Antioxidant, Antihypertensive and Lipid Lowering Properties of Fruit Vinegar Beverages

by

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Submitted in partial fulfilment of the requirements
for the degree of Master of Science

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DALHOUSIE UNIVERSITY

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Dedication

I dedicate this document to my dearest parents and my lovely wife for their support, trust, motivation & being with me at every step of this journey.

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ABSTRACT

Cardiovascular disease (CVD) is ranked as one of top leading causes of death in most industrialized countries. Recent research suggests that fruit vinegar beverages (FVB) possess beneficial effects such as antihypertensive properties, reduction of serum cholesterol and triacylglycerols (TAG). FVB made using apple, blueberry, cranberry and tomato were evaluated for their sensory, antioxidant, antihypertensive and lipid lowering properties. All four treatments demonstrated very high *in vitro* antioxidant and antihypertensive properties. These FVB were further evaluated for their hypolipidemic and antihypertensive properties using a spontaneously hypertensive rats (SHR) model with diet-induced hyperlipidemia. All four FVB significantly reduced serum TAG, elevated the high density lipoprotein (HDL)-cholesterol compared to the control. Further, all four FVB demonstrated a reduction in the diastolic blood pressure after four weeks of supplementation. Overall, the FVB exhibited lipid lowering effects and antihypertensive properties *in vivo*. Confirmation of the beneficial effects of FVB using a clinical trial is needed.

LIST OF ABBREVIATIONS USED

AAPH	2, 2′-azobis (2-amidinopropane) dihydrochloride
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid
ACE	angiotensin converting enzyme
ACUC	animal care and use committee
AD	atherogenic Diet
AIN 93-G	american institute of nutrition 93 growth
ANOVA	analysis of variance
BEH	bridged ethylene hybrid
BHT	butylated hydroxytoluene
BPS	buffer phosphate solution
BW	body weight
CE	cholesterol ester
CHD	coronary heart disease
Cu^{2+}	cupric ion
CVD	cardiovascular disease
DBP	diastolic blood pressure
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	ethylenediaminetetraacetic acid
ESI	electron spray ionization
FC	free cholesterol
Fe^{2+}	ferrous ion
Fe^{3+}	ferric ion
$FeCl_3$	ferric chloride
FRAP	ferric reducing antioxidant power
GLM	general Linear Model
HCl	hydrochloric acid
HDL-C	high density lipoprotein cholesterol
HHL	histidine-L-hippuryl-L-leucine

HMG	3-hydroxy-3-methyl-glutaryl
HPLC	high pressure liquid chromatography
IC_{50}	50% inhibitory concentration
KCl	potassium chloride
LC	liquid chromatography
LDL-C	low density lipoprotein cholesterol
LSD	least square deviation
Melo	methyl linoleate
MS	mass spectrometry
m/z	mass to charge ratio
Na_2CO_3	sodium carbonate
NaCl	sodium chloride
NaOH	sodium hydroxide
N	normality
N_2	nitrogen
nd	not detected
NO	nitric oxide
NP-40	nonyl-phenoxypolyethoxylethanol-40
ns	not significant
NSAC	nova scotia agricultural college
ORAC	oxygen-radical absorbance capacity
PAD	photodiode array detector
PPO	poly phenol oxidase
RDA	recommended daily allowance
RI	refractive index
ROS	reactive oxygen species
SBP	systolic blood pressure
SD	standard deviation
SHR	spontaneously hypertensive rats
SPE	solid phase extraction

TA	titratable acidity
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TC	total cholesterol
TCA	trichloroacetic acid
TE	Trolox Equivalent
TAG	triacylglycerols
TPI	total phenolic index
TPTZ	2, 4, 6-tripyridyl-S-triazine
UPEI	university of prince edward island
UPLC-MS/MS	ultra performance liquid chromatography coupled with tandem mass spectrometry
UTI	urinary tract infections
UV	ultra violet
VLDL	very low density lipoprotein

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CHAPTER 1.0 INTRODUCTION

Cardiovascular disease (CVD) (Chu and Liu 2004), hypertension (Tanaka et al. 2009) and diabetes (O'Keefe et al. 2008; Shahidi et al. 2008) are becoming major causes of mortality in the world. Today, 25% of the world's adult population is suffering from hypertension and the numbers are likely to increase by another 5% in the next ten years (Mittal and Singh, 2010). Plaque build-up in the coronary arteries is the main cause of CVD and hypertension (Chu and Liu 2004). Long-term elevated low-density lipoprotein (LDL) levels and oxidative modification of LDL are leading causes in the formation of plaque (Chu and Liu 2004). Dysfunction of the endothelium may affect vasodilation and is one of the key factors in the progression of atherosclerosis (Chu and Liu 2004). Several studies have revealed that a temporary increase in triacylglycerols and fatty acids has the ability to affect vasodilation (Setorki et al. 2010). In addition, poor dietary and lifestyle habits are prominent factors that contribute to the progression of the above diseases (Tanaka et al. 2009) and also enhance the prevalence of obesity, which is distinguished as a risk-factor for lifestyle-related diseases (Kondo et al. 2009).

Increasing health care cost, due to the above mentioned diseases, has led to the development of natural antioxidants and functional foods and beverages which are rich in biologically active phytochemicals. Recent statistics indicate that the average North American consumer spent approximately US $90 per year on functional foods and beverages (Granato et al. 2010). The market growth of fruit-based functional beverages increased by 60% between 1998 and 2003 and a further 40% in 2008 (Granato et al. 2010). Furthermore, vinegar drinks are the latest group of functional beverages that have

sparked the North American beverage market with promising results and have become the second largest market for vinegar; further, Canada and the US has a combined total trade value of $147.5 million in 2009 (Berry 2011).

Generally, fruit vinegar is manufactured through the conversion of sugars to alcohol by yeast fermentation and subsequent addition of acetic acid bacteria to induce the acetic acid fermentation (Kato et al. 1998). It has been reported that phytochemicals, which are plant secondary metabolites, found in fruits have been shown to exhibit the antioxidant and physiological properties that are beneficial to health (Pinsirodom et al. 2008).

Currently, there are a number of commercial fruit vinegar beverages available on the market. However, a fruit vinegar beverage targeted for the reduction of CVD, by using fruits rich in different classes of biologically active plant secondary metabolites, is rare. Therefore, this study was focused on the development and assessment of fruit vinegar beverages prepared using apples (*Malus domestica*), wild blueberries (*Vaccinium angustifolium*), cranberries (*Vaccinium oxycoccos*) and tomatoes (*Lycopersicon esculentum*). The nutritional benefits of the produced beverages were determined by analyzing and studying the phytochemical attributes. All four beverages were analyzed using ultra performance liquid chromatography mass spectrometry (UPLC/MS) to examine the distribution of major bio-active compounds. Further, distribution of sugars in the vinegar beverages was analyzed using a high performance liquid chromatography (HPLC) system with a refractive index (RI) detector. The antioxidant assays, ferric reducing antioxidant power (FRAP), and oxygen-radical absorbance capacities (ORAC), were used as general measures of antioxidant capacity of the four vinegar beverages.

Odor, level of acidity, after taste and overall acceptability of the four vinegar beverages were assessed using a sensory panel of 18 trained panelists. The ability of dietary intervention to reduce the risk of CVD and hypertension was studied using an animal model system of spontaneously hypertensive rats (SHR). Furthermore, the effects of the bioactive constituents of the beverages were investigated for their ability to inhibit angiotensin converting enzyme (ACE), a key enzyme associated with hypertension. Therefore, the results of this research study provide some preliminary understanding for future product development, focusing on the reduction of the risk of hypertension and CVD.

