THESIS

EFFECTS OF DARK MALTS, DRY HOPPING, AND FILTRATION ON XANTHOHUMOL CONTENT AND BIOACTIVITY OF AMERICAN INDIA PALE ALES

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ABSTRACT

EFFECTS OF DARK MALTS, DRY HOPPING, AND FILTRATION ON XANTHOHUMOL CONTENT AND BIOACTIVITY OF AMERICAN INDIA PALE ALES

Xanthohumol (XN), a prenylated chalcone found in hops (*Humulus lupulus* L.) has been shown to possess a wide spectrum of beneficial properties including anti-oxidant, anti-proliferative, pro-apoptotic, anti-inflammatory, anti-bacterial, anti-viral, and anti-malarial activities. Efforts have been made to increase the amount of XN in beers by modifying certain brewing ingredients and procedures. However, the effects of modifications such as addition of dark malts, dry hopping, and DE filtration on XN content and the biological activity of American India Pale Ales (IPAs) are not known. In this study, different brands of IPAs with and without addition of dark/roasted malts, dry hopping, and filtration and one non IPA as a standard were analyzed for XN, isoxanthohumol, total phenolic content, and antioxidant capacity. Isolated beer compounds and selected whole beer matrixes were used to determine the synergistic effect of beer compounds on proliferation and apoptosis of HCT 116 p53 +/+ colon cancer cells. No XN was found in the standard, and the XN content in IPAs ranged from 0.00 to 12.69 mg/L. A heavily dry hopped IPA made with increased amounts of dark malt

contained higher amounts of XN compared to other IPAs. The use of dark malts was protective against the removal of XN and other phenolics after diatomaceous earth (DE) filtration and dry hopping increased XN content in beer. Whole beer matrixes with greater levels of XN suppressed proliferation and elevated apoptosis in colon cancer cells compared with isolated XN and/or IX, indicating that the biological effect of XN can be bolstered in the presence of other beer compounds.

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ABBREVIATIONS PAGE

8PN 8-Prenylnaringenin
AOM Azoxymethane
BSG Brewers spent grain
CMV Cytomegalovirus
Da Dalton
DE Diatomaceous earth
FCR Folin-Ciocalteau reagent
FXR Farnesoid X receptor
HMW High molecular weight
iNOS Inducible nitric oxide synthases
IPA India pale ale
IQ Amino-3-methyl-imidazo[4,5-f] quinoline
IX Isoxanthohumol
LDL Low-density lipoprotein
ODF Odell dark IPA filtered
ODP Odell dark IPA prefiltered
OOF Odell IPA filtered
OOP Odell IPA prefiltered
PPA Punjabi pale ale
PPI Paulaner pils
PVPP (polyvinylpyrrolidone)
QR Quinone reductase
RGF Ranger filtered
RGP Ranger frefiltered
TEAC Trolox equivalent antioxidant capacity assay
TP Total phenolics
TRAP Telomere repeat amplification protocol assay
XN Xanthohumol
ZNR Zenith new recipe
ZOR Zenith original recipe

CHAPTER I

INTRODUCTION

Beer contains multiple compounds beneficial to health including silicone, benzoic and cinnamic acid derivatives, ferulic acid, vitamin B_6, betaine glycine, catechins, and proanthocyanidins. One compound in beer that has received much attention is the hop derived compound xanthohumol (XN). XN is the principal prenylated chalcone in the hop plant (Magalhaes et al., 2008). This compound possesses health-benefitting properties including antioxidant, anti-proliferative, pro-apoptotic, anti-inflammatory, anti-bacterial, anti-viral, and anti-malarial activities (Miranda et al., 2000; Bamforth, 2002; Gerhauser et al., 2002; Stevens et al., 2004; Gerhauser, 2005; Monteiro et al., 2008; Magalhaes et al., 2009; 2009; Sohrabvandi et al., 2010). Interestingly, a recent study showed that XN increased apoptosis and suppressed cell proliferation of human colon carcinoma cell lines HT 29 and HCT 116 at 10 μM concentrations (Hadjiolov et al., 2009). Because of the wide spectrum of beneficial properties XN possesses, XAN technology, which includes modifying brewing ingredients (using dark, roasted malts and XN-enriched hop products) and brewing procedures (wort boiling, fermentation, maturation/storage, and filtration/stabilization) has been developed to increase the amount of XN in beers (Wunderlich et al., 2005; Back, 2007). The amount of XN in commercial beers is low: 0.2 mg/L or less (Wunderlich et al., 2005). However, addition of dark malts that contain several unidentified soluble substances with a molecule size range of 600,000-300,000 Da form complexes with beer compounds and might protect

XN during the boiling process, and reduce its isomerization to IX, a compound that is not as biologically beneficial as XN (Wunderlich et al., 2005; Back, 2007). By utilizing the combination of dark malts and XN-enriched hop products, a beer with a XN content of 17.2 mg/L was developed (Wunderlich et al., 2005).

One example of a beer style that may inherently contain more XN is the India Pale Ale (IPA). More specifically, American IPA is a style of beer containing American hop varieties and often utilizes dry hopping where hops are added after the wort has been cooled and while the beer ferments (Guinard, 1990). Dry hopping contributes to hop aroma but not bitterness as alpha acids need to be isomerized during wort boiling to form the bitter tasting iso-alpha acids (De Keukeleire, 2000). Information on bioactive compounds in American India Pale Ale styled beers is limited. Although one study analyzed prenylflavonoids in different beer styles and reported XN content of 0.16 mg/L in an India Pale Ale (Stevens et al., 1999b), this amount is expected to be higher in beers that are dry hopped. In order to determine if American IPAs contain elevated amounts of XN, different brands of IPAs were analyzed for total phenolics (TP), antioxidant activity, and amounts of XN and IX. This research also addressed 1) the effect of processing techniques (dry hopping and filtration) and ingredients (dark malts) on final XN and IX contents 2) pro-apoptotic and anti-proliferative properties of isolated compounds XN and/or IX and concentrated whole beer matrixes in HCT 116 (P53+/+) human colon cancer cells.

CHAPTER II

REVIEW OF LITERATURE

1. Beer History, Variation, and Health

Beer has been a beverage of choice long before the American microbrewing boom of the latter part of the 20th century. Beer has been produced and consumed for thousands of years, although the exact origin of beer is unknown. The Greek historian, Herodotus, cited the Egyptians as making the first true beer. Other evidence suggests that Sumerians were the first beer drinkers some 10,000 years BCE (Bruce, 2002). Consumption of beer is related to a multitude of religious and cultural ceremonies and rituals and was utilized as food, medicine, and tonic (Darby et al., 1977). For example, beer was used for a variety of medicinal functions, including mouthwash, enema, vaginal douche, and for treatment of wounds. Modern analytical techniques have confirmed a multitude of inherent beer compounds, such as xanthohumol (XN) that could quite possibly be the scientific explanation for beers' medicinal use throughout history. Another reason for beer consumption is based on sanitation. Historically, both on and off land, drinking water was frequently polluted, scarce, and expensive. Thus beer and other alcoholic beverages were used instead of water. One example of this includes a British legislation, which required the masters of ships to have at least two-thirds of a gallon of beer (about three liters) for each passenger when making a transatlantic voyage (Heron, 2003). In the past, beer was consumed by everyone independent of age; however, in modern society

beer consumption is limited to those older than 16 in several European nations to 21 in the United States (Bamforth, 2002).

1.1 Beer Variations

Beer is the term used to describe a fermented alcoholic beverage in which grains (typically barley and/or wheat) provide body and fermentable sugars that are converted to alcohol by yeast. Hops impart bitterness and water assists mashing, brewing, and fermentation (Bruce, 2002). Other types of alcoholic beverages similar to beer include mead (sugars from honey), braggots (sugars from grain and honey), and melomels (sugars from honey and fruit) (Mosher, 2004). Variations of "beer" were brewed for many centuries without the use of hops. Instead, mild herbs such as rosemary, mugwort, and yarrow were used for flavoring (Ingels, 1987; Bruce, 2002). Other variations of beer were produced in different areas depending on available ingredients.

1.2 Beer and Health

While beer was used for medicinal purposes, today beer consumption has a narrower purpose. It may occasionally be an ingredient in shampoos or in a range of food recipes including soups, sauces, breads, and cakes (Schermerhorn, 1993). However, the majority of beer today is consumed as a beverage, and it is the most commonly consumed type of alcoholic beverage in the world (Kondo, 2004). Beer contributes calories, amino acids, peptides, B vitamins, phenolic compounds, and other bioactive compounds to consumers (Kondo, 2004). Because beer contains beneficial compounds, it is of interest to study its beneficial effects on human health.

Before discussing the possible health benefits of beer, it must be noted that high ethanol intake is associated with carcinogenesis and other chronic diseases (Gerhauser,

2005). Research has shown consuming 10.14 oz. or 0.3 L of beer a day reduces annual mortality for all causes of death compared to no or higher alcohol consumption (Gerhauser, 2005). This provides evidence that beer consumption, in moderation, can be beneficial to the body. Other alcoholic beverages have long been known for possessing beneficial compounds, for example, wine contains resveratrol and other phenolic compounds associated with positive effects on health. Wine receives favorable publicity, yet beer does not (Bamforth, 2002). This may be due to the fact that drinking wine is associated with having a higher socio-economic status, better healthcare, and consuming healthier foods. People who smoke daily tend to drink more beer, more coffee, and less tea (Bamforth, 2002). In the past, perhaps beer was harmful to the body because of carcinogenic volatile nitrosamines contributed by directly fired malt. This practice has been halted in the malt house, and currently malt contains only trace amounts of these compounds (Smith, 1994).

2. Beer Ingredients and Chemical Compounds

Beer is made from four essential ingredients: water, malt, hops, and yeast. Water is 90% of the composition of most beers (Bamforth, 2006). The other major ingredient in beer is malt. A few hundred grams of malt is used to make one liter of beer (De Keukeleire, 2000). Malt, typically made from barley, enhances the body of the beer. Because starches in barley need to be converted into fermentable sugars, the grain is subjected to a controlled steep and germination, during which time enzymes are formed in the barley grain kernel. These enzymes break down starch and yield fermentable sugars (De Keukeleire, 2000). Starch-to-sugar conversion is stopped by a heating process called kilning (Briggs, 1998). Kilned malt can produce pale malt with significant

enzymatic activity. Malts also can go through another process of roasting, which develops other flavors and produces a darker color. Roasted malt has limited enzyme activity and a distinct taste such as coffee and chocolate (Briggs, 1998). Some beers are brewed exclusively with pale malt. Others are darker in color because they are brewed with dark malts. During kilning and roasting, developed colors are due to caramelization of sugars and Maillard-type reactions (De Keukeleire, 2000). All malt is not created equal and chemical composition can vary widely depending on the process (kilning or roasting) and procedure (time and temperature).

2.1 Malt-Derived Compounds

Benzoic and cinnamic acid derivatives, catechins, proanthocyanidins, and flavones are found in small quantities in most beers and possess radical scavenging activity (Gerhauser, 2005). Kimura et al. (1999) showed that a malt derived compound, betaine glycine, suppressed a precursor to colon cancer, aberrant crypt formation, when induced by azoxymethane (AOM), a potent colon specific carcinogen. Malt contributes other beneficial compounds to beer: e.g., silicone, ferulic acid, and vitamin B_6. These compounds are also noted for being present in beer and generating beneficial responses in the human body (Bamforth, 2002; Casey et al., 2009). Samaras et al. (2005) showed antioxidant activity increased with the malt’s temperature and was higher for roasted than kilned malts. Phenolics were the main contributors to antioxidant activity for kilned malts. In roasted malts, Maillard reaction products were responsible for the majority of the antioxidant activity (Samaras et al., 2005).

2.2 Hop-Derived Compounds

Although 70-80% of beer polyphenolics come from malt and 20-30% from hops (Gerhauser, 2005; Gorjanovic et al., 2010), much of the current research on bioactives focuses on hop-derived compounds. The female hop plant is widely grown and cultivated for its secondary metabolites, which are used during brewing to impart bitterness and aroma (Ceh et al., 2007). There are many health-related implications for this. Hops contain bitter acids, essential oils, and prenylflavonoids, which are produced by the lupulin glands in the female hop cone (Magalhaes et al., 2008). Hops are the only source of these compounds in beer and some may be beneficial to the humans. For example, XN, isoxanthohumol (IX), 8-prenylnaringenin (8PN), alpha acids, and others all possess multiple health benefits (Gerhauser, 2005). XN, is the principal chalcone prenylflavonoid in hops (0.2-1.1% w/w) (Magalhaes et al., 2008). It was first isolated from hops by Power et al. (1913) and was called xanthohumol after “xanthos,” Greek for yellow, and “humulus,” Latin for hops. XN is reported to be slightly bitter tasting (Haseleu et al., 2010) and is also present in *Sophora flavescens* Aiton (Jung et al., 2005), a Chinese medicinal plant.

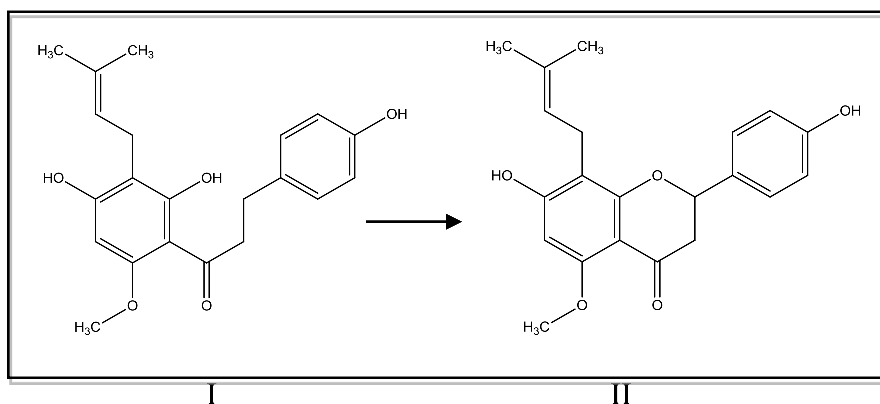

Figure 2.1 Xanthohumol (I) and Isoxanthohumol (II)

During the brewing process, XN is converted into its isomeric flavanone form, IX, which is typically the main prenylflavonoid in beer. This conversion takes place because XN has one hydroxyl available for ring closure, thus IX is formed as heat catalyzes this reaction (Stevens et al., 1999a). Two other prenylated chalcones exist in female hop cones: desmethylxanthohumol and 3′geranylchalconaringenin. These compounds also isomerize. Desmethylxanthohumol undergoes spontaneous or base-catalyzed cyclization during dry storage and forms 8PN, a bioactive compound (Stevens et al., 2004). While traces of prenylated flavonoids other than XN and IX are found in beers, the focus of this project will be on these prenylated flavonoids because of their inherent health benefits and potential final beer concentrations. XN and its isomeric form, IX have demonstrated a multitude of biological and/or pharmacological activities that are well summarized by Magalhaes et al. (2009). Potential benefits of XN have been well documented in numerous reviews (Gerhauser et al., 2002; Stevens et al., 2004; Gerhauser, 2005; Magalhaes et al., 2009).

2.2.1 Antioxidant Activity

Antioxidant activity of XN, ie. the scavenging of reactive species, was analyzed by Gerhauser et al. (2002). This investigation showed XN was 8.9 and 2.9 fold more potent scavenger than trolox at 1 mM for hydroxyl and peroxyl radicals in the ORAC (oxygen radical absorbance capacity) assay. XN was a potent scavenger of superoxide anion radicals (Gerhauser et al., 2002). Scavenging of reactive species is one way XN can play a role in preventing the development of cancer and neurodegenerative diseases such as atherosclerosis (Magalhaes et al., 2009). At concentrations of 5 and 25 µM *in vitro*, XN inhibited low-density lipoprotein (LDL) oxidation (Miranda et al., 2000). Stevens et

al. (2003) showed that prenylated chalcones, such as XN, and non-prenylated flavonone analogues, such as IX, inhibited peroxynitrite-mediated oxidation of LDL at micromolar concentrations. Prenylflavonoids are more lipophilic than phenolic acids and other polyphenols, and "may be more effective antioxidants at lipophilic surfaces such as membranes and low-density lipoprotein" (Stevens et al., 2004). The findings suggest that hop-derived prenylchalcones and prenylflavonoids found in beer might protect human LDL from oxidation (Miranda et al, 2000). Vinson et al. (2003) tested the effect of lager and dark beer on atherosclerosis in cholesterol-fed hamsters using two different concentrations (high and low) of beer solutions. At high doses, both significantly inhibited atherosclerosis, but at the low dose only the lager showed a significant decrease in atherosclerosis ($P < 0.01$) (Vinson et al., 2003). Both beers decreased LDL oxidation at high concentration. In a human study, 500 ml of beer caused an increase in plasma antioxidant activity using the telomere repeat amplification protocol assay (TRAP) ($P < 0.05$) (Ghiselli et al., 2000).

