

# BLUEBERRY FLAVONOIDS AND LIVER PROTEIN EXPRESSION IN HEALTHY RATS

By

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(Under the direction of Arthur Grider)

## ABSTRACT

Flavonoids and particularly anthocyanins have been shown to reduce inflammatory markers in both humans and animals, and blueberries are rich in anthocyanins (1-3). However, the mechanisms and other possible effects are still largely unknown. The purpose of this study was to determine the relationship between anthocyanin-rich diets and liver protein expression. In a previous study rats were fed diets with 10% whole blueberries, 10% carbohydrate-matched blueberry polyphenol extract, 1% blueberry flavonoid extract, 0.2% blueberry flavonoid extract, a carbohydrate-matched control, or AIN-93 rodent diet for three weeks (4). For this secondary analysis two-dimensional gel electrophoresis (2DE; isoelectric focusing and SDS-PAGE) was used to separate out the proteins from the liver samples. Spots with a normalized volume difference greater than or equal to 1.5 fold (a 50% increase or decrease) were considered significant. In the water-soluble protein fraction no significant spots were detected. In the fat-soluble fraction three spots were detected and identified as urate oxidase.

**INDEX WORDS:** Blueberries, Polyphenols, Flavonoids, Anthocyanins, Gel electrophoresis, Proteomics, Liver, Urate oxidase, Uricase

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## CHAPTER 1

### INTRODUCTION

Liver disease is linked to inflammatory processes in the liver that can be activated by excess alcohol consumption, poor diet, viral infection, or other factors (5, 6). Anthocyanins and other phenolics food in plants have been shown to positively affect liver health in cases of induced liver damage through alcohol or other toxins, and both anti-inflammatory and antioxidant effects have been observed (7-9). This project was designed to increase our understanding of the hepato-protective effects of anthocyanins and aid in identifying the biochemical pathways influenced by the identified proteins. Proteomic analysis was used to identify affected proteins that may not have been assessed in immunological or biochemical tests performed in other anthocyanin studies. To date, no other studies have examined anthocyanin-rich diets and their effect on the full liver proteome.

## CHAPTER 2

### LITERATURE REVIEW

#### Abstract

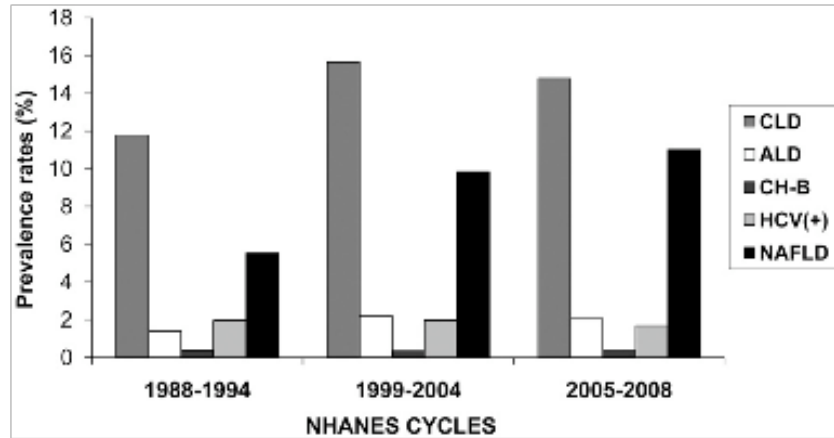
Many chronic disease processes in the liver begin with oxidative stress and inflammation that can lead to chronic pain, hepatosteatosis, fibrosis, and possible cirrhosis (5, 6) Uncontrolled oxidative stress can also lead to hepatocellular cancer through free radical damage to DNA (10). Polyphenols are a particular group of phytochemicals defined by their two aromatic rings that bridge together to form a third ring (11). Flavonoids are one class of polyphenol within which anthocyanins are a subject of particular interest, and blueberries are an anthocyanin-rich food (11, 12). Anthocyanins have been studied *in vitro* and *in vivo* for their antioxidant capacity and potential to attenuate various disease states. *In vivo* there are mixed results in both human and animal studies looking at biomarkers of liver disease and general inflammation.

#### Introduction

This chapter begins with a review of liver disease processes, including discussing recent literature on biomarkers of inflammation and liver disease. The structure of anthocyanins and their relationship to other polyphenols will be presented, and possible physiological activities of anthocyanins and anthocyanin-rich foods will be reviewed. This will include exploring both human and animal studies and stressed versus unstressed subjects with their responses to treatments.

## Liver Disease

Almost 45,000 people in the US die every year from chronic liver disease (13). Nearly 15% of the population was estimated to have some form of chronic liver disease in 2005-2008, and the prevalence of non-alcoholic fatty liver disease has more than doubled since the 1988-94 NHANES cycle as seen in Figure 2.1 (14).



**Figure 2.1** Prevalence of CLD in the US. Reprinted with permission from Younossi et al, 2011 (14). Abbreviations: alcoholic liver disease (ALD), chronic hepatitis B (CH-B), hepatitis C positive (HCV+), non-alcoholic fatty liver disease (NAFLD).

Oxidative stress and inflammation in the liver can be caused by hepatitis virus infection, alcoholism, drug-induced liver injury, physical trauma, or factors related to metabolic syndrome (15). Oxidation and inflammation are a part of normal physiological processes in the human body and are balanced by antioxidants and negative feedback mechanisms respectively. However, excessive or prolonged exposure to these stressors overloads the normal repair/protective mechanisms. This can lead to steatosis and steatohepatitis, which are reversible with intervention. However, they can progress to liver fibrosis, cirrhosis, and death from liver failure (16). Additionally, the oxidative stress associated with chronic liver disease can also lead to hepatocellular cancer, another cause of death stemming from chronic liver disease.

When the liver experiences stress, reactive oxygen species (ROS) are formed resulting in lipid peroxidation, oxidative cellular damage, and the initiation of an immune response that starts with inflammation. Leukocytes, T cells, and other immune cells are recruited to the affected area as a part of the inflammatory response (15). Proteins associated with increased inflammation include C-reactive protein (CRP) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) in serum (17). It has been proposed that NF- $\kappa$ B especially plays many roles in the development of chronic liver disease (18, 19). Although hepatocellular damage triggers inflammatory processes in the liver, it is now thought that the immune cells themselves and not the hepatocytes may be responsible for the long-term effects of liver damage, as seen in alcoholic liver disease (5). Other pro-inflammatory molecules such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), cyclooxygenase-2 (COX2), and inducible nitric oxide synthase (iNOS), are also involved and serve as biomarkers of inflammation (9, 17, 20).

NF- $\kappa$ B regulates iNOS and COX2, as well as many other proteins associated with disease processes in the liver (18). COX2 converts arachidonic acid into prostaglandins (21). The COX2-prostanoid pathway is associated with multiple liver diseases as well as having key roles in inflammation and carcinogenesis.

Under normal circumstances the damage caused by oxidative stress in the liver is constrained by glutathione S-transferase (GST), superoxide dismutase (SOD), and other antioxidant proteins. GST catalyzes the addition of a single electron to the tripeptide glutathione (GSH), and in this way assists with neutralizing ROS (22). These proteins are considered biomarkers of oxidation. However, in the case of severe or chronic exposure to stressors these antioxidants cannot fully protect the liver from damage caused by ROS and excessive inflammation. There is some evidence that iNOS is particularly active in inflammatory pathways

related to liver stress from diabetic ketosis, which has been shown to increase free radical formation *in vivo* (17). COX1 and COX2 are expressed in response to stressors such as proinflammatory cytokines, but unlike COX1, COX2 is associated with pathological conditions such as tumor development and progression (20).

Uncontrolled oxidative stress and inflammation can lead to hepatocellular cancer through the formation of highly reactive hydroxyl radicals that directly damage the DNA, increase cell proliferation, and other possible mechanisms (10). Increased NF- $\kappa$ B is associated with resistance of cancer cells to apoptosis (18). Other chronic liver diseases include alcoholic steatohepatitis, non-alcoholic steatohepatitis, and viral hepatitis infection (16). Fatty liver disease can be in the form of steatosis or steatohepatitis. Simple steatosis in the liver is not necessarily related to inflammation and can be benign, while steatohepatitis is a pathological inflammatory condition (16). However, it has been reported that in the UK 47% of steatosis cases progress to steatohepatitis (16). These diseases can also lead to other serious conditions such as liver fibrosis and cirrhosis. Fibrosis and cirrhosis are the final outcomes of liver damage that can have irreversible effects and can result in death in severe cases (23). Liver inflammation can eventually result in fibrosis, characterized by excessive proliferation and differentiation of hepatic stellate cells (HSC) into activated HSC. Activated HSC produce excess endothelial extracellular matrix in the liver that can be seen upon histological examination (23). Activated HSC can also migrate toward damaged areas to increase collagen formation at those sites.

### Anthocyanins

According to Manach et al (11), polyphenols are a class of phytochemical defined by their molecular structure, which has multiple hydroxyl groups around aromatic rings as seen in Figure 2.2. Polyphenols are frequently associated with a carbohydrate or organic acid moiety.

Among the polyphenols are the class called flavonoids, which have two aromatic rings connected by three carbons with an bridging oxygen to form a third six-membered heterocyclic ring. This class is further divided into six sub-classes that differ by variations in their heterocyclic ring as shown in Figure 2.3. These sub-classes include flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols. Anthocyanins are the glycoside form of anthocyanidins and are a form commonly seen in plants consumed by humans (24). The sugar moiety on an anthocyanins is most commonly a glucose but it can also be a galactose, rhamnose, xylose, or fructose (11, 25). Anthocyanins are known to have antioxidant effects *in vitro* (24). There are many types of anthocyanins, and the specific types vary based on the positions of their hydroxyl and methoxy (OMe) groups around three six-membered rings (24). Cyanidins are the most common type of anthocyanidins in edible plants, but other anthocyanins in foods include delphinidin, peonidin, malvidin, and petunidin (11, 25).

Biologically, polyphenols are a secondary metabolite in plants (11). Anthocyanins are present in high amounts in berries, red fruits, and many other dark-colored foods and flowers (26, 27). They give many plants their pink, dark red, purple, or blue coloring but may be colorless depending on the type of anthocyanin and the pH (11). Many factors contribute to the polyphenol content of foods. Among them are sun exposure, climate, season, soil type, soil microflora, and other factors (11, 28). Because these and other factors can vary so greatly, it is difficult to specify the amount of flavonoids or any other class of polyphenol that are present in a certain type of plant or fruit (11). The amount of total anthocyanins in blueberries can range from 250 - 5000 mg/kg depending on these factors (11).