CHAPTER 2.0 OBJECTIVES

Presently, hypertension is one of the leading causes of mortality in the world. Hypertension leads to several other diseases, CVD, stroke, diabetes, and renal failures, etc. ACE plays a significant role in regulating the blood pressure in the body by producing angiotensin II, which is a potent vasoconstrictor. Thus, ACE inhibitors have been identified as potential antihypertensive drugs which help to regulate the blood pressure in the body. In addition, these drugs are also beneficial for the prevention of CVD. However, a major drawback of taking therapeutics for a prolonged duration is the associated side effects. This phenomenon has led to an investigation of alternative plant-based bioactive compounds which have the ability to regulate hypertension and blood pressure. Fruit vinegar beverages are one of the leading food products reported from ancient times to possess health benefits such as antihypertensive properties (Kondo et al. 2001; Nakamura et al. 2010).

The research hypothesis of the current study was that dietary intervention by fruit vinegar beverages rich in phenolic acids, anthocyanins or lycopene help to maintain blood pressure and serum lipid profile at healthy levels. The overall objective was to develop and assess four types of fruit vinegar beverages, based on various bioactive plant secondary metabolite profiles, produced from fruit juices of tomato, blueberry, cranberry and apple and assess their physico-chemical, antioxidant and selected cardio-protective properties using an animal model of spontaneously hypertensive rats. The specific objectives of this research were to:

(1) Develop and optimize processing parameters for four low acid fruit vinegar beverages from apple, blueberry, cranberry and tomato;

(2) Determine the changes in antioxidant capacity and bio-active composition during the processing steps of the fruit vinegar beverage production;

(3) Evaluate the physico-chemical, antioxidant, antihypertensive properties and sensory attributes of the selected fruit vinegar beverage formulations containing 0.5%, 1.0% and 1.5% acetic acid; and

(4) Assess the effects of the fruit vinegar beverages on blood pressure and plasma and liver lipid profiles using an experimental animal model of spontaneously hypertensive rats (SHR).

CHAPTER 3.0 LITERATURE REVIEW

3.1 Vinegar Production

During the last few years, the use of vinegar as a food ingredient, as well as a functional beverage, has increased (Budak et al. 2011). Generally, vinegar production is carried out using a double fermentation process (**Figure 3.1**) (alcoholic fermentation followed by acetic acid fermentation). There are two production methods (Budak et al. 2011): (i) slow method, in which the culture of acetic acid bacteria (*Acetobacter aceti*), due to its requirement of oxygen, grows on the surface of the liquid (Budak et al. 2011; Sengun and Karabiyikli 2011); and (ii) quick method, where a submerged culture of bacteria is used and oxygen is supplied through aeration (Natera et al. 2003; Nakamura et al. 2010; Su and Chien 2010). Normally, *A. aceti* are gram-negative, aerobic, non-spore forming, rod-shaped cells. Furthermore, their sizes vary between 0.4 - 1 µm wide and 0.8 - 4.5 µm long and the optimum pH for the growth of *A. aceti* is 5 - 6.5. However, they can survive even at lower pH values between 3 and 4. Moreover, due to their high stability in the acidic environment, *A. aceti* has an ability to produce more acetic acid (Sengun and Karabiyikli 2011).

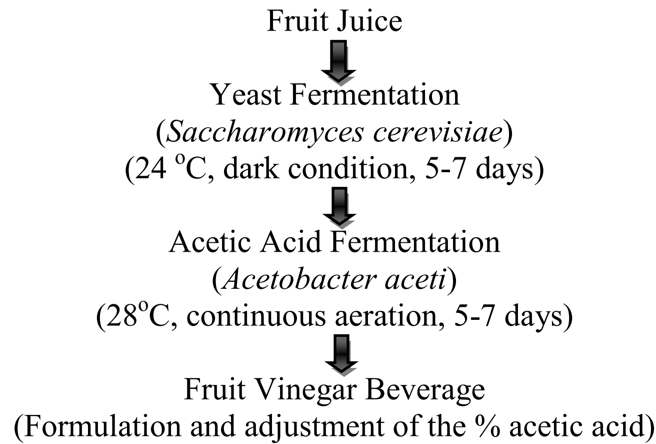


Figure 3.1: Process Flow Chart of Commercial Fruit Vinegar Production (Quick Method)

3.2 Definition of Fruit Vinegar Beverage

Fruit vinegar beverages have been categorized into two different types based on their acetic acid concentration: fruit vinegar beverage, which is low in acetic acid (< 3% v/v) and concentrated fruit vinegar beverage, which is high in acetic acid (5 - 7% v/v). These concentrated fruit vinegar beverages must be diluted 4 to 8 times with water before consumption (Chang et al. 2005). Fruit vinegar beverage is defined as a beverage that has been fermented from at least one kind of fruit, where each liter of raw material must contain more than 300 g of fruit juice (Chang et al. 2005).

3.3 Composition of Fruit Vinegar Beverage

Total sugar content of concentrated fruit vinegar beverage is normally less than 3% (v/v) and the acidity level is around 5 to 7% (v/v). Total soluble solids content and density of the fruit vinegar beverage may depend on total sugar content. Key organic acids found in fruit vinegar beverage, in addition to acetic acid, are malic, lactic, tartaric, succinic and citric acids (**Table 3.1**) (Chang et al. 2005; Natera et al. 2003). Major volatile compounds found in vinegar beverages are 2- and 3-methyl-1-butanol, 3-hydroxy-2-butanone, 2-phenylethanol, 2,3-butanediol, and isopentanoic acid, where apple vinegars are associated with a very high amount of 3-hydroxy-2-butanone produced with a slow acetification process (Natera et al. 2003). Furthermore, fruit vinegars consist of high concentrations of polyphenolic compounds (Bastante et al. 2010).

Table 3.1: Organic Acids Found in Different Vinegar Types

Type of vinegar	Sample Size (n)	acetic acid (g/L)	succinic acid (g/L)	malic acid (g/L)	tartaric acid (g/L)	citric acid (g/L)	lactic acid (g/L)
Apple	11	56.7 ± 12.8	0.1 ± 0.2	0.1 ± 0.2	nd	0.2 ± 0.4	2.0 ± 1.5
Red Wine	4	86.8 ± 29.2	0.4 ± 0.3	nd	0.1 ± 0.1	nd	1.0 ± 0.9
Balsamic	6	51.1 ± 21.5	0.4 ± 0.2	0.7 ± 0.6	1.4 ± 0.7	0.7 ± 0.7	0.8 ± 0.7

(n -number of samples for each category, nd - not detected) (Natera et al. 2003)

3.4 Health Benefits of Fruit Vinegar Beverage

Fruit vinegar beverages are well known from ancient times and used as a food product as well as medicine because of their properties (Dogaru et al. 2009; Fushimi and Sato 2005). All vinegars are solutions that primarily contain acetic acid and have been reported to possess physiological effects in humans such as antihypertensive properties (Kondo et al. 2001; Nakamura et al. 2010), enhancement of glycogen repletion in liver and muscle (Fushimi and Sato 2005; Fushimi et al. 2001), anticancer effects (Shizuma et al. 2011), stimulation of Ca^{2+} absorption (Fushimi et al. 2006) and reduction of serum cholesterol and triacylglycerols (Setorki et al. 2010; Setorki et al. 2011; Ubeda et al. 2011). Furthermore, a recent study has demonstrated that the dosage of black rice vinegar (kurosu) given to male Wistar rats, corresponding to the human dosage (recommended daily allowance of 700 mL per day per 70 kg body weight), confirmed an improvement in blood sugar levels (Shibayama et al. 2010). Moreover, a study conducted by Machado et al. (2011) showed that functional and nutritional quality results of grape juice and vinegars have the ability to alleviate a plasmatic total polyphenol content one hour after ingestion in humans. Some other constituents of fruit vinegar beverages are anthocyanins (cyanidin-3-glucoside), flavonols (quercetin glycosides), flavanols (catechin, epicatechin), vitamins, mineral salts, amino acids and non-volatile organic acids (tartaric, citric, and malic) (Machado et al. 2011). Moreover, these organic acids have the ability to influence flavor, color and aroma of the final product or the stability and microbiological control of these beverages (Zhang et al. 2011). These compounds can also act as antioxidants, enzyme inhibitors and inducers, inhibitors of receptors, and enhancers or

suppressors of gene expression (Kris-Etherton 2004; Pinsirodom et al. 2008; Setorki et al. 2010). Furthermore, Iizuka et al. (2010) found that polyphenols (catechins) present in apple vinegar have the ability to inhibit the LDL oxidation *ex-vivo* in endothelial cells.