2.2.2 Antiproliferative/Anticarcinogenic/Antiangiogenesis and Anti-inflammatory Activity

XN was found to be 10 to 200-fold more active than resveratrol in preventing carcinogen-induced preneoplastic mammary lesion formation, which is the precursor stage of mouse mammary tumorigenesis (Gerhauser et al., 2002). Ferk et al. (2010) showed XN and IX limited these preneoplastic lesions as well as DNA damage in liver and colon cells by beneficially affecting heterocyclic aromatic amine amino-3-methyl-imidazo[4,5-f] quinoline (IQ). XN was found to increase apoptosis and supress cell proliferation of human colon carcinoma cell lines HT 29 and HCT 116 at 10 μM concentrations (Hadjiolov et al., 2009). These observed effects of XN on colon cancer

cells can be attributed to XN's ability to increase activation of caspase 3 enzymes, inhibit DNA synthesis, and increase induction of cell cycle arrest in S-phase (Miranda et al., 2000; Hadjiolov et al., 2009).

Futhermore, XN decreases the activity of the nuclear factor kappa B (NFkB) pathway, inhibiting both breast cancer and host cells (Monteiro et al., 2008). Prenylated flavanones were also found to enhance quinone reductase (QR) activity as an indication for elevated detoxification of toxic and reactive chemical species (Gerhauser et al., 2002). In an *in vivo* study using rats, XN reduced tumor-induced neovascularisation by 33% in comparison with the untreated control group (Albini et al., 2005). Prenylated flavanones had anti-inflammatory potential by inhibiting inducible nitric oxide synthases (iNOS) induction and COX-1 activity (Gerhauser, 2005).

2.2.3 Anti-bacterial Activity

Although the anti-bacterial activity of hop resins has been documented and summarized by Magalhaes et al. (2009), XN's role in this activity is lesser known. One study compared the antimicrobial activity of several hop components against strains of *Streptococcus* (*S. mutans, S. salivarius,* and *S. sanguis)* with some essential oils commonly used in commercially available mouthwashes (Bhattacharya et al., 2003). At concentrations of 50 µg per disc, XN (nmol) showed inhibitory affects against all three strains of bacteria. A study by Natarajan et al. showed XN, and other hop-derived compounds, possessed a positive co-action against certain Gram-positive and Gram-negative bacteria when paired with antibiotics in *in vitro* experiments (Natarajan et al., 2008).

2.2.4 Anti-viral Activity

XN was shown to have anti-viral activity against certain DNA and RNA viruses. Specifically, this compound was found to have inhibitory affects against HIV-1-induced cytopathic effect and to inhibit HIV-1 replication in peripheral blood mononuclear cells (Bhattacharya et al., 2003). XN also showed anti-viral activities against cytomegalovirus (CMV), which demonstrates XN possesses anti-herpes virus activity at concentrations of 1.6 mg/L (Buckwold et al., 2004). Because of these findings, XN might prove to be a useful compound in the fight against several virus infections.

2.2.5 Antifungal Activity

XN was found to be one of the most potent of all hop compounds against certain human pathogenic fungi (*Trichophyton mentagrophytes, Trichophyton rubrum, and Mucor rouxianus*) (Mizobuchi et al., 1984). While XN was only a weak inhibitor of *Mucor rouxianus,* at a concentration of 3.13 ug/mL, XN was found to inhibit the growth of both *Trichophyton mentagrophyte* and *Trichophyton rubrum* more efficiently than the positive control griseofulvin.

2.2.6 Anti-malarial Activity

Malaria (caused by parasites *Plasmodium falciparum* and *Plasmodium vivax)* is one of the most common infectious diseases and is responsible for more than one million deaths annually (Nowakowska, 2007; Magalhaes et al., 2009).While certain strains of the disease can be treated by anti-malarial drugs, such as chloroquine, certain drug-resistant strains require other forms of defense (Nowakowska, 2007; Magalhaes et al., 2009). One study by Herath et al. (2003) showed XN was active against D6 and W2 strains of *P. falciparum* that are chloroquine sensitive and resistant, respectively. A similar study by

Frolich et al. (2005) showed XN was also protective against chloroquine-sensitive strain poW and the multiresistant clone Dd2.

2.2.7 Anti-obesity Activity

Prenylated flavonones could reduce the risk of metabolic syndrome, since they had anti-obesity effects in high fat diet-consuming rodents (Miura et al., 2005). Chalcones, such as XN, were found to have a beneficial effect on farnesoid X receptor (FXR). FXR is a selective bile acid receptor modulator, which lowers serum or hepatic triglyceride levels, regulates the genes involved in bile acid transport, and plays a role in the regulation of carbohydrate metabolism (Nozawa, 2005).

2.2.8 Estrogenic Activity

Hops have been reported to possess estrogenic properties (Stevens et al., 2004). It was recently shown that XN does not possess intrinsic estrogenic potential and IX is a weak estrogen agonist (Stevens et al., 2004). 8-Prenylnaringenin (8PN) has been identified as one of the most potent phytoestrogens. Rat studies confirmed 8PN is an estrogen agonist in female reproductive organs (Diel et al., 2004). Other estrogen binding studies and *in vitro* investigations have shown that 8PN and other prenylated flavones are selective estrogen receptor modulators. XN might have an influence on estrogenic potential because it can undergo cyclization to IX and be metabolized by liver enzymes to form 8PN (Nikolic, 2005).

XN and humulone (an alpha acid) were shown to be strong inhibitors of bone resorption (Stevens et al., 2004). Although scientific literature is not yet supporting using hops for treatment of premenopausal problems, such as hot flashes and prevention of

osteoporosis, there is a possibility of its application for prevention of these conditions in the future (Bowe et al., 2006).

2.2.9 Other Hop-Derived Constituents and Health Benefits

Other hop-derived beer constituents include alpha acids. While female hop cones contain both beta and alpha acids, beta acids are extremely sensitive to oxidation and do not survive the brewing process. During wort boiling, alpha acids go into solution and are isomerized into iso-alpha acids. Iso-alpha acids represent one of the most abundant classes of polyphenolic compounds in beer and are present in concentrations of up to 100 mg/L in extremely bitter English ales (Stevens et al., 2004). Humulone is the primary alpha acid occurring in most hops and possesses many beneficial health properties including: radical-scavenging, lipid peroxidation inhibitory activity, inhibition of bone resorption, anti-inflammatory activity, tumor suppression, and prevention of angiogenesis (Stevens et al., 2004). In addition, isohumulones are the dual agonists of both PPARα and PPARγ and have been found to improve insulin sensitivity in high fat diet-fed mice with insulin resistance or in patients with type 2 diabetes (Nozawa, 2005).

2.2.10 XN Toxicity

Presented evidence suggests XN possesses positive biological activity. Only a handful of studies have addressed XN consumption and toxicity. The most recent study by Dorn et al. (2010) investigated liver function and homeostasis of mice receiving a daily XN dose of approximately 1000 mg/kg b.w. for three weeks. Results were void of any signs of toxic effects on the lung, heart, thymus, spleen, kidney, liver, and colon upon macroscopical, histological and serum analysis (Dorn, 2010). This is in line with other safety studies on XN, with one exception. A study by Hussong et al. (2005) addressed the

toxicity and influence on fertility and offspring development on rats fed 1000 mg XN/kg body weight (b.w.) per day. Results revealed reduced liver weights (30–40%) in rats receiving this elevated dose of XN in comparison with the control group. While this indicates weak hepatotoxicity, no other negative biological of physiological effects were found (Hussong et al., 2005).

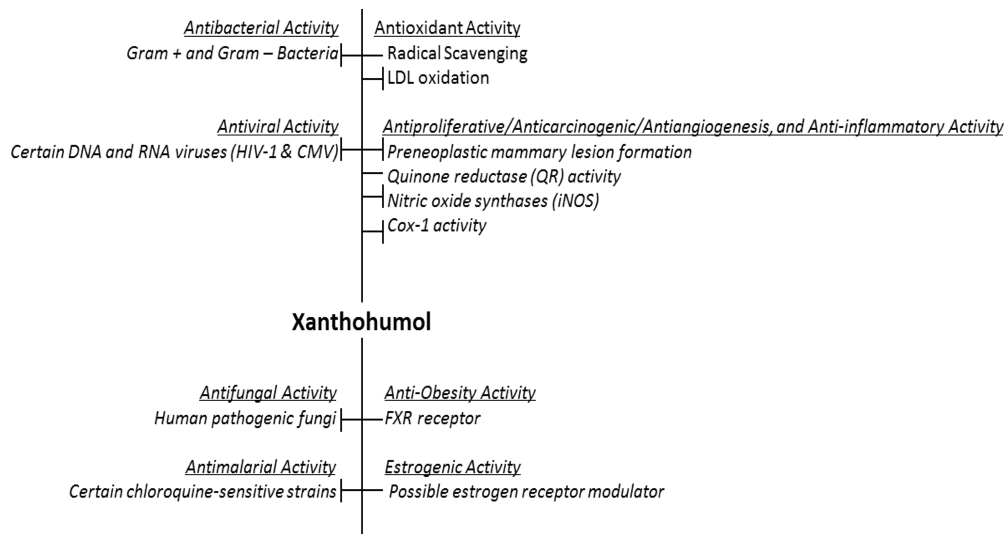

Figure 2.2 Summary of XN biological properties

2.2.11Continuing XN and Beer Research

Exact concentrations of XN needed for health-promoting effects in humans are not known and require further pharmacological experiments. Studies on the pharmacodynamics, pharmacokinetics, and metabolites of XN indicate the compound is minimally recovered after intake likely resulting from low absorption, rapid excretion, and/or extensive metabolization (Yilmazer et al., 2001; Hanske et al., 2010). This metabolization of XN can be attributed to both phase I and II enzymes as well as human intestinal microbiota (Hanske et al., 2010). Furthermore, it is known that in very acidic conditions, such as in the stomach, XN can undergo cyclization to IX. Compared with IX,

XN forms fewer metabolites during incubation with human liver microsomes and types of XN metabolites produced differ depending on the species of microsomes (Nikolic et al., 2005). Thus, the multiple biological effects reported after XN administration must be taken with caution as the complex nature of XN metabolites workings within the body have yet to be investigated.

The actual amount of XN found in beer ranges from between 0.02 mg/L to 10 mg/L (Biendl et al., 2004a; Wunderlich et al., 2005) while the concentration of IX can range from 0.0 to 3.5 mg/L (Stevens et al., 1999b). This large range is due to usage of specific ingredients and manipulation of the brewing process. The rationale behind research on XN in beers is that currently the only way Westernized society's consumers are getting any of it is through beer consumption. If XN can have beneficial properties on human health, effort should be directed into maximizing its amount in the final product and understanding underlying molecular mechanisms for biological activity.

3. Outline of Beer Production

3.1 Milling

When discussing beer and bioactive compounds, it is advantageous to have an understanding of the beer-making process. Thus, a brief overview of the main brewing procedures are outlined. Beer production over the years has developed into a highly automated science that still incorporates some amount of art and human effort. The essential ingredients of beer include malt, water, hops, and yeast. The brewing process involves manipulation of these ingredients, thermal processing, and fermentation. The brewing process begins with creating a grain bill with types of malted grains. These grains must be milled before the mash-in process begins in order to crack the husk

surrounding the internal starches, sugars, and enzymes, making them readily available in the mashing process (Lewis et al., 2001). The husk should be cracked but kept intact as much as possible, as the intact husk will help with filtration of the wort.

3.2 Mashing

After the grain is milled, the mash in process begins, and the malted and milled grains are combined with water to form a thick “oatmeal-like” consistency. In addition to barley malt, adjuncts are often added to the mash. These adjuncts can act as substrates for malt enzymes (Bamforth, 2006) and increase the amount of fermentable sugars. Adjuncts also impart unique flavor and color to the final beer. During mashing, grain enzymes continue to break down the starch and complex sugar matrix of the hydrated malted grains. Mashing temperatures and equipment can vary depending on the method used: infusion, decoction, double or temperature programmed mashing (Lewis et al., 2001). Once sufficient time has passed and conversion of grain starches to fermentable sugars has leveled off, the sweet liquid, called wort, is slowly drawn off the bottom of the grain bed and re-circulated back to the top of the grain bed to clarify the wort. The grain bed, especially the hulls, acts as a filter bed, so that only the wort is removed and continues on to the next process. Next, the lautering process begins and the sweet wort is drawn off the bottom and a small amount of hot water is added to the top of the grain bed in order to rinse out and utilize all of the sweet wort. The contents remaining in the mashing vessel, called brewers spent grain (BSG), can be utilized as products for animal feed, cultivation of mushrooms, and nutraceuticals among other uses (Mussatto et al., 2006).

3.3 Boiling

The sweet wort is transferred into a separate kettle and boiled. Boiling is done for several reasons. It concentrates the wort by evaporating water, evaporates volatiles in wort, improves wort color and flavor formation, stabilizes and sterilizes the wort, inactivates all residual enzymes, precipitates out unwanted protein-polyphenol compounds, and is beneficial for extraction of hop bittering compounds (Bamforth, 2000; Lewis et al., 2001). Hops are added during boiling at different times to control high foaming (early in the boil), to promote beer bitterness (throughout the boil), and to provide aroma and flavor (late in the boil) (Lewis et al., 2001). Compounds in hops, specifically alpha acids, are isomerized by a heat-catalyzed event. This changes the 6-carbon ring to a 5-carbon ring (Lewis et al., 2001). Alpha acids are insoluble in beer and when isomerized, the iso-alpha acids are soluble and contribute desirable bitter qualities to beer.

3.4 Fermentation

After boiling, the wort is whirlpooled, which is a centrifugal action that helps collect hot break (undesirable flocculated proteins) and hop residues in the center of boil kettle (Lewis et al., 2001). The clear wort is drawn off the bottom and ideally all of the unwanted hot trub (a combination of hot break and hop residues) is left behind. Next the wort is cooled to pitching yeast temperatures, which range between 21.1-26.7°C (70-80°F) for ale yeast strains and 7.2-12.8°C (45-55°F) for lager yeast strains (Anonymous, 2010). Typically, yeast is pitched at a concentration of 10^7 cells/mL (Lewis et al., 2001). The yeast cells grow and multiply producing ethanol, carbon dioxide, and other complex flavors from fermentable sugars in the wort. Fermentation can be divided into several

stages. The main fermentation, called primary fermentation, is when the majority of ethanol is produced. This is often followed by a slower process at lower temperatures (Bamforth, 2000). This secondary fermentation allows for yeast to scavenge any oxygen present, allows time for yeast sedimentation, encourages formation of chill haze particles, and matures the flavor of beer (Bamforth, 2000; Lewis et al., 2001). Secondary fermentation may also be called conditioning or maturation. After fermentation, beer can go through a clarification process, such as sedimentation, centrifugation, and/or filtration. These processes can remove particles from the beer, sometimes beneficial compounds. For example, polyvinylpolypyrrolidone (PVPP) has been found to remove multiple polyphenolic compounds (Whittle et al., 1999).

3.5 Beer Styles

The manipulation of the brewing process and/or brewing ingredients will produce a different beer and/or style. For example, adding roasted malts to a pale malt bill will produce a darker beer, perhaps a stout or porter-styled beer. Using a bottom fermenting yeast will produce a lager whereas a top fermenting yeast which will produce an ale. Changes to the actual procedure will produce a different beer as well. For example, certain beers, especially those produced in Germany, are produced under Reinheisgebolt. This is the German (Bavarian) Purity Law established in 1516, which mandates that only barley (and sometimes wheat) malt, water, hops, and yeast may be used to produce beer using standard German brewing practices: grains are mashed, wort is boiled, and hops are only added to the wort during boil (Wunderlich et al., 2005). Because of this, certain styles, such as the American India Pale Ale, in which hops are added to the beer during fermentation, are not producible. Other techniques include high-gravity brewing, which is

when wort possesses a higher than normal sugar concentration (15—20°P original gravity) (Stewart, 2010). Dilution with water (usually carbon filtered and deoxygenated) is required at a later stage in processing to obtain sales-gravity beer (Lewis et al., 2001). Using high gravity brewing can be used to meet increased production demands without significant expansion of brewing, fermenting, and storage facilities. Additionally, high gravity brewing can help increase beneficial compounds, such as XN, in the final beer (Stevens et al., 1999a).

3.5.1 Low-alcohol beer

Normal beer contains 2.5-13% ethanol (v/v) (Sohrabvandi et al., 2010)**.** Recently, there has been an increased demand for non-alcoholic beers (also called low-alcohol beer or near beer). This is likely due to consumer sets that enjoy the taste and organoleptic qualities of beer, yet do not want to consume ethanol for health, religious, or safety reasons. In the United States, non-alcoholic beers must contain less than 0.5% alcohol by volume and beer falling within this parameter can be produced by utilizing many different techniques. These techniques can be broken up into two categories: restricted alcohol fermentations and alcohol removal. The first category focuses on reducing the amount of fermentable sugars in the fermenting liquid or altering fermentation yeast. Examples of this method include using high mashing temperatures to inactivate enzymes that break down malt starch matrixes, using a yeast strain that is unable to produce or produces very little amounts of ethanol, and simply not pitching yeast into wort. Once alcohol is produced in normal fermenting beers, certain procedures can be utilized to remove the inherent alcohol. Distillation, water vapor/gas stripping under vacuum, dialysis, reverse osmosis, and osmotic distillation are all examples of this technique

(Sohrabvandi et al., 2010). The production of non-alcoholic beers can result in improper sensory characteristics, such as off flavors/defects and immature flavor profiles. While the sensory characteristics may be lacking, many non-alcoholic beers are available on the market, and there is certainly room for another, especially if it possesses improved flavor and biological beneficial profiles.