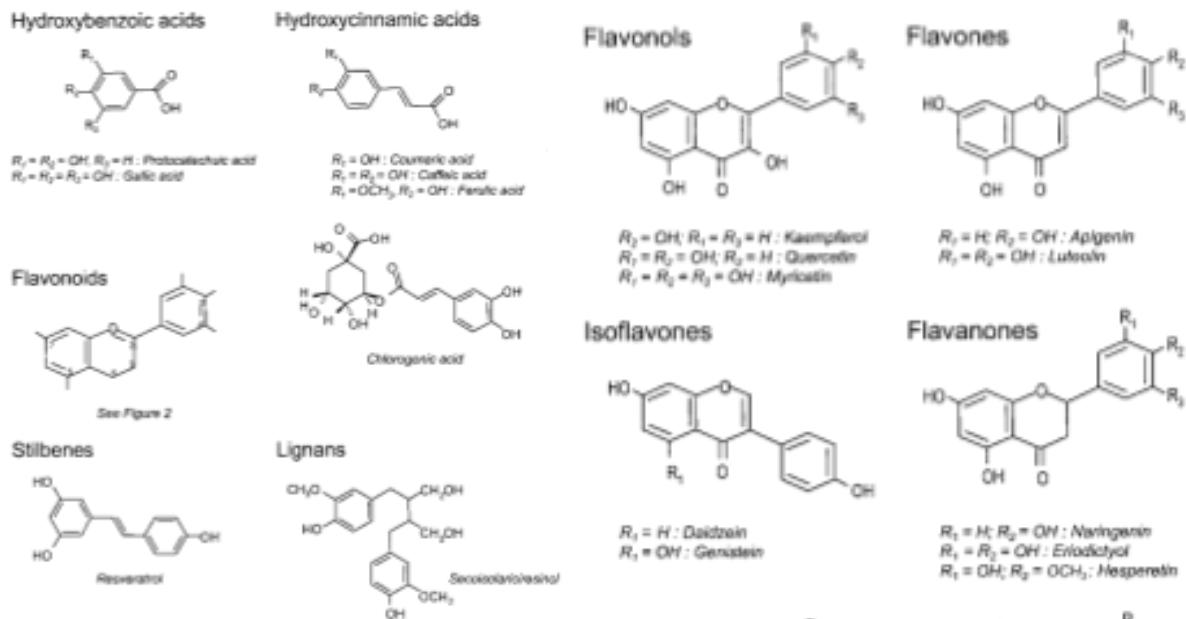


FIGURE 1. Chemical structures of polyphenols.

**Figure 2.2** (above) Polyphenol molecular structures. The molecular structures of different types of polyphenols, defined by the number of aromatic rings, their relationship, and the location of hydroxyl groups. **Figure 2.3** (right) Flavonoid molecular structures. The molecular structures of different flavonoids, including anthocyanidins, the non-glycosylated form of anthocyanins. Both figures from Manach et al 2004 (11).

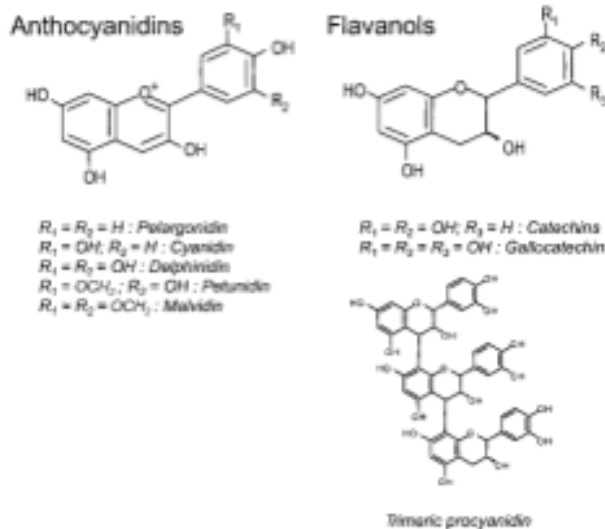


FIGURE 2. Chemical structures of flavonoids.

The amounts of each type of anthocyanin found in blueberries varies by cultivar as well as environmental factors already mentioned (28). It was previously thought that organic varieties of berries would have a greater anthocyanin content caused by increased environmental stress on organically grown products, and plants under more stressful conditions appeared to have greater polyphenol content (11). However, this is not consistently supported in the research (28). Organic versus conventional growing methods do not appear to affect anthocyanin type, content,



or *in vitro* anti-oxidant capacity (28). You et al (28) demonstrated this by using blueberries of the same cultivars from the same farm, some of which were grown organically and others conventionally. One cultivar showed a significantly higher polyphenol content in the organic berries, and another cultivar showed a significant difference favoring the conventionally grown berries.

Antioxidant effects *in vivo* are more difficult to assess because they appear to occur both directly through anthocyanin oxidation and indirectly through the up-regulation of antioxidant proteins. Borges et al (27) used high-performance liquid chromatography (HPLC) to look at *in vitro* antioxidant activity in five types of berries and found that blueberries had the second highest antioxidant activity (after black currants). However, a large portion of the antioxidant capacity (AOC) of black currants was due to vitamin C, while most of the AOC of blueberries was due to the anthocyanins. In many studies examining the effects of anthocyanins *in vivo* anthocyanin-rich foods or extracts are used that do not separate the anthocyanins from the flavonoid or polyphenol fractions. Part of the reason for this may be to show the benefits of whole foods or juices, but also there are studies suggesting that the benefits of polyphenols are greater in combination (4). Blueberries are a good choice for dietary anthocyanin antioxidant studies because most of the AOC of the fruit is from the anthocyanins and very little from vitamin C and other flavonoids (27).

Bioavailability of anthocyanins from the diet appear to be low but also highly variable (29, 30). Although an analysis by Del Bo et al (26) did not detect anthocyanins in the liver of rats, other studies have detected anthocyanins in the liver, brain, and other organs (29, 31). Talavera et al (31) detected high levels of methylated anthocyanins in the livers of rats after 15 days of a high anthocyanin diet, and Kalt et al (29) detected anthocyanins in the livers of pigs

after a 4-week blueberry-supplemented diet. Regardless of detectable levels from liver tissue, it is clear that *in vivo* anthocyanins do have an effect on protein expression in the liver (1, 4, 7-9, 32-35). However, these studies focus on specific liver proteins of interest and do not use proteomic methods.

Zamora-Ros et al (25) determined that in Europe the average total anthocyanidin consumption is about 29-33 mg/d based on data from the European Prospective Investigation into Cancer and Nutrition study (EPIC), and flavonoid intake in the US is thought to be less. In animal studies, anthocyanin doses for treatment are often calculated as a proportion of body weight. When the human consumption data is considered in this way, average consumption is well under 1 mg/kg/d. Yang et al (36) compared dietary sources of antioxidants in the US and found that total flavonoids provided 39% of the total antioxidant capacity of the average diet, second only to vitamin C (41%). When considering human intake it also must be considered if anthocyanins and other flavonoids available in whole fruits are still available after processing berries for products like juice. According to Kechinski et al (12), the half-life of anthocyanins in blueberry juice when heated to 70°C is  $8.60 \pm 1.54$  h. This indicates that after pasteurization blueberry juice would still be rich in intact anthocyanins. Furthermore, Buckow et al (37) indicate that the total anthocyanin content and total phenolic content are higher in pasteurized blueberry juice than unpasteurized juice after 42 days of shelf storage. This is thought to be due to the inactivation of degrading enzymes that occurs with pasteurization.

#### Effects of Anthocyanins in Stressed Subjects

There are already many studies on the antioxidant properties of anthocyanins. Immunological studies have shown that inflammation can be linked to many chronic diseases, including liver disease (38). Hogan et al (32) used diet-induced obesity as a model for a human

pro-inflammatory disease state, as measured by C-reactive protein and other biomarkers in the plasma. Anthocyanin-rich grape extracts reduced C-reactive protein levels by 15.5% in obese mice on a high fat diet compared to a group with the same diet and no grape extract supplementation (32). Similarly, Poudyal et al (39) fed a high-fat, high-carbohydrate diet to Wistar rats, a model of metabolic syndrome. Anthocyanin-rich purple carrot juice improved hepatic structure and function when compared to a  $\beta$ -carotene treatment and controls (39). Hou et al (1) induced liver damage in rats by diluted ethanol administered intragastrically for 45 days, supplementing some with anthocyanins. The anthocyanin-supplemented groups had a better profile for antioxidant enzymes such as glutathione peroxidase (GSH-Px), SOD, and GST as well as a reduced adverse effect of alcohol consumption on the liver as seen histologically (1).

In addition to studies using dietary methods (intragastric feeding, etc.) to cause oxidative stress *in vivo*, direct injection is another method that can be used to create an animal model of liver disease or oxidative stress. Hwang et al (9) induced liver damage in rats using dimethylnitrosamine (DMN) to model the inflammation and liver fibrosis caused by alcohol-induced liver damage, toxin exposure, or viral hepatitis (9). DMN diluted in saline was injected (10 mg/kg/d) in each rat three times per week for four weeks. 0, 50, 100, or 200 mg/kg/d of pure anthocyanins from the purple sweet potatoes were administered intragastrically to different groups. Rats fed anthocyanins at either 100 or 200 mg/kg/d had significantly increased GST activity compared to the control, and a dose-dependent distribution was observed related to GST and GSH levels as markers of antioxidant activity. Anti-inflammatory activity was assessed by measuring COX2 and iNOS levels, which were significantly lower in the same two treatment groups, again demonstrating a dose-dependent response. Zhang et al (35) also examined the effects of purple sweet potato extract *in vivo*. Mice were divided into four groups, creating a

factorial design. Two groups were treated with D-galactose for 8 weeks to initiate an inflammatory process in the liver. It was found that 4 weeks of treatment with the anthocyanin-rich extract (100mg/kg/d) after the initial 8-weeks of D-galactose suppressed inflammatory processes in histological cross-sections of the group that received both the D-galactose and the anthocyanin treatment. This study demonstrated that the anthocyanin-rich sweet potato fraction was able to suppress the up-regulation of the inflammatory markers NF- $\kappa$ B, iNOS, and COX2 using western blot analysis ( $P < 0.01$  for all). Furthermore, there was a significant reduction in SOD levels, suggesting reduced oxidative stress with the anthocyanin-rich treatment.

Alternatively, Domitrovic and Jakovac (33) did not measure inflammatory markers but instead looked at liver function serum biomarkers, such as aspartate transaminase (AST) and alkaline phosphatase (ALP), as well as histological methods to determine the anti-fibrotic activity of the anthocyanin delphinidin in mice. Tetrachloride-induced hepatotoxicity was used to initiate fibrosis, and delphinidin increased ALP and AST in mice. These findings agree with Shin and Moon (8) and with Hwang et al (9), showing reduced fibrosis after treatment with anthocyanin-rich grape skins and purified anthocyanins respectively following DMN-induced fibrosis.

In a human study by Traustadóttir et al (2) consumption of anthocyanin-rich tart cherry juice (119 mg/d for 14 d) reduced oxidative stress in the arm of elderly individuals during an ischemia-reperfusion test. In this double-blind, placebo-controlled crossover design study reduced F<sub>2</sub>-isoprotane levels were observed in plasma after several successive restrictions of the arm with a blood pressure cuff and reperfusion with the release of cuff tension. Spormann et al (30) studied the effects of an anthocyanin-rich juice on dialysis patients. Patients consumed a berry juice blend for 4 wks and stressed vs. Non-stressed individuals were compared for changes

in GSH and reduced DNA damage. Similar changes in GSH and reduction of DNA damage were observed. The full effect on blood values was seen after one week, suggesting that anthocyanins/polyphenols at amounts normally found in berry juices can have a positive effect on the body's ability to cope with oxidative stress.