3.4.1 Atherosclerosis

CVD is one of the major causes of mortality worldwide. CVD is associated with high blood cholesterol levels, high blood pressure, atherosclerosis, obesity, smoking, etc. (Budak et al. 2011). Atherosclerosis is currently one of the primary causes of death in the developed world (Setorki et al. 2011). The scavenger receptor-facilitated mechanism of oxidized LDL by macrophages leads to foam cell formation and when developed further, it will cause plaque formation, which plays an important role in the initiation and progression of atherosclerosis (Iizuka et al. 2010). Furthermore, high levels of LDL and oxidative modification of LDL in the vasculature is believed to be a significant factor contributing to atherosclerosis, coronary heart disease (CHD), and ischemic stroke (Lotito and Frei 2004; Iizuka et al. 2010). Flavonoids serve as an antioxidant and are one cluster of bioactive compounds studied extensively to explain biological functions and health outcomes, with emphasis on atherosclerosis (Kris-Etherton 2004; Lotito and Frei 2004; Setorki et al. 2010). Recent studies found that high doses of vinegar (> 5 mL per serving) can result in a significant decline in total serum cholesterol (TC) and LDL concentrations in experimental rabbits (Setorki et al. 2010). In addition, Budak et al. (2011) found a significant reduction in steatosis in the rats treated with apple cider vinegars (1% total acidity; 157 μL of apple cider vinegar in 843 μL of water) orally for 7 weeks when compared to control group. Iizuka et al. (2010) demonstrated that dietary

intervention of 800 mg polyphenols in concentrated balsamic vinegar has the ability to reduce the LDL oxidation both *in vitro* and *in vivo*. Furthermore, they observed a significant reduction of LDL oxidation after one hour of consumption for the same dosage of balsamic vinegar in humans.

3.4.2 Hypertension

Hypertension is one of the major independent risk factors for arteriosclerosis, stroke, myocardial infarction and renal disease (Honsho et al. 2005). Blood pressure reduction is helpful in the prevention of stroke and other adverse vascular events, including heart failure (Tanaka et al. 2009). Scientists in the field of food science and technology have discovered functional foods, including the vinegar beverages, which may have self-regulating mechanisms of blood pressure levels (Honsho et al. 2005). A beverage prepared from wine and rice vinegar has demonstrated antihypertensive effects in rats (Kondo et al. 2001). A studied dose (3 mL/kg body weight) of the beverage prepared using wine vinegar and grape juice reduced the heart rate and mean blood pressure of the pentobarbital-anesthetized rats (Sugiyama et al. 2003). These results suggest that this new vinegar beverage may be valuable for people who are concerned about palpitation and/or hypertension (Sugiyama et al. 2003). Furthermore, a new beverage made from red wine vinegar and grape juice decreased the ACE activity by 21% on the renin-angiotensin aldosterone system (RAAS) *in vivo* in a spontaneously hypertensive rat (SHR) model (Honsho et al. 2005). A single (3 g/kg body weight) dose and continuous administration (8 weeks; 10% (w/w) of diet) of black malt vinegar have shown a significant hypotensive effect in SHR (Odahara et al. 2008).

3.5 Market Potential for Fruit Vinegar

Traditionally, vinegar products have been used as a food flavoring ingredient (rice vinegar, wine vinegar and apple cider vinegar) but this has changed recently to a health food or functional beverage (Ou and Chang 2009). A survey has indicated that health conscious consumers have expressed their interest in drinking fruit vinegar beverages, increasing the market demand for fruit vinegar beverage products in Taiwan (Ou and Chang 2009). A recent report produced by the Nielsen Company (2010) indicates that global vinegar sales have been increasing with specialty vinegars (balsamic, red wine and fruit vinegars) leading the way with 45% of the market value and 12% of the unit value. Furthermore, Canada's vinegar imports have consistently increased over the past few years, by 11% between 2008 and 2009 (Berry 2011). The emergence of these new fruit vinegar products has prompted the change of names of vinegars, wine to grape and cider to apple, with these now being categorized as fruit vinegars (Ou and Chang 2009). It is quite possible that in the near future, innovative vinegar products from Canada (e.g. Saskatoon berry, icewine, pumpkin, maple syrup, and dessert vinegars) may be well received in the North American marketplace (Berry 2011).

3.5.1 North American Vinegar Market

North America is the second largest market for vinegar, following Europe (Berry 2011). The United States vinegar market is similar to that in Canada and consumer tastes and vinegar uses are comparable (Berry 2011). Italy remains the largest exporter of vinegar to the United States and its products were worth a total of US $71 million in

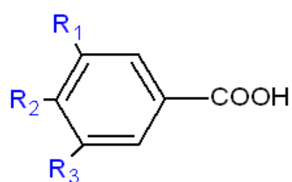
2009. Spain (US $8.5 million), France (US $4.7 million), Japan (US $4.4 million) and Philippines (US $1.8 million) followed Italy as foremost vinegar suppliers to the United States. Canadian vinegar producers must contend with high quality European varieties and inexpensive Asian products in the American marketplace (Berry 2011).

3.5.2 Fruits Available in Canada and Nova Scotia

An ample variety of fruits are grown commercially in Canada, including apples (farm gate value in Canada ~US $ 150 million), tender fruits (peaches, pears, plums and cherries) and berries. Apples are Canada's principal fruit crop in terms of tonnage; however, due to declining apple prices over the last few years and the growth of the blueberry industry, blueberries are now the most valuable crop, with a farm gate value of ~ US $ 150 million (Statistics Canada 2012). According to statistics in 2012, Nova Scotia produced 33,657 metric tons of apples, 14,873 metric tons of blueberries and 933 metric tons of cranberries with farm gate values of the fruits 12.1, 22.3 and 1.2 million dollars, respectively. Furthermore, Nova Scotia produces 75 metric tons of tomatoes, with a farm gate value of 0.13 million dollars (Statistics Canada 2012).

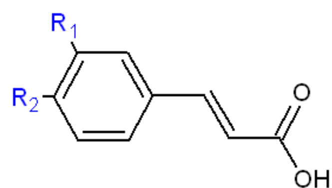
3.6 Bioactive Compounds in Fruits

Over the past decade, researchers, food manufacturers and food processing agencies have become increasingly interested in polyphenols (Manach et al. 2004). This has encouraged an understanding of the importance of polyphenols in several areas e.g.: agriculture, ecology and food selection, nutrition, medicines and pharmaceuticals (Shoji 2007). In addition to having antioxidant properties, polyphenols have several other specific biological actions, yet poorly understood (Manach et al. 2004). Polyphenols have been grouped according to their structures (**Figure 3.2**) as either non-flavonoids or flavonoids. The non-flavonoids are comprised of phenolic acid derivatives, lignans, stilbenes, and, hydrolysable tannins (Shoji 2007). Flavonoids can be further categorized into groups such as flavanones, flavonols, flavones, flavanols, isoflavones, and anthocyanidins, on the basis of the hydroxylation of phenolic rings, glycosylation, and acylation with phenolic acids (Shoji 2007). In fact, most important dietary phenolics are the phenolic acids, polyphenols and flavonoids; thus, flavonoids are the most studied group (Dillard and German 2000).