4. Critical Steps to Increase Beneficial Compounds in Beer:

Current brewing methods for commercial beers contain 5% or less of the XN content found in the hops used to make the beer (Wunderlich et al., 2005). Another researcher found that in trial brews the overall yield of XN was 22-30%, although the majority was present in the isomeric IX form (Stevens et al., 1999a). The amount of XN in commercial beers lies at relatively low levels: 0.2 mg/L or less (Wunderlich et al., 2005). Specific concentrations of XN needed for health-promoting effects are not known and require further pharmacological experiments, but the fact remains that currently the only way consumers are currently getting any amounts of this compound is through beer consumption. Thus, research has been conducted to increase amounts of XN in beer. Recent efforts focused on modifying brewing ingredients and procedures within Reinheisgebolt, thus dry hopping is not warranted or allowed. Wunderlich et al. (2005) developed a practice, called XAN technology, to increase XN content in beer. Xan Technology includes using enriched hop products, dark, roasted malts, and modifying specific brewing practices, which are explained below. An additional practice that could be utilized to increase XN content in final beer is dry hopping, although this hypothesis has yet to be proven.

4.1 XN-Enriched Hop Products

Another new development being used to increase XN content in beer is XN-enriched hop products. XN fractionates with pure resin during ethanol extraction but is not extracted by carbon dioxide, thus the amount of XN in various commercial hop products differs depending on the extraction process (Biendl et al., 2004b). For example Biendl et al. (2004b) found that pellets recover 95%, ethanol extract recovers 95%, and CO_2 extract recovers less than 5% XN in the original hop cone. Hopsteiner, an international company specializing in hops and hop products, carries five specialized products containing increased quantities of XN, and these products have been used in multiple experiments (Biendl et al., 2004a; Wunderlich et al., 2005). Utilizing enriched hop products resulted in an increase in XN content in final beer. Beer with more than 10 mg XN/L was produced (Wunderlich et al., 2005).

4.2 Roasted Malts

XAN technology involves using dark, roasted malts in addition to pale malts in the grain bill. Researchers (Walker et al., 2003; Wunderlich et al., 2005) have shown the use of dark, roasted malts has a positive effect on XN recovery in the final beer. Wunderlich et al. (2005) reported several unidentified soluble substances with a molecule size range of 600,000-300,000 Da (dalton: unit used for recording mass on a molecular scale) contained in dark malts form complexes with and protect XN during the boiling process and reduce its isomerization to IX. Perhaps HMW (high molecular weight) melanoidins, a Millard reaction compound, are the specific malt derived substances responsible for this increase. Coghe et al. (2006) reported HMW melanoidins (> 70,000 Da) might likely be less soluble in wort. If melanoidins form a complex with XN and are less soluble in wort, XN would be protected during the boiling process.

No matter what the exact compound responsible for protecting XN, a replacement of 10% pale malt with roasted malt (and use of XN-enriched hop products) resulted in a beer with a XN concentration of 17.2 mg/L (Wunderlich et al., 2005). Interestingly, the complex, symbiotic relationship of XN and certain roasted malt substances had a protective measure against certain types of filtration. Wunderlich and Back (2007) reported XN content in beer with roasted malts was reduced to a minimum reduction of 50% after PVPP filtration, while almost all XN was removed in pale beers receiving PVPP filtration.

4.3 Boiling, Cooling, Filtration, and Fermentation

XAN technology requires a boiling regimen that lasts for less than one hour and calls for the addition of hops in the last five minutes of the boil. In addition, XAN technology calls for high gravity brewing and the addition of cool water to bring down the wort temperature to pitching temperatures. One reason behind these last four recommendations is to reduce the amount of heat the hops are exposed to, as heat catalyzes the isomerization of XN into IX (Stevens et al., 1999a). Utilizing high gravity brewing (production of wort with a higher concentration of sugars) increases the solubility of XN and other prenylfavonoids, which keeps them in solution and increases the content in the final beer (Stevens et al., 1999a). XAN technology also recommends altering other steps beyond the wort boil, including fermentation and filtration/stabilization. Reduced yeast pitching rates and recycling of yeast are recommended to improve XN recovery. However, this research shows the impact of yeast on XN concentrations is not clear (Wunderlich et al., 2005). XAN technology negates the use of krausening, the term used for adding in fresh wort to fermented beer before

bottling as a way to induce natural carbonation, although the justification for this is not explained (Wunderlich et al., 2005). Finally, XAN technology calls for minimal filtration. This is because many filtration methods, specifically PVPP, activated carbon, and even centrifugation, drastically reduces the amount of XN in the final beer (McMurrough et al., 1995; Wunderlich et al., 2005).

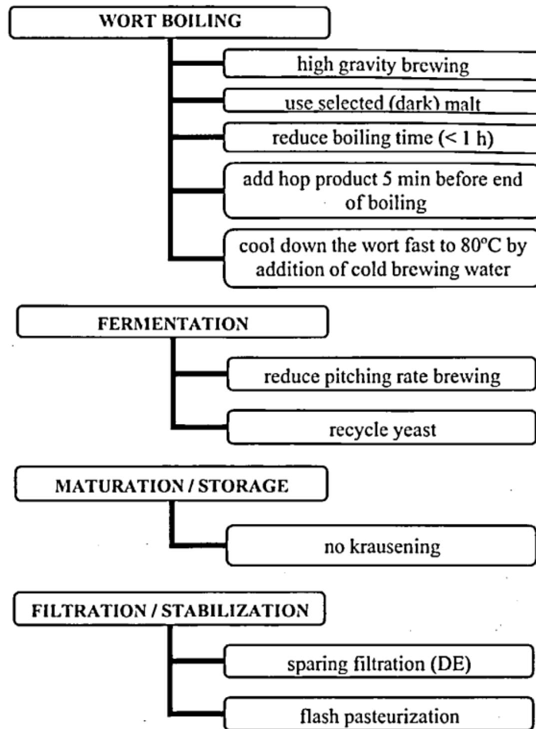

Figure 2.3 Xan Technology summary (Magalhaes et al., 2009)

4.4 Explanation of Other XN Losses

While increasing the amount of XN in hop products might logically contribute to an increased amount of XN in the final beer product, this is not the case as the majority of XN is isomerized to IX during the boiling process. Moreover, the bolstering affects XAN technology can have on the final XN content in beer cannot completely counteract the losses throughout the brewing process. Efforts to quantify XN content in all brewing ingredients and throughout the entire brewing/fermentation process yielded information

on the causes for XN losses. Reasons include incomplete extraction from hops into wort, adsorption to insoluble malt proteins, adsorption to yeast cells and cold trub, and isomerization to IX (Stevens et al., 1999a). Interestingly in Stevens' experiment, 27% of XN was missing from the total amounts measured in the final products. "Missing" amounts of XN were also seen in other experiments as well (Magalhaes et al., 2008).

5. Knowledge Gaps: Current Research

Beer recipes and styles have morphed since their inception thousands of years ago and often without any restrictions, such as purity laws. Thus, there exists a multitude of beer styles on the international market that do not adhere to German purity regulations. However, the current research has focused on increasing XN contents in beer produced under the German purity law. One example of a beer that may contain more XN than the average beer is the India Pale Ale (IPA) style beer. The IPA was first produced in Victorian England and it possesses a more pronounced hop flavor and aroma due to utilizing higher amounts of hops. Utilizing hops in high amounts helped protect beer from spoilage during transportation to India, hence its namesake. Furthermore, the American IPA has a prominent to intense hop aroma and many versions are dry-hopped. Dry-hopping is a brewing term to describe when hops are added after the wort has cooled and while the beer ferments (Guinard, 1990). Dry hopping contributes to hop aroma but not the perceived bitterness as alpha acids need to be isomerized during wort boiling to form the bitter tasting iso-alpha acids (De Keukeleire, 2000). Thus, the result of dry hopping is a beer with intense hop aroma. Because the dry hops are not exposed to heat, in theory, their inherent XN should be preserved, although some losses from insufficient extraction and adsorption into cold trub are expected. Future research should focus on identifying

amounts of XN in American IPAs, evaluate the effect (on XN content in final beer) of adding dark malt to an American IPA formula, and investigate the effects of filtration on XN contents in IPA styled beers. Currently, little research has been done to address the amount of XN and IX in American IPAs or any other dry hopped beers. While one study analyzed prenylflavonoids in different beer styles and reported an XN content of 0.16 mg/L in an India Pale Ale (Stevens et al., 1999b), this amount is expected to be higher in beers that are dry hopped.

Objectives

- To determine amounts of XN and IX content in samples of American IPAs.
- To determine if adding dark malt to an American IPA formula had an effect on XN content in final beer.
- To investigate the effect of DE filtration on final XN contents in American IPAs.
- To evaluate the difference in XN content in a non dry hopped and a dry hopped beer formula.
- To determine the total phenolic and antioxidant capacity of beer samples.
- To identify the effect of isolated beer compounds and whole beer matrix on proliferation and apoptosis of HCT 116 p53 +/+ colon cancer cells.

CHAPTER III

EFFECTS OF DARK MALTS, DRY HOPPING, AND FILTRATION ON XANTHOHUMOL CONTENT AND BIOACTIVITY OF AMERICAN INDIA PALE ALES

Abstract

Xanthohumol (XN), a prenylated chalcone found in hops (*Humulus lupulus* L.) has been shown to possess a wide spectrum of beneficial properties. Efforts have been made to increase the amount of XN in beers by modifying certain brewing ingredients and procedures. However, the effects of modifications such as addition of dark malts, dry hopping, and DE filtration on XN content and the biological activity of American India Pale Ales (IPAs) are not known. In this study, different brands of IPAs with and without addition of dark/roasted malts, dry hopping, and filtration and one non IPA were analyzed for XN, isoxanthohumol (IX), total phenolic content, and antioxidant capacity. Isolated beer compounds and selected whole beer matrixes were used to determine the synergistic effect of beer compounds on proliferation and apoptosis of HCT 116 p53 +/+ human colon cancer cell lines. The XN content in IPAs ranged from 0.0 to 12.69 mg/L. A heavily dry hopped IPA made with increased amounts of dark malt contained higher amounts of XN compared to other IPAs. The use of dark malts was protective against the removal of XN and other phenolics after diatomaceous earth (DE) filtration and dry hopping increased XN content in beer. Whole beer matrixes with greater levels of XN suppressed proliferation and elevated apoptosis in colon cancer cells compared with

isolated XN and/or IX, indicating that the biological effect of XN can be bolstered in the presence of other beer compounds.

1. Introduction

Beer contains multiple compounds beneficial to health including silicone, benzoic and cinnamic acid derivatives, ferulic acid, vitamin B6, betaine glycine, catechins, and proanthocyanidins. One compound in beer that has received much attention is the hop derived compound xanthohumol (XN). XN is the principal prenylated chalcone in the hop plant (Magalhaes et al., 2008). This compound has been found to possess a number of health-benefitting properties including antioxidant, anti-proliferative, pro-apoptotic, anti-inflammatory, anti-bacterial, anti-viral, and anti-malarial activities (Miranda et al., 2000; Bamforth, 2002; Gerhauser et al., 2002; Stevens et al., 2004; Gerhauser, 2005; Monteiro et al., 2008; Magalhaes et al., 2009; 2009; Sohrabvandi et al., 2010). A recent study showed that XN (10 μM) increased apoptosis and reduced cell proliferation of human colon carcinoma cell lines HT 29 and HCT 116 at 10 μM concentrations (Hadjiolov et al., 2009). Because of the wide spectrum of beneficial properties XN possesses, XAN technology, which includes modifying brewing ingredients (using dark, roasted malts and XN-enriched hop products) and brewing procedures (wort boiling, fermentation, maturation/storage, and filtration/stabilization) has been developed to increase the amount of XN in beers (Wunderlich et al., 2005; Back, 2007). The amount of XN in commercial beers is low: 0.2 mg/L or less (Wunderlich et al., 2005). However, addition of dark malts that contain several unidentified soluble substances with a molecule size range of 600,000-300,000 Da form complexes with beer compounds and might protect XN during the boiling process and reduce its isomerization to IX, a

compound that is not as biologically beneficial as XN (Wunderlich et al., 2005; Back, 2007). By utilizing the combination of dark malts and XN-enriched hop products, a beer with a XN content of 17.2 mg/L was developed (Wunderlich et al., 2005). XAN technology focuses on enriching XN content in beer produced under Reinheitsgebolt, the German purity law, which mandates hops are added during wort boil. This process exposes XN to heat, which isomerizes it to IX.

One example of a beer style that may inherently contain more XN is the India Pale Ale (IPA). More specifically, American IPA is a style of beer containing American hop varieties and often utilizes dry hopping where hops are added after the wort has been cooled and while the beer ferments (Guinard, 1990). Dry hopping contributes to hop aroma but not bitterness as alpha acids need to be isomerized during wort boiling to form the bitter tasting iso-alpha acids (De Keukeleire, 2000). Information on bioactive compounds in American IPA beers is limited. One study analyzed prenylflavonoids in different beer styles and reported XN content of 0.16 mg/L in an India Pale Ale (Stevens et al., 1999b), this amount is expected to be higher in beers that are dry hopped.

We hypothesized that 1) American IPAs will have higher amounts of XN, TP and antioxidant capacity compared to non IPA style beers and addition of dark malts and dry hopping increases the XN content, TP, and antioxidant activity 2) Beer matrix will have higher anti-proliferative and pro-apoptotic properties *in vitro* compare to isolated XN and/or IX. To test these hypotheses different brands of IPAs were analyzed for total phenolics (TP), antioxidant activity, and amounts of XN and IX. This research also addressed the effect of processing techniques (dry hopping and filtration) and ingredients (dark malts) on final XN and IX contents, and pro-apoptotic and anti-proliferative

properties of isolated compounds XN and/or IX, as well as whole beer matrixes in HCT 116 (P53+/+) colon cancer cells. Results indicated that the amount of XN, TP, and antioxidant capacity of IPAs vary widely depending on the ingredients and processes used. However, certain IPAs contained much higher amounts of XN than were previously reported (Stevens et al., 1999b). Finally, beer matrix with a mixture of bioactive compounds along with XN and IX showed potent anti-proliferative and pro-apoptotic properties compared to isolated XN and/or IX at similar concentrations.

2. Materials and Methods

2.1 Materials

Formic acid, Folin-Ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid (Trolox), and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical were purchased from Sigma (St. Louis, MO). Na_2CO_3 was purchased from Mallinckrodt (Paris, KY) and Gallic acid was purchased from Acros Organics (North Hampton, NH). XN and IX standards were purchased from Enzo Life Sciences (Plymouth Meeting, PA) and chlorogenic acid was procured from Indofine (Hillsborough, NJ). For the cell culture assays, McCoy's 5A modified medium, Dulbecco's modified Eagle's medium F-12, bovine serum albumin, and sodium bicarbonate were purchased from Sigma (St. Louis, MO). Fetal bovine serum, streptomycin/penicillin mix and 5% activated charcoal were obtained from Fisher Scientific (North Hampton, NH). Caspase-Glo 3/7 was purchased from Promega Corporation (Madison, WI). IPAS were donated by breweries in Fort Collins, CO. The non IPA was purchased at a liquor store in Fort Collins, CO.

2. 2. Beer Preparation

A total of 10 different beers were analyzed in this study. The effect of addition of dark malts on XN content in final beer was determined by collecting beer samples made from original Odell IPA recipe and from altering the Odell IPA recipe to contain 8.7% more darker malts. The effect of DE filtration on final XN contents in American IPAs was analyzed by collecting samples before and after filtration for three beer types. Finally, two different versions of the Zenith IPA, one dry hopped and the other non-dry hopped were tested to determine the effect of dry hopping on the final XN content. Different types of IPAs used in this study and information on beer preparation of each of these IPAs is presented in Table 2.1.

New Belgium Brewing Company's Ranger IPA contained a grain bill that consisted of two-row Pale and Crystal 120 malt. Both T-90 pellets and CO_2 extract were used for hop additions during the brew. The beer was dry hopped with Cascade hops. Chinook and Simcoe hops were also used. The Ranger had 70 IBUs (international bitterness units), 185 calories, and 6.5% ABV (alcohol by volume).

The Odell IPA contained Pale, Munich, Crystal, and Cara malts. Columbus (Hopsteiner- 14.4% alpha acids), Chinuk (Hop Union 10.5% alpha acids), Perle (Hop Union 7.7% alpha acids 3.4% beta acids) Amerillo (Hop Union 8.2% alpha acids), and Centennial (Hopsteiner 8.0% alpha acids) were added during and right after the boil. All hops used were pelleted. After several days of fermentation, three types of pelleted hops were added to the fermentation vessel. Other attributes of this beer include 70 IBUs and 7.0% ABV.

Table 2.1. IPA samples and preparation method in September, 2010.