#### Effects of Anthocyanins in Non-Stressed Subjects

A human study by Weisel et al (3) used healthy, young male subjects and found that supplementing the diet with anthocyanin/polyphenol-rich juice for reduced oxidative DNA damage and showed an increase in serum reduced GSH (3). In this study the subjects consumed 700 mL/d of berry-grape juice blend for 4 weeks. However, weekly blood collections showed that the full effect could be seen after the first week of treatment (3), in concordance with the timing of effects seen by Spormann et al (30). Furthermore, both studies also show results treating with normal fruit juices without concentrating the anthocyanins artificially. Kaspar et al (40) used whole anthocyanin-rich purple-flesh potatoes and compared them to yellow potatoes and white potatoes. The purple potato treatment has about 92 mg/d of anthocyanins, but this did not have a significant effect on blood markers over other diet groups despite some improvements that were noted. One reason for this may be that the participants were not asked to avoid berries or purple foods such as red wine during the 6-week treatment period as they were in the study by Weisel et al (3), so as the changes in the control group suggest, other dietary factors may have been involved.

Karlsen et al (41) used a different approach, giving anthocyanin pills (300 mg/d) instead of juices in healthy individuals (n = 118). The benefit of this study design is that the anthocyanins are a more likely cause of any changes observed because the capsules contained only anthocyanins with no other flavonoids, vitamin C, or other constituents that may be present

in juices or whole berries. Anthocyanin treatments showed decreased serum interleukin-8 and RANTES, which are both pro-inflammatory cytokines regulated by NF- $\kappa$ B. Interferon- $\alpha$  (IFN $\alpha$ ), which is an inducer of NF- $\kappa$ B activation, was also down-regulated. However, the capsule treatment method used by Karlsen et al (41) still contained a daily dose of anthocyanins that would probably be too high to consume in a normal diet.

Hassimotto and Lajolo (34) used healthy male Wistar rats to determine the long-term effects (35 days treatment) of supplementation with either anthocyanins or ellagitannins from blackberries. It was determined that anthocyanins had a greater effect on glutathione peroxidase, an anti-inflammatory protein, in the liver; and ellagitannins had a greater effect on the brain. Dulebohn et al (4) used male Sprague-Dawley rats to examine the effects of anthocyanins on the liver. A 28%, 27%, and 24% increase in GST activity was seen with preparations of whole blueberry, blueberry polyphenols, and 1% blueberry flavonoids (4). However, this increase in GST activity did not reach significance ( $p = 0.51$ ). There was a significant reduction of 22.5% in DNA damage in the liver with the 1% flavonoid diet. Liver samples from these animals were used in the current study.

#### Proteomics and Phytochemical Studies

Silvestri et al (42) assert that proteomic analysis has become a critical method for learning about new biochemical mechanisms and identifying biomarkers. Few studies have used two-dimensional gel electrophoresis (2DE) in analyses of polyphenols. Mouat et al 2005 (43) looked at the effects of the polyphenol quercetin on SW480 human colon carcinoma cells. Type II cytoskeletal keratin, an annexin family protein, and ubiquinone were all identified as proteins altered by exposure to quercetin (43). Currently, there are no analyses using 2DE analysis to study the effects of anthocyanin-rich foods or extracts on liver protein expression. This method

has been used successfully to analyze livers in an animal study of green tea polyphenols, confirming the results of cited *in vitro* studies associated with green tea (44). Green tea polyphenols are rich in catechins and flavanols. This study targeted the liver to assess possible antioxidants, although osteoporosis was the primary pathology; and the rats were ovariectomized. In another study, grape seed extract, which is high in anthocyanidins, has shown effects in the brains of rats (45). After treatment with grape seed extract, three proteins were identified which may be implicated in brain health for the first time in addition to confirming existing biomarker research on the subject. The identified proteins were actin, NFL-M (neurofilament protein), and RIKEN cDNA NM 029554 (45). Two-dimensional gel electrophoresis has been used in many other nutrition research contexts (46-48), but not to examine the effects of anthocyanins, flavonoids, or polyphenols on liver health.

## CHAPTER 3

### BLUEBERRY FLAVONOIDS AND LIVER PROTEIN EXPRESSION IN HEALTHY RATS<sup>1</sup>

<sup>1</sup>Duncan, N.D., A. Grider, and K. Wickwire. To be submitted to *Research Journal of Phytochemistry*.



## Abstract

The purpose of this study was to determine the relationship between an anthocyanin-rich diet and liver protein expression in healthy rats. In a previous study male Sprague-Dawley rats were fed diets with 10% whole blueberries, 10% carbohydrate-matched blueberry polyphenol extract, 1% blueberry flavonoid extract, 0.2% blueberry flavonoid extract, a carbohydrate-matched control, or AIN-93 rodent diet for three weeks (4). In this secondary analysis the liver samples were analyzed with proteomic analysis methods. Isoelectric focusing and SDS-PAGE was used to separate the proteins from the liver samples into fat-soluble and water-soluble fractions. 2DE densitometry software (Phoretix) was used for comparing spot volumes. Spots with a normalized volume difference greater than or equal to 1.5 fold (a 50% increase or decrease) were considered significant ( $p < 0.05$  in all cases). In the water-soluble protein fraction no significant spots were detected. In the fat-soluble fraction three spots were detected, and each of them was identified as urate oxidase using mass spectrometry.

## Introduction

Chronic liver disease begins with oxidative stress and inflammation in the liver, and extensive stress can lead to significant DNA damage, structural damage, and hepatocellular cancer (15, 49). Anthocyanin-rich diets have been shown to significantly reduce DNA damage, pro-inflammatory biomarkers, and histological liver damage as well as increase anti-inflammatory response in laboratory animals (1, 4, 9, 32, 39). Furthermore, short-term human trials have also shown reductions in pro-inflammatory markers and DNA damage with anthocyanin-rich juice blends (2, 30). Anthocyanins come from the flavonoid fraction of polyphenols; and they give plants a dark purple, red, or blue coloring (11). Fruits and vegetables with these colors are often high in anthocyanins and include black raspberries, red grapes,

blueberries, purple sweet potatoes, and many other foods (27). Blueberries in particular are high in anthocyanins, and *in vitro* techniques have found that blueberry flavonoids provide an even greater antioxidant capacity (AOC) than the blueberry vitamin C content (27).

The physiological benefits of anthocyanin-rich foods are not limited to their AOC, and changes in the expression and activity of liver proteins have been noted (7, 8). The mechanisms by which dietary polyphenols are able to influence protein expression are not well understood, and there have been no studies to date examining the effects of anthocyanin-rich diets on the full liver proteome. In the current study, we examined which liver proteins are affected by consumption of an anthocyanin-supplemented diet. We hypothesized that increased anthocyanin consumption will result in down-regulation of inflammatory markers and up-regulation of antioxidant proteins. In addition to looking at the relationship between an anthocyanin-rich blueberry diet and liver protein expression, we compared differences in expression between anthocyanin-rich blueberry flavonoid fractions and polyphenol fractions.

In a previous study by Dulebohn et al. (4) rats were fed diets with 10% freeze-dried whole blueberries, 10% carbohydrate-matched blueberry polyphenol extract, 1% blueberry flavonoid extract, 0.2% blueberry flavonoid extract, a carbohydrate-matched control, or an AIN-93 purified rodent-diet control for three weeks. Liver and colon mucosa samples were analyzed for their GST activity, quinone reductase activity, and UDP-glucuronosyltransferase activity. Also, DNA damage in the liver and lymphocytes were also assessed. A 28%, 27%, and 24% increase in GST activity was seen with preparations of whole blueberry, blueberry polyphenols, and 1% blueberry flavonoids. However, this increase in GST activity did not reach significance ( $p = 0.51$ ). There was a significant reduction of 22.5% in DNA damage in the liver with the 1% flavonoid diet. Liver samples from these animals were used in the current study.

In this secondary analysis, liver tissue samples were analyzed with 2DE methods. Isoelectric focusing and SDS-PAGE were used to separate out the proteins from the liver samples. Phoretix densitometry software was used to analyze the differences in protein expression with the different treatments. Spots with a normalized volume difference greater than, or equal to, 1.5 fold (a 50% increase) will be considered significant. Data will be analyzed by ANOVA, and a post hoc analysis will be performed using Statistical Analysis Software. Mass spectrometry (MALDI TOF) will be used to identify the significant protein spots.

This study explores which liver proteins were affected by consumption of an anthocyanin-supplemented diet, beyond the results of protein assays in the primary analysis. The study design is aimed at determining the relationship between anthocyanin consumption and liver protein expression as well as comparing changes in liver protein expression between anthocyanin-rich blueberry flavonoid fractions and polyphenol fractions. Based on the findings of Dulebohn et al., 2008 and others it is hypothesized that increased anthocyanin consumption will result in down-regulation of inflammatory markers and up-regulation of antioxidant proteins.

## Materials and Methods

The rats for this study were raised and studied in a previous project with the approval of the University of Georgia Institutional Animal Care and Use Committee (4). Sprague-Dawley male rats were fed one of six diets during the three-week trial: 10% dried whole blueberries, blueberry polyphenol extract and sugars equivalent to 10% whole dried blueberry diet, 1% blueberry flavonoid extract, 0.2% blueberry flavonoid extract, a carbohydrate control, or an AIN-93 rodent diets as a control. After 21 days the rats were euthanized using CO<sub>2</sub>. Livers were removed, sectioned, and stored at -80°C until analysis.

The livers were stored at -80°C prior to this study. A total of 21 liver samples were pulverized in liquid nitrogen using a mortar and pestle, and the powdered samples stored at -80°C until their preparation for 2DE. Some of the six treatment groups contained four samples, and some of them contained three samples. The identities of each group were blinded until the conclusion of sample analysis.

Sample preparation for 2DE: The powdered liver samples were weighed out at 100-200 mg into Sample Buffer II (Investigator 2D Electrophoresis System Manual) and treated with a protease inhibitor cocktail (Sigma-Aldrich). The samples were then sonicated to break up the tissue and further liquefy the sample. Samples were centrifuged at 14000g for 30 minutes. After centrifugation the supernatant containing the water soluble protein fraction was removed from the pellet portion and placed into a separate tube. The supernatant and pellet samples were then stored at -80°C.

The RC-DC Assay (Bio-Rad) was used to determine the concentration of the samples (50). Lyophilized bovine plasma (Bio-Rad) was used to generate the standard curve. Aliquots of the supernatant were diluted to fit within the standard curve. Samples were analyzed with a

Beckman Coulter DU 800 Spectrophotometer at 750 nm wavelengths to determine the protein concentration of the samples. The supernatant samples were analyzed individually in duplicate for a total of 42 complete gels. The fat-soluble pellet fractions were analyzed initially by pooling the samples by treatment group based on the concentration of protein in each sample so that the protein contribution from each pellet was equal in the pooled sample. After pooling these samples, analysis of all six pooled samples was performed in duplicate for a total of twelve gels. Pooled fat-soluble samples were analyzed using the same method as the individual water-soluble samples. After differences were detected (but before any identification), another more detailed analysis was performed on the fat-soluble pellets that matched the design of the water-soluble fraction analysis. Therefore, 42 individual gels resulted from the fat-soluble analysis in addition to the 12 pooled fat-soluble samples and the 42 gels from the water soluble fractions.