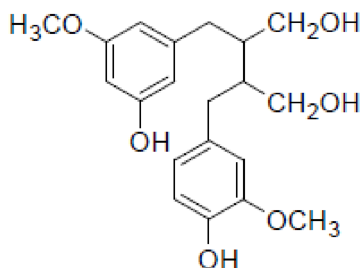
Hydroxybenzoic acid

R_1=R_2=OH, R_3=H: Protocatechuic acid
R_1=R_2=R_3=OH: Gallic acid

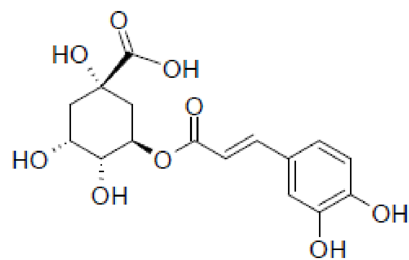


Hydroxycinnamic acid

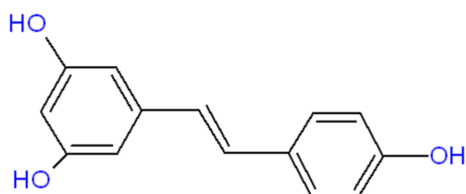
R_1=OH: Coumaric acid
R_1=R_2=OH: Caffeic acid
R_1=OCH_3, R_2=OH: Ferulic acid



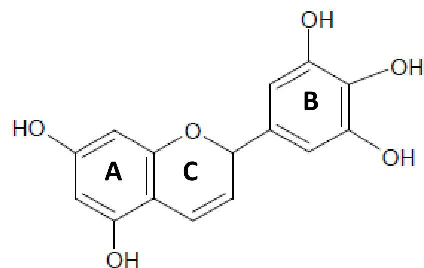
Lignans
Secoisolarciresinol



Phenolic acid
Chlorogenic acid



Stilbenes
Resveratrol



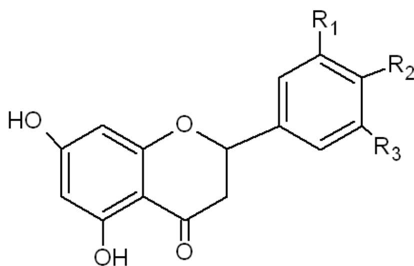
Flavonoids

Figure 3.2: Chemical Structures of Polyphenols (Manach et al. 2004)

3.6.1 Flavonoids

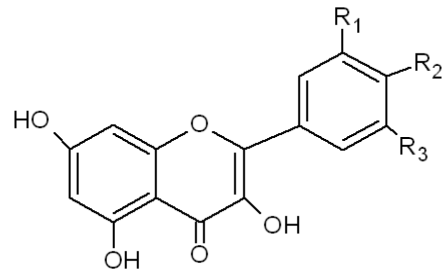
Flavonoids can be divided into six sub-classes (**Figure 3.3**) including: flavanones, flavonols, flavones, flavanols, isoflavones, and anthocyanidins (Manach et al. 2004). In general, flavonoids are either multiple colors or colorless, depending upon their structures

and are mostly present as glycosides with relatively low solubility in water (Shoji 2007). Flavonols, which mainly accumulate in the outer and aerial tissues (skin and leaves) of the plant, are the most common flavonoids in foods and their biosynthesis is stimulated by light (Manach et al. 2004). The key representatives of the flavonols are quercetin and kaempferol which are present at relatively low concentrations in fresh fruits (Manach et al. 2004). Flavonoids share a common ring structure, consisting of two aromatic rings (A and B) that are bound together by three carbon atoms that form an oxygenated heterocycle (ring C).



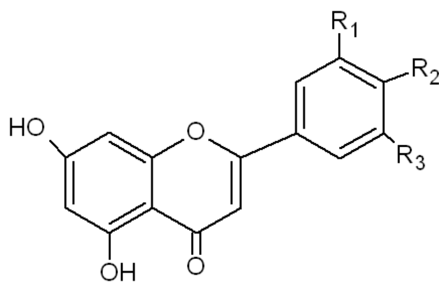
Flavanones

R_1=H, R_2=OH: Naringenin
R_1=R_2=OH: Eriodictyol
R_1=OH, R_2=OCH_3: Hesperetin



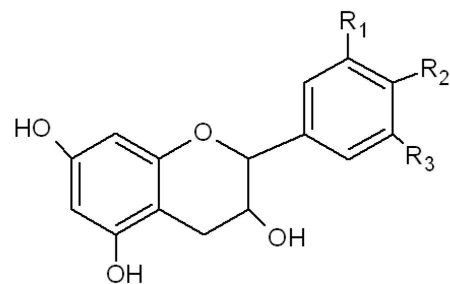
Flavonols

R_1=R_3=H, R_2=OH: Kampferol
R_1=R_2=OH, R_3=H: Quercetin
R_1= R2=R3=OH: Myricetin



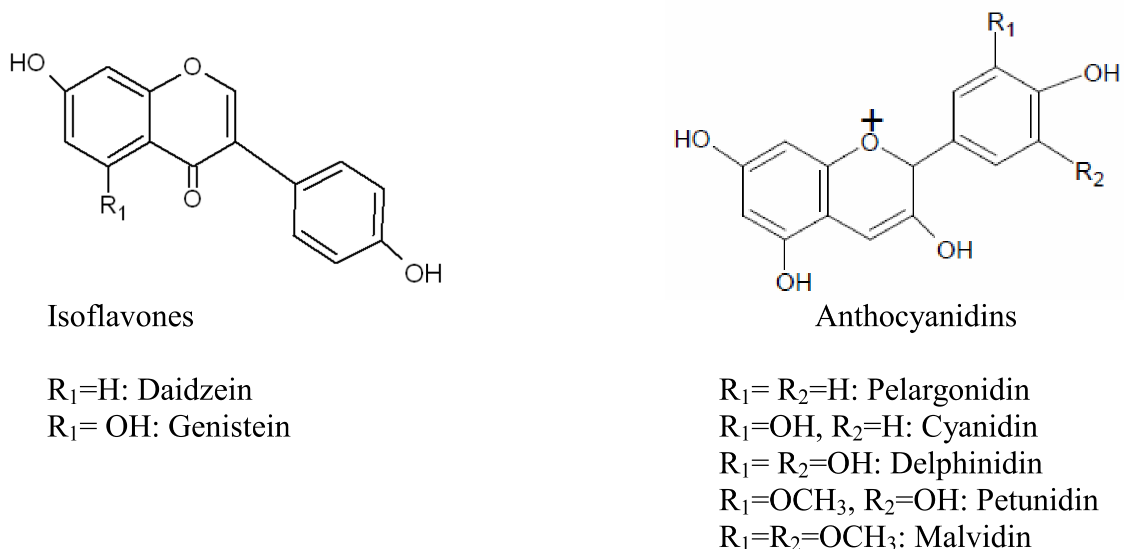
Flavones

R_1=H, R_2=OH: Apigenin
R_1=R_2=OH: Lutoolin



Flavanols

R_1= R_2=R_3=OH: Gallocatechin
R_1=R_2=OH, R_3=H: Catechin



Isoflavones

R_1=H: Daidzein
R_1= OH: Genistein

Anthocyanidins

R_1= R_2=H: Pelargonidin
R_1=OH, R_2=H: Cyanidin
R_1= R_2=OH: Delphinidin
R_1=OCH_3, R_2=OH: Petunidin
R_1=R_2=OCH_3: Malvidin

Figure 3.3: Chemical Structure of Flavonoids (Manach et al. 2004)

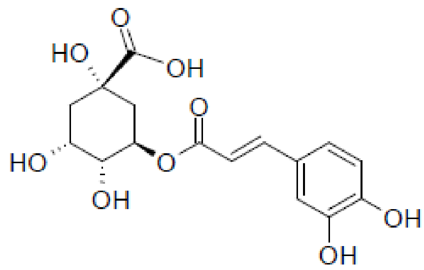
3.7 Raw Materials for the Fruit Vinegar Beverage Production

Some studies have shown a relationship between a reduced risk of CVD and fruit consumption and these protective effects could come from dietary fibres, micro-constituents, phenolic compounds, etc. (Decorde et al. 2008; Dogaru et al. 2009). The most widespread raw materials for production of fruit vinegar beverages are apple and grape, which provide a rich body and a superior flavor to the final product. Other commonly used fruits are black currant, raspberry and tomato (Kato 1998). Usually, balsamic vinegar is manufactured from the concentrated juice (30º Brix), and it is dark brown in color and its flavor is rich, sweet and complex. However, the commercial balsamic vinegar that is available in supermarkets is normally finalized using red wine vinegar or concentrated grape juice mixed with vinegar (Ugliano et al. 2002).

