lager beer was not evaluated for shelf-life stability. Even though this value is four times greater that the largest value found in the current study it has been postulated that (+) - catechin concentration may be dependent on the brewing process or the types of malt used and that results in the variation of concentrations in beer.

Table 8 shows the (+) - catechin and (-) - epicatechin content of the four non-alcoholic and one alcoholic beer beverages expressed in mg/340 mL (beer serving size), which indicate the concentration of flavonoids obtained from a typical beer serving size. The values for the (+) - catechin content ranged from 0.01 mg/340 mL to 0.2 mg/340 mL in the non-alcoholic beer at day 0. The (+) - catechin content ranged from 0.02 mg/340 mL to 0.18 mg/L up to 30 days and up to 60 days the range was from not detectable to 0.17 mg/340 mL. This illustrates a decrease in the (+) - catechin content in a typical beer serving as shown by the range of (+) - catechin concentrations in the non-alcoholic beer beverages over the 60 days storage. A similar pattern was observed in the alcoholic beer Leinenkugel`s® in that (+) - catechin decreased from initially at 0.04 mg/340 mL (day 0) to 0.01 mg/340 mL (day 60). (-) - Epicatechin which was observed only at day 0 ranged from 0.11 mg/340 mL to 0.30 mg/340 mL in the non-alcoholic beer and was 0.23 mg/340 mL in the alcoholic beer beverage Leinenkugel`s®. The values for the flavonoid content shown in Table 8 may alert consumers to the concentrations available in a typical beer serving and that would also indicate to the consumer how many beer beverages they may need to consume if greater health benefits are to be attained. The quantities of the flavonoids present per beer serving may not contribute significantly to the health benefits from beer.

Table 8

Flavanoid Content: [(+) - Catechin and (-) - Epicatechin Concentration] of the Four Non-Alcoholic and One Alcoholic Beer Beverages expressed in mg/340 mL (beer serving size) [1].

Beer Samples	(+) - Catechin (mg/340 mL)			(-) - Epicatechin (mg/340 mL)		
	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60
Beck`s®[2]	0.01 ± 0.00[A]	0.02 ± 0.00[A]	nd[4]	0.15 ± 0.10	nd	nd
Clausthaler®[2]	0.05± 0.00[A]	0.06 ± 0.00[A]	0.03 ± 0.00[A]	0.11 ± 0.00	nd	nd
Kaliber®[2]	0.09 ± 0.00[A]	0.07 ± 0.010[A]	0.04 ± 0.00[B]	0.26 ± 0.00	nd	nd
O'Doul's®[2]	0.20 ± 0.00[A]	0.18 ± 0.02[A]	0.17 ± 0.04[A]	0.30 ± 0.01	nd	nd
Leinenkugel`s®[3]	0.04 ± 0.00[A]	0.03± 0.00[A]	0.01 ± 0.00[B]	0.23± 0.00	nd	nd
Average	0.05 ± 0.07	0.04 ± 0.06	-	0.12 ± 0.08	-	-

Values followed by different upper case letters in each row are significantly different ($p < 0.05$),[1] Mean of three trials ± standard deviation (SD), [2] Non-alcoholic beer, [3] Alcoholic beer, [4]nd= Concentration below the detection limit.

The decrease in individual polyphenols is also reported by Jandera et al. (2005) who evaluated the change in beer antioxidants at different stages in brewing during storage. The study analyzed experimental samples of sweet wort, hopped wort,

fresh beer, and aged beer (storage duration for the aged was not reported). Results indicated that the concentrations of antioxidants such as (+) - catechin and (-) - epicatechin were approximately constant or slightly increased during the beer brewing process. However, the antioxidant concentrations decreased during storage as was evident in the aged beer. For instance, (+) - catechin decreased from an initial value 1.80 mg/L to 1.46 mg/L after an undisclosed storage, and (-) - epicatechin decreased from initially at 0.76 mg/L to 0.47 mg/L after storage. This is comparable to the results in this study, since for example Kaliber® non-alcoholic beer had the (+) - catechin concentration decrease from 0.27 mg/L initially to 0.12 mg/L up to 60 days storage, and Leinenkugel`s® alcoholic beer had an initial (+) - catechin concentration of 0.12 mg/L and then decreased to 0.04 mg/L up to day 60. This suggests that the decrease in the flavonoid, (+) - catechin may be expected in stored beer because another source in current literature reports a decrease in (+) - catechin over an undisclosed storage time and temperature. Therefore, consumers may expect a decrease in (+) - catechin over refrigerated storage although the total polyphenols were shown to increase and the beer would still be good to consume.

Figure 9 shows that O'Doul's® non-alcoholic beer (0.58 mg/L) was significantly greater in the (+) - catechin than all the beer beverages when the means of the (+) -catechin content of the beer beverages were compared at day 0. Kaliber® (0.27 mg/L) was significantly greater than Beck`s® (0.04 mg/L), Clausthaler® (0.16 mg/L), and Leinenkugel`s® (0.12 mg/L). This implies that O'Doul's® non-alcoholic beer had the greatest content of the flavonoid which may also explain that it would be more preferable to consume the beverage and obtain more beneficial antioxidants compared to the other beer beverages. Also, Leinenkugel`s®, alcoholic beer which had the greatest total polyphenol was surprisingly lower in the (+) - catechin content. The explanations for the low (+) - catechin content in Leinenkugel`s® were not reviewed in the current study. Beck`s®, non-alcoholic beer had the lowest level of (+) - catechin and also had a total polyphenol level that was significantly lower than the other beer beverages at day 0. This suggests that Beck`s® may contain the least amount of beneficial phytonutrients when compared to the other beer beverages in the current study. Health wise, Beck`s® non-alcoholic beer would not contribute as much as other beer beverages in the current study

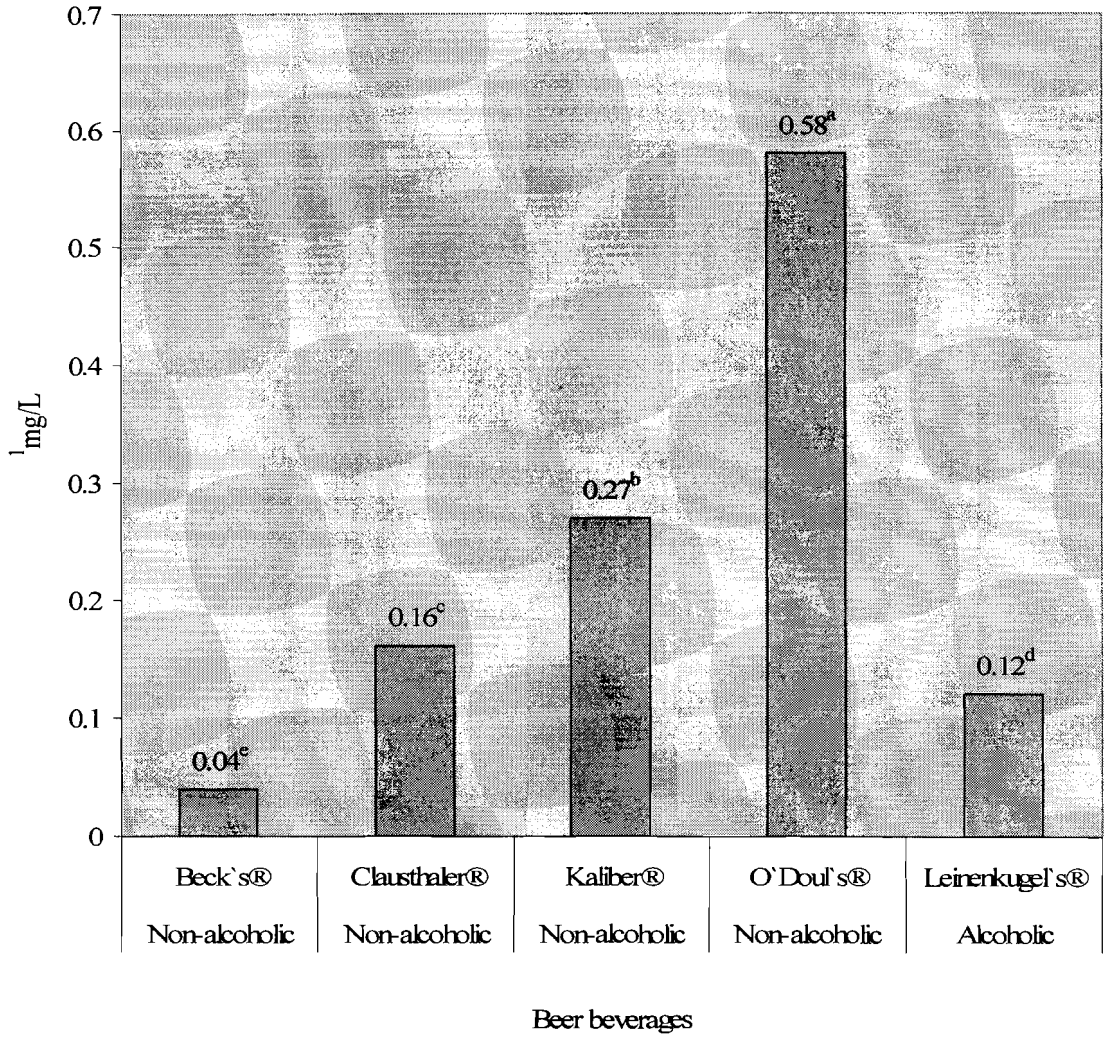


Figure 9. Average (+) – catechin content (mg/L) in the beer beverages at day 0. Values followed by different lower case letters are significantly different, ($p<0.05$). [1]milligram per liter (+) – catechin.

Figure 10, shows the comparison of means of the beer beverages at day 30 using the Student t-test. O'Doul's® non-alcoholic beer beverage (0.53 mg/L) had a significantly greater (+) - catechin content than all the other beer beverages. The (+) - catechin content of Kaliber® (0.20 mg/L) and Clausthaler® (0.17 mg/L) were significantly greater than Leinenkugel`s® (0.08 mg/L) and Beck`s beer® (0.05 mg/L). Overall up to 30 days of beer storage, O'Doul's® non-alcoholic beer beverage had the greatest (+) - catechin content in comparison to all the other beer beverages and this is expected because initially at day 0 this beverage had the greatest content of the flavonoid. This may also indicate that the level of (+) - catechin in O'Doul's® did not decrease more than the other beverages. Leinenkugel`s®, alcoholic beer was still significantly lower than the non-alcoholic beer beverages O'Doul's®, Kaliber® and Clausthaler®, the same trend being shown at day 0. Beck`s®, did not significantly change up to 30 days suggesting that the beer had the same health benefits from (+) – catechin as at day 0.