IPA	Brewing Company	Malts	Hops	Filtration	Other Ingredients	Specifications
Ranger Prefiltered (RGP)	New Belgium Brewing Company	Two-row Pale and Crystal 120	T-90 pellets and CO_2 extract			Dry Hopped: amt. unknown
Ranger Filtered (RGF)	New Belgium Brewing Company	Two-row Pale and Crystal 120	T-90 pellets and CO_2 extract	DE Filtration		Dry Hopped: amt. unknown
IPA Prefiltered (OOP)	Odell Brewing Company	Pale, Munich, Crystal, and Cara	Pelleted Columbus, Chinuk, Perle, and Centennial			Dry Hopped: 0.055 oz. for 0.3 L of beer
IPA Filtered (OOF)	Odell Brewing Company	Pale, Munich, Crystal, and Cara	Pelleted Columbus, Chinuk, Perle, and Centennial	DE Filtration		Dry Hopped: 0.055 oz. for 0.3 L of beer
Dark IPA Prefiltered (ODP)	Odell Brewing Company	Pale, Munich, Crystal, Cara, ESB, Carafa, and Amber	Pelleted Columbus, Chinuk, Perle, and Centennial			Dry Hopped: 0.055 oz. for 0.3 L of beer
Dark IPA Filtered (ODF)	Odell Brewing Company	Pale, Munich, Crystal, Cara, ESB, Carafa, and Amber	Pelleted Columbus, Chinuk, Perle, and Centennial	DE Filtration		Dry Hopped: 0.055 oz. for 0.3 L of beer
Zenith Original Recipe (ZOR)	Equinox Brewing Company	Pale, Aromatic, Munich, Carapils, Crystal/Caramel, and Debittered black	Pelleted Columbus, Golding, Amarillo, Simcoe, and Centennial		Biofine Clear	2 oz. pelleted Centennial hops were added at the beginning of the whirlpool
Zenith New Recipe (ZNR)	Equinox Brewing Company	Pale, Aromatic, Munich, Carapils, Crystal/Caramel, and Debittered black	Pelleted Columbus, Golding, Amarillo, Simcoe, and Centennial		Biofine Clear	Dry hopped: 0.016 oz. per 0.3 L of beer
Punjabi Pale Ale (PPA)	CooperSmith's Pub and Brewing	Pale, light and medium Crystal	Pelleted Centennial, Columbus, and Cascade			Dry hopped: 0.014 oz. per 0.3 L of beer

The Odell Dark IPA was a scaled down version (5 barrels) of the Odell IPA. The original grain bill was altered to contain 8.7% more dark malt. The grain bill was as follows: 55 lbs ESB (Extra Special British), 250 lbs Pale, 20 lbs Munich, 20 lbs 100-120 Crystal, 15 lbs Cara, 11 lbs Carafa III, 5 lbs Amber. Three-hundred and fifty g Columbus (Hopsteiner- 14.4% alpha acids) hops were added at 90 minutes left in the boil, 350 g of Chinuk (Hop Union 10.5% alpha acids) hops were added at 45 minutes, 1000 g of Perle

(Hop Union 7.7% alpha acids 3.4% beta acids) hops were added at the end of the boil, 1360 g of Amerillo (Hop Union 8.2% alpha acids) hops and 360 g of Centennial (Hopsteiner 8.0% alpha acids) hops were added before the knock out. All hops used were pelleted. After a several days of fermentation, 2.2 lbs each of three types of pelleted hops were added to the fermentation vessel. Other attributes of the Dark IPA include 70 IBUs and 7.7% ABV. For the first three beers, samples were taken from various stages of transfer before filtration (RGP, OOP, and ODP) and before final bottling (RGF, OOF, and ODF) to address the effect of DE filtration on XN content.

Equinox Brewing Company's Zenith IPA contained 7.75 lbs Pale malt, 1 lb Aromatic malt, 0.90 lb Munich malt, 0.4 lb Carapils Malt, 0.40 lb crystal/caramel 30°L (Degrees Lovibond, indicating darker malts), and 0.4 lb Debittered black malt. A total of 0.85 oz of Columbus pelleted hops were steeped in the wort during the boil, 1 oz. pelleted Golding hops were added at 15 minutes left in the boil, 1 oz. pelleted Amarillo hops were added at 10 minutes left in the boil, and 1 oz. Simcoe hops were added at 5 minutes left in the boil. For ZOR, 2 oz. of pelleted Centennial hops were added at the beginning of the whirlpool. For ZNR, 1 oz. pelleted Centennial hops were added at the beginning of the whirlpool, and 1 oz of whole leaf hops were added during fermentation. The beers were chilled and fermented with British ale yeast. Prior to bottling, the fining agent Biofine Clear was used in a ratio of 30 mL per 300 gallons of beer to remove undesired sedimentation. Beer specifications include: OG: 1.058, FG 1.012, ABV 6.0%, IBU 75. Four samples of each beer were taken at different time points of keg tapping.

CooperSmith's Punjabi Pale Ale (PPA) was created with Pale malts and light and medium Crystal malts for the grain bill. Centennial, Columbus, and Cascade pelleted

hops were used and added in the following amounts and times: 10 lbs in kettle, 2.5 lbs at 90 minutes, 2.5 lbs at 30 minutes, 2.5 lbs at 5 minutes, 2.5 lbs at 1 minute, 3.5 lbs in whirlpool, and 3 pounds in aging tank. This beer contained 56 IBUs and 6.8% ABV and was brewed in 9 barrel batches. Four samples of the beer were taken at different time points of keg tapping.

Paulaner Pils (PPI) was a German-style Pilsner (pils) beer and contained 4.9% alcohol by volume (ABV). German Pilsners are typically made with Pilsner malts, German hop varieties, and German lager yeasts (Pavsler et al., 2009). Compared to the other beers, it was the lightest in color and highest in carbonation. It was the only beer purchased at a liquor store and samples were collected randomly from four bottles from the same 6-pack packaging.

2.3 Sample Preparation for Analytical Assays

Beer samples collected at breweries were transported on ice to the lab. All samples were stored at -80°C in 50 mL falcon tubes. The samples were thawed at 4°C and centrifuged at 1600 rpm for 20 minutes, and the supernatant was collected for analysis. Though some studies used solid phase extraction to extract bioactive compounds from beers before determining the content of XN (Magalhaes et al., 2008), a recent study showed a reduction in the XN recovery after C18 solid phase extraction of dark beers (Wunderlich et al., 2009; Haseleu et al., 2010). Thus, in this study the supernatant after centrifugation of beer was directly used for antioxidant activity, total phenolic content and LC-MS analysis. Beer samples were filtered and evaporated under nitrogen gas to remove ethanol prior to cell culture assays.

2.4. Identification of XN and IX Using LC/MS/MS

A Waters Acquity UPLC system with a BEH C18 column (1.7 µM, 1.0 x 100 mm) was used for chromatographic separation. Samples were eluted using a gradient mobile phase for 16 min at a flow rate of 140 µL/min. Solvent A was 5% acetonitrile, 5% water, 0.1% formic acid and solvent B was 95% acetonitrile, 5% water, 0.1% formic acid. Solvent A flow (100%) was maintained for 1 min followed by a gradient run from 100% A to 100% B over 10 minutes and maintained at 100% B for 2 min followed by 100% A for 3 min to re-equilibrate the column to original condition. The column and auto-sampler were maintained at 50°C and 5°C, respectively.

Column eluent was infused into a Micromass Q-Tof fitted with an electrospray source. Data was collected in negative polarity MS/MS mode, scanning from 50-1000 with a 0.5 second scan time and a 0.1 second interscan delay. Calibration was performed prior to sample analysis via infusion of sodium formate solution, with mass accuracy within 3 ppm. The capillary voltage was held at 2200V, the source temp at 130°C, and the desolvation temperature at 300°C with nitrogen desolvation gas flow rate of 300 L/hr. The quadrupole was held at collision energy of 18 electron volts for fragmentation. Both internal standard and target analytes were quantified using MS/MS fragmentation to improve specificity. Chlorogenic acid, an internal standard, isolated using mass 353.08, with product ion 191.06 eluting at 2.7 minutes served as the target for quantitation. Both IX and XN were quantified using the parent ion 353.14 and targeting the fragment ion 233.08 at retention times 6.01 and 7.84 minutes, respectively. Each beer type was tested in quadruplicate with two technical replications for each sample, for a total of eight replications. All sample areas were standardized using chlorogenic acid peaks as the

spiking rate was tenfold above detectable amounts of chlorogenic acid found inherently in the beer samples. Because detectable amounts of XN and IX were found in the beer matrix in which the dose response curve was prepared, these native areas were subtracted from all seven points before the dose-response curve was calibrated. The XN and IX dose response curve (338, 675, 1250, 2500, 5000, 10000, and 20000 ng/mL) had R^2 values of 0.999 and 0.992, respectively.

2.5 Total Phenolics Assay

The Folin-Ciocalteau reagent (FCR) assay was used to measure the total phenolic content of beer samples (Reddivari et al., 2007). The FC reagent was a mixture of phosphomolybdate and phosphotungstate and when exposed to samples, inherent phenolic compounds donated their electrons to the phosphomolybdic/phosphotungstic acid complexes imparting color change. Gallic acid was used to set the standard curve ($R^2>0.99$). Stored samples were diluted and tested in duplicate with four technical replications for each sample, for a total of eight replications. Samples were added to a 96 micro-well plate (35 µl per sample). Then 150 µl of 0.2 M Folin-Ciocalteau reagent was added to each well. After being shaken and left to stand for 5 minutes, 115 µl 7.5% Na_2CO_3 was added to all wells. Plates were held at room temperature for 1 hour before being read by a spectrophotometer (Synergy 2, BioTek, Winooski, VT) at a wavelength of 765 nm. Values were expressed as milligrams Gallic acid equivalent (GAE) per liter of sample ± SE.

2.6 Antioxidant Capacity Assay

The ABTS Trolox Equivalent Antioxidant Capacity assay (TEAC) was used to measure total antioxidant activity of the beer samples (Reddivari et al., 2007). This assay

measures the loss of color to the ABTS blue-green chromophore (free radical) when an antioxidant is added. A mother solution of ABTS was prepared by mixing 8 mM ABTS solution and 3 mM of potassium persulfate, which reacted in the dark for 12 hours. Then a working solution was made fresh every time by adding 5 mL of the mother solution to 45 mL of phosphate buffer.

Samples were diluted so that the absorbance reading was between 0.1-1.5. Diluted samples (10 µl) and ABTS working solution (290 µl)were mixed in a 96 micro-well plate and read in the spectrophotometer at 734 nm. Each beer type was tested in quadruplicate with four technical replications (16 replications in total). Results were expressed in milligrams TEAC per liter of sample ± SE.

2.7 Cell Lines

Human colon carcinoma cell line HCT-116 p53 +/+, gift from Dr. Bert Vogelstein, was maintained at 37ºC in 5% CO_2 jacketed incubator in McCoy's 5A modified medium supplemented with 2.0 g/L sodium bicarbonate, 0.11 g/L sodium pyruvate, 4.5 g/L glucose, 100 mL/L fetal bovine serum, and 10 mL/L antibiotic antimycotic solution.

2.8 Cell Proliferation Assay

Four beers were selected based on the content of XN or IX. OOP and ODP (with the highest XN content, ZNR (with the highest IX content), and PPI (with the lowest XN content) were used for *in vitro* assays. Centrifuged beer samples were filtered using a 0.2 µm nylon filter membrane and evaporated to completion under nitrogen gas. Then 1 mL of media was added to dissolve beer compounds. Cells were plated at a density of 5.0 X 10^4 per well in a 12-well plate. After 24 hours, growth medium was replaced with 2.5%

serum stripped Dulbecco's modified Eagle's medium F-12 containing either four beer samples in three concentrations (5, 10, 15 µg/mL GAE), or XN/IX at highest beer concentration (12.69 ng/mL), (2 ng/mL), XN + IX (12.69 ng/mL + 1 ng/mL), and XN at a concentration that previously has shown anti-proliferative activity (3.5 µg/mL) (Hadjiolov et al., 2009) were added. Ethanol was used as the control, as isolated compounds were dissolved in 80% ethanol. After 24 h of incubation with the treatments, cell proliferation (cell number) was measured using a Cellometer Auto T4 counter (Nexcelom Bioscience, Lawrence, MA). Results were expressed as means ± SEs.

2.9 Apoptosis

Cells were plated at a density of 5.0 X 10^4 per well in 12-well plates. After 24 h, growth media was replaced with Dulbecco's modified Eagle's medium F-12 media containing 2.5% charcoal stripped serum, and four beers were tested at three different concentrations (5, 10, 15 µg GAE /ml). After 24 h, 15,000 cells for each treatment were transferred to a 96-well white luminescence plate. Media was added to each well to make a total 200 µl followed by 100 µl of Caspase-Glo 3/7. The cells were incubated for 30 minutes in the dark and shaken thoroughly for one minute before the luminescence reading. Results were expressed as means ± SEs.

2.10. Statistical Analysis

Differences among means were calculated using Tukey's Honest Significant Difference (HSD) test using SPSS Software. For all comparisons, P-values ≤ 0.05 were considered significant. ANOVA was performed using SPSS. Pearson correlation coefficients were determined using the SAS Proc Corr procedure.

3. Results and Discussion

Ten different beers were analyzed for XN, IX, total phenolic content, and antioxidant activity. Of the ten beers tested, eight contained detectible amounts of XN (Figure 3.1 and Figure 3.2). ODP and ODF contained the highest amounts of XN: 12.69 and 12.62 mg/L, respectively. XN contents in the original IPA recipes were lower ($p \leq 0.05$) than the dark IPA: OOP (11.18 mg/L) and OOF (9.94 mg/L). Much lower amounts of XN were found in the other four beer samples: RGP (0.67 mg/L), RGF (0.44 mg/L), ZOR (0.15 mg/L), and ZNR (0.26 mg/L). Many IPAs tested in this study showed higher levels of XN compared to the earlier reports of 0.16 mg/L XN (Stevens et al., 1999b), which supports our hypothesis.

3.1 Effect of Addition of Roasted Malts, Filtration, and Dry Hopping on XN Content

The grain bill of the Odell IPA original recipe (OOP and OOF) was altered to contain 8.7% more roasted malts to prepare ODP and ODF. Beers with additional dark malts (ODP and ODF) showed the highest amount of XN (12.69 and 12.62 mg/L, respectively). Substances (600,000-300,000 Da) in roasted malts might form complexes with XN to protect it from isomerization to IX during wort boiling (Wunderlich et al., 2005; Back, 2007; Wunderlich et al., 2009).

The amount of XN in pre-filtered and filtered beers was dependent on the types of IPA. Results indicate DE filtration removed 34.3% of the XN content in the Ranger beer and 11.10% in the Odell IPA. The addition of dark malts reduced the removal of XN upon filtration and only a 0.55% reduction in XN content in Odell Dark IPA was observed. The data support our hypothesis that the use of dark malts decreases the removal of XN during filtration, as was observed in PVPP (polyvinylpyrrolidone)

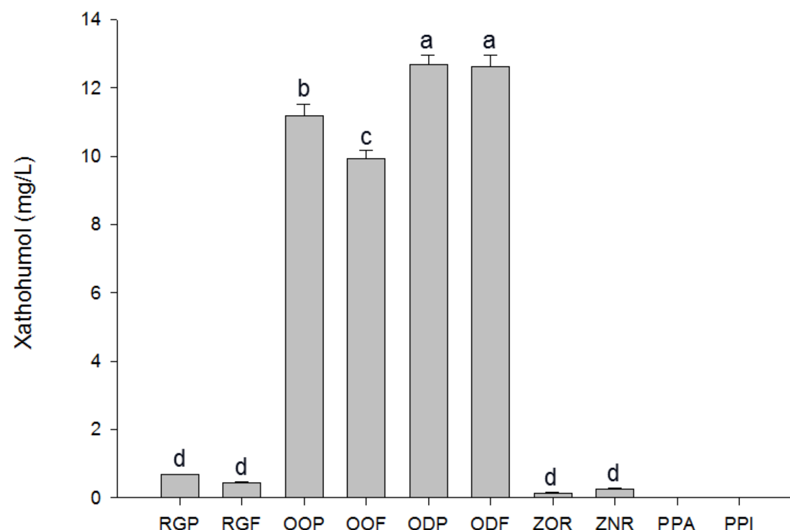

Figure 3.1. XN content of beer samples using UPLC/MS/MS. Ranger Pre-DE filtration (RGP*); Ranger Post-DE Filtration (RGF*); Odell IPA Pre-DE filtration (OOP*); Odell IPA Post-DE Filtration (OOF*); Odell Dark IPA Pre-DE filtration (ODP*); Odell Dark IPA Post-DE Filtration (ODF*); Zenith Original Recipe (ZOR); Zenith New Recipe (ZNR*); Punjabi Pale Ale (PPA*); and Paulaner Premium Pils (PPI). Data expressed as the mean of eight replications ± SE. Different letters on the bars represent significant differences at $p \leq 0.05$ in XN content among the beer samples. *Indicates dry hopped beers.

filtration (Wunderlich S., 2007).