**Reduction/Alkylation:** A Reduction/Alkylation assay was used to prepare the samples for isoelectric focusing (IEF). 12.5  $\mu$ L of tributyl phosphate (TBP) were added to each sample for reduction and allowed to incubate at room temperature for 1 hour. 15  $\mu$ L of iodoacetamide were added for alkylation, the samples were allowed to incubate for 1.5 hours. After incubation samples are centrifuged for 5 minutes at 14000g. The supernatant is removed and 1 mL of cold acetone was added for acetone precipitation. The acetone-supernatant mixture was stored in the -20°C freezer overnight.

**Isoelectric focusing:** This procedure will separate the proteins in each sample based on their isoelectric point (pI) across an immobilized pH gradient (IPG) gel strip. 7 cm gel strips with a pH range 3 – 10 will be rehydrated in 125  $\mu$ L of solution containing the samples and protein solubilization buffer (PSB) overnight. The buffer was composed of ampholytes (Bio-lyte), TBP, PSB powder, PSB diluent, and bromophenol blue. The strips, infused with one sample on each

were run in a Hoefer IEF100 using the preprogrammed protocol for 7 cm IPG strips. This program starts at 500 V and reaches a maximum of 6000 V during IEF. The IPG strips were frozen at -80°C until the second dimension procedure has been set up.

Second dimension denaturing SDS-PAGE: The samples were separated in a second dimension by SDS-PAGE. This procedure separated proteins from the IPG strips across a polyacrylamide gel slab based on their molecular weight. The gels were prepared according to page 21 of the Hoefer Electrophoresis Application Guide following the instructions for a 10% acrylamide gel preparation: this preparation contains 30% acrylamide monomer, 1.5 M Tris base, 10% SDS, 10% ammonium persulfate, and TEMED (Bio-Rad). The glass plates were 16 cm x 18 cm with 1.5 mm spacers, and the polyacrylamide gel is poured between them with a 1 cm layer of butanol on top and allowed to solidify for at least one hour. The butanol was drained and rinsed away with deionized water. IPG strips from the IEF are allowed to thaw for 15 minutes and then 2 mL equilibration buffer and 10 $\mu$ L TBP are added to each strip. The equilibration buffer was composed of 1.5 M Tris HCl, 10% SDS, urea, bromophenol blue, and anhydrous glycerol. The blue color created a “dye front” that allowed visible movement of the sample from the IPG strip to the 2D gel. The gels were run fully immersed in tank buffer at a steady 30 mA per gel for about four hours or until the dye reaches 1.5 to 2 inches from the bottom of the gel. The tank buffer is composed of Tris, glycine, and SDS all dissolved in deionized water. Gels were fixed for 30 minutes in a solution of 40% methanol and 10% acetic acid before draining and rinsing with deionized water for 10 minutes on the orbital shaker three times. The gels were stained overnight on the orbital shaker with blue silver stain (51) composed of 0.12% Coomassie Blue G-250, 10% phosphoric acid, 10% ammonium sulfate, and 20% methanol. The gels were then de-stained with deionized water for at least 24 hours.

The images of the gels were digitized by scanning into a computer using a HP Scanjet Automatic Document Feeder and HP Scanjet software. They were analyzed with Phoretix software (Nonlinear Dynamics, Newcastle, UK), comparing volumes of similar spots. Similar spots were those that were in the same location on different gels, and therefore were expected to be the same protein. The Phoretix software helped match up spots that were similar and gave them a number as a label. Measurement of spot volume was achieved by (the software) measuring the surface area and color density of each spot to determine the spot volume. Significant spots, those with volume differences greater than or equal to 1.5-fold, were excised from the original 2D gels for further analysis. The excised spots were digested with trypsin in preparation for mass spectrometry. Peptide masses extracted from each spot were then obtained by MALDI-TOF mass spectrometry. The masses are then compared to a database at <http://www.matrixscience.com/> to identify the proteins.

The power analysis of this study was determined by using the existing 21 liver samples, which will be duplicated for a total of 42 gels. The power was 60% for 42 samples split among 6 treatment groups. This analysis was performed using G\*Power analysis software. Data was analyzed using one-way ANOVA. Spot volume was assessed as an average volume of the similar spots in a single treatment group, and the initial statistical analysis determined if there are any significant differences between similar spots in any of the treatment groups. Post hoc analysis was done with multiple comparisons t-test using Statistical Analysis Software for Windows version 9.1. Spots with at least 1.5-fold difference in volume will be considered significant ( $P \leq 0.05$ ). The post-hoc analysis determined which specific treatment groups had a significant difference from other specific groups; this provided more specific information than the ANOVA. Notably, the study was still blinded during the entire spot evaluation and statistical

analysis process. The researchers performing the analysis only knew the 6 treatment groups as letters A through F. Therefore, the control groups were unknown, and all groups were compared to all other groups for each similar spot.

### Results

The water-soluble proteins showed no significant differences between the controls and blueberry extract treatment groups ( $p > 0.05$ ). In the initial pooled analysis of the fat-soluble samples found eight spots that were significant between groups. The eight spots from the fat-soluble pooled analysis did not undergo identification by mass spectrometry. Instead, another analysis of individual fat-soluble fraction samples was performed. The mass spectrometry analysis of the non-pooled fat-soluble fraction samples identified three spots that were significantly different between groups (Figure 3.1). The three spots were arbitrarily labeled as numbers 183, 188, and 332 (Figure 3.2). These assigned numbers cannot be compared to the numbers assigned to the eight spots from the pooled samples because the numbering is different for each series analyzed. Mass spectrometry identified all three spots as urate oxidase. Post hoc analysis revealed that the three spots somewhat differed in the way they were affected by the six treatments, as shown in Figures 3.4-3.6; Figure 3.3 illustrates the overall changes in urate oxidase. It is possible that there were other spots of uricase present on the gels that did not change and therefore were not detected, but out of the altered uricase, Figure 3.3 shows the significant effects. Significant up-regulation of uricase is seen in all spots and their averaged values for the whole blueberries and the carbohydrate control with blueberry polyphenols diet. None of spots indicated a significant difference between the 0.2% flavonoid fraction and the 1% flavonoid fraction, and both blueberry flavonoid fractions were statistically the same as the control AIN-93 diet.