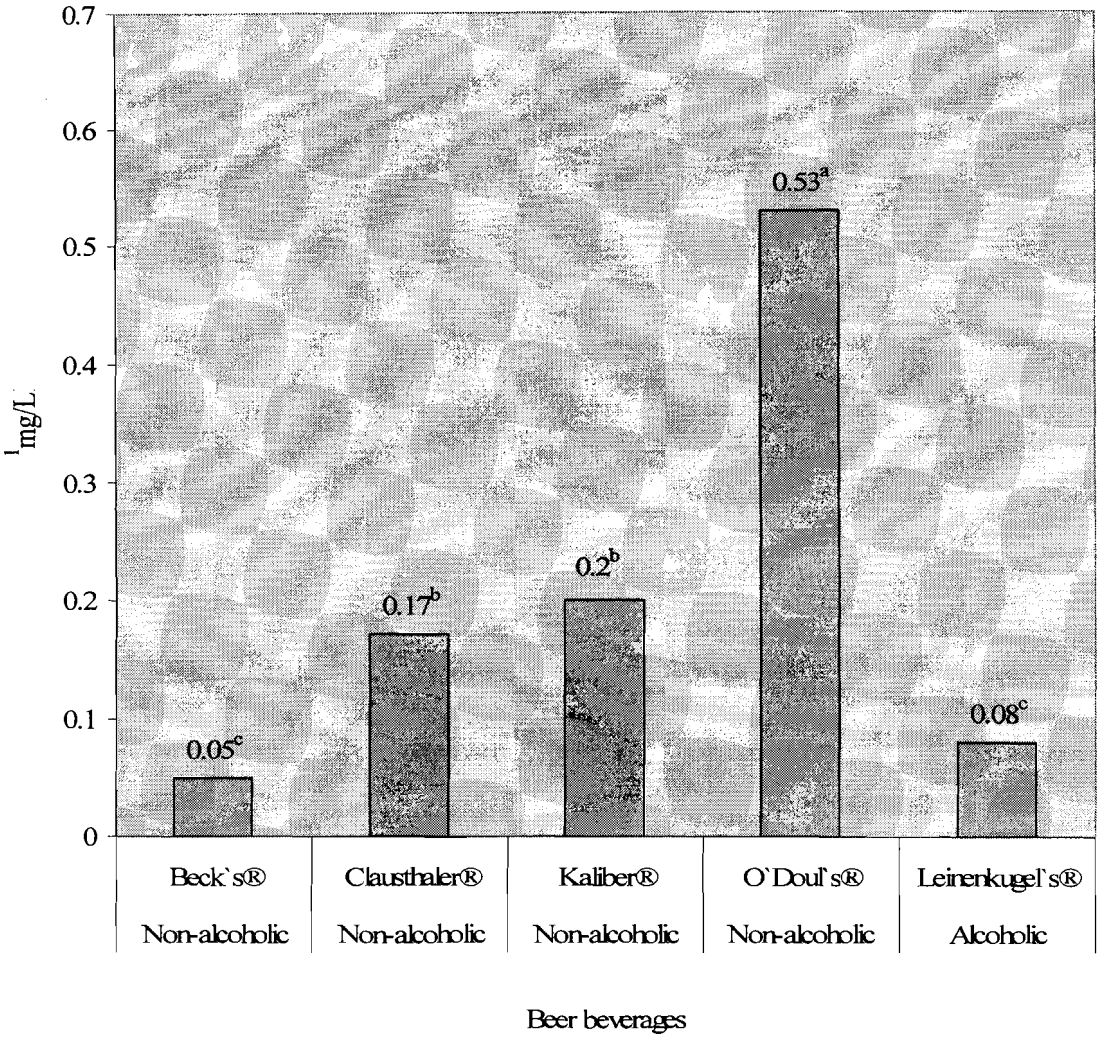


Figure 10. Average (+) – catechin content (mg/L) in the beer beverages at day 30. Values followed by different lower case letters are significantly different, ($p<0.05$). [1]milligram per liter (+) – catechin.

Figure 11 shows that at day 60, O'Doul's® (0.52 mg/L) was significantly greater than all the beer beverages and Kaliber® (0.12 mg/L), Clausthaler® (0.08 mg/L), Leinenkugel`s® (0.04 mg/L) were not significantly different from each other although Kaliber® had a slightly greater (+) - catechin content than Clausthaler® and Leinenkugel`s® beer beverages. The results suggest that the (+) - catechin content up to 60 days in the beer beverages Kaliber®, Clausthaler® and Leinenkugel`s® may have decreased at the same rate. However up to day 30, the non-alcoholics, Kaliber® and Clausthaler® were significantly greater than Leinenkugel`s®, it might have been expected for the trend to be kept up to day 60 but this observation may suggest that (+) - catechin in the non-alcoholics, Kaliber® and Clausthaler® may have depleted more than Leinenkugel`s®, as storage continued up to 60 days. O'Doul's® had a greater quantity of the flavonoid initially compared to the other beer beverages, therefore it might be expected for the beer to continue being significantly greater and may be healthier to consume than the other beer beverages.

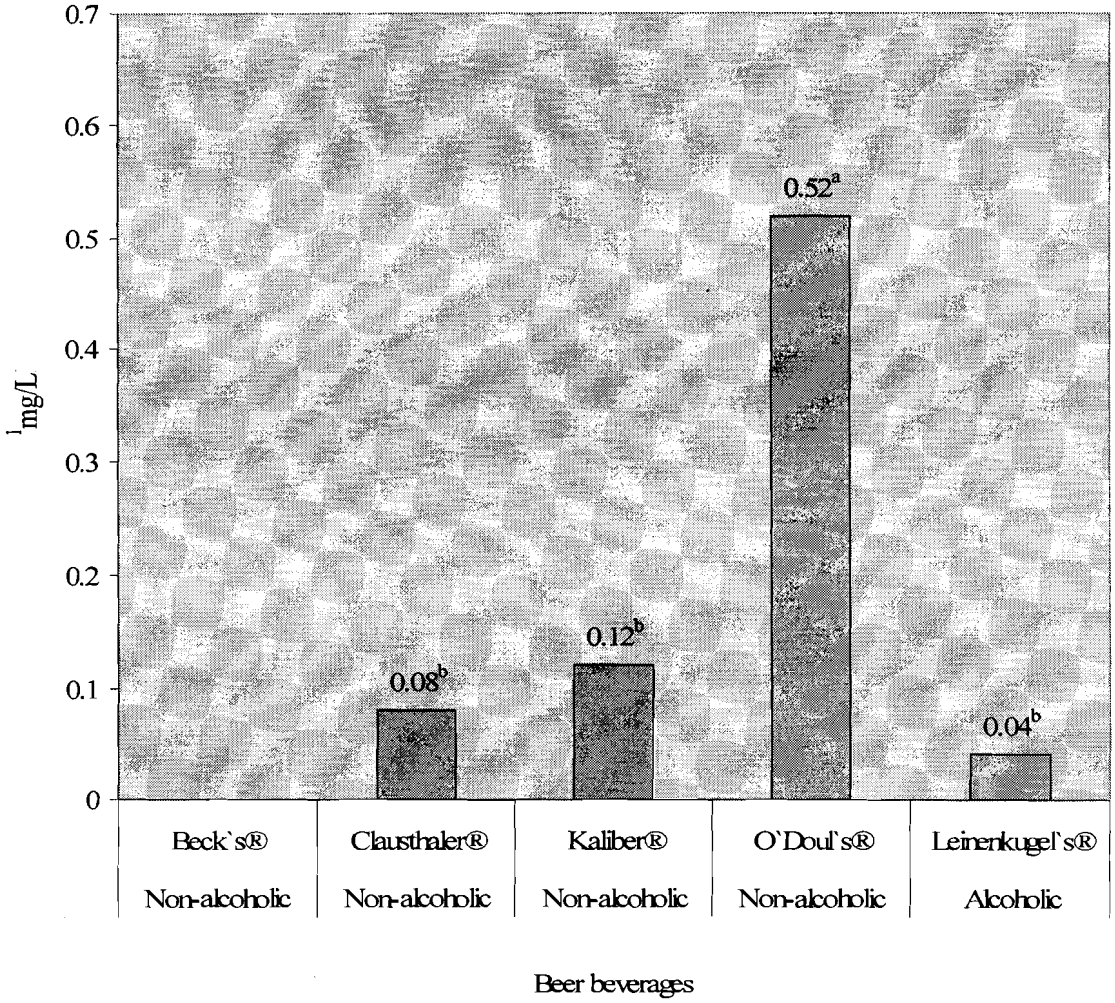


Figure 11. Average (+) – catechin content (mg/L) in the beer beverages at day 60. Values followed by different lower case letters are significantly different, ($p<0.05$). [1]milligram per liter (+) – catechin.

Figure 12 indicates the comparison of means of (-) - epicatechin content in the beer beverages at day 0 and shows that the flavonoid was significantly greater in O'Doul's® (0.88 mg/L) and Kaliber® (0.77 mg/L) than the other non-alcoholic beer beverages Beck`s® (0.43 mg/L) and Clausthaler® (0.33 mg/L). Leinenkugel`s®, alcoholic beer (0.69 mg/L) was significantly greater than Clausthaler®. Therefore, the non-alcoholic beer beverages with the greatest health benefits in terms of the flavonoid (-) - epicatechin are O'Doul's® and Kaliber®. The component (-) - epicatechin varied in the beer beverages, this might be as a result of the different types of a malts which were used in brewing the beers. Malts from various barley species differ in their flavonoid content and for the beer beverages O'Doul's® and Kaliber®, probably a malt containing greater levels of (-) - epicatechin was used in the brewing process (Shahidi & Naczk, 1995).