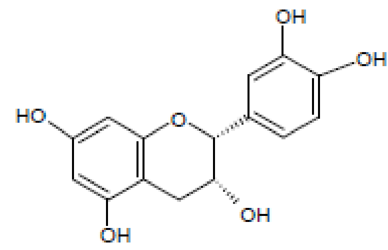
However, in every year, a large fraction of harvested fruit is discarded due to deformations, over production and lack of post-harvest management; therefore, these bio resources could be used in the production of fruit vinegars or as functional beverages (Ubeda et al. 2011).

3.7.1 Apple

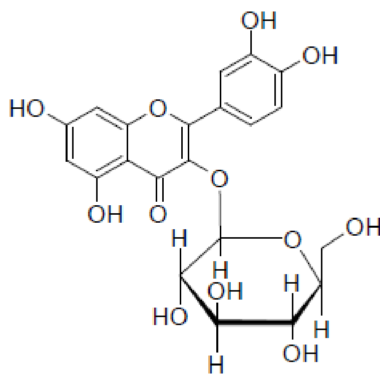
"An apple a day keeps the doctor away". This saying has encouraged many researchers to investigate the "magic" ingredient of the apple that may reveal the soundness of this expression (Taso et al. 2005). Apples are one of the foremost sources of dietary flavonoids that demonstrate the strongest associations with decreased mortality (Boyer and Liu 2004). Furthermore, a study done by Decorde et al. (2008) demonstrated that apples have the ability to prevent diet-induced atherosclerosis in hamsters. Apples are one of the main sources of flavonoids in the western diet and contain as much as 2g of phenols per kilogram fresh weight (Lotito and Frei 2004). Apple polyphenols contain mainly polyphenolic acid derivatives and other flavonoids (Budak et al. 2011) and these phenolic compounds are the most active constituents in apple (Nakamura et al. 2010). There are five major groups of polyphenolic compounds in apple; hydroxycinnamic acids, flavan-3-ols, anthocyanins, flavonols, and dihydrochalcones (Kathiirvel and Rupasinghe 2012). The amount of bioactive substances in apples is higher than in other tree fruits i.e. peach and pear (Leontowicz et al. 2004).



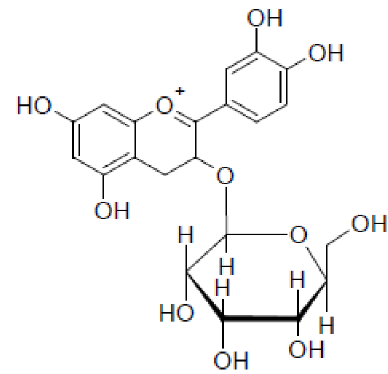
Chlorogenic acid



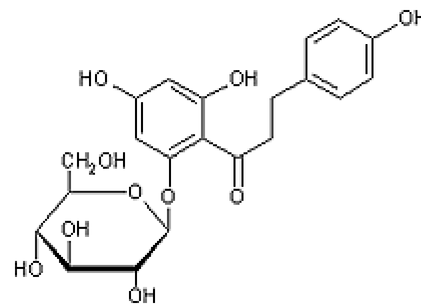
(-) Epi-catechin



Quercetin-3-glucoside



Cyanidin-3-galactoside



Phloridzin

Figure 3.4: Major Bioactive Compounds of Apples

3.7.1.1 Flavonols: Quercetin glycosides

A major class of phytochemicals commonly found in fruits and vegetables are the flavonoids (Boyer and Liu 2004). Apple is one of the major sources of flavonoids in the European and North American diet (Wach et al. 2007). Quercetin glycocides are one of the most abundant flavonoid sub-classes present in fruits and vegetables (Taso et al. 2005). Substantial amounts of iso-quercetin (quercetin-3-glucoside) have been found in apple and pear peels (Taso et al. 2005). Since the skin is an important source of quercetin glycocides, any endorsement of apple consumption should imply the inclusion of the skin (Rupasinghe and Kean 2008; Rupasinghe et al. 2010).

3.7.1.2 Flavan-3-ols: Epicatechin and Catechin

Flavanols, which make up a major class of apple polyphenols, correspond to between 71 and 90% of the polyphenolic compounds in apples (Vrhovsek et al. 2004). Epicatechin is found at high concentrations in apples, blackberries, cherries, pears and raspberries (Williamson and Manach 2005). Apple peels typically contain catechin and epicatechin, but these compounds are found in much lower concentrations in the apple flesh (Boyer and Liu 2004). In human intervention studies, catechins have showed higher plasma antioxidant activity, reduced plasma lipid peroxide and malondialdehyde concentrations and greater resistance of LDL to oxidation (Williamson and Manach 2005).

3.7.2 Blueberry and Cranberry

Berries are rich sources of both anthocyanins and flavonoid glycosides (Su and Chien 2007). Anthocyanins comprise the largest group of natural, water-soluble, plant pigments present in edible berries (Basu et al. 2010; Zafra-Stone et al. 2007) and anthocyanins are associated with higher antioxidant activity (Su and Chien 2007). Berry flavonoids are best known for their antioxidant and anti-inflammatory actions (Huntley 2009). Blueberries have ORAC values ranging between 14 - 45.9 µmol/g, depending on variety (Neto 2007). Furthermore, blueberries contain the following major anthocyanins: malvidin-*O*-galactoside, delphinidin-*O*-galactoside, malvidin-*O*-glucoside, cyanidin-*O*-glucoside, cyanidin-*O*-galactoside, etc. (Lee et al. 2002). A recent study also indicated that feeding a blueberry diet (3g blueberry powder per 100 g diet) for 8 weeks reduced the development of hypertension in a rat model system (Shaughnessya et al. 2009). Even though it has been reported that blueberries have favorable effects on metabolic syndrome and Type 2 diabetes in animal models, human intervention studies investigating the similar effects are inconclusive (Basu 2010).

Cranberries are one of the three commercially important fruits native to North America, along with the blueberry and Concord grape (McKay and Blumberg 2007) and are primarily produced in the United States (85%) and Canada (15%). Cranberries are comprised of three major classes of flavonoids (flavonols, anthocyanins, and proanthocyanidins), catechins, hydroxycinnamic and other phenolic acids, and triterpenoids (Neto 2007b). Major anthocyanins found in cranberries are galactoside of cyanidin, and peonidins and the content varies between cultivars, with a range of 25 - 65

mg/100g ripe fruit at harvest (Nato 2007b). Furthermore, cranberries are one of the leading sources of quercetin on a weight basis and contain total flavonols around 20 - 30 mg/100g of fresh fruit weight (Nato 2007b). In addition, researchers have found that cranberries inhibit adhesion of *Escherichia coli* to uroepithelial cells and reduce their ability to proliferate in the urinary tract (Nato 2007b). They further inhibit adhesion of *Helicobactoer pylori* to gastrointestinal mucosa, preventing gastric and duodenal ulcers (McKay and Blumberg 2007; Nato 2007b), preventing some urinary tract infections (Jepson and Craig 2007).