In addition to the use of dark malts, both the Odell Dark IPA and the Odell IPA are heavily dry hopped (6.6 lbs of dry hops were added to 5 barrels of beer, which is equal to 0.055 oz. for 0.3 L of beer). This ratio of dry hops to beer is likely the highest of all beers tested in this study (ZNR: 0.016 oz. and PPA: 0.014 oz. of dry hops per 0.3 L of beer). RGP and RGF were also dry hopped but the amounts and type of hops (T-90 pellets and CO_2 extract) were not known. The low amount of XN in RGP and RGF (0.67 and 0.44 mg/L, respectively) may be due to the use of small amount of dry hops and/or

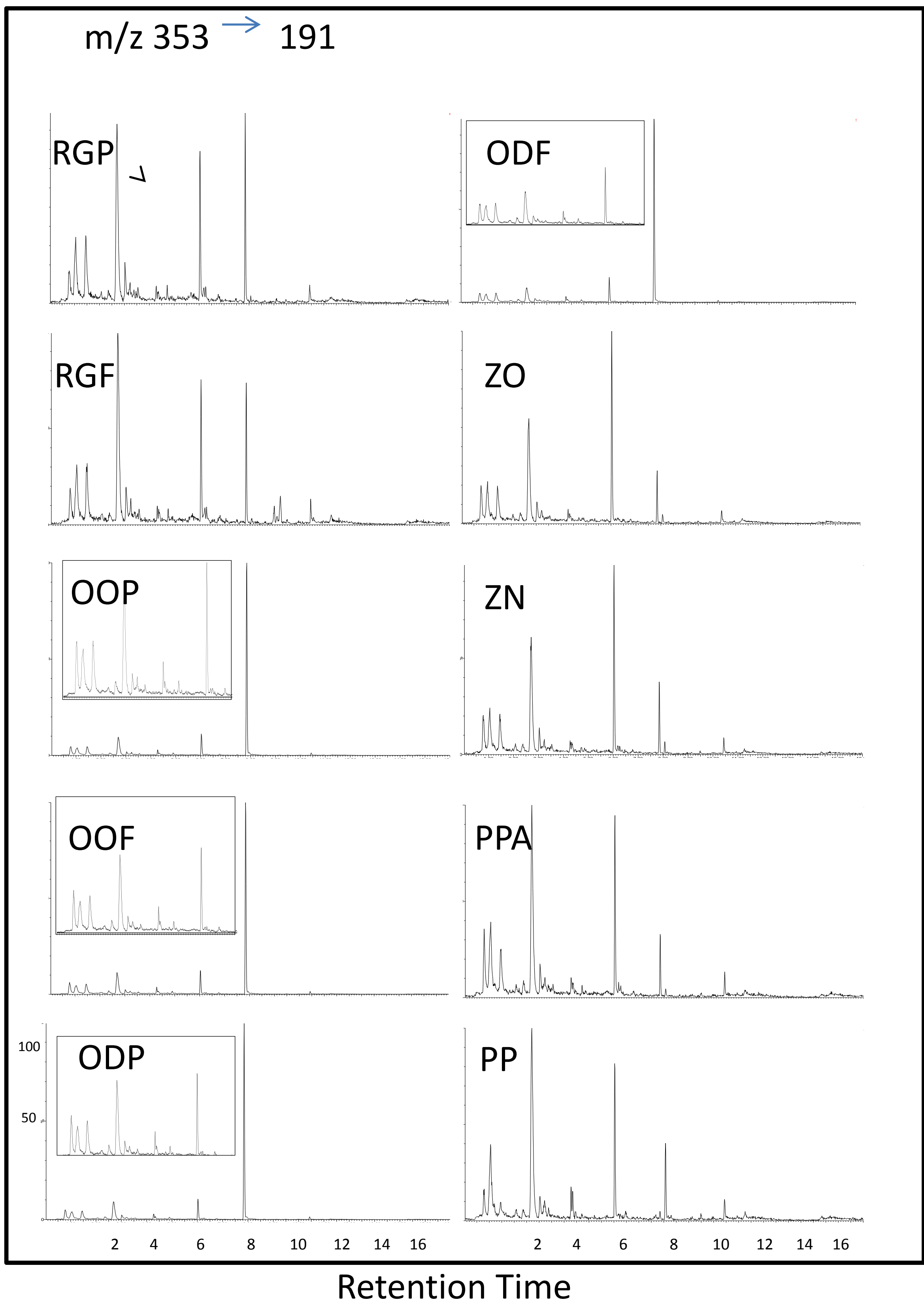

Figure 3.2 UPLC-MS/MS chromatograms. Analysis of chlorogenic acid (rt. 2.7 min), IX (rt. 6.01 min), and XN (rt. 7.84 min). For further details on UPLC run and interpretation, see section 2.3. Call out boxes show the peak intensities at reduced scale.

CO_2 extract for dry hopping. This is because CO_2 hop extraction recovers less than 5% XN in the original hop cone (Biendl et al., 2004b).

ZNR was prepared with 1 oz of dry hops while the ZOR contained the same amount of hops but all hops were added before the wort was cooled. This change in hop addition did result in a significant difference in XN content of ZNR (0.26 mg/L) and ZOR (0.15 mg/L). It is surprising that both Zenith beers did not contain more XN considering they were prepared with debittered black malt. These beers were both treated with Biofine Clear, a product that removes yeast and other haze forming particles based on colloidal silicon dioxide. Biofine Clear treatment might be associated with removing polyphenolic compounds, such as XN. Other explanations for varying amounts of XN in dry hopped beers analyzed in this study could include differences in the temperature and time of hop and beer contact during fermentation.

3.2 Effect of Addition of Roasted Malts, Filtration, and Dry Hopping on IX Content.

A variation in IX content was observed in the beers sampled (Figure 3.3). The IX content was lowest in RGF (0.30 mg/L) and highest in ZNR (1.82 mg/L). The use of roasted malts did increase the amount of IX observed as OOP contained 0.92 mg IX/L and ODP contained 1.08 mg IX/L. Filtration did not necessarily reduce the amount of IX in dark IPAs. In ODP the IX content actually increased from 1.08 to 1.27 mg/L. However, a slight decrease was seen in the other beers after filtration (RGP: 0.49; RGF: 0.30; OOP: 0.92; OOF: 0.86). All differences in IX content before and after filtration of beers were not statistically different.

The amount of IX was expected to be higher in certain beers because of the small amount of XN detected. For example, PPA contained no detectable amounts of XN even

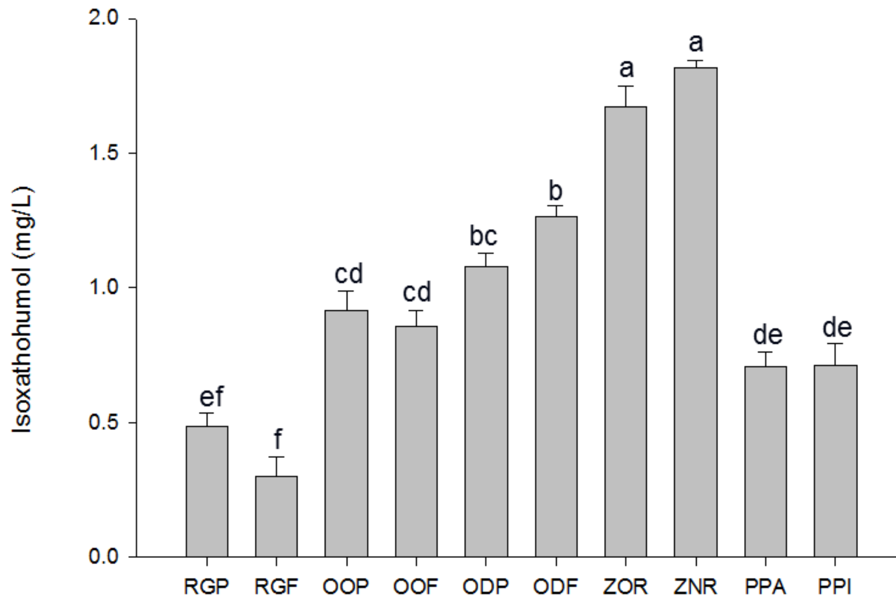

Figure 3.3. IX content of beer samples using UPLC/MS/MS. Ranger Pre-DE filtration (RGP*); Ranger Post-DE Filtration (RGF*); Odell IPA Pre-DE filtration (OOP*); Odell IPA Post-DE Filtration (OOF*); Odell Dark IPA Pre-DE filtration (ODP*); Odell Dark IPA Post-DE Filtration (ODF*); Zenith Original Recipe (ZOR); Zenith New Recipe (ZNR*); Punjabi Pale Ale (PPA*); and Paulaner Premium Pils (PPI). Data expressed as the mean of eight replications ± SE. Different letters on the bars represent significant differences at $p \leq 0.05$ in IX content among the beer samples. *Indicates dry hopped beers.

though it was dry hopped with 3 lbs. of pelleted malts for a 9 barrel batch. This beer also contained low amounts of IX. Possible reasons for low amounts of these compounds in this beer include poor extraction from the pelleted hops or that perhaps the pelleted hops did not contain XN, although XN is not typically lost in the pelletizing process (Biendl et al., 2004b). For other beers, such as ZOR and ZNR, the low amount of XN could be the result of the isomerization of XN to IX as amounts were statistically higher than all other beers. Although the difference in IX content between ZOR and ZNR was not statistically

significant, a trend was observed that the amount of IX was lower in ZOR than in ZNR. The reverse was expected as all the hops in ZOR were exposed to heat and 1 oz. of hops in ZNR were not. Perhaps the hops added during dry hopping contained inherent amounts of IX due to isomerization, which could account for this finding.

3.3 Variation in TP Content among Beers Sampled

The TP content of all IPAs style was significantly higher than that of PPI, the German-style Pilsner: 190.47 mg/L GAE (Figure 3.4). This is likely due to IPAs containing more hops and a larger variation in malts than do Pilsners. The highest TP content in the IPAs was observed in ODP (504.35 mg/L GAE) and could be attributed to the fact that this beer contained the highest amount of dark mats, was not filtered, and was heavily dry hopped. ODF had a slight reduction in TP content (482.90 mg/L GAE), indicating DE filtration resulted in a reduction of total phenolics in beer. Upon filtration the Odell IPA contained a significantly lower total phenolic content: 480.74 mg/L GAE

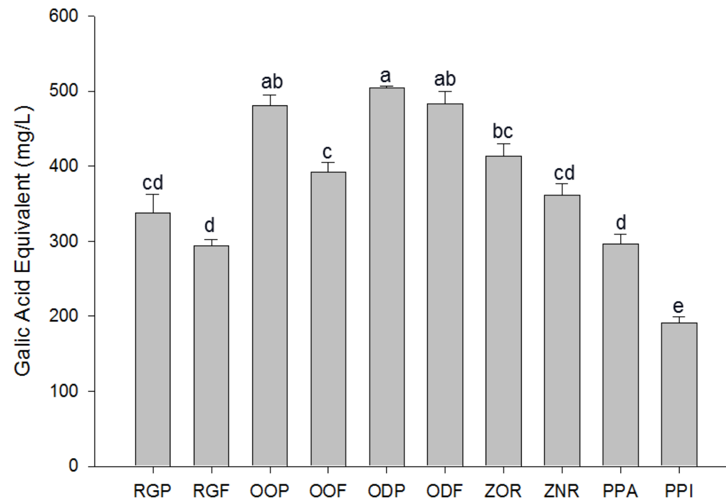

Figure 3.4. TP content analyzed by the Folin-Ciocalteau reagent assay. Ranger Pre-DE filtration (RGP*); Ranger Post-DE Filtration (RGF*); Odell IPA Pre-DE filtration (OOP*); Odell IPA Post-DE Filtration (OOF*); Odell Dark IPA Pre-DE filtration (ODP*); Odell Dark IPA Post-DE Filtration (ODF*); Zenith Original Recipe (ZOR); Zenith New Recipe (ZNR*); Punjabi Pale Ale (PPA*); and Paulaner Premium Pils (PPI).

Data expressed as the mean of eight replications ± SE. Different letters on the bars represent significant differences at $p \leq 0.05$ in TP content among the beer samples. *Indicates dry hopped beers

was reduced to 392.47 mg/L GAE. These results indicate that the addition of roasted malts could also minimize the removal of other phenolic compounds besides XN from the beer matrix upon DE filtration. TP content in RGF was slightly reduced compared to RGP, but this reduction was not statistically significant. ZOR contained less XN and IX compared to ZNR, but this did not correlate with total phenolic content (360.81 and 413.22 mg GAE/L, respectively). Even though some of the IPAs did not contain increased levels of XN as was expected, these beers still showed significant TP content. This may be due to the presence of other hop derived compounds. For example, RGP and RGF were made with both CO_2 hop extracts and pelleted hops. While the CO_2 extract recovers less than 5% XN in the original hop cone (Biendl et al., 2004b), other hop derived compounds such as alpha and beta acids are captured and preserved in this extract (Hopsteiner, 2010). Given that hop derived compounds besides XN are present in the CO_2 hop extracts it is not surprising the Ranger beers had similar total phenolic content as that of ZOR, ZNR and PPA.

3.4 Variation in ABTS Reducing Equivalent Capacity

The highest antioxidant capacity value was observed for ODP: 1327.38 mg/L TEAC (Figure 3.5). The antioxidant capacity of PPI (836.45 mg/L TEAC) was not significantly different from PPA (1035.75 mg/L TEAC), ZNR (958.65 mg/L TEAC), and ZOR (1095.0 0mg/L). This indicates that malt and hop derived compounds inherent in Pilsner-style beers contributed to the antioxidant capacity, although this amount is

statistically lower than beers containing darker malts and greater amounts of hop products.

Antioxidant capacity of RGP and RGF were 1323.98 and 1115.49 mg/L TEAC, respectively. These results indicate other compounds besides phenolic compounds contribute to antioxidant capacity in beer samples. This can be due to the fact that certain hop derived phenolic compounds, such as alpha acids, transform into trans-and cis-iso-alpha acids isocohumulone, cis-isocohumulone, trans-isohumulone, cis-isohumulone, trans-isoadhumulone, cis-(Intelmann et al., 2009). These compounds are not accounted

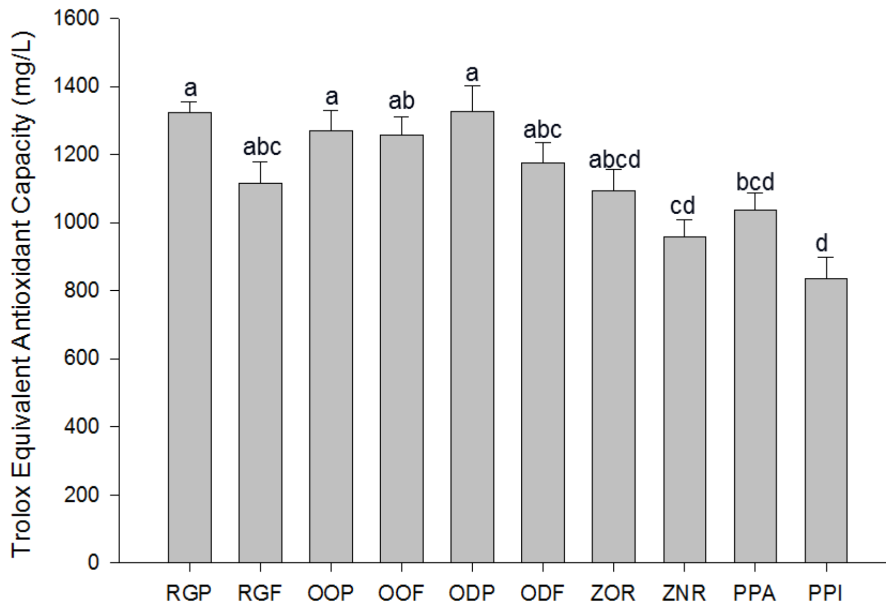

Figure 3.5 Antioxidant capacity analyzed by the ABTS Trolox Equivalent Antioxidant Capacity Assay. Ranger Pre-DE filtration (RGP*); Ranger Post-DE Filtration (RGF*); Odell IPA Pre-DE filtration (OOP*); Odell IPA Post-DE Filtration (OOF*); Odell Dark IPA Pre-DE filtration (ODP*); Odell Dark IPA Post-DE Filtration (ODF*); Zenith Original Recipe (ZOR); Zenith New Recipe (ZNR*); Punjabi Pale Ale (PPA*); and Paulaner Premium Pils (PPI). Data expressed as the mean of eight replications ± SE. Different letters on the bars represent significant differences at $p \leq 0.05$ in antioxidant capacity among the beer samples. *Indicates dry hopped beers

for in the total phenolics assay, but could play a role in antioxidant capacity. This could be the reason why RGP and RGF had a higher antioxidant capacity than total phenolic content compared to other beers.