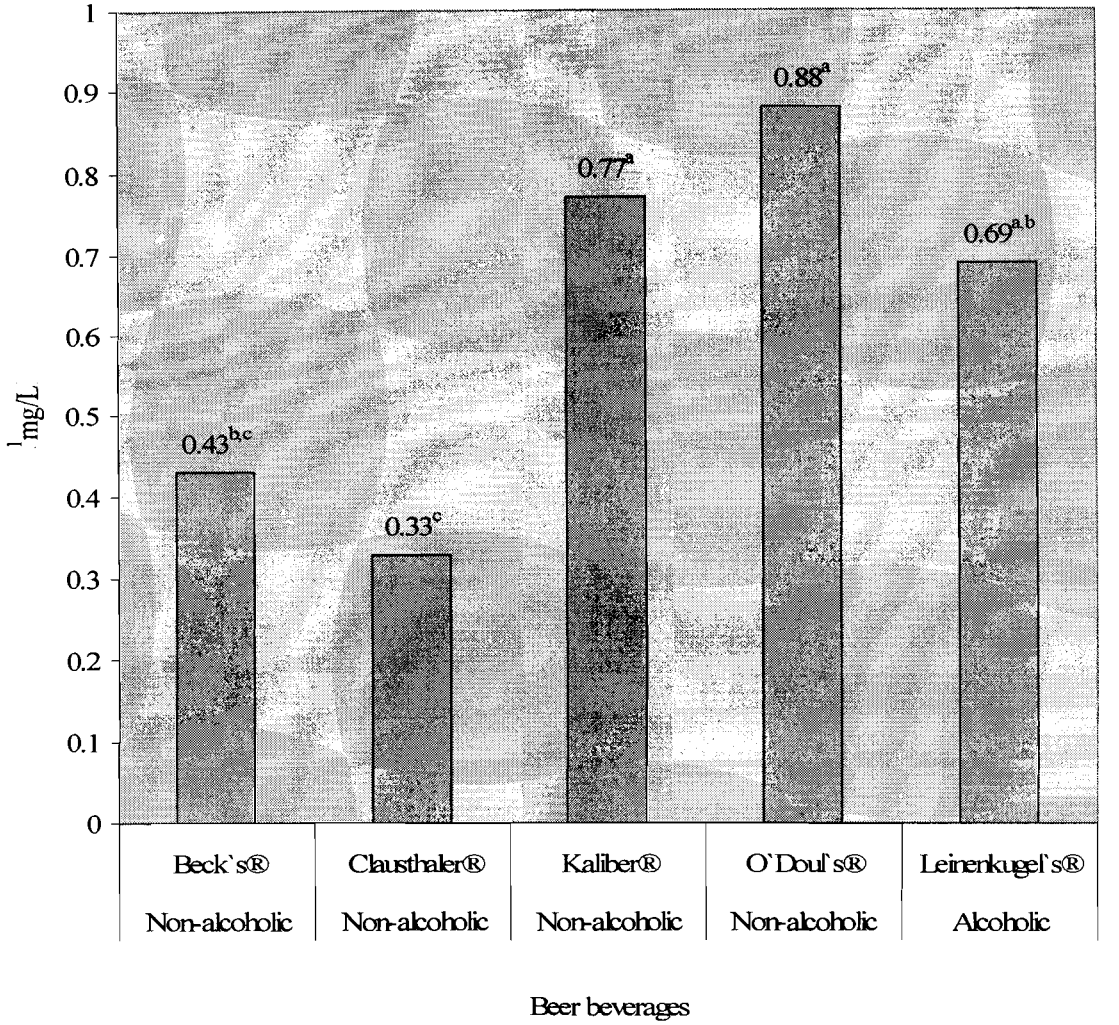


Figure 12. Average (-) - epicatechin content (mg/L) in the beer beverages at day 60. Values followed by different lower case letters are significantly different, ($p<0.05$). [1]milligram per liter (-) - epicatechin.

Chapter V: Summary and Conclusions

The current study aimed at evaluating the effect of household storage, 32° F (0° C), on the composition of phytonutrients, total polyphenols and flavonoids [(+) - catechin and (-) - epicatechin] contained in non alcoholic versus alcoholic beer beverages and also determine the concentrations of phytonutrients in non-alcoholic beer. It is already known that beer has beneficial phytonutrients such as (+) - catechin and (-) - epicatechin, and that they mainly arise from the raw materials used in the brewing process. Additionally, limited research has shown that certain phytonutrients in beer change over refrigerated storage. It has not been determined however if all non- alcoholic beer beverages change similarly in their content of phytonutrients over a storage duration. The beer phytonutrients were studied at day 0, 30 and 60 and the analyses were carried out in triplicates for each beer beverage type. The beer beverages under study included four non-alcoholic types (Beck`s®, Clausthaler®, Kaliber® and O'Doul's®). The alcoholic beer type was Leinenkugel`s® Creamy Dark Lager.

Overall, it was found that there was a significant increase in the total polyphenols in the alcoholic beer beverage, Leinenkugel`s® after 30 days. However, no significant change was noted in any non-alcoholic beer beverage after 30 days of storage. Although after 60 days of refrigerated storage, there was a significant increase in the total polyphenol content of all the beverages except for Beck`s® (non-alcoholic beer) and Leinenkugel`s® (alcoholic beer). This finding indicates that the beer phytonutrients may have continued to form during storage in all the beer beverages. For example O'Doul's® non-alcoholic beer had a total polyphenol content of 485 mg/L at day 0 and 505 mg/L after 60 days. The increase indicates that if O'Doul's® non-alcoholic beer is consumed after 2 months of refrigerated storage, greater health benefits may be attained than if consumed at day 0. The results also showed that the dark lager beer,

Leinenkugel`s® had greater total polyphenol content than the other beer beverages. The darker beer beverage indicates more phytonutrients and greater health benefits. Therefore, in order to get more beneficial beer phytonutrients from the non-alcoholic beer types, O'Doul's® may be a preferred beverage to consume because the beer was darker than the other non-alcoholic types. The study showed that storing beer at household refrigerated temperatures does not destroy the beneficial phytonutrients and therefore consumers may still enjoy health benefits from consuming stored non-alcoholic beer. In addition, non-alcoholic beer beverages were also shown to contain significant levels of beneficial phytonutrients as alcoholic beer such as in O'Doul's®, suggesting that people who have restricted their intake of alcoholic beverages or who do not prefer to consume alcohol may drink the non-alcoholic beer and attain similar benefits from the phytonutrients.

The (+) - catechin content generally decreased in all the beer beverages at 60 days of refrigerated storage. At day 60, the (+) - catechin content could not be detected in Beck`s® non-alcoholic beer indicating that this phytonutrient had decreased to below the detection limit. O'Doul's® non-alcoholic beer had the greatest (+) - catechin content than all the beer beverages which may be as a result of the malt used in the brewing process because barley malt also has an influence on the (+) - catechin concentration in beer. (-) -Epicatechin was detected at day 0 and could not be identified in the beer beverages at day 30 and 60. This may have been due to interaction with other polyphenols of differing polarities as the beer aged and possibly the methods of analyses may have not been sensitive and highly selective in detecting the (-) - epicatechin content at day 30 and 60.

The decrease in the flavonoid, (+) - catechin after 60 days of refrigerated storage might be as a result of the flavonoid acting as antioxidants in beer flavor stability during beer storage.

During beer storage, staling of beer may occur as a result of oxidation and this is a free radical process in which molecular oxygen is converted to reactive oxygen species which oxidizes beer components into free radicals. The free radicals in beer react further, resulting in the formation of carbonyl compounds such as aldehydes and ketones. The carbonyl compounds give rise to the cardboard-like flavor of stale beer. Therefore (+) - catechin may display its antioxidant properties in beer by terminating the free radical formation process thereby preventing the formation of off-flavor compounds and beer flavor stability is increased. Therefore, it might be expected for beer flavonoids such as (+) - catechin to depreciate with storage although other beer polyphenols may increase. The results also suggest that, (+) - catechin may not be present in substantial amounts as beer ages and that there are other polyphenols present in beer which may be studied over shelf-life. Therefore, further research on the current project could look at ways in which other beer polyphenols may be analyzed.

Recommendations Based on the Determination of Total Polyphenols Content

The analysis of total polyphenol content was done by means of the Folin- Ciocalteu reagent and the method has been used in several studies for evaluating total phenolic content of beer. The total polyphenols were reported as mg gallic acid equivalents per liter which is the commonly used standard. It will be important to measure the polyphenols using (+) - catechin as the standard and compare if the same results can be obtained. (+) - Catechin is the second most used standard after gallic acid and can be advantageous in comparing flavonoid partitioning in samples.

The Folin-Ciocalteu method used in this study was an alkaline method and it took more than two hours to perform the two-step analysis and prepare the reagents used in the experiment. It would be recommended to use a faster Folin procedure in order to minimize error and reduce the time required for the analysis (Vinson et al., 2001). This can be achieved by using a commercially available Folin-Ciocalteu reagent that contains phosphoric acid and is done under acidic conditions in which polyphenols are stable (Vinson et al., 2001). It will also be important to evaluate free polyphenols in beer beverages. In addition, there are interferences that might occur when measuring total polyphenols. For example, sulfites, used as beer stabilizers are interfering substances. Interferences by sulfites may be removed by adjusting the pH of beer to 3.0 using acetate buffer and adding acetaldehyde (Vinson et al., 2001). This was not done in this study and further research can determine if interferences by sulfites occur when measuring total polyphenols in non-alcoholic beer.

Recommendation Based on the Determination of (+) - Catechin and (-) - Epicatechin Content

The HPLC method for the analyses of the flavonoids could detect (-) - epicatechin at day 0. At day 30 and 60, it became increasingly difficult to identify (-) - epicatechin. This might have been as a result of polymerization of individual polyphenols in the beer which needed sensitive and selective methods to detect the peaks. For example, using a multi channel CoulArray detector, which is compatible with gradient elution (enables separation of different compounds in a single run), would be an important step to take in the analyses of beer components (Jandera et al., 2005).

More importantly, using a sensitive and selective detector such as mentioned above, requires no special sample pretreatment (Jandera et al., 2005). In this study a photodiode-array detector was used with gradient elution but the polyphenols had to be extracted from the beer using a 500- mg L-C18 Sep-Pak® cartridge. Another method of analyses to employ will be using a more non polar organic solvent to elute extracted polyphenols from the cartridge, such as acetonitrile and compare results (Jandera et al., 2005). Methanol was used in this study because it is a good solvent to use for extracting polyphenols of low degree of polymerization such as (+) - catechin and (-) - epicatechin.

There are other polyphenols that are formed during the storage as evidenced by the increase in total polyphenol content in the beer beverages. It will be important to analyze the different polyphenols that may be formed during storage. This can be done by studying different beer polyphenols retention times, running the standards and determining if any of the beer polyphenols will be matching the standards.

Recommendation Based on the Shelf-Life Study

The shelf-life study was done using beer beverages stored at 32° F. Further research could study the stability of the phytonutrients using ambient room temperature over 60 days. During storage the flavor of the beer changes and further research could evaluate through sensory evaluation the flavor characteristics in the non-alcoholic beer over shelf-life.

References

Ames, B. N., Shigenga, M. K., & Gold, L. D. (1993). DNA lesions inducible DNA repair and cell division: The three key factors in mutagenesis and carcinogenesis. *Environmental Health Perspective, 101,* 35-44.

American Heart Association. (2003). *Heart attack and angina statistics*. Retrieved March 23, 2006 from http://www.americanheart.org/presenter.

Bamforth, C. W. (2004). *Beer: Health and nutrition.* Oxford, UK: Blackwell Science.

Bors, W., Heller, W., Michel, C., & Saran, M. (1990). Flavonoids as antioxidants, determination of radical scavenging efficiencies. *Methods in Enzymology, 186*, 343-355.

Bourne, L., Paganga, G., Baxter, D., Hughes, P., & Rice-Evans, C. (2000). Absorption of ferulic acid from low-alcohol beer. *Free Radical Research, 32(3),* 273-280.

Bravo, L. (1998). Polyphenols: Chemistry, dietary sources, metabolism and nutritional significance. *Nutritional Reviews, 56,* 317-333.

Denke, M. A. (2000). Nutritional and health benefits of beer. *The American Journal of Medical Sciences, 320*(5), 320-326.

Ferguson, L. R. (2000). Role of plant polyphenols in genomic stability. *Mutation Research, 47,* 89-111.

Garcia, A. A., Grande, C. B., & Gandara, S. J. (2004). Development of a rapid method based on solid phase extraction and liquid chromatography with ultraviolet absorbance detection for the determination of polyphenols in alcohol-free beers. *Journal of Chromatography A, 1054*, 175-180.

Gasowski, B., Leontowicz, M., Leontowicz, H., Katrich, E., Lojek, A., Ciz, M., et al. (2004). The influence of beer with different antioxidant potential on plasma lipids, plasma antioxidant capacity, and bile excretion of rats fed cholesterol-containing and cholesterol free- diets. *Journal of Nutritional Biochemistry, 15,*527-533.