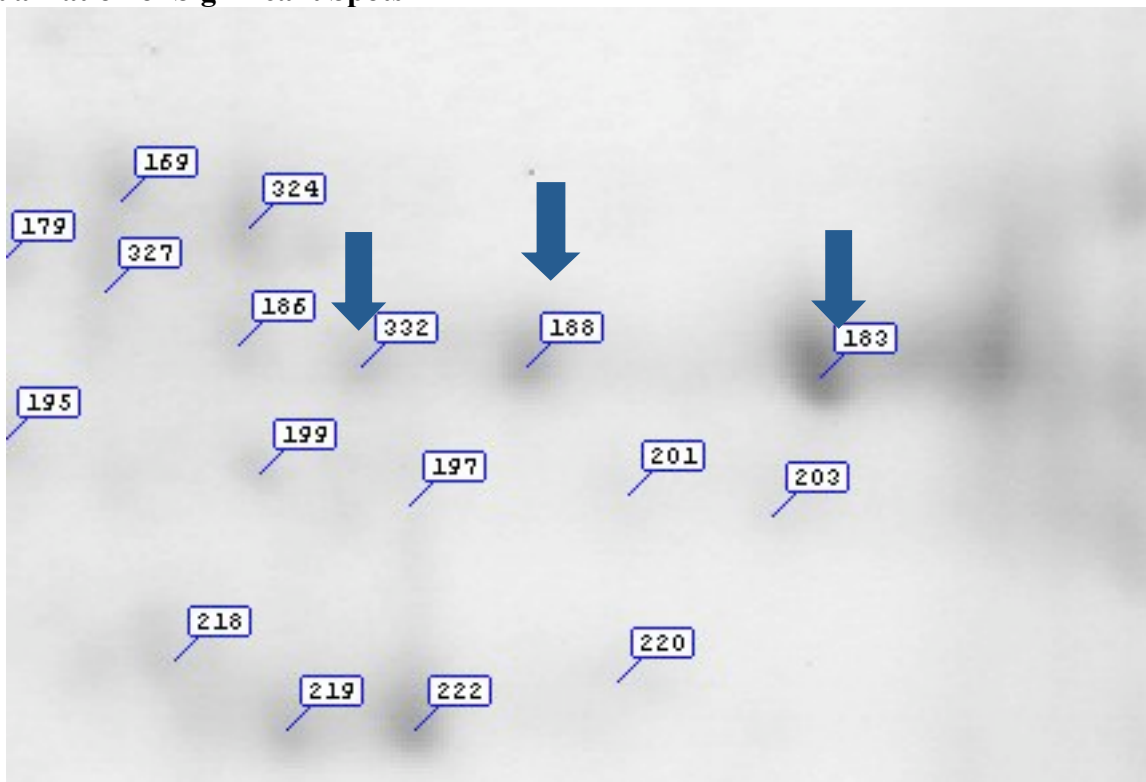


### Mass Spectrometry Results Chart

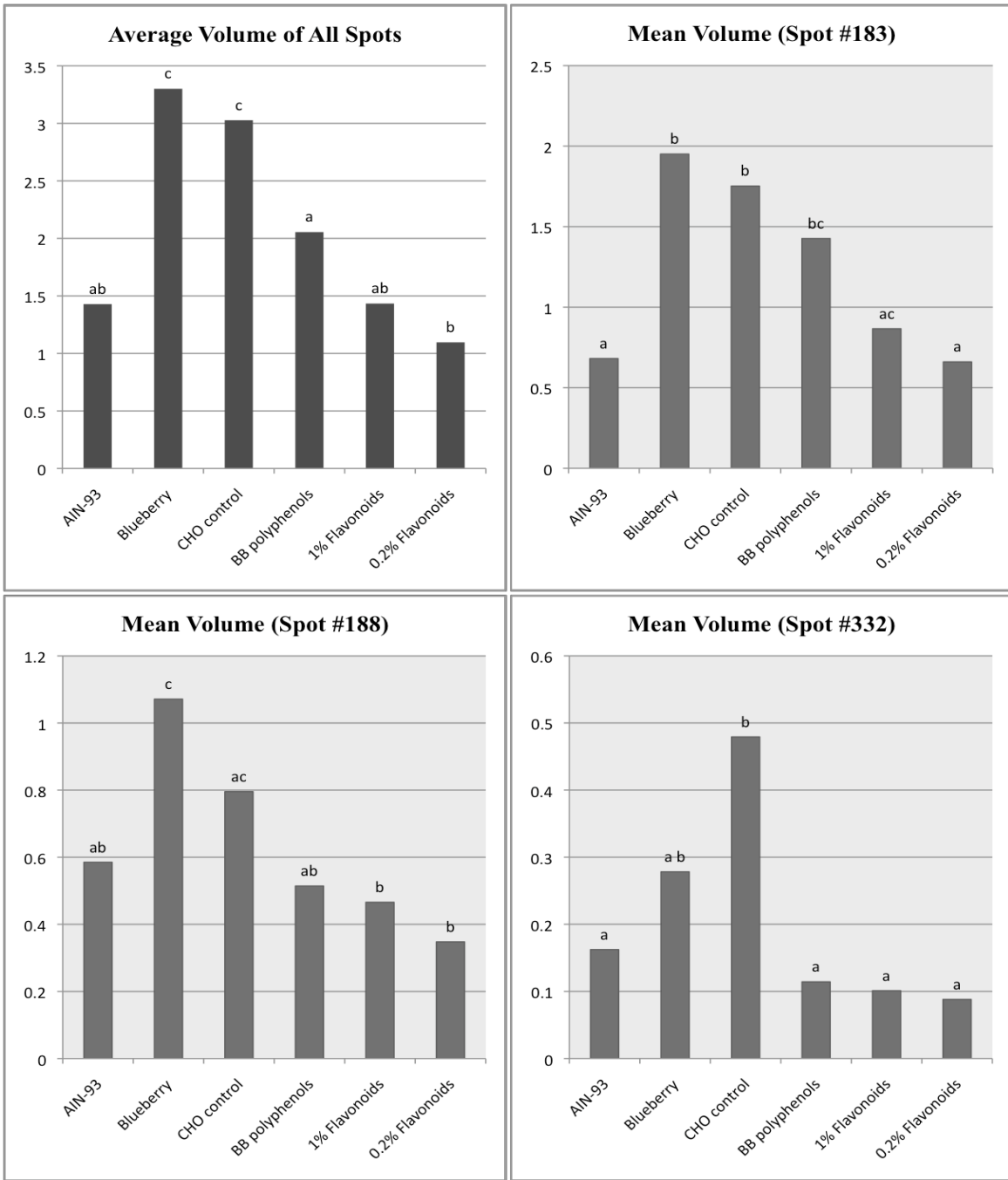
Phoretix Spot #	Protein Name	Accession No.	Protein MW	Protein PI	Pep Count	Protein Score	Protein Score C.I.%	Total Ion Score	Total Ion C.I.%	Best Ion Score	Best Ion C.I.%
183	Urate oxidase [Rattus norvegicus]	gi 56971244	35140	8.2	20	734	100	638	100	89	100
188	Urate oxidase [Rattus norvegicus]	gi 56971244	35140	8.2	18	515	100	439	100	81	100
332	Urate oxidase [Rattus norvegicus]	gi 56971244	35140	8.2	17	597	100	528	100	80	100

**Figure 3.1** Mass Spectrometry Results Chart. A brief review of the results provided by The Proteomics and Mass Spectrometry Core Laboratory of Georgia Health Sciences University.

### Visualization of Significant Spots



**Figure 3.2** Three significant spots were found: Spot numbers 183, 188, and 332.



**Figure 3.3** Averaged volume of all spots. Shows the average of all three spots, since they were all identified as the same protein, urate oxidase. **Figures 3.4-3.6** Mean volume. show the differences between treatment groups for each spot. Treatments that share a letter (a-c) are not significantly different, and significance was determined by  $P < 0.05$ .

## Discussion

The results of this study detected only uricase. Although this protein does play an indirect role in antioxidant capacity through the breakdown of the antioxidant urate, these findings do not suggest that flavonoid or anthocyanin presence in the diet had a role in uricase expression. Previous work by Mouat et al., 2005 detected significant spots from the same protein family (in that case, the annexin family) that were suspected to be different isoforms of the same protein, although mass spectrometry was unable to identify one of the spots (43).

Urate oxidase, also called uricase, is responsible for the breakdown of uric acid in many non-human mammals, including rats and mice (52). Urate is a product of purine degradation, two-thirds of which comes from the degradation of guanine and adenosine from the breakdown of endogenous RNA, DNA, and ATP (52, 53). The remaining one-third of purines comes from the diet (52). Purines are degraded into xanthine, which xanthine oxidase will oxidize to uric acid or urate (52, 54). Urate is thought to be taken up in the liver by GLUT9, which was formerly thought to transport fructose (55). In the peroxisomes of the hepatocytes uricase degrades urate into a much more water-soluble product, allantoin (56, 57). In this study, uricase spots were detected in the fat-soluble fraction of the liver protein. This confirms biochemical findings that peroxisomal uricase from rat liver is highly insoluble in water (58). In uricase-deficient mice kidney damage could be seen in pups as early as 6 days after birth, and these mice developed chronic inflammation of the kidney with clinical findings and pathology similar to hyperuricemia nephropathy seen in humans (57).

Uricase is protein formed from a single gene (UOX), which is present in most animals. It is not surprising that uricase was found in this study, since rats are one of the many animals that have the active form of the UOX gene (59). Additionally, the detection of uricase has been

accomplished previously elsewhere using proteomic methods in a study of sea bream fish livers (60). The gene for uricase exists in an inactivated form in humans and other hominids but also appears to have been deactivated separately in the bird/reptile line and in gibbons (59). Instead, humans manage urate levels through the renal system (52). The benefit of this is that uric acid is the primary circulating antioxidant in the human body, potentially facilitating increased longevity (56). Another theory is that uric acid's structural similarity to caffeine causes a stimulant effect in humans, increasing cognitive abilities (59). A third theory suggests that loss of urate oxidase helped hominids maintain their blood pressure when the diet may have been lower in sodium and purines (59). Keebaugh and Thomas 2010 (59) have reviewed the genetics details of purine metabolism, comparing multiple species. Their findings do not conclude any specific reason for the gene's loss. They do report that it was probably lost 15 million years ago in the hominid line and suggest that this loss was probably beneficial.

However, current clinical and epidemiological research suggests that the human lack of actively expressed uricase leads to a higher risk for diseases and conditions associated with hyperuricemia such as gout, nephropathy, and cardiovascular disease (55, 57, 61-65). In humans, the normal circulating level of urate is only slightly lower than the *in vitro* solubility limit of about 6.8-7 mg/dL at 37 deg C (56, 57, 63). The result is that relatively small increases in blood urate levels can lead to gout, which involves the precipitation of urate microcrystals in the joint spaces. Gout presents as painful inflammation and swelling that can occur in single or multiple joints. During "flare-ups" patients require medications to lower circulating urate levels and relieve pain (66). One treatment for gout is treatment with recombinant uricase (rasburicase) from the bacterium *Aspergillus flavus* as well as nonrecombinant uricase treatment options used more frequently in Europe (56). Serum uric acid has also been positively associated with

hypertension as an independent risk factor in epidemiological studies, especially in younger adult populations (67, 68). Some trials have also suggested a causative role for uric acid in hypertension, even when renal function is optimal (69). This is thought to be caused by increased endothelial cell proliferation, increased expression of inflammatory cytokines, and uric acid mediated reduction of vascular nitric oxide levels (69, 70). Due to the known and suspected causative effects of high serum uric acid levels in humans, the potential for rodent models of hyperuricemia with suppressed urate oxidase expression, and its therapeutic uses in humans – uricase remains a topic of continued research and interest to the scientific community (54, 57).

Despite the ongoing examination of uricase in clinical and animal research, there are few studies looking at the effect of polyphenols on uricase expression or activity. Hwa et al 1994 (54) used this drug and found that one oral dose of 100 mg/kg anthocyanin extract from purple sweet potatoes significantly reduced the uric acid levels in mice, but the decreased presence of uric acid may mean an increase in uricase activity or decrease in xanthine oxidase. When looking at uricase expression, xanthine oxidase should also be considered (54). Since xanthine oxidase produces urate in the purine degradation process, and lowered expression of this enzyme could potentially lead to less need for uricase and a resultant lower expression. In vitro anthocyanidins and flavones both inhibited xanthine oxidase through mixed, non-competitive inhibition (71). Results from Bao et al 2008 (72) show decreased expression of xanthine oxidase in mice with induced kidney damage treated over the course of only five days with anthocyanin-rich bilberry extract.

In humans, a study of apple polyphenols suggests that the effects of fructose in the fruit and not the polyphenols themselves were the cause of antioxidant effects, since both the carbohydrate control and the polyphenol rich juice increased uric acid levels (73). Although this

study is in conflict with similar studies looking at serum antioxidant capacity (2, 40, 74), it measures serum uric acid levels as others do not – providing a valuable measure of the human body’s primary circulating antioxidant (56). However, this does not give a strong indication as to whether xanthine oxidase activity had changed. Another reason for differing results may be the lower flavonoid and anthocyanin content of apples, which have more polyphenols in the form of quercetin (73). Modun et al 2008 (75) also found that urate levels were higher after treatments of polyphenol-rich red wine. However, this study also found an independent effect of polyphenols on ferric-reducing antioxidant power (FRAP). Again, anthocyanins are not the dominant flavonoid in this treatment, and catechin levels were measured accordingly.

In this study the most prominent finding was the increased expression of urate oxidase in treatment groups containing a greater proportion of monosaccharides (crude blueberries, carbohydrate match, and blueberry polyphenols with carbohydrates to match) compared to the other diets (Figure 3.7). This result applies only to spot #183, but since spot #183 contains more protein than the other two spots combined this effect is noted. It is known that a higher fructose diet can increase urate levels in rats and therefore increase the physiological need for urate oxidase, although it is unknown whether high serum urate concentration can directly stimulate uricase expression. There are many studies looking at the effects of fructose on liver health (76). There are criticisms that many of these studies do not use realistic dietary doses of fructose, but Roncal-Jimenez et al., 2011 (77) found that even realistic doses of high sucrose can induce fatty liver in male Sprague-Dawley rats, independent of excess

**TABLE 1** Composition of diets<sup>1</sup>

	diet 1	diet 2	diet 3	diet 4	diet 5	diet 6
	Control	Crude blueberry	Carbohydrate match	Crude polyphenol	1% flavonoid	0.2% flavonoid
Ingredient	g/kg diet					
casein	200.0	200.0	200.0	200.0	198.0	199.6
cornstarch	529.0	529.0	529.0	529.0	524.0	528.4
soybean oil	70.0	70.0	70.0	70.0	69.3	69.7
fiber	50.0	33.0	50.0	50.0	49.5	49.9
mineral mix	35.0	35.0	35.0	35.0	34.7	34.9
vitamin mix	10.0	10.0	10.0	10.0	9.9	10.0
L-cysteine choline bitartrate	3.0	3.0	3.0	3.0	3.0	3.0
sucrose	2.5	2.5	2.5	2.5	2.5	2.5
fructose	100.0	18.0	26.2	24.3	99.0	99.8
glucose	-	-	37.0	28.5	-	-
blueberries	-	-	37.0	28.5	-	-
polyphenols	-	100.0	-	-	-	-
flavonoids	-	-	-	20.0	-	-
	-	-	-	-	10.0	2.0

<sup>1</sup>Diet 1 is an AIN-93M diet, Diet 2 is 10% dried blueberries, which are 17% fiber and 82% sugar. Added fiber and sucrose were adjusted so total values would match control. Diet 3 contains sugars to match the composition in blueberries, 10% sucrose and 45% each of glucose and fructose. Diet 4 contains crude polyphenol extract and sugars to match the 10% blueberry diet. Diet 5 consists of 99% control diet and 1% purified flavonoids consisting mainly of anthocyanins, Diet 6 is 99.8% control diet and 0.2% purified flavonoids consisting mainly of anthocyanins.