Table 3.2: Major Classes of Phenolic Phytochemicals Identified in Cranberries (McKay and Blumberg 2007)

Class	Subclass	Compound
Phenolic acid	Hydroxybenzoic acid	Benzoic acid
	Hydroxycinnamic acid	p-Coumaric acid
		Sinapic acid
		Caffeic acid
		Ferulic acid
Flavonoids	Flavonols	Quercetin glycoside
		Myricetin
	Flavan-3-ols	Proanthocyanidins
		Epicatechin
	Anthocyanins	Cyanidin glycoside
		Peonidin glycoside
Stilbenes		Resveratrol

3.7.2.1 Anthocyanins

Anthocyanins are a diverse group of water-soluble flavonoids produced by plants (Durst and Wrolstad 2002; Pergola et al. 2005) which largely determine the colors of flowers and fruits. Anthocyanin color pigments range from orange, red, blue, purple, etc. (Koponen et al. 2008; Corrales et al. 2009; Verbeyst et al. 2011). These anthocyanins are

mostly abundant in the skin, except for certain types of red fruit, in which they also occur in the flesh (cherries and strawberries) (Chaovanalikit and Wrolstad 2004; Lee and Wrolstad 2004; Manach et al. 2004). Anthocyanin levels of a fruit can vary due to several factors, including storage conditions, stage of maturity, and environmental factors such as light, temperature, agronomic practices, and various stresses (Srivastava et al. 2007; Fischer et al. 2011). Major naturally occurring anthocyanidins are pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Dillard and German 2000).

Anthocyanins exhibit a high antioxidant capacity due to higher number of hydroxyl groups within the structure (Decorde et al. 2008). Like other flavonoids, anthocyanins are powerful free radical scavengers, which show antioxidant activity, anti-mutagenic properties *in vivo* in lipid environments such as emulsified methyl linoleate (MeLo), human LDL and liposome (Kahkonen et al. 2003; Dillard and German 2000). Furthermore, anthocyanins exhibit a wide array of antioxidant and therapeutic benefits and also facilitate the prevention of auto-oxidation of lipids in addition to lipid peroxidation (Zafra-Stone et al. 2007). These protective features of the anthocyanins suggest that it could be used as a therapeutic agent to overcome some oxidative-damage-induced diseases (Pergola et al. 2006). Thus, consumption of berry fruits and their contribution to the enhancement of cardiovascular health is a subject of considerable importance (Basu et al. 2010). Due to antioxidant efficacy, the anthocyanins found in cranberries and blueberries could play a major role in the inhibition of oxidative damage linked to vascular diseases and cancer (Neto 2007). Studies conducted by Iizuka et al. (2010) have found that anthocyanins have the ability to inhibit LDL oxidation.

Table 3.3: Anthocyanins in Food (Manachet al. 2004)

Source (Serving Size)	Polyphenol content	
	mg/kg per fresh weight	mg/serving
Blackberry (100 g)	1000 – 4000	100 – 400
Black currant (100 g)	1300 – 4000	130 – 400
Blueberry (100 g)	250 – 5000	25 – 500
Rhubarb (100 g)	2000	200
Red wine (100 mL)	200 – 350	20 – 35

2.7.3 Tomato

Tomatoes, and tomato products, are important dietary sources of antioxidants (Paran et al. 2009). Intake of tomatoes and tomato-based products resulted in an inverse relationship between the risk of cancer, where strongest evidence was for the decline in tumors of the prostate, lung and stomach (Riccioni et al. 2008). Short-term, daily oral supplementation of carotenoid-rich tomato extract significantly decreased systolic blood pressure (SBP) and diastolic blood pressure (DBP) and decreased the levels of lipid per-oxidation products (Engelhard et al. 2006).

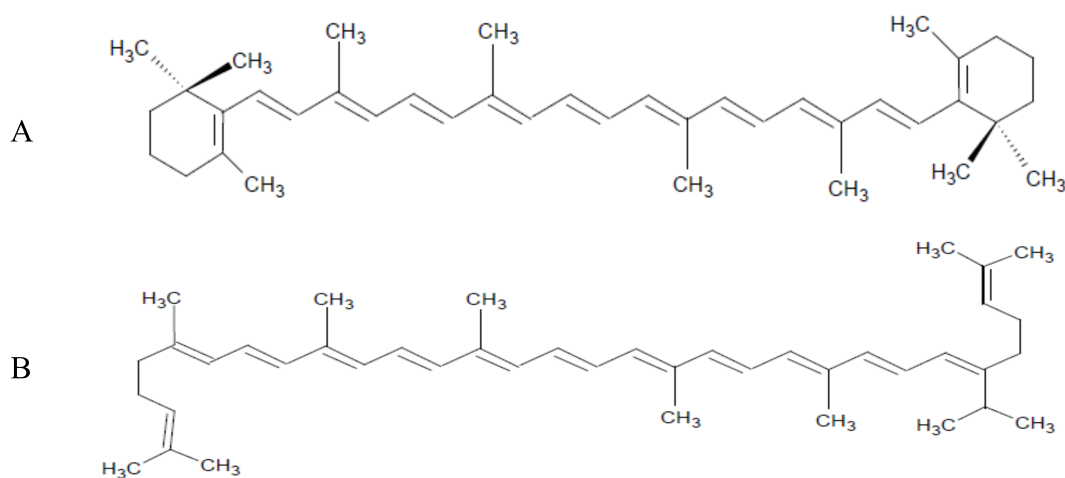


Figure 3.5: Structure of lycopene (A) and beta carotene (B)

3.7.3.1 Lycopene

More than 80% of lycopene consumed in the United States is derived from tomato products (Clinton 1998). The abundant conjugated double bonds of lycopene can scavenge peroxyl radicals, making them powerful antioxidants. Compared with other carotenoids, lycopene has been shown *in vitro* to have more potent antioxidant properties (Wang et al. 2006). Lycopenes are also relatively stable during food processing and cooking than the other fruits; for instance berries (Clinton 1998). Moreover, lycopene contributes to the protective outcome of vegetable consumption on myocardial infarction risk (Kohlmeier et al. 1997). However, any direct evidence for a relationship between lycopene supplementation and reduction risk of CHD (Riccioni et al. 2008) and the beneficial effect of lycopene on insulin action and glucose tolerance (Wang et al. 2006) are still lacking.

Although the fruit vinegar beverages have already entered the North American market, their potential health benefits are not well renowned. Therefore, the subsequent studies will be investigating the potential health benefits of the fruit vinegar beverages in connection with hypertension and lipid lowering abilities.

CHAPTER 4.0 PRODUCTION OF FRUIT VINEGAR BEVERAGES AND IMPACT OF FERMENTATION ON THE BIO-ACTIVE COMPOUNDS

4.1 ABSTRACT

Bio-active compounds present in fruits are important contributors in the human diet. Recently consumer demand for bio-active compounds and antioxidant-rich beverages has been growing. Though the fruit vinegar beverages have entered the North American functional beverage market, their health promotion constituents are not well documented. Therefore, the present study investigated the changes in the physico-chemical properties, total antioxidant capacity and selected major bio-active constituents of four different fruit vinegar beverages, in relation to the processing/fermentation steps. The results indicated that there was a decrease in pH and an increase of titratable acidity during the fermentation process. Both apple and blueberry vinegars exhibited an increase in antioxidant capacity but a decrease was observed in cranberry vinegar after alcoholic fermentation. Most of the bio-active concentrations were reduced ($p < 0.05$) after the alcoholic fermentation process, while the changes after the acetic acid fermentation were relatively low. The yeast metabolism seems to convert the bio-active compounds of juice into different forms during the fermentation process. Further investigations are required to identify yeast metabolites of phenolic constituents of fruit juices and to identify biological properties and health benefits of fruit vinegar products.

Key words – Fruit vinegars, Fermentation, bio-active composition, antioxidant capacity

4.2 INTRODUCTION

Phenolic compounds are important components in fruits. The potential health benefits of these compounds have been widely reported (Raczkowska et al. 2011). Recently, much attention has been paid to the antioxidant properties of polyphenols, which seem to protect tissues against reactive oxygen species (ROS) and lipid peroxidation (Raczkowska et al. 2011). Furthermore, increasing health care costs has led to the development of biological active phytochemical and antioxidant-rich functional foods and beverages as health promoting food products. Recent surveys disclosed that consumers are demanding value-added food products with health promoting characteristics (Berry 2011). Fruit vinegar beverages are well known from ancient times and used as a food product and medicine because of their functional properties (Dogaru et al. 2009). In the past few years, the importance of vinegar as a food product, over its traditional use, has increased (Natera et al. 2003; Ubeda et al. 2011).