Ghiselli, A., Natella, F., Guidi, A., Montanari, L., Fantozzi, P., & Scaccini, C. (2000). Beer increases plasma antioxidant capacity in humans. *Journal of Nutritional Biochemistry, 11*, 76-80.

Gorinstein, S., Caspi, A., Zemser, M., & Trakhtenberg, S. (2000). Comparative contents of some phenolic in beer, red and white wines. *Nutrition Research, 20,* 131-139.

Gorinstein, S., Zemser, M., Weiz, M., Haruenkit, R., & Trakhtenberg, S. (1998a). The influence of dry matter of different alcoholic beverages on lipids, proteins, and antioxidant activity in serum of rats. *Journal of Nutritional Biochemistry. 9,*131-135.

Gorinstein, S., Zemser, M., Weiz, M., Halevy, S., Martin-Belleso, O., & Trakhtenberg, S. (1998b). The influence of alcohol-containing and alcohol-free beverages on lipid levels and lipid peroxides in serum rats. *Journal of Nutritional Biochemistry, 9,* 682-686.

Gorinstein, S., Zemser, M., Lichman, I., Berebi, A., Kleipfish, A., Libman, I., et al. (1997). Moderate beer consumption and blood coagulation in patients with coronary artery disease. *Journal of Internal Medicine, 241*, 47-51.

Hertog, M. G. L., Kromhout, D., Aravanis, C., Blackburn, H., Buzina, R., Fidanza, F., et al. (1995). Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Archive in Internal Medicine, 155,* 381-386.

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

Hosokawa, N., Hosokawa, Y., Sakai, T., Yoshida, M., Marui, N., Nishino, H., et al. (1990). Inhibitory effect of quercetin on the synthesis of a possibly cell- cycle related 17-kDa protein, in human colon cancer cells. *International Journal of Cancer, 45*, 1119-1124.

Jandera, P., Skerikova,V., Rehova, L., Hajek, T., Baldrianova, L., Skopova, G. et al. (2005). RP-HPLC analysis of phenolic compounds and flavonoids in beverages and plants extracts using a CoulArray dectector. *Journal of separation science, 2,* 1005-1022.

Kondo, K. (2004). Beer and health: Preventive effects of beer components on lifestyle – related diseases. *Biofactors, 22,* 303-310.

Lugasi, A. (2003). Polyphenol content and antioxidant properties of beer. *Acta Alimentaria, 32*(2), 181-192.

Lugasi, A., & Hovari, J. (2003). Antioxidant properties of commercial alcoholic and non-alcoholic beverages. *Nahrung/Food, 47*, 79-86.

Lapcik,O., Hill, M., Hampl, R., Wahala, K., & Adlercreutz, H. (1998). Identification of isoflavonoids in beer. *Steroids, 63*, 14-20.

Madigan, D., McMurrough, I., & Smyth, M. R. (1994). Determination of proanthocyanidins and catechins in beer and barley by high lipid performance liquid chromatography. *The Analyst, 119*, 863-868.

McMurrough, I., Madigan, D., Kelly, R. J., & Smyth, M. R. (1996). The role of flavanoid polyphenols in beer stability. *Journal of the American Society of Brewing Chemists, 54*, 141-148.

Miranda, C. L. , Stevens, J. F., Ivanov, V., McCall, M., Frei, B., Deinzer, M. L. et al. (2000). Antioxidant and prooxidant actions of prenylated and nonprenylated chalcones and flavanones *in vitro. Journal of Agriculture Food Chemistry, 48*, 3876-3884.

Miranda, C. L., Stevens, J. F., Helmrich, A., Henderson, M. C., Rodriguez, R. J., Yang, Y. H. et al. (1999). Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (*humulus lupulus*) in human cancer cell lines. *Food and Chemical Toxicology, 37*, 271-285.

Merriam-Webster Online Dictionary. (n. d.). Retrieved November, 30, 2006 from http://www.m-w.com/

Moll, M. (1991). *Beer and coolers*: *Including low alcohol and non-alcoholic beers.* England: Intercept Ltd.

New Scientist. (2005). *Mystery compound found in beer fights cancer.* 185(2483), 17. Retrieved October 4, 2005, from: Ebsco Host Database.

Plumb, G. W., De Pascual-Teresa, S., Santos-Buelga, C., Cheynier, V., & Williamson, G. (1998). Antioxidant properties of catechins and procyanthocyanidins: Effect of polymerisation, galloylation and glycosylation. *Free Radical Research, 29,* 351- 358.

Rice-Evans, C. A., Miller, N. J., Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free radical biology and medicine. 20*, 933-956.

Rivero, D., Perez-Magarino, S., Gonzalez-Sanjose, G., Valls-Belles, V., Codener, P., & Muniz, P. (2005). Inhibition of induced DNA oxidative damage by beers: Correlation with the content of polyphenols and melanoidins. *Journal of Agricultural Food Chenistry, 53,* 3637-3642.

Robertson, G. L. (1993). *Food packaging: Principles and practice*. New York: Marcel Dekker.

Ross, J. A., & Kasum, C. M. (2002). Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Annual Review Nutrition, 22*, 19-34.

Shahidi, F., & Naczk, M. (1995). Food phenolics: Sources, chemistry, effects and applications. Lancaster, PA: Technomic Publishing Company.

Scalbert, A., & Williamsom, G. (2000). Dietary intake and bioavailability of polyphenols. *Journal of Nutrition, 130*, 2073S-2085S.

Singleton, V. L., Orthofer, R., Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu Reagant. *Methods in Enzymology, 299*, 153-178.

Singleton, V. L., Rossi, J. A. (1965). Colometric of total phenolis with phosphomolibdic-phosphtungistic acid reagent. *American Journal of Enology and Viticulture, 16*, 144-158.

Stevens, J. F., & Page, J. E. (2004). Xanthohumol and related prenylflavonoids from hops and beer: To your good health. *Phytochemistry, 65,* 1317-1330.

Stevens, J. F., Miranda, C. L., Wolthers, K. R., Schimerlik, M., Deinzer, M.L., & Buhler, D. R. (2002). Identification and *in vitro* biological activities of hop proanthocyanidins Inhibition of nNOS activity and scavenging of reactive nitrogen species. *Journal of Agricultural and Food Chemistry, 50,* 3435-3443.

Yoshida, M., Sakai, T., Hosokawa, N., Marui, N., Matsumoto, K., Fujioka, A., et al. (1990). The effect of quercetin on cell cycle progression and growth of human gastric cancer cells. *FEBS letters, 260*, 10-13.

Uddin, S., & Choudhry, M. A. (1995). Quercetin, a bioflavonoid, inhibits the DNA synthesis of human leukemia cells. *Biochemistry and molecular biology international, 36*, 545-550.

Vinson, J. A., Mandarano, M., Hisrt, M., Trevithick, J. R., & Bose, P. (2003). Phenol antioxidant quantity and quality in foods: Beers and the effect of two types of beer on an animal model of atherosclerosis. *Journal of Agricultural Food Chemistry, 51,* 5528-5533.

Vinson, J. A., Proch, J., & Bose, P. (2001). Determination of quantity and quality of polyphenol antioxidants in foods and beverages. *Methods in Enzymology, 335,*103-114.

Walters, M. T., Heasman, A. P., & Hughes, P. S. (1997a). Comparison of (+) - catechin and ferulic acid as natural antioxidants and their impact on beer flavor stability. Part 1: Forced-aging. *Journal of the American Society of Brewing Chemists, 55*(2), 83-89.

Walters, M. T., Heasman, A. P., & Hughes, P. S. (1997b).Comparison of (+) - catechin and ferulic acid as natural antioxidants and their impact on beer flavor stability. Part 2: Extended storage trials. *Journal of the American Society of Brewing Chemists, 55*(3), 91-98.

Wikipedia, the free encyclopedia. (n. d). Retrieved November, 30, 2006 from http://en.wikipedia.org/wiki/Wikipedia

Appendices

The data and standard curves from the test for total polyphenol (TPP) content are shown in Appendix A through Appendix E. For the flavonoid content, measurements used for standard curves are shown in Appendix F through Appendix G. The chromatograms for the standards; (+) - catechin and (-) - epicatechin, O'Doul's® non-alcoholic beer and Leinenkugel`s® alcoholic beer at day 0 are shown in Appendix H through Appendix J and data is shown in Appendix K through Appendix S.

Appendix A

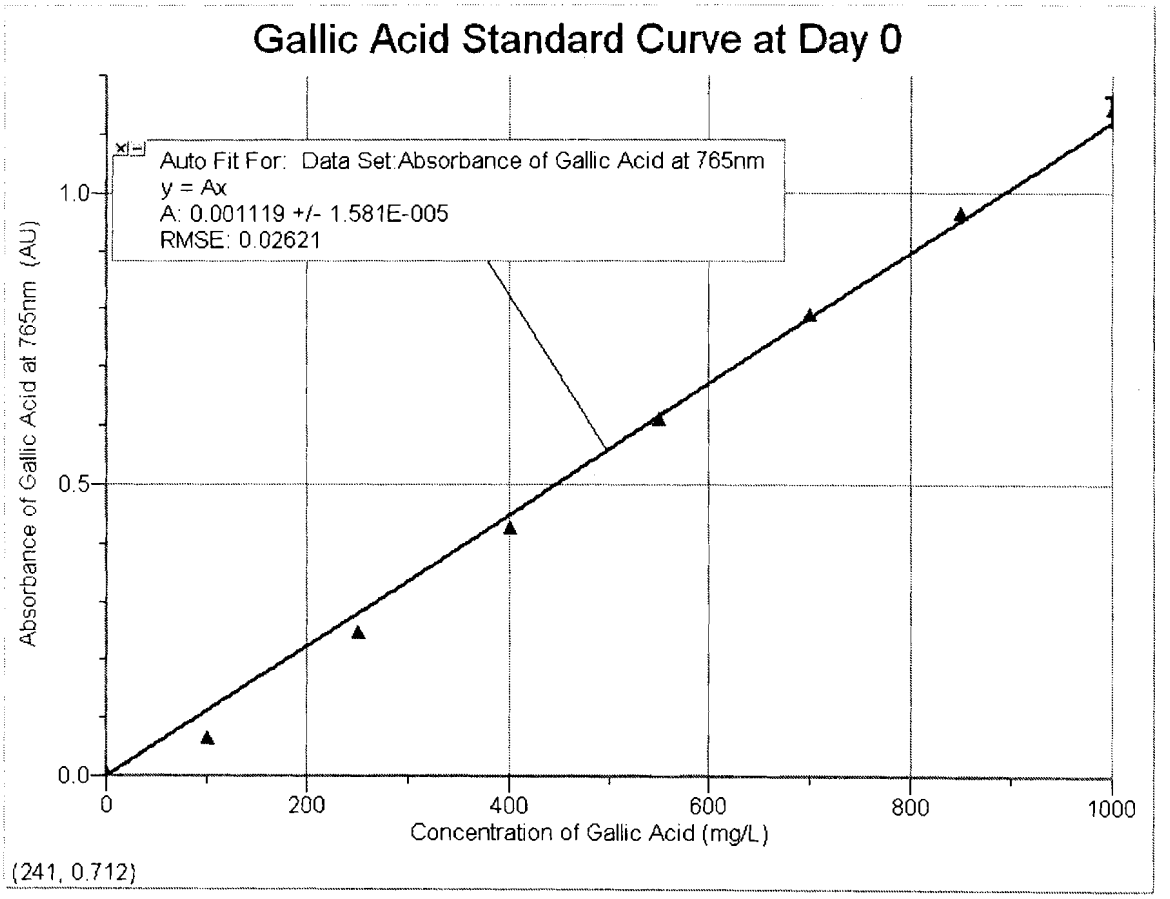
Gallic Acid Concentration and Absorbance Measurements Used for the Standard Curves