**Figure 3.7** Detailed description of rodent diets, as published in the primary analysis by Dulebohn et al., 2008 (4).

energy intake when compared to a diet with more complex carbohydrate. The related inflammation and tissue damage observed by Roncal-Jimenez et al., 2011 may lead to higher urate levels as the purine metabolism becomes more active in attempt to degrade DNA and RNA from damaged or dying cells. Additionally, inflammation is catabolic in nature, and additional ATP will be turned over in the process. This may contribute even more substrate to purine degradation, urate production, and potential uricase up-regulation. In the present study, fatty liver was not observed in the liver specimens, and the dose of dietary simple sugars was lower than the 40% sucrose (20% from fructose) seen in the study by Roncal-Jimenez et al., 2011 (4, 77). A more subtle reiteration of this same physiological chain of events would explain the increased urate oxidase observed. Although the rats from this study were healthy, the small amount of

additional monosaccharide sugars in three of the treatment groups may have put a small additional burden on the liver compared to the group consuming slightly more disaccharide sugars. However, this is not true of the summed total significance of the three spots, so other possibilities should be explored.

When the volumes of the three uricase spots are combined for analysis, the carbohydrate match loses its significance, and only the crude blueberry diet and the carbohydrate match are significantly different from the AIN-93 diet. The AIN-93 diet also has a different fiber profile from the blueberry diet. The fiber content of the AIN-93 diet is 100% cellulose, which is an insoluble fiber. Although the fiber profile of the blueberries in this study was not analyzed, frozen blueberries generally are about 30% soluble fiber (78). A study of blueberry supplementation compared to probiotic bacteria supplementation in Sprague-Dawley rats shows that whole blueberry prevents liver injury to the same extent as *Lactobacillus* and *Bifidobacterium* strains (79). The effect of whole blueberry on colonic flora may also play a role in liver function and therefore affect purine metabolism, although the relationship between fiber, probiotics, and purine metabolism has not been established. It may be that diets with more complex carbohydrates versus saccharides and more insoluble fiber versus soluble lead to less purine breakdown and a consequent down-regulation of uricase expression. Ultimately, polyphenols not appear to play a role in purine metabolism in healthy livers, but other components of the diet appear to have an effect.



## CHAPTER 4

### SUMMARY AND CONCLUSIONS

#### Overall Purpose

The purpose of this study was to look at the effects of dietary anthocyanin-rich extracts and their effects on the liver proteome. This is important because although there is mounting evidence that anthocyanins have varying effects on the liver and the serum markers, the mechanisms are not fully known. Additionally, with the abundance of functional proteins in the liver, it is important to examine what processes are being affected that may not have been examined by substance-specific assays. Processes of the liver other than those most actively examined may also be impacting human and animal health.

#### Major Findings

This study did not detect any proteins that were significantly different between the polyphenol or flavonoid groups and the AIN-93 diet control. However, in the highest protein spot the two treatment groups that contained more simple sugars (whole blueberries, carbohydrate match, and blueberry polyphenols with carbohydrates to match the blueberry diet) showed significantly higher levels of urate oxidase (uricase) compared to the other treatment groups. When the results of all uricase spots detected are combined, the blueberry polyphenols group loses its significance.

#### Limitations

The samples for this secondary analysis were limited to only the liver sections provided by a previous study, and 3 of the intended 24 samples were not recovered. Also from the

previous study, the rats were only fed their test diets for a period of three weeks, and more time may have been needed to see changes in the liver proteome. Additionally, the rats were not stressed, so this also may have made short-term results more difficult to detect. Two-dimensional gel electrophoresis studies are limited by their low sensitivity. These methods are not capable of detecting small changes in protein values, even if the physiological effect is significant. Also, the use of 7 cm isoelectric focusing strips to cover the entire physiological pH range (versus longer strips or strips targeted on specific pH ranges) limited the amount of separation that could be achieved in the first dimension and led to some groups of spots bleeding into one another. This makes analysis very difficult. Variations in fiber and carbohydrate profiles may have also been confounding factors.

#### Implications and Future Research

The results seen here draw attention to the potential relationship between dietary sugars, fiber, and the purine degradation pathway. These relationships are in need of further study, as alterations in uricase expression will have an effect on oxidative stress and inflammatory response in the mice and rats used in polyphenol research. For future polyphenol animal examining the liver, the amount of simple sugars in addition to the total carbohydrate should be accounted for. An attempt should also be made to match the fiber profile. Since uric acid itself is an antioxidant, the activity of uricase is likely to affect the outcome of animal studies observing antioxidant or anti-inflammatory processes and should be taken into consideration. Use of uricase knock-down mice may be warranted in some studies.

## REFERENCES

1. Hou Z, Qin P, Ren G. Effect of anthocyanin-rich extract from black rice (*Oryza sativa* L. Japonica) on chronically alcohol-induced liver damage in rats. *J Agric Food Chem* 2010;58:3191-6.
2. Traustadottir T, Davies SS, Stock AA, et al. Tart cherry juice decreases oxidative stress in healthy older men and women. *J Nutr* 2009;139:1896-900.
3. Weisel T, Baum M, Eisenbrand G, et al. An anthocyanin/polyphenolic-rich fruit juice reduces oxidative DNA damage and increases glutathione level in healthy probands. *Biotechnol J* 2006;1:388-97.
4. Dulebohn RV, Yi W, Srivastava A, Akoh CC, Krewer G, Fischer JG. Effects of blueberry (*Vaccinium ashei*) on DNA damage, lipid peroxidation, and phase II enzyme activities in rats. *J Agric Food Chem* 2008;56:11700-6.
5. Byun JS, Jeong WI. Involvement of hepatic innate immunity in alcoholic liver disease. *Immune Netw* 2010;10:181-7.
6. Molendi-Coste O, Legry V, Leclercq IA. Dietary lipids and NAFLD: suggestions for improved nutrition. *Acta Gastroenterol Belg* 2010;73:431-6.
7. Bishayee A, Mbimba T, Thoppil RJ, et al. Anthocyanin-rich black currant (*Ribes nigrum* L.) extract affords chemoprevention against diethylnitrosamine-induced hepatocellular carcinogenesis in rats. *J Nutr Biochem* 2010.
8. Shin MO, Moon JO. Effect of dietary supplementation of grape skin and seeds on liver fibrosis induced by dimethylnitrosamine in rats. *Nutr Res Pract* 2010;4:369-74.
9. Hwang YP, Choi JH, Yun HJ, et al. Anthocyanins from purple sweet potato attenuate dimethylnitrosamine-induced liver injury in rats by inducing Nrf2-mediated antioxidant enzymes and reducing COX-2 and iNOS expression. *Food Chem Toxicol* 2011;49:93-9.
10. Halliwell B. Oxidative stress and cancer: have we moved forward? *Biochem J* 2007;401:1-11.
11. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 2004;79:727-47.
12. Kechinski CP, Guimaraes PV, Norena CP, Tessaro IC, Marczak LD. Degradation kinetics of anthocyanin in blueberry juice during thermal treatment. *J Food Sci* 2010;75:C173-6.

13. Jiaquan Xu KDK, Sherry L. Murphy, , Tejada-Vera aB. Deaths: Final Data for 2007. *National Vital Statistics Report* 2010;58.
14. Younossi ZM, Stepanova M, Afendy M, et al. Changes in the Prevalence of the Most Common Causes of Chronic Liver Diseases in the United States from 1988 to 2008. *Clin Gastroenterol Hepatol* 2011;9:524-530.
15. Adams DH, Ju C, Ramaiah SK, Uetrecht J, Jaeschke H. Mechanisms of immune-mediated liver injury. *Toxicol Sci* 2010;115:307-21.
16. Moore JB. Non-alcoholic fatty liver disease: the hepatic consequence of obesity and the metabolic syndrome. *Proc Nutr Soc* 2010;69:211-20.
17. Stadler K, Bonini MG, Dallas S, Duma D, Mason RP, Kadiiska MB. Direct evidence of iNOS-mediated in vivo free radical production and protein oxidation in acetone-induced ketosis. *Am J Physiol Endocrinol Metab* 2008;295:E456-62.
18. Bharti AC, Aggarwal BB. Nuclear factor-kappa B and cancer: its role in prevention and therapy. *Biochem Pharmacol* 2002;64:883-8.
19. Luedde T, Schwabe RF. NF-kappaB in the liver-linking injury, fibrosis and hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol* 2011;8:108-18.
20. Martin-Sanz P, Mayoral R, Casado M, Bosca L. COX-2 in liver, from regeneration to hepatocarcinogenesis: what we have learned from animal models? *World J Gastroenterol* 2010;16:1430-5.
21. Hu KQ. Cyclooxygenase 2 (COX2)-prostanoid pathway and liver diseases. *Prostaglandins Leukot Essent Fatty Acids* 2003;69:329-37.
22. Armstrong RN. Glutathione S-transferases: reaction mechanism, structure, and function. *Chem Res Toxicol* 1991;4:131-40.
23. Lalor PF, Faint J, Aarbodem Y, Hubscher SG, Adams DH. The role of cytokines and chemokines in the development of steatohepatitis. *Semin Liver Dis* 2007;27:173-93.
24. Castaneda-Ovando A, Pacheco-Hernández MdL, Páez-Hernández ME, Rodríguez JA, Galán-Vidal CA. Chemical studies of anthocyanins: A review. *Food Chemistry* 2009;113:859-871.
25. Zamora-Ros R, Knaze V, Lujan-Barroso L, et al. Estimation of the intake of anthocyanidins and their food sources in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. *Br J Nutr* 2011:1-10.
26. Del Bo C, Ciappellano S, Klimis-Zacas D, et al. Anthocyanin absorption, metabolism, and distribution from a wild blueberry-enriched diet (*Vaccinium angustifolium*) is affected by diet duration in the Sprague-Dawley rat. *J Agric Food Chem* 2010;58:2491-7.