3.5 Cell Proliferation and Apoptosis

XN is well known for its biological benefits and has been found to inhibit colon cancer cell proliferation at 10 mM concentrations (3.5 μg/mL) (Hadjiolov et al., 2009). However, no studies have focused on the synergistic effect of beer compounds in relation to colon cancer cell proliferation and cell death. This study tested four beer samples at three different concentrations on colon cancer lines HCT-116 p53 +/+ as well as pure XN

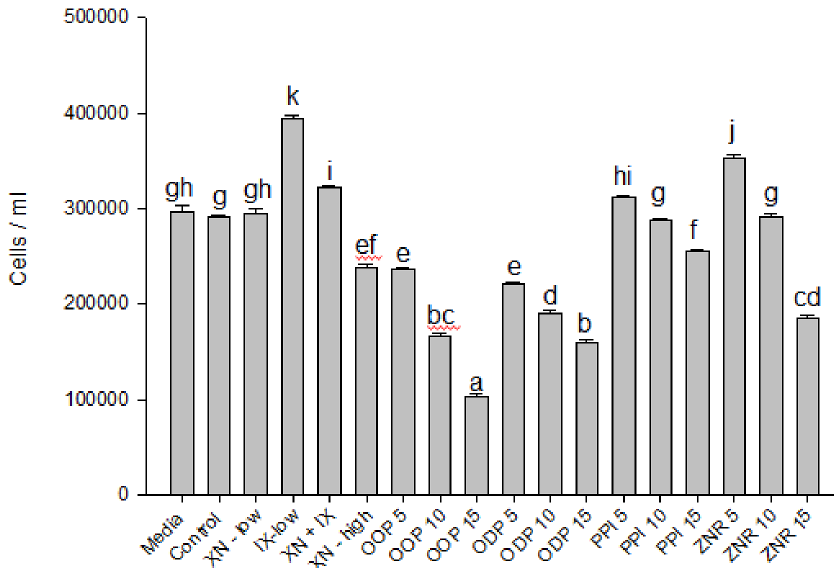

Figure 3.6. Anti-proliferative effects of isolated vs. whole beer matrixes on HCT-116 p53 +/+ human colon cancer cells. XN at highest beer concentration (12.69 ng/ml); IX at highest beer concentration (2 ng/ml); XN and IX at beer with highest XN concentration (12.69 ng/ml and 1 ng/ml, respectively); XN at (3.5 μg/ml); and beer samples: Odell IPA Pre-DE filtration (OOP); Odell Dark IPA Pre-DE filtration (ODP); Zenith New Recipe (ZNR); and Paulaner Premium Pils (PPI) at GAE concentrations of 5, 10, and 15 μg/μL. Data expressed as the mean of three replications ± SE. Different letters on the bars represent significant differences at $p \leq 0.05$ in cell proliferation among the beer samples.

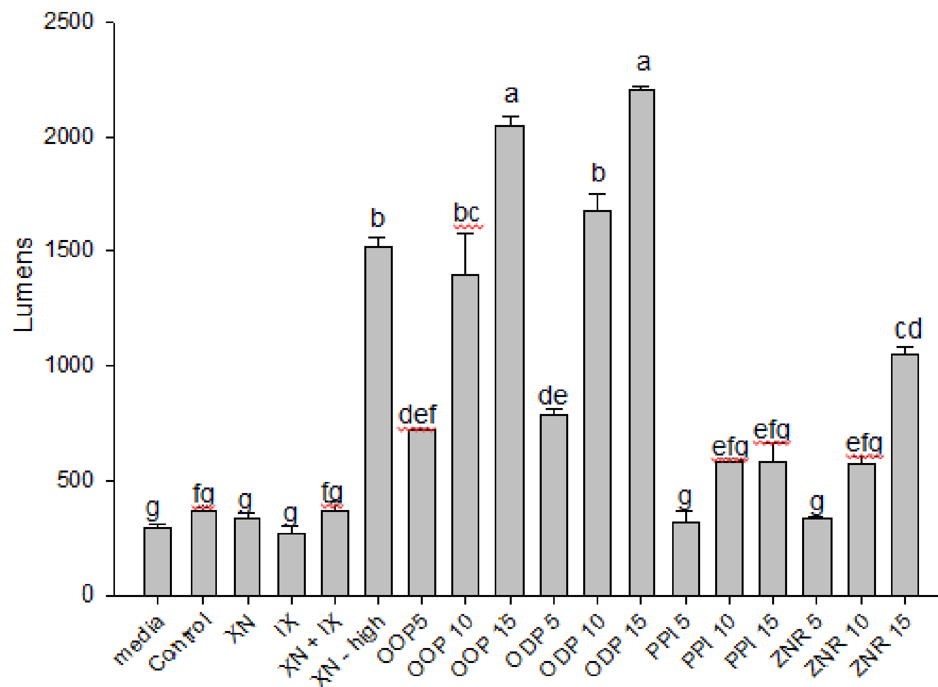

Figure 3.7. Proapoptotic effects of isolated vs. whole beer matrixes on HCT-116 p53 +/+ human colon cancer cells. XN at highest beer concentration (12.69 ng/ml); IX at highest beer concentration (2 ng/ml); XN and IX at beer with highest XN concentration (12.69 ng/ml and 1 ng/ml, respectively); XN at (3.5 µg/ml); and beer samples: Odell IPA Pre-DE filtration (OOP); Odell Dark IPA Pre-DE filtration (ODP); Zenith New Recipe (ZNR); and Paulaner Premium Pils (PPI) at GAE concentrations of 5, 10, and 15 µg/µL. Data expressed as the mean of three replications ± SE. Different letters on the bars represent significant differences at $p \leq 0.05$ in cell apoptosis among the beer samples.

and/or IX. Results indicated that IPAs differed in their suppression of cancer cell proliferation (Figure 3.6) and elevation of apoptosis (Figure 3.7).

IPAs with higher amounts of XN contained greater anti-proliferative and pro-apoptotic properties and the effect was dose dependent. ODP and OOP were more potent in suppressing cell proliferation and elevating apoptosis. The highest amounts of XN in these beer matrixes are only 12.69 and 11.18 ng/mL, respectively, and both suppressed ($p \leq 0.05$) cell proliferation and elevated apoptosis compared to XN and/or IX at the similar concentrations. These results indicate that XN in combination with other inherent beer

compounds is approximately 200 times more potent than isolated XN in reducing cell cancer growth. The observed effect of beer on colon cancer cells can be attributed to XN's ability to increase activation of Caspase 3/7 enzyme, inhibit DNA synthesis, and induce cell cycle arrest in S-phase (Miranda et al., 2000; Hadjiolov et al., 2009).

3.6 Correlation Coefficients among Different Parameters

Significant positive correlations were observed between XN and other parameters such as TP, antioxidant capacity, and apoptosis (Table 3.1). Significant negative correlations were observed between XN and cell proliferation. However, no correlation was seen between IX and all other parameters analyzed.

Table 3.1 Correlation coefficients and p-values between antioxidant activity (ABTS), total phenolics (TP), xanthohumol (XN), isoxanthohumol (IX), colon cancer cell proliferation (P), and Apoptosis (A)

	TP	XN	IX	P	A
ABTS	0.852* (0.0004)	0.857* (0.0004)	0.008 (0.9802)	-0.796* (0.0019)	0.876* (0.0002)
TP		0.878* (0.0002)	0.276 (0.3851)	-0.901* (0.0001)	0.953* (0.0001)
XN			0.172 (0.5931)	-0.785* (0.0025)	0.970* (0.0001)
IX				-0.207 (0.5190)	0.033 (0.9180)
P					-0.863* (0.0003)

* = Significant ($p < 0.05$)

4. Conclusions

Samples of IPAs from different breweries and one German Pilsner were analyzed for total phenolic, antioxidant capacity, XN, and IX content to determine the effects of

filtration, addition of dark malts, and dry hopping. By adding dark, roasted malts into an already intensely dry hopped beer, a XN content of 12.62 mg/L was achieved after DE filtration. This research confirmed that certain IPAs on the market, especially those that are intensely dry hopped (0.055 oz. of hops per .3 L of beer), contained an increased amount of XN compared to the German Pilsner and beers with a XN concentration of 0.2 mg/L. Beer made with increased amounts of dark malts contained significantly more XN, and pre-filtered beer contained more XN than filtered beer. The decrease in XN content due to filtration was significantly reduced in beer that contained increased dark malts. The recipe that contained dry hops had significantly more XN than the recipe without dry hops. Finally, cell culture assays confirmed beer mixtures with high amounts of XN suppressed cell proliferation and elevated apoptosis better than isolated components.

It is possible that elevated amounts of XN in combination with other inherent beer compounds could possibly provide beneficial effects, such as increasing anti-oxidant activity, anti-proliferative and pro-apoptotic properties. While this study showed the combination of increasing roasted malts and dry hopping a beer during fermentation produced a product with an elevated XN content (without the use of XN-enriched hop products), additional information may enhance the implementation of this synergistic effect. While dark malts were added to produce ODP/ODF, the exact type of malts used could have been optimized in terms of potential to protect XN. Malts possessing the same color can be produced from different methods, which vary in the development of roasted substances, compared to Maillard reaction compounds. As reported by Wunderlich and Back (2007), protection of XN does not necessarily increase as the color of the malt intensifies. Furthermore, complete information on hop ingredients and the details of the

brewing process for beers analyzed in this study would provide greater insight as to how to optimize XN content in IPAs. Perhaps the process of dry hopping beers could be used to increase the sensory characteristics and prenylated flavonoid content of specific beer styles, such as low-alcohol beers. There are already non-alcoholic beverages tapping into the niche market of XN-enriched products; Beverages Xan Wellness and Xan Tea produced by TA XAN Plc are examples of products with elevated XN content for the purpose of enhancing health, but they are not widely available. The unification of consumers' awareness of and desire for functional foods and XNs known biological benefits quite logically points to the development of future XN-enriched products.

REFERENCES

Albini, A., R. Dell'Eva, R. Vene, N. Ferrari, D. R. Buhler, *et al.* (2005). "Mechanisms of the Antiangiogenic Activity by the Hop Flavonoid Xanthohumol: Nf-Kappa B and Akt as Targets." Faseb Journal **19**(14): 527.

Anonymous. (2010). "Technical Information." While Labs Pure Brewers Yeast, Acessed on Aug. 8 2010, from http://www.whitelabs.com/beer/craft_technical_info.html.

Back, S. W. a. W. (2007). Brewing Technological Limits of Xanthohumol Enrichment in Beer. 31. European Brewery Convention Congress. Venice, Italy.

Bamforth, C. W. (2000). "Brewing and Brewing Research: Past, Present and Future." Journal of the Science of Food and Agriculture **80**(9): 1371-1378.

Bamforth, C. W. (2002). Nutritional Aspects of Beer: A Review, Nutrition Research. **22:** 227-237.

Bamforth, C. W. (2006). Scientific Principles of Malting and Brewing. St. Paul, Minn., American Society of Brewing Chemists.

Bhattacharya, S., S. Virani, M. Zavro and G. J. Haas (2003). "Inhibition of *Streptococcus Mutans* and Other Oral Streptococci by Hop (*Humulus Lupulus* L.) Constituents." Economic Botany **57**(1): 118-125.

Biendl, M., F. J. Methner, G. Stettner and C. J. Walker (2004a). "Brewing Trials with a Xanthohumol Rich Hop Product." Brauwelt **144**(9--10): 236.

Biendl, M., M. Virant, P. Varju and E. B. C. A. Committee (2004b). "Determination of Iso-Alpha-Acids, Alpha- and Beta-Acids in Isomerised Hop Pellets by Hplc." Journal of the Institute of Brewing **110**(3): 242-243.

Bowe, J., X. F. Li, J. Kinsey-Jones, A. Heyerick, S. Brain, *et al.* (2006). "The Hop Phytoestrogen, 8-Prenylnaringenin, Reverses the Ovariectomy-Induced Rise in Skin Temperature in an Animal Model of Menopausal Hot Flushes." Journal of Endocrinology **191**(2): 399-405.

Briggs, D. E. (1998). Malts and Malting. London, Blackie Academic.

Bruce, J. (2002). "Analysis of Anions in Beer Using Ion Chromatography." Journal of Automated Methods & Management in Chemistry **24**(4): 127-130.

Buckwold, V. E., R. J. H. Wilson, A. Nalca, B. B. Beer, T. G. Voss, *et al.* (2004). "Antiviral Activity of Hop Constituents against a Series of DNA and Rna Viruses." Antiviral Research **61**(1): 57-62.

Casey, T. R. and C. W. Bamforth (2009). "Silicon in Beer and Brewing." Journal of the Science of Food and Agriculture **90**(5): 784-788.

Ceh, B., M. Kac, I. J. Kosir and V. Abram (2007). "Relationships between Xanthohumol and Polyphenol Content in Hop Leaves and Hop Cones with Regard to Water Supply and Cultivar." International Journal of Molecular Sciences **8**(9): 989-1000.

Darby, W. J., L. Grivetti and P. Ghalioungui (1977). Food : The Gift of Osiris / William J. Darby, Paul Ghalioungui, Louis Grivetti, London ; New York : Academic Press.

De Keukeleire, D. (2000). "Fundamentals of Beer and Hop Chemistry." Quimica Nova **23**(1): 108-112.

Diel, P., R. B. Thomae, A. Caldarelli, O. Zierau, S. Kolba*, et al.* (2004). "Regulation of Gene Expression by 8-Prenylnaringenin in Uterus and Liver of Wistar Rats." Planta Medica **70**(1): 39-44.

Gerhauser, C. (2005). "Beer Constituents as Potential Cancer Chemopreventive Agents." European Journal of Cancer **41**(13): 1941-1954.

Gerhauser, C., A. Alt, E. Heiss, A. Gamal-Eldeen, K. Klimo*, et al.* (2002). "Cancer Chemopreventive Activity of Xanthohumol, a Natural Product Derived from Hop." Molecular Cancer Therapeutics **1**(11): 959-969.

Ghiselli, A., F. Natella, A. Guidi, L. Montanari, P. Fantozzi*, et al.* (2000). "Beer Increases Plasma Antioxidant Capacity in Humans." Journal of Nutritional Biochemistry **11**(2): 76-80.

Gorjanovic, S. Z., M. M. Novakovic, N. I. Potkonjak, I. Leskosek-Cukalovic and D. Z. Suznjevic (2010). "Application of a Novel Antioxidative Assay in Beer Analysis and Brewing Process Monitoring." Journal of Agricultural and Food Chemistry **58**(2): 744-751.

Guinard, J.-X., Woodmansee, R.D., Billovits, M.J., Hanson, L.G., Gutierrez, M.-J., Snider, M.L., Miranda, M.G. and Lewis, M.J. (1990). The Microbiology of Dry Hopping. Technical Quarterly, Master Brewers Association of the Americas. **27:** 83-89.

Hadjiolov, N. and N. Frank (2009). "Xanthohumol and Sulforaphane Induce Apoptosis and Inhibit Proliferation of Ht29 and Hct 116 Colon Cancer Cells." Comptes Rendus De L Academie Bulgare Des Sciences **62**(9): 1175-1182.

Hanske, L., G. Loh, S. Sczesny, M. Blaut and A. Braune (2010). "Recovery and Metabolism of Xanthohumol in Germ-Free and Human Microbiota-Associated Rats." Molecular Nutrition & Food Research **54**(10): 1405-1413.

Haseleu, G., A. Lagemann, A. Stephan, D. Intelmann, A. Dunkel*, et al.* (2010). "Quantitative Sensomics Profiling of Hop-Derived Bitter Compounds Throughout a Full-Scale Beer Manufacturing Process." Journal of Agricultural and Food Chemistry **58**(13): 7930-7939.

Hopsteiner. (2010). "Hop Products." from http://www.hopsteiner.com/products.html.

Ingels, A. (1987). "'Gruit' Herbs and Beer Made with Them." Voedingsmiddelentechnologie **20**(3): 12-16.

Intelmann, D., G. Haseleu and T. Hofmann (2009). "Lc-Ms/Ms Quantitation of Hop-Derived Bitter Compounds in Beer Using the Echo Technique." Journal of Agricultural and Food Chemistry **57**(4): 1172-1182.

Jung, H. J., S. S. Kang, S. K. Hyun and J. S. Choi (2005). "In Vitro Free Radical and Onoo- Scavengers from Sophora Flavescens." Archives of Pharmacal Research **28**(5): 534-540.

Kondo, K. (2004). "Beer and Health: Preventive Effects of Beer Components on Lifestyle-Related Diseases." Biofactors **22**(1-4): 303-310.

Lewis, M.-. and T. W. Young (2001). Brewing. New York, Kluwer Academic/Plenum Publishers.

Magalhaes, P. J., D. O. Carvalho, J. M. Cruz, L. F. Guido and A. A. Barros (2009). "Fundamentals and Health Benefits of Xanthohumol, a Natural Product Derived from Hops and Beer." Natural Product Communications **4**(5): 591-610.

Magalhaes, P. J., P. Dostalek, J. M. Cruz, L. F. Guido and A. A. Barros (2008). "The Impact of a Xanthohumol-Enriched Hop Product on the Behaviour of Xanthohumol and Isoxanthohumol in Pale and Dark Beers: A Pilot Scale Approach." Journal of the Institute of Brewing **114**(3): 246-256.

McMurrough, I., D. Madigan and M. R. Smyth (1995). "Adsorption by Polyvinylpolypyrrolidone of Catechins and Proanthocyanidins from Beer." Journal of Agricultural and Food Chemistry **43**(10): 2687-2691.