Gallic Acid Concentration and Absorbance Measurements used for the Standard Curves

Gallic acid concentration (mg/L)	Absorbance(AU) at day 0	Absorbance(AU) at day 30	Absorbance(AU) at day 60	Mean ± SD
100	0.0628	0.0565	0.0641	0.0611 ± 0.00
250	0.2444	0.2245	0.2373	0.2354 ± 0.01
400	0.4232	0.4124	0.4235	0.4197 ± 0.01
550	0.6069	0.5811	0.5991	0.5957 ± 0.01
700	0.7864	0.7617	0.7606	0.7696 ± 0.01
850	0.9605	0.9113	0.9265	0.9328 ± 0.03
1000	1.1369	1.0898	1.0914	1.1060 ± 0.03

Appendix B

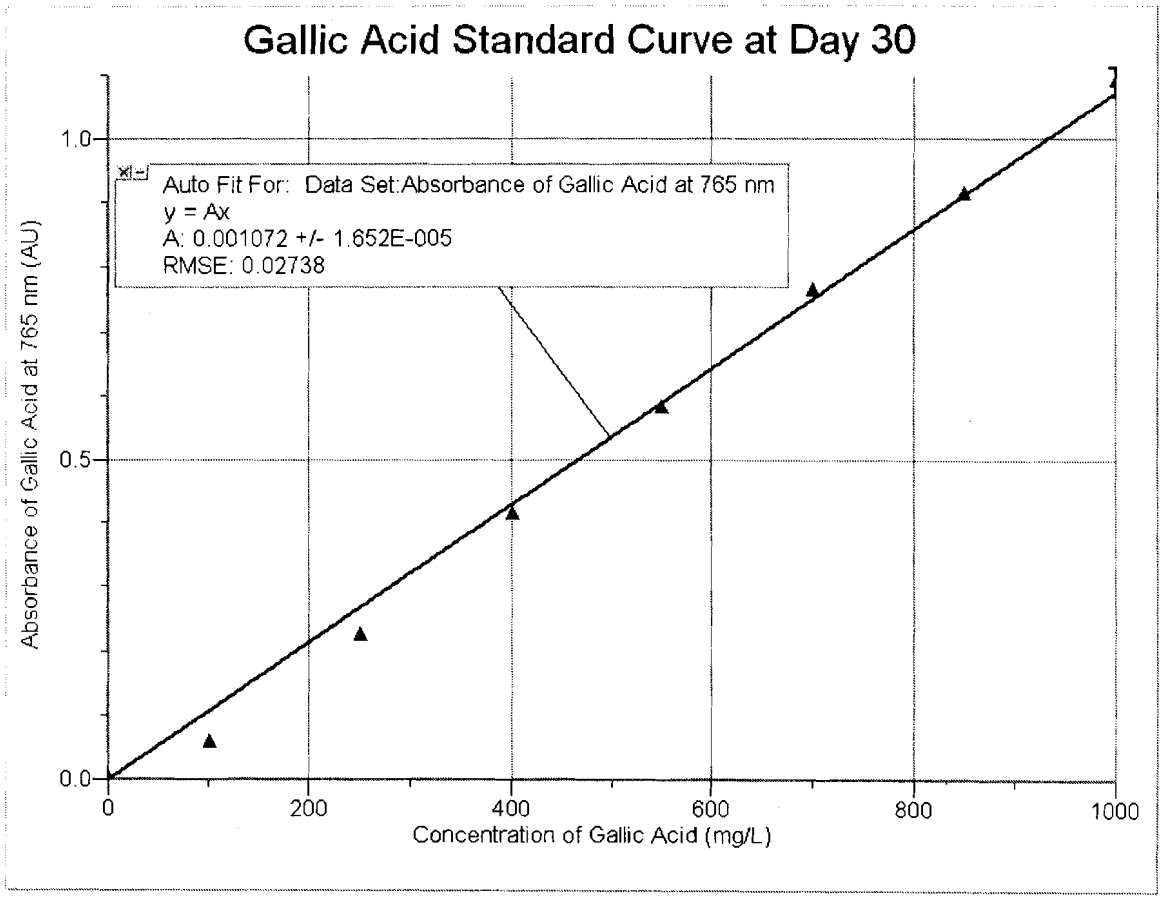
Standard Curve for Total Polyphenol Content of Beer Beverages at Day 0



Standard Curve for Total Polyphenol Content of Beer beverages at Day 0.

Appendix C

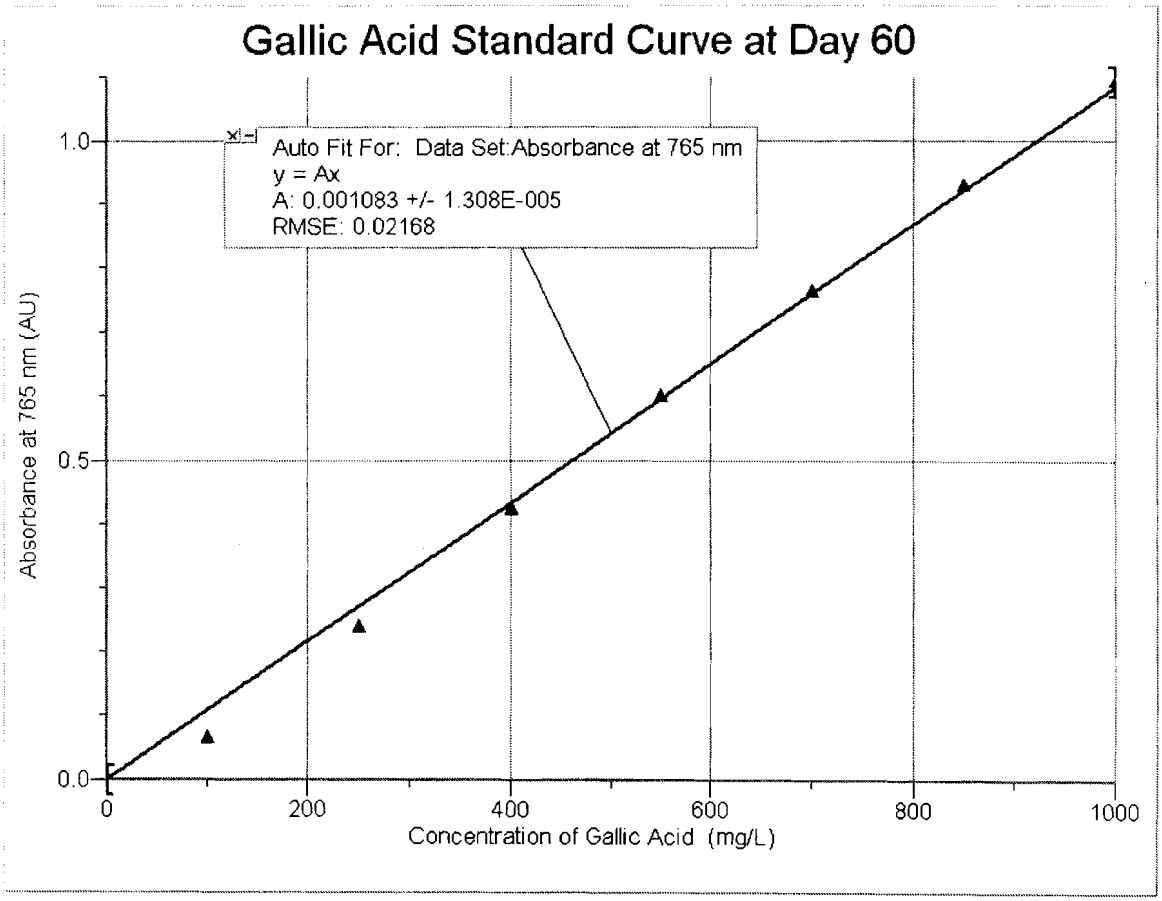
Standard Curve for Total Polyphenol Content of Beer Beverages at Day 30



Standard Curve for Total Polyphenol Content of Beer Beverages at Day 30.

Appendix D

Standard Curve for Total Polyphenol Content of Beer Beverages at Day 60



Standard Curve for Total Polyphenol Content of Beer Beverages at Day 60.

Appendix E

Total Polyphenol Content (TPP) Measurements of Beer Beverages at Day 0, 30 and 60

Beer beverage	Replicates	TPP (mg/L) at day 0	TPP (mg/L) at day 30	TPP (mg/L) day 60
Beck`s® Non-alcoholic	1	243	234	251
	2	227	232	244
	3	217	233	251
	Mean ± SD	229 ± 13	233 ± 1	249 ± 4
Clausthaler® Non-alcoholic	1	273	276	279
	2	274	275	279
	3	266	275	285
	Mean ± SD	271± 4	275± 1	281± 3
Kaliber® Non-alcoholic	1	276	268	279
	2	259	261	286
	3	259	264	285
	Mean ± SD	265 ± 10	264 ± 4	283 ± 4
Leinenkugel`s® Alcoholic	1	619	675	662
	2	632	668	678
	3	615	665	664
	Mean ± SD	622 ± 9	669 ± 5	668± 9
O'Doul's® Non-alcoholic	1	476	481	502
	2	490	481	509
	3	488	477	503
	Mean ± SD	485 ± 8	480 ± 2	505 ± 4

Appendix F

Retention Times and Peak Areas for (+) – Catechin and (-) Epicatechin at Day 0

	(+) - Catechin			(-) - Epicatechin		
[1]Conc mg/L	[2]RT (mins)	[3]PA at 278 nm(μV*Sec)	[3]PA at 290 nm(μV*Sec)	[2]RT (mins)	[3]PA at 278 nm(μV*Sec)	[3]PA at 290 nm(μV*Sec)
10	18.398	84200	34200	20.981	77500	29100
20	18.435	160000	65300	20.956	143000	55800
30	18.476	280000	97900	20.980	253000	84400
40	18.590	369000	135000	21.016	336000	116000
50	18.514	450000	169000	21.053	412000	145000

[1]Concentration in mg/L,

[2]Retention time (minutes),

[3]Peak area (μV*Sec).

Appendix G

Retention times and Peak areas for (+) - Catechin (-) - Epicatechin at Day 30

	(+) - Catechin			(-) - Epicatechin		
[1]Conc mg/L	[2]RT (mins)	[3]PA at 278 nm(μV*Sec)	[3]PA at 290 nm(μV*Sec)	[2]RT (mins)	[3]PA at 278 nm(μV*Sec)	[3]PA at 290 nm(μV*Sec)
10	18.118	92600	33500	20.592	82900	28300
20	18.118	181000	66100	20.608	163000	56000
30	18.111	283000	103000	20.599	253000	86400
40	18.150	367000	133000	20.622	330000	113000
50	18.154	457000	165000	20.612	407000	138000

[1]Concentration in mg/L,

[2]Retention time (minutes),

[3]Peak area (μV*Sec).