27. Borges G, Degeneve A, Mullen W, Crozier A. Identification of flavonoid and phenolic antioxidants in black currants, blueberries, raspberries, red currants, and cranberries. *J Agric Food Chem* 2009;58:3901-9.
28. You Q, Wang B, Chen F, Huang Z, Wang X, Luo PG. Comparison of anthocyanins and phenolics in organically and conventionally grown blueberries in selected cultivars. *Food Chemistry* 2010;125:201-208.
29. Kalt W, Blumberg JB, McDonald JE, et al. Identification of anthocyanins in the liver, eye, and brain of blueberry-fed pigs. *J Agric Food Chem* 2008;56:705-12.
30. Spormann TM, Albert FW, Rath T, et al. Anthocyanin/polyphenolic-rich fruit juice reduces oxidative cell damage in an intervention study with patients on hemodialysis. *Cancer Epidemiol Biomarkers Prev* 2008;17:3372-80.
31. Talavera S, Felgines C, Texier O, et al. Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. *J Agric Food Chem* 2005;53:3902-8.
32. Hogan S, Canning C, Sun S, Sun X, Zhou K. Effects of Grape Pomace Antioxidant Extract on Oxidative Stress and Inflammation in Diet Induced Obese Mice. *J Agric Food Chem* 2010.
33. Domitrovic R, Jakovac H. Antifibrotic activity of anthocyanidin delphinidin in carbon tetrachloride-induced hepatotoxicity in mice. *Toxicology*;272:1-10.
34. Hassimotto NM, Lajolo FM. Antioxidant status in rats after long-term intake of anthocyanins and ellagitannins from blackberries. *J Sci Food Agric* 2010;91:523-31.
35. Zhang ZF, Fan SH, Zheng YL, et al. Purple sweet potato color attenuates oxidative stress and inflammatory response induced by d-galactose in mouse liver. *Food Chem Toxicol* 2009;47:496-501.
36. Yang M, Chung SJ, Chung CE, et al. Estimation of total antioxidant capacity from diet and supplements in US adults. *Br J Nutr* 2011:1-11.
37. Buckow R, Kastell A, Terefe NS, Versteeg C. Pressure and temperature effects on degradation kinetics and storage stability of total anthocyanins in blueberry juice. *J Agric Food Chem*;58:10076-84.
38. Proctor MJ, Morrison DS, Talwar D, et al. An inflammation-based prognostic score (mGPS) predicts cancer survival independent of tumour site: a Glasgow Inflammation Outcome Study. *Br J Cancer* 2011.
39. Poudyal H, Panchal S, Brown L. Comparison of purple carrot juice and beta-carotene in a high-carbohydrate, high-fat diet-fed rat model of the metabolic syndrome. *Br J Nutr* 2010;104:1322-32.

40. Kaspar KL, Park JS, Brown CR, Mathison BD, Navarre DA, Chew BP. Pigmented potato consumption alters oxidative stress and inflammatory damage in men. *J Nutr* 2010;141:108-11.
41. Karlsen A, Retterstol L, Laake P, et al. Anthocyanins inhibit nuclear factor-kappaB activation in monocytes and reduce plasma concentrations of pro-inflammatory mediators in healthy adults. *J Nutr* 2007;137:1951-4.
42. Silvestri E, Lombardi A, de Lange P, et al. Studies of complex biological systems with applications to molecular medicine: the need to integrate transcriptomic and proteomic approaches. *J Biomed Biotechnol* 2011;2011:810242.
43. Mouat MF, Kolli K, Orlando R, Hargrove JL, Grider A. The effects of quercetin on SW480 human colon carcinoma cells: a proteomic study. *Nutr J* 2005;4:11.
44. Shao C, Chen L, Lu C, Shen CL, Gao W. A gel-based proteomic analysis of the effects of green tea polyphenols on ovariectomized rats. *Nutrition* 2010.
45. Kim H, Deshane J, Barnes S, Meleth S. Proteomics analysis of the actions of grape seed extract in rat brain: Technological and biological implications for the study of the actions of psychoactive compounds. *Life Sciences* 2006;78:2060-2065.
46. Fu L, Liu Q, Shen L, Wang Y. Proteomic study on sodium selenite-induced apoptosis of human cervical cancer HeLa cells. *Journal of Trace Elements in Medicine and Biology* 2011;25:130-137.
47. Grider A, Mouat MF, Scrimgeour AG. Consumption of a moderately Zn-deficient and Zn-supplemented diet affects soluble protein expression in rat soleus muscle. *J Nutr Biochem* 2007;18:753-9.
48. Mouat MF, Greenspan P, Byerley LO, Grider A. Zinc uptake into MCF-10A cells is inhibited by cholesterol depletion. *J Nutr Biochem* 2003;14:74-80.
49. Hou DX. Potential mechanisms of cancer chemoprevention by anthocyanins. *Curr Mol Med* 2003;3:149-59.
50. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-75.
51. Candiano G, Bruschi M, Musante L, et al. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* 2004;25:1327-33.
52. Lee SJ, Terkeltaub RA. New developments in clinically relevant mechanisms and treatment of hyperuricemia. *Curr Rheumatol Rep* 2006;8:224-30.
53. Tipton PA. Urate to allantoin, specifically (S)-allantoin. *Nat Chem Biol* 2006;2:124-5.

54. Hwa KS, Chung DM, Chung YC, Chun HK. Hypouricemic Effects of Anthocyanin Extracts of Purple Sweet Potato on Potassium Oxonate-induced Hyperuricemia in Mice. *Phytother Res* 2011.
55. Anzai N, Jutabha P, Amonpatumrat-Takahashi S, Sakurai H. Recent advances in renal urate transport: characterization of candidate transporters indicated by genome-wide association studies. *Clin Exp Nephrol* 2011.
56. Bieber JD, Terkeltaub RA. Gout: on the brink of novel therapeutic options for an ancient disease. *Arthritis Rheum* 2004;50:2400-14.
57. Wu X, Wakamiya M, Vaishnav S, et al. Hyperuricemia and urate nephropathy in urate oxidase-deficient mice. *Proc Natl Acad Sci U S A* 1994;91:742-6.
58. Chu R, Usuda N, Yeldandi AV. Truncational analysis of rat urate oxidase in insect cells. *Ann N Y Acad Sci* 1996;804:777-80.
59. Keebaugh AC, Thomas JW. The evolutionary fate of the genes encoding the purine catabolic enzymes in hominoids, birds, and reptiles. *Mol Biol Evol* 2010;27:1359-69.
60. Rufino-Palomares E, Reyes-Zurita FJ, Fuentes-Almagro CA, de la Higuera M, Lupianez JA, Peragon J. Proteomics in the liver of gilthead sea bream (*Sparus aurata*) to elucidate the cellular response induced by the intake of maslinic acid. *Proteomics* 2011;11:3312-25.
61. Wu CW, Terkeltaub R. What do epidemiologic studies tell us about hyperuricemia and cardiovascular disease and death? *Curr Rheumatol Rep* 2005;7:211-2.
62. Takayama S, Kawamoto R, Kusunoki T, Abe M, Onji M. Uric acid is an independent risk factor for carotid atherosclerosis in a Japanese elderly population without metabolic syndrome. *Cardiovasc Diabetol*;11:2.
63. Fels E, Sundy JS. Refractory gout: what is it and what to do about it? *Curr Opin Rheumatol* 2008;20:198-202.
64. George J, Struthers AD. The role of urate and xanthine oxidase inhibitors in cardiovascular disease. *Cardiovasc Ther* 2008;26:59-64.
65. George J, Struthers AD. Role of urate, xanthine oxidase and the effects of allopurinol in vascular oxidative stress. *Vasc Health Risk Manag* 2009;5:265-72.
66. Bieber J, Terkeltaub R. The role of hypertension in cyclosporine-induced hyperuricemia. *Curr Rheumatol Rep* 2004;6:215-6.
67. Sundstrom J, Sullivan L, D'Agostino RB, Levy D, Kannel WB, Vasan RS. Relations of serum uric acid to longitudinal blood pressure tracking and hypertension incidence. *Hypertension* 2005;45:28-33.

68. Kansui Y, Ohtsubo T, Goto K, et al. Association of serum uric acid with blood pressure in Japanese men. Cross-sectional study in work-site group. *Circ J* 2011;75:2827-32.
69. Feig DI, Soletsky B, Johnson RJ. Effect of allopurinol on blood pressure of adolescents with newly diagnosed essential hypertension: a randomized trial. *JAMA* 2008;300:924-32.
70. Mazzali M, Kanellis J, Han L, et al. Hyperuricemia induces a primary renal arteriolopathy in rats by a blood pressure-independent mechanism. *Am J Physiol Renal Physiol* 2002;282:F991-7.
71. Costantino L, Rastelli G, Albasini A. Anthocyanidines as inhibitors of xanthine oxidase. *Pharmazie* 1995;50:573-4.
72. Bao L, Yao XS, Tsi D, et al. Protective effects of bilberry (*Vaccinium myrtillus* L.) extract on KBrO<sub>3</sub>-induced kidney damage in mice. *J Agric Food Chem* 2008;56:420-5.
73. Godycki-Cwirko M, Krol M, Krol B, et al. Uric acid but not apple polyphenols is responsible for the rise of plasma antioxidant activity after apple juice consumption in healthy subjects. *J Am Coll Nutr* 2010;29:397-406.
74. Crozier A, Jaganath IB, Clifford MN. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat Prod Rep* 2009;26:1001-43.
75. Modun D, Music I, Vukovic J, et al. The increase in human plasma antioxidant capacity after red wine consumption is due to both plasma urate and wine polyphenols. *Atherosclerosis* 2008;197:250-6.
76. Nomura K, Yamanouchi T. The role of fructose-enriched diets in mechanisms of nonalcoholic fatty liver disease. *J Nutr Biochem* 2012;23:203-8.
77. Roncal-Jimenez CA, Lanaspa MA, Rivard CJ, et al. Sucrose induces fatty liver and pancreatic inflammation in male breeder rats independent of excess energy intake. *Metabolism* 2011;60:1259-70.
78. USDA. National Nutrient Database for Standard Reference, Release 24. Nutrient Data Laboratory 2011.
79. Osman N, Adawi D, Ahrne S, Jeppsson B, Molin G. Endotoxin- and D-galactosamine-induced liver injury improved by the administration of *Lactobacillus*, *Bifidobacterium* and blueberry. *Dig Liver Dis* 2007;39:849-56.



## APPENDICES

## APPENDIX A: ASSAYS

### RC-DC Protein Assay

1. Pull samples out of -80 freezer.
2. Determine the amount of sample to use in assay.
3. Label 1.5 ml microcentrifuge tubes for assay. (single samples – no duplicates)
4. Set up standard curve. (Standard is in BioRad box in freezer #1.)

<b>Conc. ug/ml</b>	<b>Protein</b>	<b>Water</b>
0	0 $\mu$ l	25 $\mu$ l
0.146	0.146 $\mu$ l	22.5 $\mu$ l
0.409	0.409 $\mu$ l	18 $\mu$ l
0.73	0.73 $\mu$ l	12.5 $\mu$ l
1.46	1.46 $\mu$ l	0 $\mu$ l

5. Prepare Reagent A' by adding 5  $\mu$ l of DC Reagent S to 250  $\mu$ l of DC Reagent A. (Calculate amount needed by multiplying # samples by 127  $\mu$ l.) Mix and set aside.
6. Pipet 25  $\mu$ l of standards and samples into clean, dry, labeled microcentrifuge tubes
7. Add 125  $\mu$ l RC Reagent k into each tube and vortex.
8. Incubate tubes for 1 minute at room temperature.
9. Add 125  $\mu$ l RC Reagent II into each tube and vortex.
10. Centrifuge the tubes at 15,000 x g for 3-5 minutes. (5 minutes using the Eppendorf microcentrifuge.)
11. Discard the supernatant using a Pasteur pipet, then invert the tube onto a paper towel to drain.
12. Add 125  $\mu$ l RC Reagent I into each tube and vortex.
13. Incubate tubes for 1 minute at room temperature.