Miranda, C. L., J. F. Stevens, V. Ivanov, M. McCall, B. Frei*, et al.* (2000). "Antioxidant and Prooxidant Actions of Prenylated and Nonprenylated Chalcones and Flavanones in Vitro." Journal of Agricultural and Food Chemistry **48**(9): 3876-3884.

Miura, Y., M. Hosono, C. Oyamada, H. Odai, S. Oikawa*, et al.* (2005). "Dietary Isohumulones, the Bitter Components of Beer, Raise Plasma Hdl-Cholesterol Levels and Reduce Liver Cholesterol and Triacylglycerol Contents Similar to Ppar Alpha Activations in C57bl/6 Mice." British Journal of Nutrition **93**(4): 559-567.

Mizobuchi, S. and Y. Sato (1984). "A New Flavanone with Antifungal Activity Isolated from Hops " Agricultural and Biological Chemistry **48**(11): 2771-2775.

Monteiro, R., C. Calhau, A. O. E. Silva, S. Pinheiro-Silva, S. Cuerreiro*, et al.* (2008). "Xanthohumol Inhibits Inflammatory Factor Production and Angiogenesis in Breast Cancer Xenografts." Journal of Cellular Biochemistry **104**(5): 1699-1707.

Mussatto, S. I., G. Dragone and I. C. Roberto (2006). "Brewers' Spent Grain: Generation, Characteristics and Potential Applications." Journal of Cereal Science **43**(1): 1-14.

Natarajan, P., S. Katta, I. Andrei, V. B. R. Ambati, M. Leonida*, et al.* (2008). "Positive Antibacterial Co-Action between Hop (Humulus Lupulus) Constituents and Selected Antibiotics." Phytomedicine **15**(3): 194-201.

Nikolic, D., Y. M. Li, L. R. Chadwick, G. F. Pauli and R. B. van Breemen (2005). "Metabolism of Xanthohumol and Isoxanthohumol, Prenylated Flavonoids from Hops (Humulus Lupulus L.), by Human Liver Microsomes." Journal of Mass Spectrometry **40**(3): 289-299.

Nowakowska, Z. (2007). "A Review of Anti-Infective and Anti-Inflammatory Chalcones." European Journal of Medicinal Chemistry **42**(2): 125-137.

Nozawa, H. (2005). "Xanthohumol, the Chalcone from Beer Hops (Humulus Lupulus L.), Is the Ligand for Farnesoid X Receptor and Ameliorates Lipid and Glucose Metabolism in Kk-a(Y) Mice." Biochemical and Biophysical Research Communications **336**(3): 754-761.

Pavsler, A. and S. Buiatti (2009). Lager Beer. Beer in Health and Disease Prevention. V. R. Preedy. Burlington, MA, Academic Press**:** 31-43.

Reddivari, L., A. L. Hale and J. C. Miller, Jr. (2007). "Determination of Phenolic Content, Composition and Their Contribution to Antioxidant Activity in Specialty Potato Selections." American Journal of Potato Research **84**(4): 275-282.

Samaras, T. S., P. A. Camburn, S. X. Chandra, M. H. Gordon and J. M. Ames (2005). "Antioxidant Properties of Kilned and Roasted Malts." Journal of Agricultural and Food Chemistry **53**(20): 8068-8074.

Schermerhorn, C. (1993). Great American Beer Cookbook. Brewers Publication: Boulder, CO, Association of Brewers.

Smith, N. A. (1994). "Nitrate Reduction and N-Nitrosation in Brewing " Journal of the Institute of Brewing **100**(5): 347-355.

Sohrabvandi, S., S. M. Mousavi, S. H. Razavi, A. M. Mortazavian and K. Rezaei (2010). "Alcohol-Free Beer: Methods of Production, Sensorial Defects, and Healthful Effects." Food Reviews International **26**(4): 335-352.

Stevens, J. F. and J. E. Page (2004). "Xanthohumol and Related Prenylflavonoids from Hops and Beer: To Your Good Health!" Phytochemistry **65**(10): 1317-1330.

Stevens, J. F., A. W. Taylor, J. E. Clawson and M. L. Deinzer (1999a). "Fate of Xanthohumol and Related Prenylflavonoids from Hops to Beer." Journal of Agricultural and Food Chemistry **47**(6): 2421-2428.

Stevens, J. F., A. W. Taylor and M. L. Deinzer (1999b). "Quantitative Analysis of Xanthohumol and Related Prenylflavonoids in Hops and Beer by Liquid Chromatography Tandem Mass Spectrometry." Journal of Chromatography A **832**(1-2): 97-107.

Stewart, G. G. (2010). "High-Gravity Brewing and Distilling-Past Experiences and Future Prospects." Journal of the American Society of Brewing Chemists **68**(1): 1-9.

Vinson, J. A., M. Mandarano, M. Hirst, J. R. Trevithick and P. Bose (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 and Food Chemistry **51**(18): 5528-5533.

Walker, C. J., C. Fernandez-Lence and M. Biendl (2003). "Studies on the High Xanthohumol Content in Beers of the Stout/Porter Type." Brauwelt **143**(50): 1709-1712.

Whittle, N., H. Eldridge, J. Bartley and G. Organ (1999). "Identification of the Polyphenols in Barley and Beer by Hplc/Ms and Hplc/Electrochemical Detection." Journal of the Institute of Brewing **105**(2): 89-99.

Wunderlich, S., M. Biendl, A. Zurcher and W. Back (2009). "Reduced Xanthohumol Recovery after Solid Phase Extraction." Brewing Science **Vol. 62**(July/ August).

Wunderlich, S., A. Zurcher and W. Back (2005). "Enrichment of Xanthohumol in the Brewing Process." Molecular Nutrition & Food Research **49**(9): 874-881.

Wunderlich S., W. B. (2007). Brewing Technological Limits of Xanthohumol Enrichment in Beer. 31st European Brewery Convention Congress, Venice, Italy.

Yilmazer, M., J. F. Stevens, M. L. Deinzer and D. R. Buhler (2001). "In Vitro Biotransformation of Xanthohumol, a Flavonoid from Hops (Humulus Lupulus), by Rat Liver Microsomes." Drug Metabolism and Disposition **29**(3): 223-231.

APPENDIX I

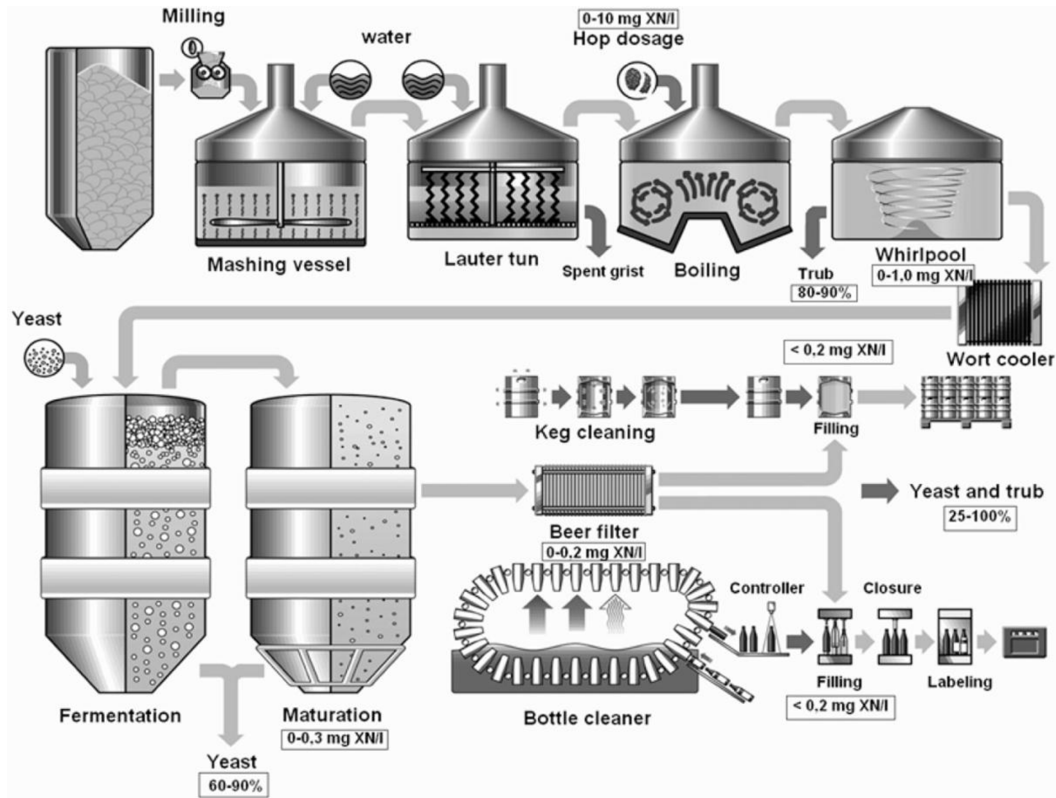

Figure A-1 Outline of beer production

Wunderlich et al. (2005) Mol Nutr Food Res 49(9): 874-881

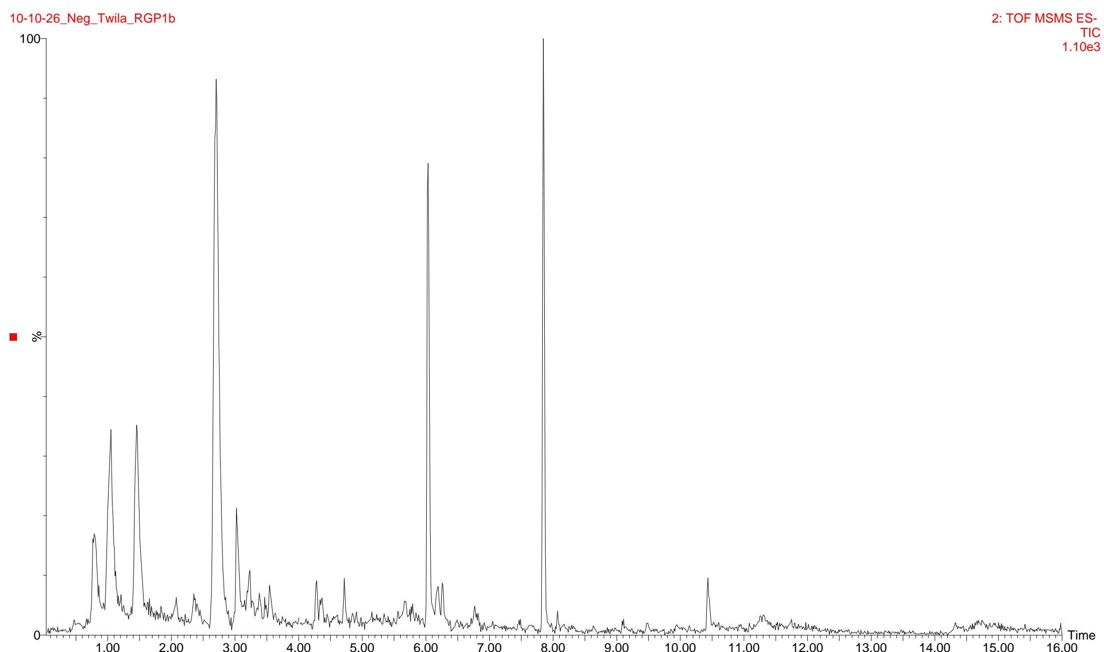

Figure A-2 Chromatogram for Ranger Prefiltered (RGP)

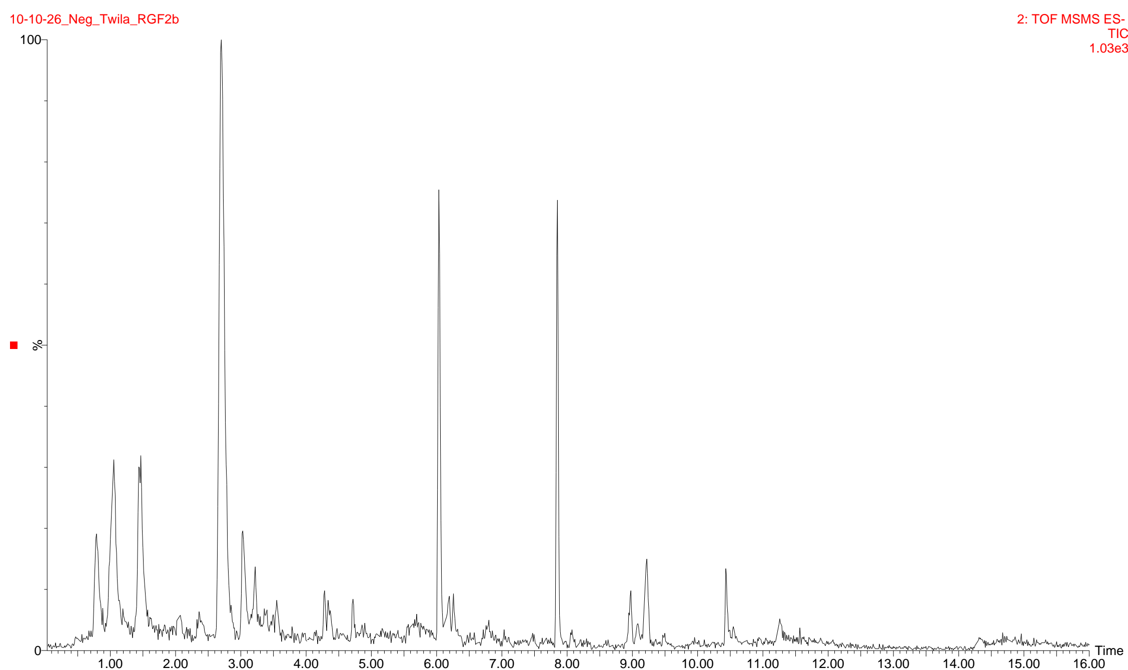

Figure A-3 Chromatogram for Ranger Filtered (RGF)

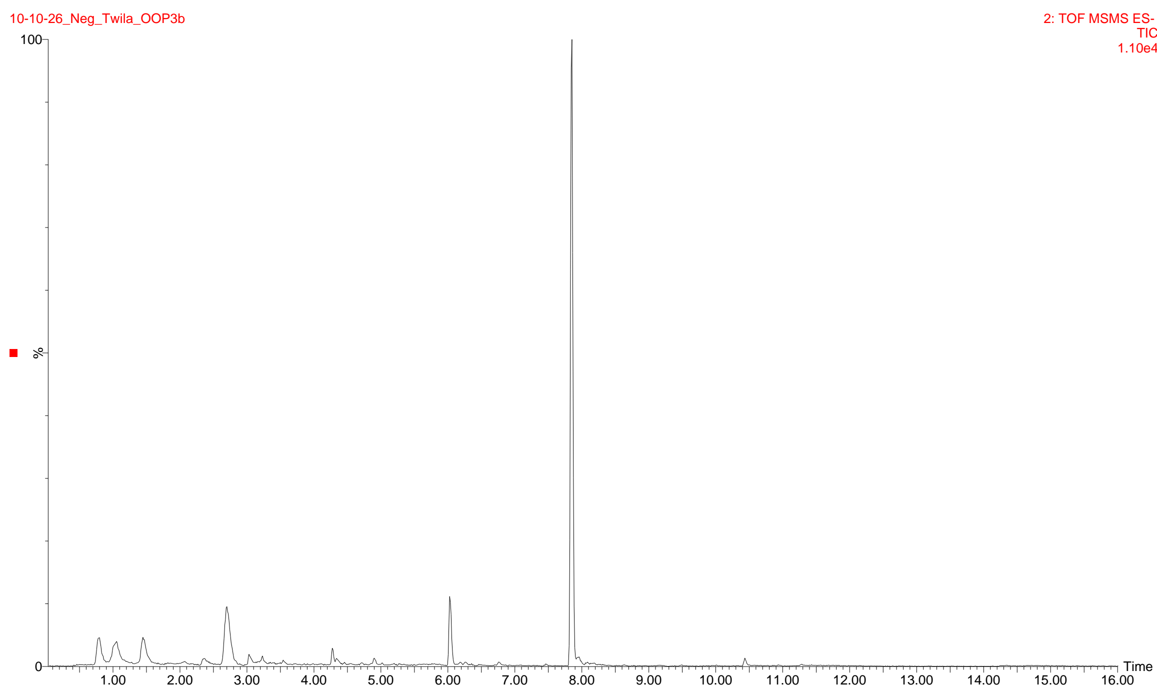

Figure A-4 Chromatogram for Odell IPA Prefiltered (OOP)