Appendix H

Retention Times and Peak areas for (+) - Catechin (-) - Epicatechin at Day 60

	(+) - Catechin			(-) - Epicatechin		
[1]Conc mg/L	[2]RT (mins)	[3]PA at 278 nm(μV*Sec)	[3]PA at 290 nm(μV*Sec)	[2]RT (mins)	[3]PA at 278 nm(μV*Sec)	[3]PA at 290 nm(μV*Sec)
10	17.959	93 300	33 200	20.552	81 800	28 200
20	17.964	189 000	68 700	20.548	168 100	57 300
30	18.009	273 100	98 400	20.606	243 000	82 600
40	18.012	366 300	131 700	20.625	326 100	110 400
50	18.016	461 300	165 700	20.596	407 900	138 600

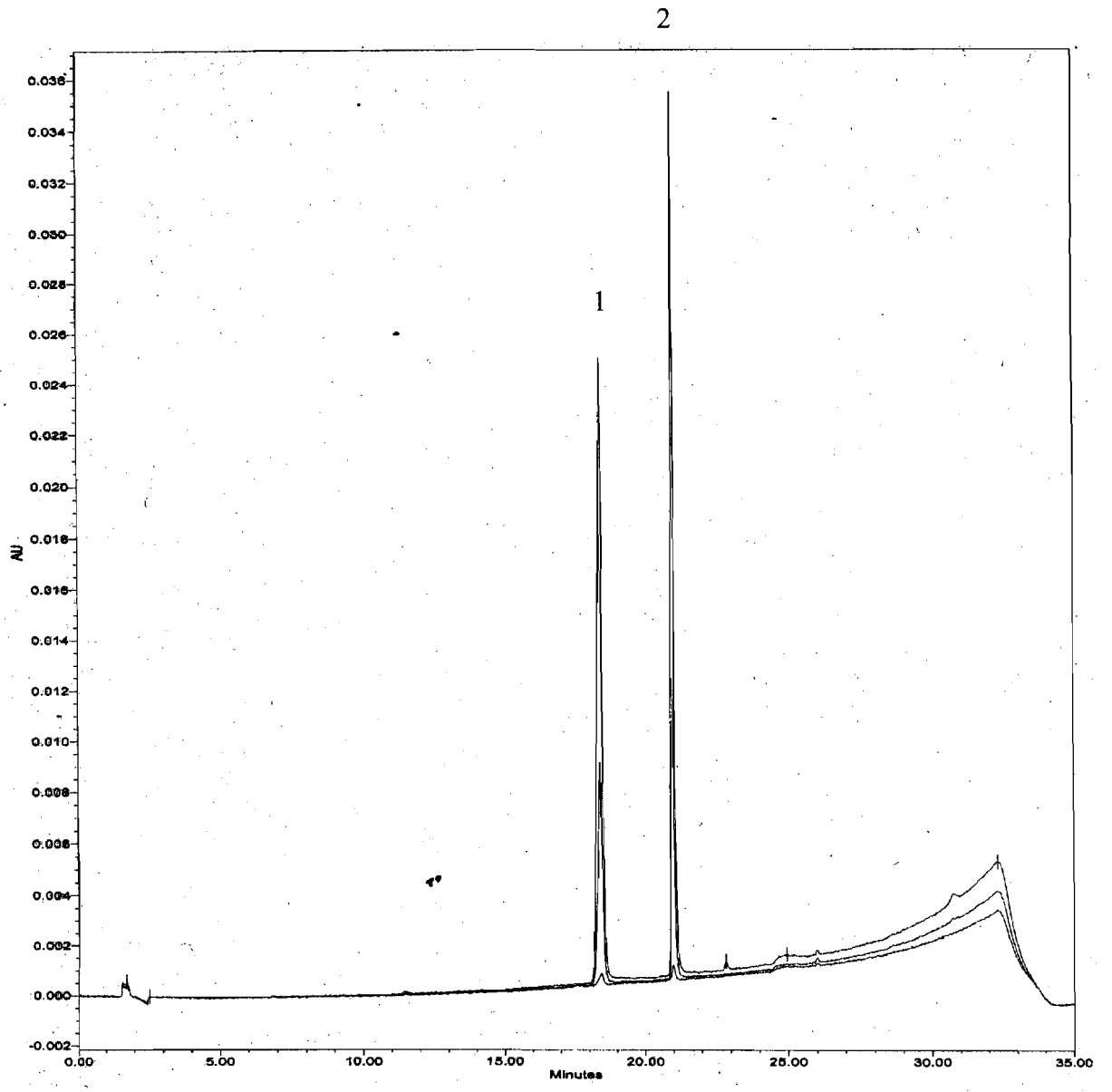
[1]Concentration in mg/L,

[2]Retention time (minutes),

[3]Peak area (μV*Sec).

Appendix I

Standard Chromatogram for (+) - Catechin and (-) - Epicatechin at Day 0



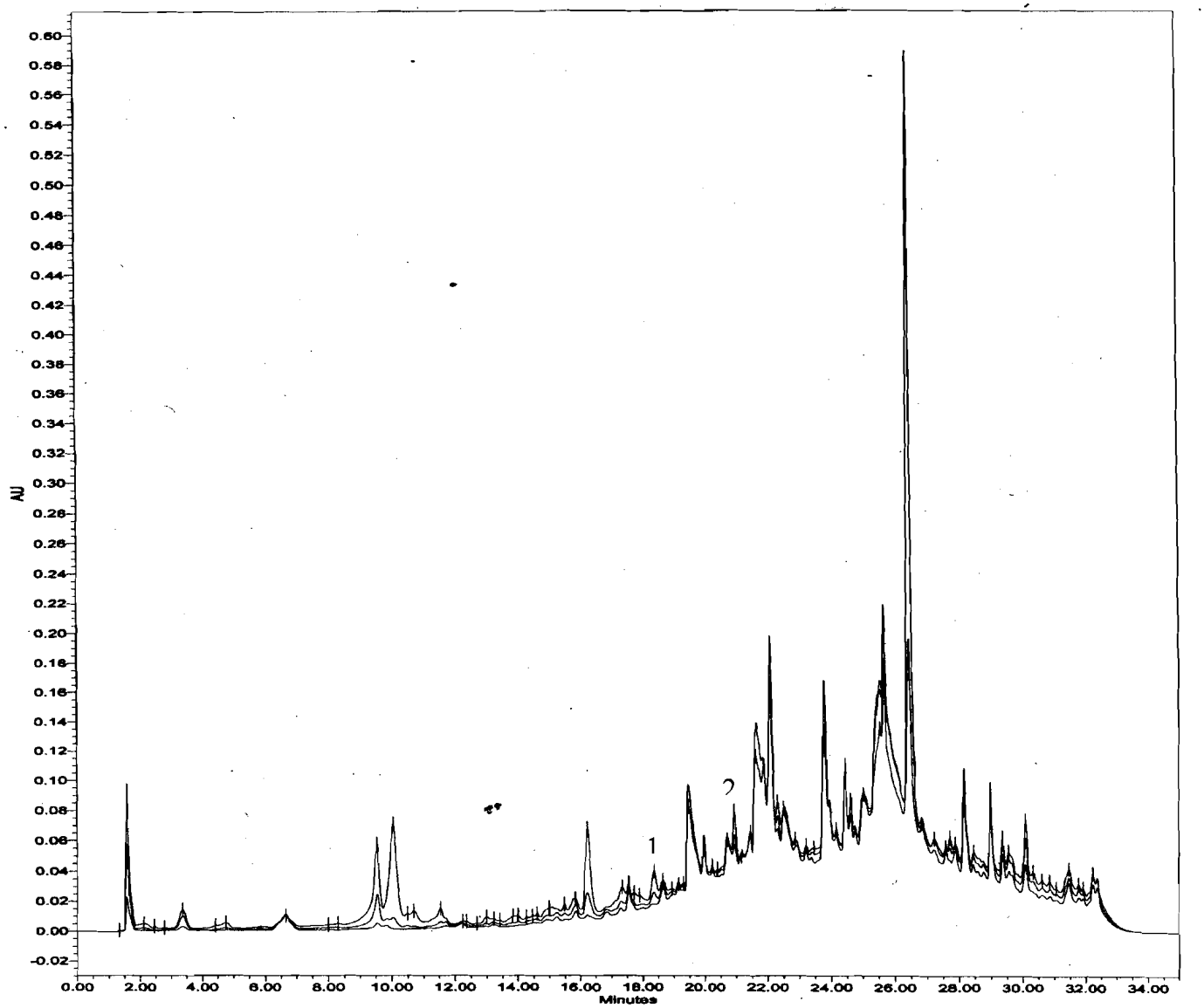
Standard Chromatogram for (+) - Catechin and (-) - Epicatechin at Day 0.

1 = (+) - Catechin,

2 = (-) - Epicatechin.