14. Add 40  $\mu$ l RC Reagent ii into each tube and vortex.
15. Centrifuge the tubes at 15,000 x g for 3-5 minutes.
16. Discard the supernatant using a Pasteur pipet, then invert the tube onto a paper towel to drain.
17. Add 127  $\mu$ l Reagent A' to each microfuge tube and vortex.
18. Incubate the tubes at room temperature for 5 minutes or until precipitate is completely dissolved.
19. Vortex before proceeding to next step.
20. Add 1 ml DC Reagent B to each tube and vortex immediately. (Use repeat pipetor.)
21. Incubate the tubes at room temperature for 15 minutes.

After the 15 minute incubation, absorbances can be read at 750 nm. The absorbance will be stable for at least 1 hour.

#### Reduction/Alkylation

1. Allow samples to come to room temperature.
2. Add 25  $\mu$ l of TBP per ml of sample. TBP is found on top shelf of freezer 3. Vortex. (Reduction)
3. Incubate for 1 hour at room temperature.
4. Resuspend Iodoacetamide in 0.6 ml of water. Located in the white box in refrigerator 2.
5. Add 30  $\mu$ l of Iodoacetamide per ml of sample. Vortex. (Alkylation) Discard any unused portion of Iodoacetamide.
6. Incubate for 1.5 hr. at room temperature.
7. Centrifuge samples at full speed for 5 minutes using the Eppendorf tabletop centrifuge.
8. Remove supernatant to new, labeled tube.

#### Acetone Precipitation

1. Aliquot samples into microcentrifuge tubes containing 200  $\mu$ l each. Add 800  $\mu$ l of ice cold Acetone (found in the flammables refrigerator, freezer section).
2. Place samples into the -20 C freezer for 30 minutes or overnight.

3. Remove samples from the freezer and spin for 20 minutes at 10,000 using the refrigerated tabletop centrifuge.
4. Aspirate off the supernatant using a Pasteur pipet.
5. Allow samples to air dry (inverted on rack) for 5 minutes.
6. Add 125  $\mu$ l of protein solubilization buffer and vortex to dissolve protein pellet. (May take a while)
7. Spin samples in the Eppendorf table top centrifuge for 10 minutes to pellet out any undissolved particles.
8. Place samples in to the IEF tray and proceed with gel rehydration.

#### 1<sup>st</sup> Dimension Gel Rehydration (Hoefer)

1. Remove strip gels from the freezer (Door of Freezer 3).
2. Clean tips of blunt forceps.
3. Pipette sample along the back edge of the well.
4. Remove plastic stripping from the gel.
5. Place the gel strip with the 3-10 on the left side (+) and the gel side down, into the well.
6. Add 1 ml of mineral oil on top of each strip (oil prevents dehydration).
7. Add an additional 1 ml of mineral oil on top of each strip.
8. Place the rehydration tray into the Hoefer instrument, close the lid and allow it to rehydrate for at least 8 hours or overnight.

#### Running 1<sup>st</sup> Dimension Gels (Hoefer)

1. Insert the IEF tray onto the far right side of the cold plate. Align the (+) and (-) marks on the tray with the corresponding marks on the IEF instrument.
2. Slide the tray to the left under the clamping tabs. (Tabs improve the contact, and heat transfer, between the tray and the cold plate.)
3. Using forceps, load the rehydrated IPG strips into the focusing tray, gel side up. The (+) end of the strips should be on the left.
4. Align the (+) end of the strips with the alignment mark in the tray.

5. Add 50  $\mu$ l of water to wicks and gently blot off any excess water. (Will need two wicks per gel strip.)
6. Apply electrode wicks on top of each end of the IPG strips. Lay the strips so they overlap the gel by 2-3 mm, and extend off the end of the IPG strip. (The wick absorbs ions collecting at the ends of the IPG strips.)
7. Place the (+) electrodes, centered, on top of the overlapping region of wicks on gels to make contact with the IPG strip.
8. Connect the (+) electrode to the (+) terminal.
9. Place the (-) electrode on top of the electrode wicks so that the wire is centered in the area of overlap between the wicks and the IPG strip.
10. Connect the (-) electrode to the (-) terminal.
11. Add 60 ml of mineral oil to the tray. This prevents the strips from drying out during the run.
12. Close the lid.
13. Turn on the machine.
14. Highlight the desired protocol and press RUN.
15. Confirm the number of IPG strips to focus. (Use the knob to change the value.)
16. Press RUN again to start the IEF.
17. IEF instrument beeps at the start of each step and when the run is finished.

### IEF Voltages

The protocols are preprogrammed. They are to be used as guidelines. Each protocol has a 1-hour, 1000 V hold step to maintain sharp bands once focusing is complete. This step is not necessary and can be removed. It can also be extended as desired.

Use Program 1: 7 cm broad & basic

Delay 0:00, Delay temp 20 °C, Run temp 20 °C, 500  $\mu$ A, 6000 V, 0.5 W

1. Step 1, Gradient volt, 500 V, 0:30 Hrs
2. Step 2, Gradient volt, 1000 V, 0:30 Hrs
3. Step 3, Gradient volt, 6000 V, 0:30 Hrs
4. Step 4, Constant volt, 6000 V, 8000 Vhrs
5. Step 5, Constant volt, 1000 V, 1:00 Hr

### Preparing Gels

1. Dr. Grider normally uses 12.5% gels. Use the chart on p.21 of the Hoeffer Electrophoresis Application Guide. For 2 gels use the top table and for a single gel use the second table.
2. Combine 25 ml Monomer solution (Refrigerator 1), 15 ml 4x Running Gel Buffer (door refrigerator 1, 1.5M Tris HCl buffer), 0.6 ml 10% SDS (shelf over work area) and 19.1 ml distilled water.
3. Using a stir bar mix the contents in a 125 ml side-arm flask.
4. Stopper the flask and de-gas for at least 15 minutes. (Place 125 ml flask into plastic cup on stirplate for stability)
5. Add 300  $\mu$ l of ammonium persulfate (plastic box on top shelf of freezer 3) and 20  $\mu$ l of TEMED (shelf over pH meter, more in flammables cabinet). Mix.
6. Gels need to be poured immediately after adding ammonium persulfate and TEMED.

### Pouring Gels

1. Clean plates and spacers with 100% ETOH. (All surfaces)
2. Make a sandwich with a glass plate, spacers, gel guide, and another glass plate.
3. Place on an even surface and attach side clamps. (Movable bar on same side as screws)
4. Tighten screws securely and check that glass plates are even on the bottom. Remove the gel guide.
5. Place glass plate sandwich firmly into casting tray.
6. Insert cams until flush with clamps. Turn to tighten. If cams are not flush with clamps, gel will leak out.

7. Using a serological pipet add 30 ml of gel to each set of glass plates.
8. Add a layer of water-saturated butanol across the top of each gel. (On counter by buffer chambers)
9. Allow gels to set up for at least an hour before loading or adding a stacking gel.

#### Loading Gels

1. Rinse off butanol layer by flushing tops of gels with distilled water.
2. Invert the gel casting trays onto absorbent paper.
3. Unthaw 1<sup>st</sup> dimension gels for 15 minutes. (Use timer)
4. Add 2 ml of equilibration buffer (located in door of upright freezer) and 10 ul/ml of TBP (located on top shelf of upright freezer) per lane.
5. Shake on orbital shaker for 20 minutes. (Use timer)
6. Set up gels so danger sign on bottom of gel casting trays is upright. (For ease in identifying which gels contain which samples if running multiple gels.)
7. Remove strip from tray. Cut plastic tab off of positive end of strip. Place positive end of strip to left edge of gel with plastic to the back and gel to the front. Add second strip with positive plastic tab removed flush with negative end of first strip.
8. Move cams from bottom of gel casting tray to top after attaching the buffer chamber to top of gels. (Red electrode to the left). Caution: Be sure cams are flush with side of buffer chamber and the rubber gaskets are in place or the buffer will leak out.)
9. Add 600 ml of buffer to the buffer chamber after setting gels into Hoeffer apparatus. (Buffer is prepared by adding 60 ml 10 X TGS up to 600 ml with distilled water. Use fleaker to prepare in)
10. Place top on chamber. Turn on circulator and stir plates.
11. Plug buffer lids into power source and set at 60 mA to run 2 gels in 4 hours or at 30 mA to run 1 gel in 4 hours.
12. Check gel periodically and stop gel when the dye front has run for 8 cm from the top.

#### Fixing Gels

1. Turn off power source, circulator and stir plates.
2. Remove gels from chamber.

3. Remove gels from between glass plates and place into plastic trays. (Cut off a corner to distinguish between gels when running two gels.)
4. Add fixing solution of 40% methanol and 10% acetic acid.
5. Fix for 30 minutes. (Use orbital shaker)
6. After the 30 minute fix, pour off the fixing solution in to the hazardous waste container.
7. Rinse the gels with distilled water.
8. Wash 3 times for 10 minutes each time with distilled water.

#### Staining a Gel

1. Pour off distilled water after last wash.
2. Add enough Coomassie Blue Silver stain to cover the gels.
3. Place gels on orbital shaker overnight.

#### De-staining a Gel

1. Pour off stain into the sink.
2. Rinse with distilled water then pour off.
3. Add fresh distilled water and allow gels to destain until done.



## APPENDIX B: SOLUTIONS

### Preparation of Protein Solubilization Buffer

1. Components
  - a. ampholytes – brown tube in door of refrigerator 1
  - b. TBPene – clear vial in small Styrofoam holder, top shelf of freezer 3
  - c. PSB diluent – plastic bottle in green box in refrigerator 2.
  - d. PSB powder – plastic bottle with blue marbles on shelf over scale
  - e. Bromophenol blue – well marked Ziploc bag on top shelf of freezer 3
2. Weigh out 1 g of PSB powder into small plastic weigh boat.
3. Add 1.1 ml of PSB diluent.
4. Add teeny stirbar and stir until powder is dissolved.
5. Add 20  $\mu$ l TBPene, 20  $\mu$ l biolyte and 5  $\mu$ l bromophenol blue for 2 ml of solubilization buffer.
6. Mix on stir plate.
7. Place in tube and stopper until ready to use.

### Colloidal Blue Silver Stain

1. Combine 100 ml water, 100 ml phosphoric acid and 50.5 g ammonium sulfate.
2. Stir to dissolve the ammonium sulfate. (Can add more water.)
3. Add 1.2 g Coomassie blue G-250.
4. Dissolve all solids. (Stir for a while.)
5. Add water to 800 ml.
6. Add 200 ml methanol.

7. Store in brown bottle on shelf near scales or near orbital shaker.

Stock Tank Buffer (10X TGS)

1. Add 30.28 g Tris (FW 121.1), 144.13 g glycine, and 10 g SDS to ~600 ml distilled water.
2. Stir on stir plate by vacuum apparatus.
3. When dissolved, bring up to 1000 ml and store in plastic bottle by the pH meter. Store at room temperature for 1 month. Not necessary to check pH.

Tank Buffer is 0.025 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.3

(P. 20 Hoeffler Protein Electrophoresis Application Guide)

Lower Tank Buffer

200 ml of 10X TGS Buffer to 1800 ml distilled water. (2 L per apparatus)

Upper Tank Buffer

60 ml of 10X TGS Buffer to 540 ml distilled water. (600 ml per apparatus)