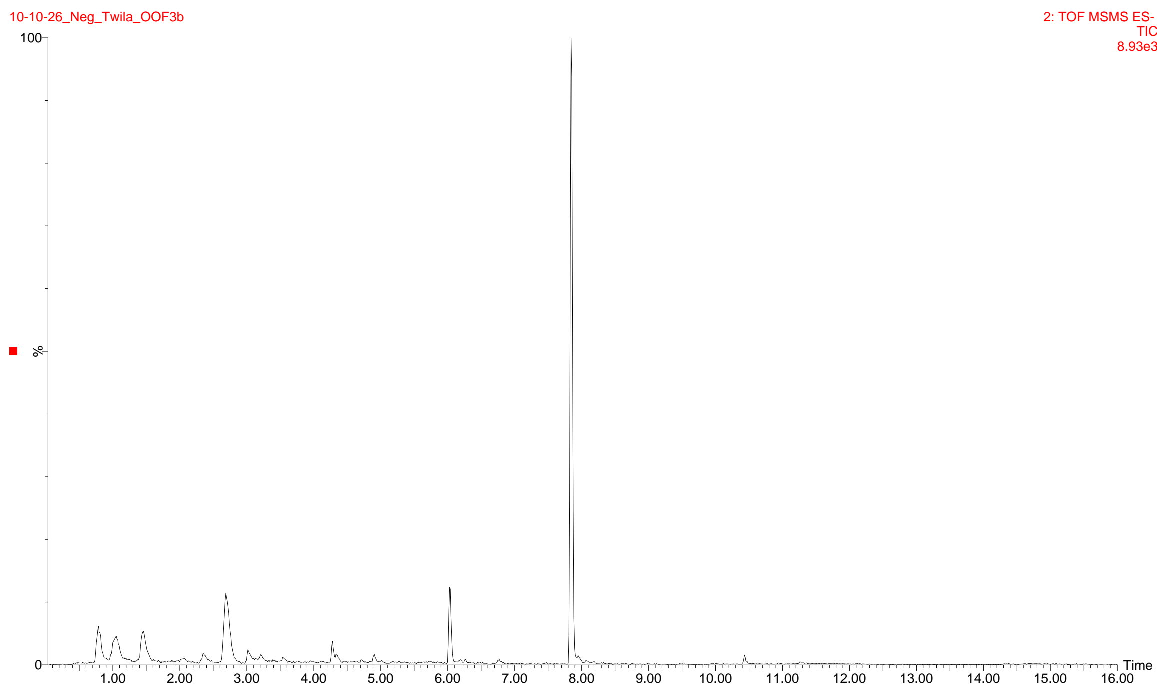

Figure A-5 Chromatogram for Odell IPA Filtered (OOF)

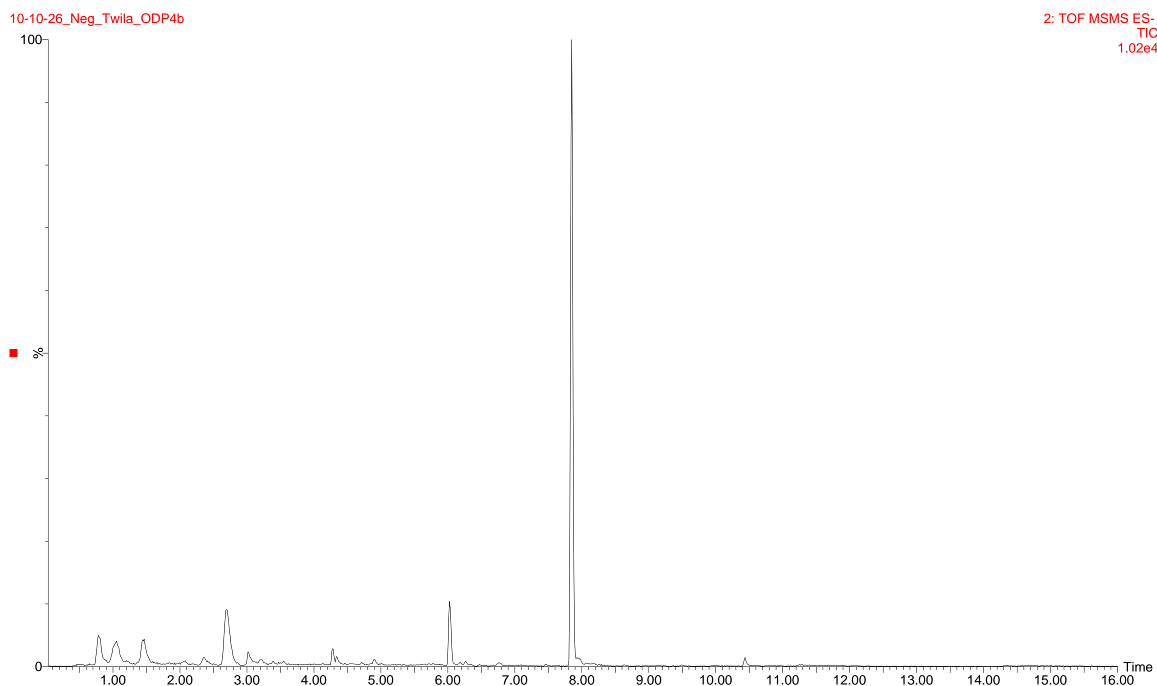

Figure A-6 Chromatogram for Odell Dark IPA Prefiltered (ODP)

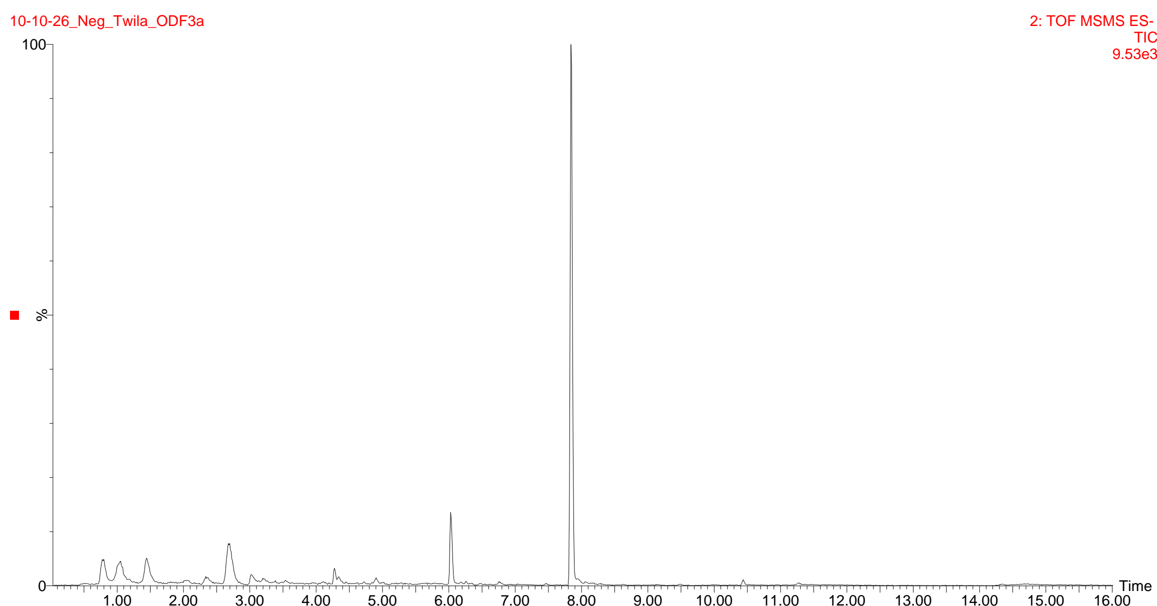

Figure A-7 Chromatogram for Odell Dark IPA Filtered (ODF)

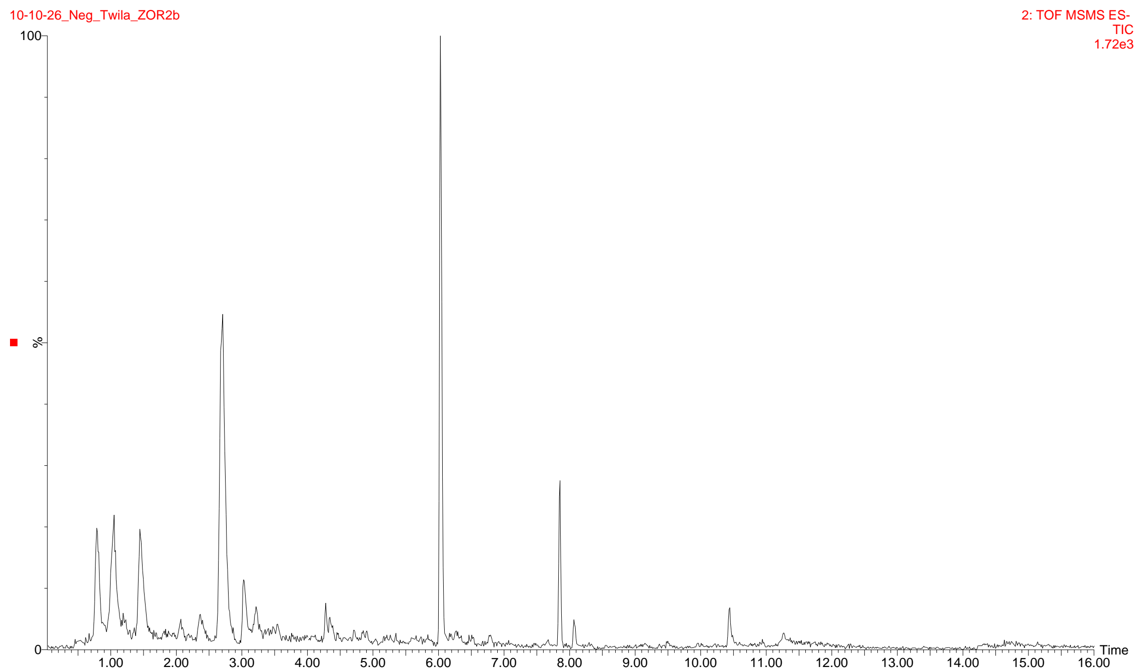

Figure A-8 Chromatogram for Zenith Original Recipe (ZOR)

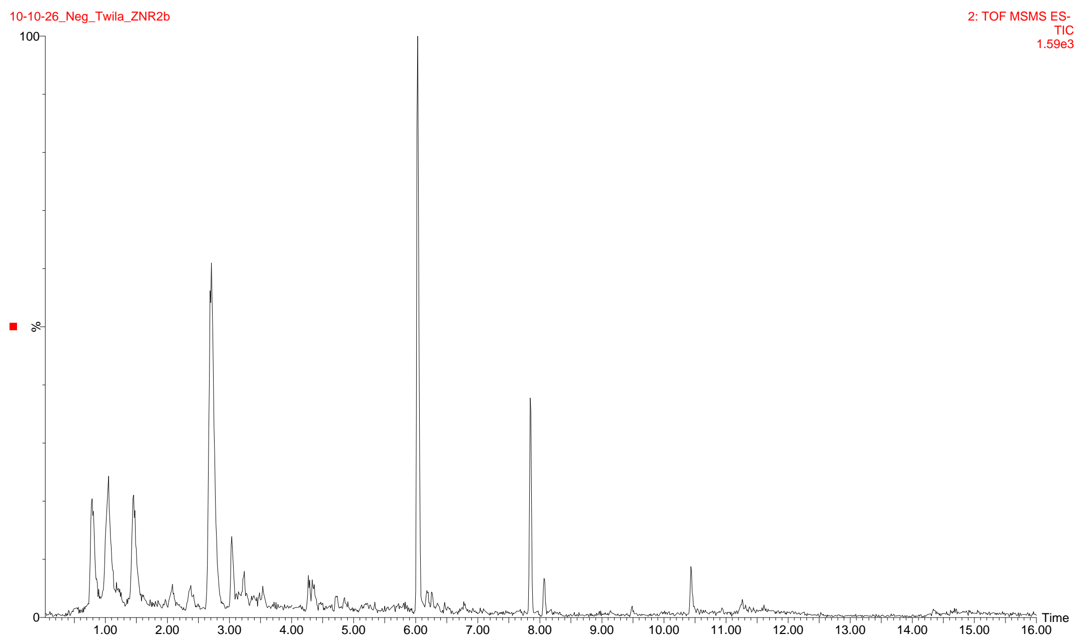

Figure A-9 Chromatogram for Zenith New Recipe (ZNR)

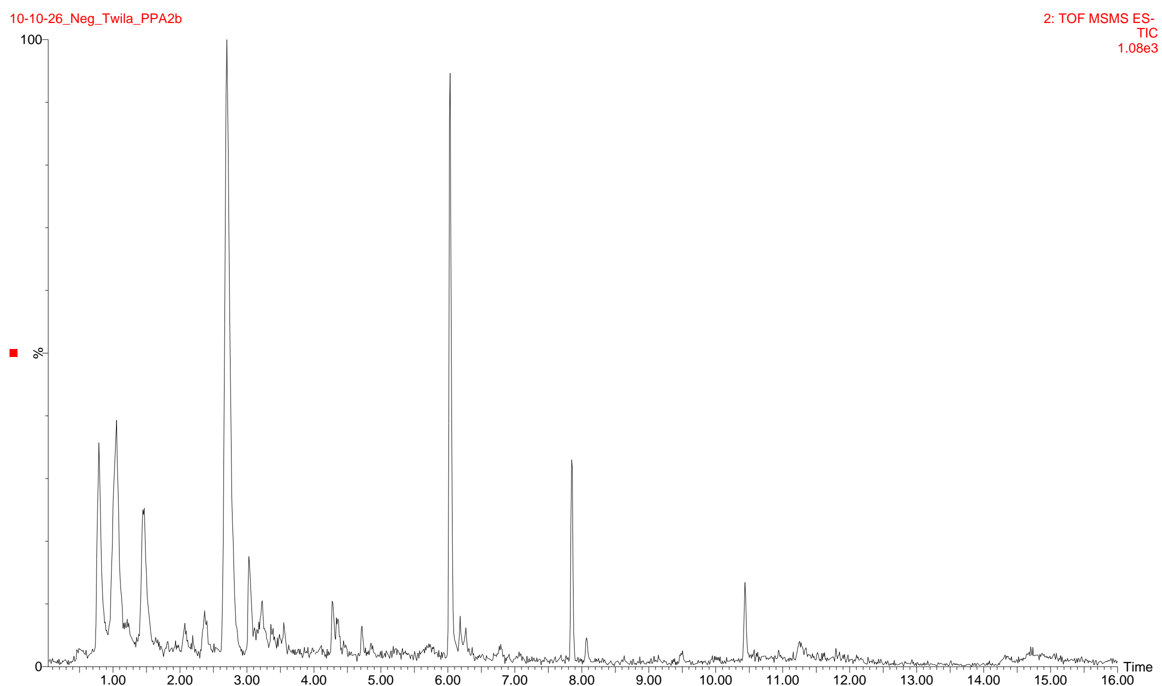

Figure A-10 Chromatogram for Punjabi Pale Ale (PPA)

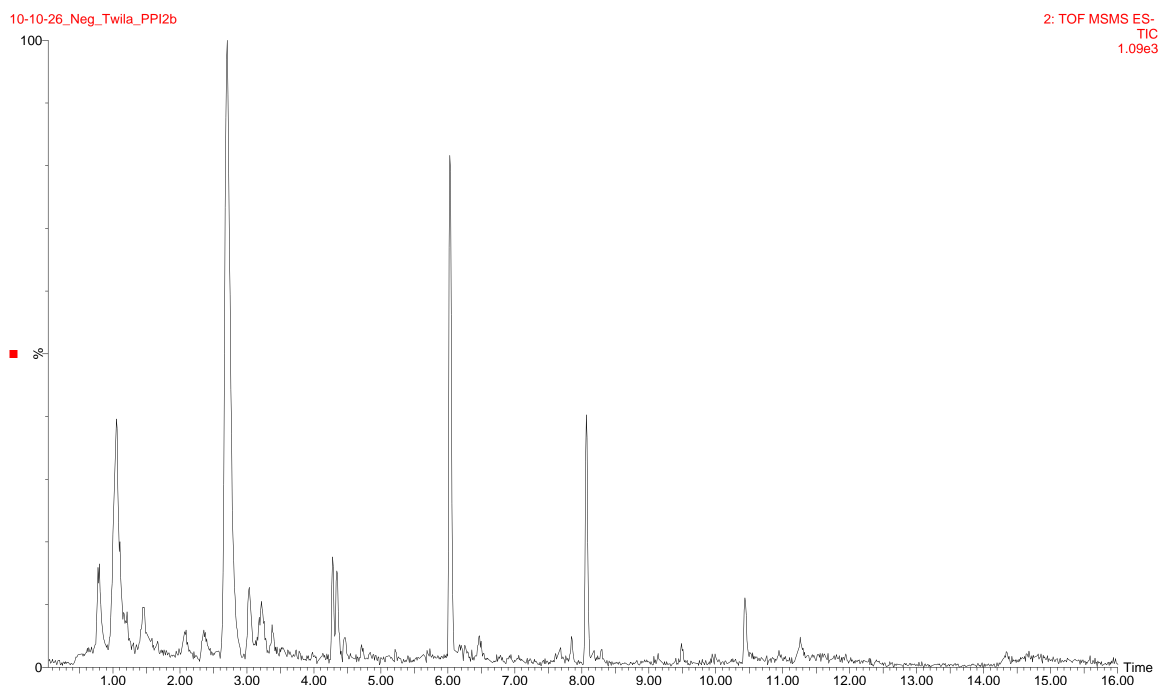

Figure A-11 Chromatogram for Paulaner Pilsner (PPI)