Generally, fruit vinegar is manufactured from a variety of raw materials (white and red wine, cider, malted barley, honey, pure alcohol, etc.) through the conversion of sugars to alcohol by yeast fermentation and subsequent addition of acetic acid bacteria to induce the acetic acid fermentation (Natera et al. 2003; Sengun and Karabiyikli 2011). Methods of making vinegar can be divided in two groups: (i) slow methods in which the culture of acetic acid bacteria, due to its requirement of oxygen, grows on the surface of the liquid; and (ii) quick processes in steel tanks with a submerged culture of bacteria, where the oxygenation is favored by agitation (Budak et al. 2011; Sengun and Karabiyikli 2011). However, the industrial production of vinegar is largely done using the semi-

continuous process where a fraction of the total volume of submerged culture is withdrawn periodically (Yang et al. 2011). Basic processing in manufacturing fruit vinegar beverages includes juice processing, winemaking, and vinegar making. The phenolic compositions of the final products depend mainly on the processing techniques, such as crushing, pressing, filtration, and fermentation (Su and Chien 2007).

Although only a few researches on fruit vinegar beverages have been technologically advanced, a consolidated industry in fruit vinegar beverages has been established in Europe, Japan and China. However, to date, no or limited scientific data have been reported in the literature about fruit vinegar beverage manufacture in North America. In addition, there is limited information on the changes in bio-active composition and concentration of fruit vinegar beverages during the processing steps. Therefore, the present study was focused on the assessment of changes in (i) the physico-chemical properties, (ii) total antioxidant capacities, and (iii) bio-active composition of four different fruit vinegar beverages, prepared using wild blueberries (*Vaccinium angustifolium*), cranberries (*Vaccinium oxycoccos*), apples (*Malus domestica*) and tomato (*Solanum lycopersicum*) after the alcoholic and acetic acid fermentation processes.

4.3 MATERIALS & METHODS

4.3.1 Samples and Chemical Reagents

Fresh apples and fresh tomato were purchased from the local grocery store (Sobeys, Truro, NS, Canada) and cranberry juice (concentrated ~ 35° Brix) was purchased from a commercial cranberry juice manufacturer (Cranberry Acres, Berwick, NS, Canada) while blueberry juice (100% juice) was obtained from a commercial blueberry juice manufacturer (Van Dyke, Caledonia, Queens County, NS, Canada). Sodium hydroxide solution, acetonitrile (HPLC grade, > 99.8%), methanol (HPLC grade, > 99.8%), formic acid (HPLC grade, > 99.8%), anthocyanin and non-anthocyanin standards were all received from Sigma Aldrich (Oakville, ON, Canada). C18 columns were obtained from Chromatographic Specialties (Brockville, ON, Canada).

4.3.2 Sample Preparation

Fruit vinegar beverages were produced using a modified process of Su and Chein (2010) and Nakamura (2010) as follows (**Figure 4.1**): selection of quality raw materials (apples and tomato), washing and then pressing of the fruits using an X1 hydraulic plate presser (Model JVH 56C17F5323J, Marathon, WI, USA). To obtain 4L of the juice sample, a total of ~ 8 kg of apples or tomato fruits were required (~ 50% yield from the X1 hydraulic plate presser). The obtained juice sample was filtered using four layers of cheesecloth and then adjusted or concentrated to approximately 20° Brix (added sucrose to a final sugar concentration ~ 120 g/L depending on the type of fruit). In general, alcoholic fermentation is usually carried out until all the sugars were converted into

ethanol. These juice samples (4 L) were then subjected to controlled alcohol fermentation in fermentation vats using yeast (*Saccharomyces cerevisiae*) to obtain the desired final alcohol concentration between 2% to 5% (v/v). Alcoholic fermentation required five to seven days depending on the type of fruit (24 ^{o}C, in dark condition) (Su and Chein 2010). Fermented juice was filtered again through four layers of cheesecloth to remove yeast from the fermentation system. Once the alcohol level reached between 2% to 5% (v/v), alcoholic fermentation was stopped and acetic acid fermentation was initiated. This was carried out using the quick method where the submerged culture of bacteria (from previously produced vinegar) was used (volume ratio of 3 fermented juice: 2 acetic acid culture) and continuous oxygen supply was manipulated through aeration. Acetic fermentation was carried out in 2L volume glass containers in three replicates. Acetic acid fermentation continued for five to six days, depending on the type of fruit (28 ^{o}C, continuous aeration). The titratable acidity (%) was monitored daily until the required level of acidity (2% v/v) was obtained.