Appendix J

O'Doul's® Non-Alcoholic Beer Chromatogram at Day 0

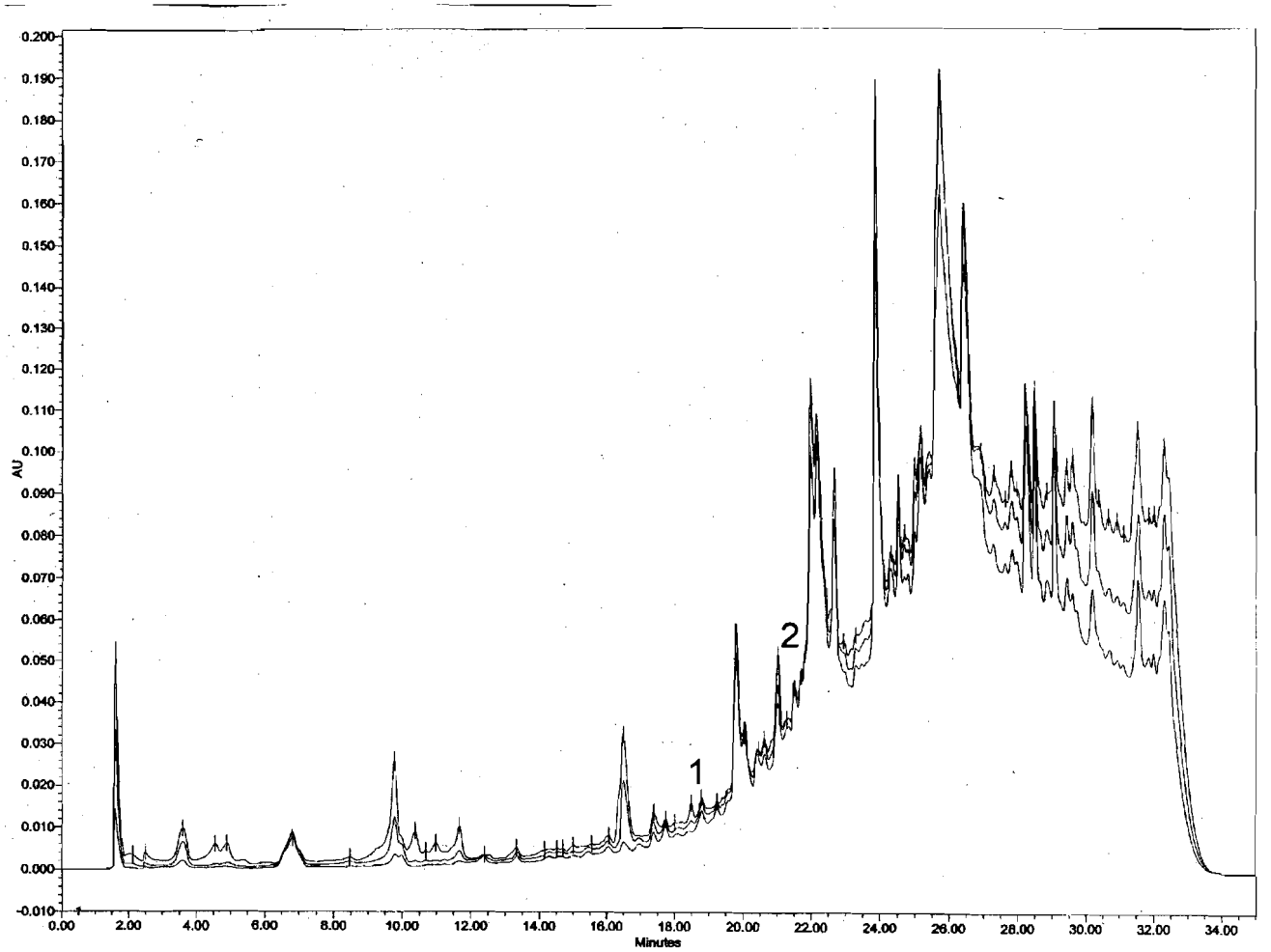


1 = (+) - Catechin,

2 = (-) - Epicatechin

Appendix K

Leinenkugel`s® Creamy Dark Lager Chromatogram at Day 0

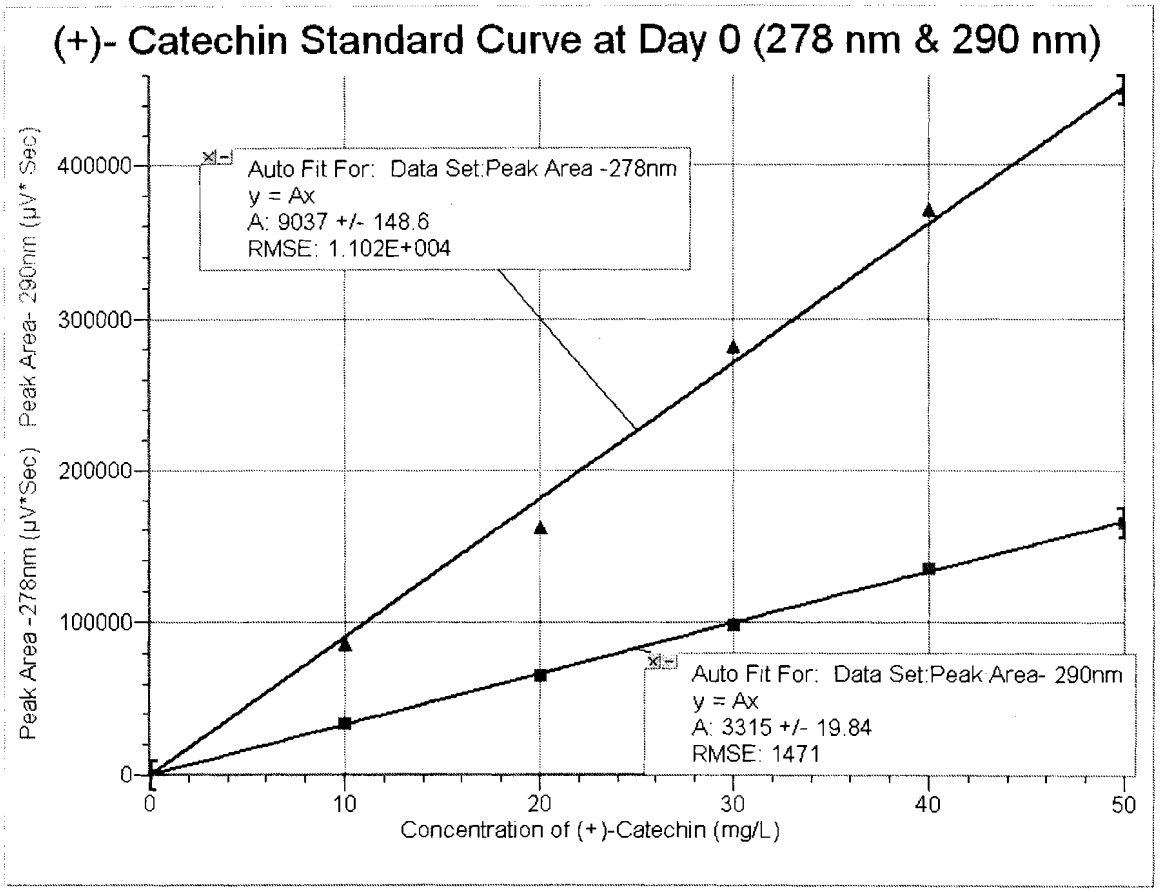


1= (+) - Catechin,

2 = (-) - Epicatechin.

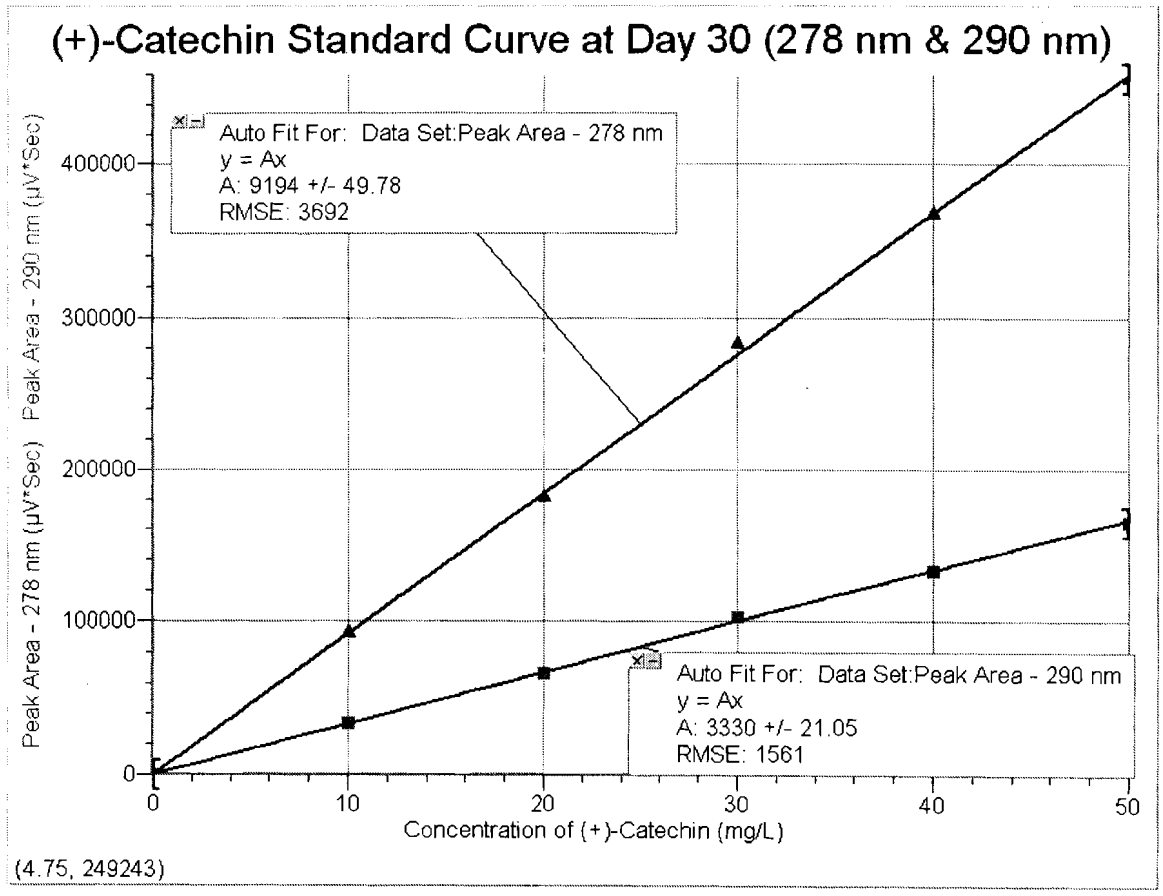
Appendix L

(+) - Catechin Standard Curve at Day 0


(+)- Catechin Standard Curve at Day 0 (278 nm & 290 nm)
Auto Fit For: Data Set:Peak Area -278nm
y = Ax
A: 9037 +/- 148.6
RMSE: 1.102E+004
Auto Fit For: Data Set:Peak Area- 290nm
y = Ax
A: 3315 +/- 19.84
RMSE: 1471
Peak Area -278nm (µV*Sec) Peak Area- 290nm (µV* Sec)
Concentration of (+)-Catechin (mg/L)
0
100000
200000
300000
400000
0
10
20
30
40
50

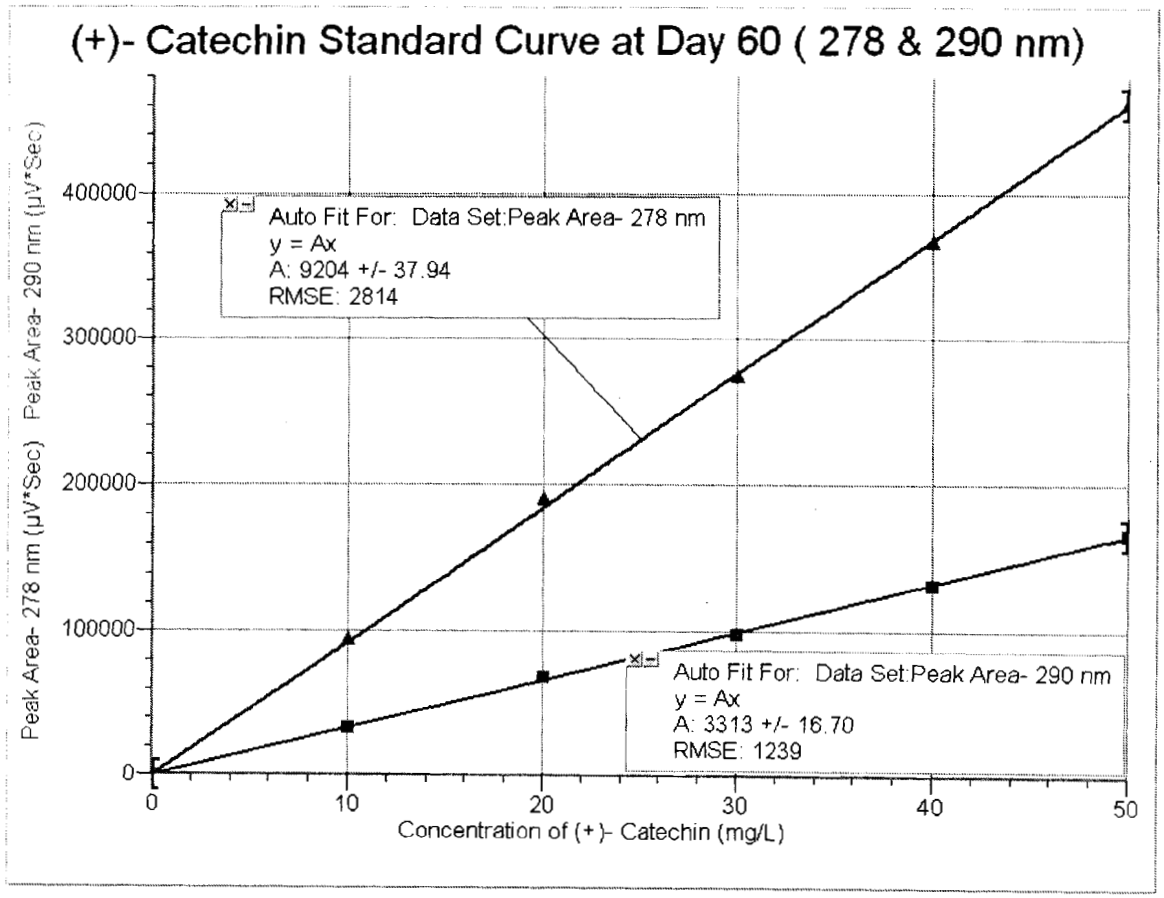
Appendix M

(+) - Catechin Standard Curve at Day 30


(+)-Catechin Standard Curve at Day 30 (278 nm & 290 nm)
Auto Fit For: Data Set:Peak Area - 278 nm
y = Ax
A: 9194 +/- 49.78
RMSE: 3692
Auto Fit For: Data Set:Peak Area - 290 nm
y = Ax
A: 3330 +/- 21.05
RMSE: 1561
Peak Area - 278 nm (µV*Sec) Peak Area - 290 nm (µV*Sec)
400000
300000
200000
100000
0
0
10
20
30
40
50
Concentration of (+)-Catechin (mg/L)
(4.75, 249243)

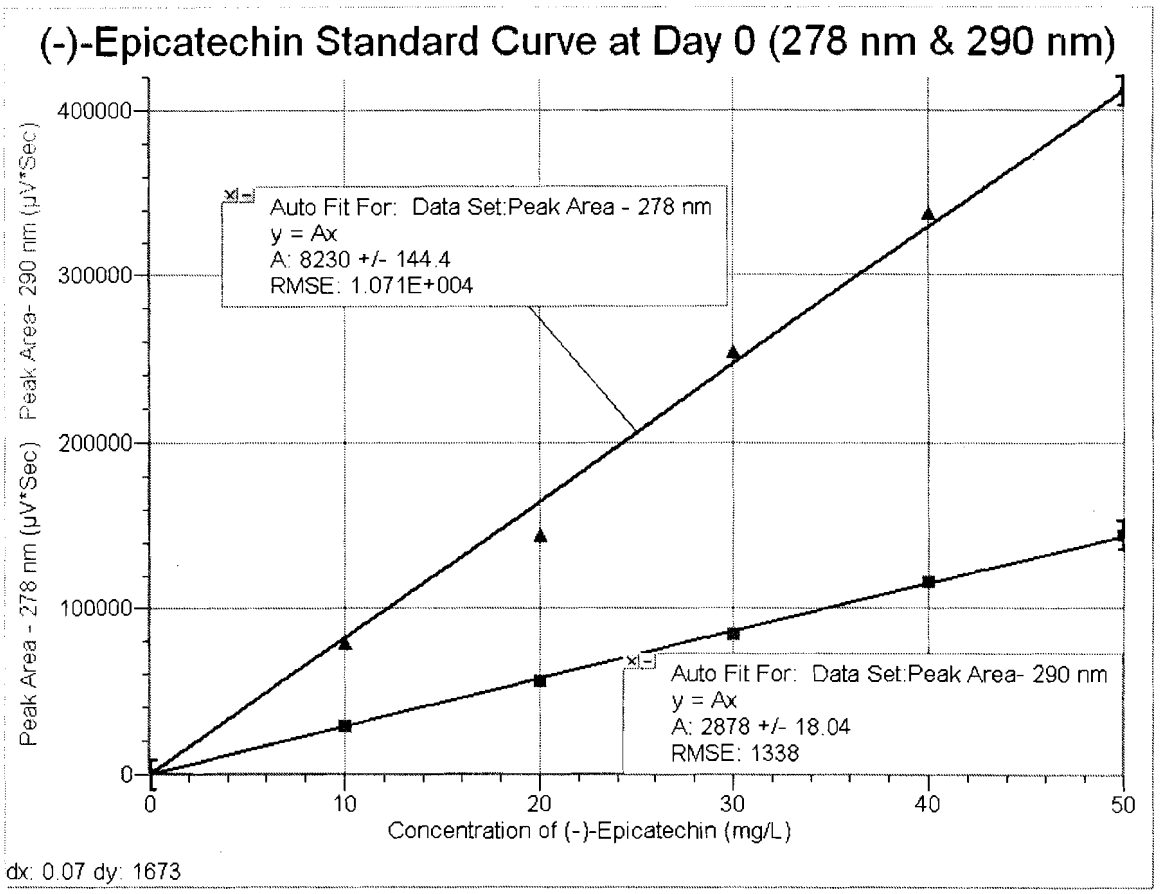
Appendix N

(+) - Catechin Standard Curve at Day 60


(+)- Catechin Standard Curve at Day 60 (278 & 290 nm)
Peak Area- 278 nm (µV*Sec) Peak Area- 290 nm (µV*Sec)
400000
300000
200000
100000
0
Auto Fit For: Data Set:Peak Area- 278 nm
y = Ax
A: 9204 +/- 37.94
RMSE: 2814
Auto Fit For: Data Set:Peak Area- 290 nm
y = Ax
A: 3313 +/- 16.70
RMSE: 1239
0
10
20
30
40
50
Concentration of (+)- Catechin (mg/L)

Appendix O

(-) - Epicatechin Standard Curve at Day 0


(-)-Epicatechin Standard Curve at Day 0 (278 nm & 290 nm)
Peak Area - 278 nm (µV*Sec) Peak Area- 290 nm (µV*Sec)
400000
300000
200000
100000
0
Auto Fit For: Data Set:Peak Area - 278 nm
y = Ax
A: 8230 +/- 144.4
RMSE: 1.071E+004
Auto Fit For: Data Set:Peak Area- 290 nm
y = Ax
A: 2878 +/- 18.04
RMSE: 1338
0
10
20
30
40
50
Concentration of (-)-Epicatechin (mg/L)
dx: 0.07 dy: 1673

Appendix P

Measurements of (+) - Catechin Content of Beer Beverages at Day 0

Beer beverage	Replicates	[1]RT at 278 nm (mins)	[2]PA (μV*Sec)	[3]Conc at 278 nm (mg/L)
Beck`s®	1	18.55	5515	0.04
Non-alcoholic	2	18.451	13000	0.04
	3	18.456	11200	0.04
Clausthaler®	1	18.55	5515	0.04
Non-alcoholic	2	18.451	13000	0.04
	3	18.456	11200	0.04
Kaliber®	1	18.39	63100	0.28
Non-alcoholic	2	18.404	64800	0.28
	3	18.417	59800	0.24
Leinenkugel`s®	1	18.502	27000	0.12
Alcoholic	2	18.622	26700	0.12
	3	18.592	23400	0.12
O'Doul's®	1	18.382	140000	0.6
Non-alcoholic	2	18.471	136000	0.6
	3	18.388	130000	0.56

[1] Retention times (minutes),

[2] Peak area (μV*Sec),

[3]Concentration of (+) - catechin

Appendix Q

Measurements of (+) - Catechin Content of Beer Beverages at Day 30

Beer beverages	Replicates	[1]RT at 278 nm (mins)	[2]PA (μV*Sec)	[3]Conc at 278 nm (mg/L)
O'Doul's®	1	18.178	105000	0.48
Non-alcoholic	2	18.178	126000	0.56
	3	18.199	106000	0.48
Kaliber®	1	18.297	51600	0.24
Non-alcoholic	2	18.200	48900	0.20
	3	18.195	40000	0.16
Beck`s®	1	18.204	13100	0.04
Non-alcoholic	2	18.239	13400	0.04
	3	18.300	14800	0.08
Clausthaler®	1	18.284	38500	0.16
Non-alcoholic	2	18.225	37500	0.16
	3	18.262	41600	0.20
Leinenkugel`s®	1	18.253	17800	0.08
Alcoholic	2	18.226	17900	0.08
	3	18.196	20000	0.08

[1] Retention times (minutes),

[2]Peak area (μV*Sec),

[3]Concentration of (+) - catechin

Appendix R

Measurements of (+) - Catechin Content of Beer Beverages at Day 60

Beer Beverage	Replicates	[1]RT at 278 nm (mins)	[2]PA (μV*Sec)	[3]Conc at 278 nm (mg/L)
Beck`s®	1	18.234	43000	0.2
Non-alcoholic	2	18.245	46700	0.2
	3	18.256	42600	0.2
Clausthaler®	1	18.029	19700	0.08
Non-alcoholic	2	18.038	15800	0.08
	3	18.046	16400	0.08
Kaliber®	1	17.950	27900	0.12
Non-alcoholic	2	17.971	27400	0.12
	3	17.970	24200	0.12
Leinenkugel`s®	1	18.035	8200	0.04
	2	18.034	6850	0.04
Alcoholic	3	18.017	8860	0.04
O'Doul's®	1	17.818	137000	0.6
Non-alcoholic	2	17.958	96500	0.44
	3	17.961	92200	0.44

[1]Retention times (minutes),

[2] Peak area (μV*Sec),

[3] Concentration of (+) - catechin

Appendix S

Measurements of (-) - Epicatechin Content of Beer Beverages at Day 0

Beer Beverage	Replicates	[1]RT at 278 nm (mins)	[2]PA (μV*Sec)	[3]Conc at 278 nm (mg/L)
Beck`s®	1	21.040	170000	0.8
Non-alcoholic	2	20.998	52000	0.24
	3	20.994	47000	0.24
Clausthaler®	1	20.990	69100	0.32
Non-alcoholic	2	20.995	75000	0.36
	3	21.003	70600	0.32
Kaliber®	1	20.973	166000	0.8
Non-alcoholic	2	20.980	165000	0.76
	3	20.983	164000	0.76
Leinenkugel`s®	1	21.023	143000	0.68
Alcoholic	2	21.079	151000	0.72
	3	21.135	142000	0.68
O'Doul's®	1	20.963	176000	0.84
Non-alcoholic	2	20.066	189000	0.88
	3	21.007	191000	0.92

[1] Retention times (minutes),

[2] Peak area (μV*Sec),

[3]Concentration of (-) - epicatechin.