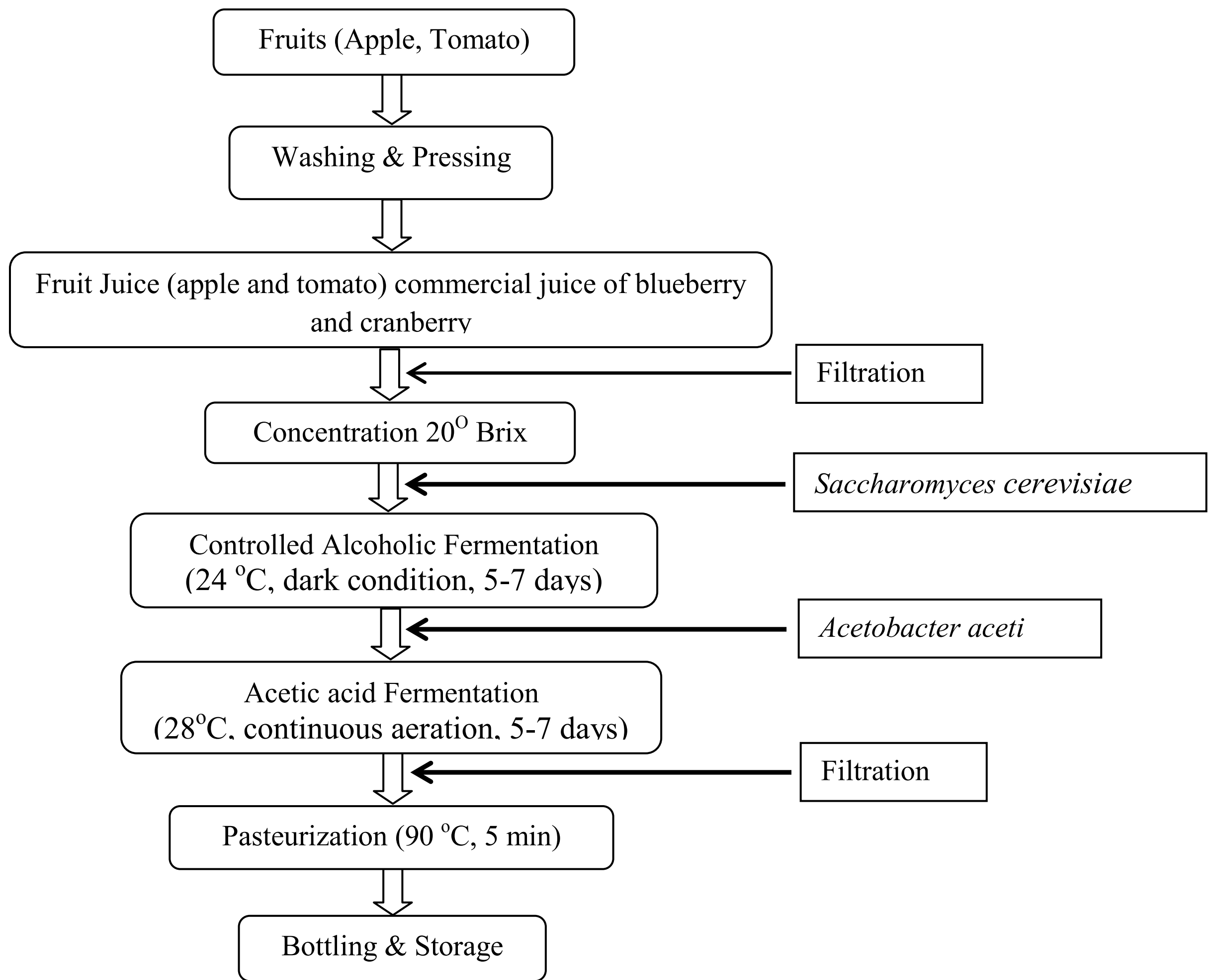


Figure 4.1: Process Flow Diagram of Fruit Vinegar Beverage Production

After the fermentation, juice samples were again filtered through four layers of cheesecloth to remove sediments (Su and Chien 2010). Filtered fruit vinegar beverage samples were then pasteurized using a batch type pasteurizer (Model SK-620X-BLT, Advantage, Greenwood, IN, USA) at 90°C for 5 minutes. Immediately after pasteurization, samples were bottled into sterilized plastic containers (4 L) and stored at -20 ^{o}C until the sensory analysis was carried out.

4.3.3 Assessment of pH and Titratable Acidity During the Stages of Fermentation

All the samples were measured for pH using a pH meter (Model Accumet 10, Denver Instruments Co, Arvada, CO, USA) that had been calibrated to pH 4.0 and 7.0. Titratable acidity of the samples (2 mL) was measured using a semi-automated titrator (Model DMP 785, Metrohm Ltd, Herisau, Switzerland) with 0.1N NaOH as the titrant to an endpoint of pH 8.2. Titratable acidity (%) was expressed in terms of acetic acid equivalents which is the most prominent acid in vinegar beverages.

4.3.4 Assessment of Antioxidant Properties at Different Stages of Fermentation

4.3.4.1 Oxygen Radical Absorbance Capacity (ORAC) Assay

The principle of ORAC assay is the decay of fluorescein, which is measured for the reduction of fluorescence over the time (Cao et al. 1993). Free radicals can degrade the fluorescence ability with time and this could be terminated by antioxidants. This assay was carried out using a modified standard method for a 96-well FLUOstar OPTIMA micro-plate reader equipped with an injection port system (BMG Labtech Inc., Offenburg, Germany) (Rupasinghe et al. 2008). The fluorescein sodium salt (0.957 μM) as well as samples and standards were prepared in 75 mM phosphate buffer solution (K_2HPO_4/NaH_2PO_4, pH 7.0). Trolox was used as the standard for ORAC assay with concentrations of 50, 100, 300, 500, 700, and 900 μM. Thirty five microliters of each sample or standard were placed in the wells of the 96-well micro-plate (COSTAR 3915, Fisher Scientific, Ottawa, ON, Canada) and 130 μL of the fluorescein was pipetted into

the wells using a micro pipette. The plate was warmed to 37°C for five minutes and 35 μL of pre-warmed peroxyl radical generator, 2,2'-Azobis(2-amidinopropane)dihydrochloride (AAPH) solution was injected into the wells, using the injection port system. The microplate was shaken for 3s after each injection of AAPH and prior to each reading. The plate was kept at 37 °C throughout the experimental time of approximately 50 minutes. Excitation (λ_{ex} 490 nm) and emission (λ_{em} 510 nm) readings were taken at two minute time intervals.

4.3.5 Solid Phase Extraction (SPE) Method for Fruit Juice/Vinegar Samples

Sample preparation for the UPLC analysis was carried out using a C18 (Varian Bond Elute, Chromatographic specialists, Brockville, ON, Canada) SPE column tube. Initially, the column was conditioned by using 3 mL of 100% methanol, followed with 3 mL of water to remove the excess methanol. Then, 6 mL of juice/vinegar sample was loaded and filtered through the column. After the loading step, the sample was washed with 9 mL of water, followed by elution of the phenolics using 3 mL of 100% methanol. The elute was filtered through 0.45 μm filter and collected into 2 mL amber colored UPLC auto sampler vials and used for the UPLC analysis.

4.3.6 UPLC Analysis

Bio-active compounds present in fruit juice/fruit vinegar samples were analyzed using the UPLC system (Model Waters Aquity CHA, Waters Corp, Milford, MA, USA) which was equipped with an Aquity BEH C_{18} (100 mm x 2.1 mm, 1.7 μm) column and C_{18} guard column. The flow rate of the UPLC system was 0.3 mL/min, with a total run

time of 12 min per one sample and an injection volume of 2 μL with a limit of detection of 0.05 mg/L. All the standard samples were prepared in methanol and their concentrations were used as follows: 0.20 - 20 mg/L of catechin, epicatechin, epigallocatechin, chlorogenic acid, caffeic acid, ferulic acid, phloridzin, quercetin, quercetin-3-galatoside, quercetin-3-rhamnoside and quercetin-3-glucoside. The anthocyanin standard samples were prepared in methanol and their concentrations were used as follows: 0.25 - 25 mg/L of cyanidin-3-glucoside, malvidin-3-glucoside, delphinidine-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, cyanidin-3-galactoside, malvidin-3-galactoside, delphinidine-3-galactoside, petunidin-3-galactoside and peonidin-3-galactoside. All the samples were analyzed using the method described by Rupasinghe et al. (2008).

4.3.6.1 Solvent Conditions:

Non-anthocyanin phenolics were quantified using the mobile phase of 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B). A linear gradient profile was used with the following proportions of Solvent A applied at time t (min): (t, A %): (0, 94%), (2, 83.5%), (2.61, 83%), (2.17, 82.5%), (3.63, 82.5%), (4.08, 81.5%), (4.76, 80%), (6.75, 20%), (8.75, 94%), (12, 94%).

Anthocyanins were quantified, as described below; the mobile phases were 5% (v/v) formic acid in water (solvent A) and 5% (v/v) formic acid in methanol (solvent B). The linear gradient profiles used were as follows: (t, B %): (0-10%), (8-30%), (17-40%), (19-40%), (20-10%), (22-10%).

4.3.6.2 MS-MS Analysis:

MS-MS analysis was carried out using a micro-mass Quattro micro API MS/MS system, as described by Rupasinghe et al. (2008). Electro spray ionization, in negative ion mode (ESI-), was used for the analysis of the flavonols, flavan-3-ol, and phenolic acid compounds. Mass spectrometry conditions used for the analysis were as follows: capillary voltage 3000 V, nebulizing gas (N_2) at a temperature of 375 °C at a flow rate of 0.35 mL/min. The cone voltage (25-50 V) was optimized for each compound. Individual compounds were identified using the multiple reactions monitoring mode (MRM), using specific precursor-production transition: m/z 301→105 for quercetin, m/z 463→301 for quercetin-3-glucoside and quercetin-3-galactoside, m/z 448→301 for quercetin-3-rhamnoside, m/z 289→109 for catechin, m/z 290→109 for epicatechin, m/z 305→125 for epigallocatechin, m/z 441→169 for epicatechingallate, m/z 457→169 for epigalloatechingallate, m/z 331→242 for malvidin-3-glucoside, m/z 303→229 for delphinidin-3-glucoside, m/z 317→245 for petunidin-3-glucoside. Individual specific compounds of cranberry were identified using the single reactions monitoring mode (SIM), using specific precursor-production transition: m/z 317 for myricitin, m/z 163 for p-coumaric acid and m/z 153 protocatechuric acid. External standards and calibration curves were used to quantify each analyte.

4.3.7 HPLC Analysis of Lycopene and Beta-Carotene

4.3.7.1 Lycopene and Beta-Carotene Extraction

Lycopene and beta-carotene extraction was carried out according to the method described by Diaz et al. (2010) and Nardo et al. (2009), with some modifications. Tomato juice/tomato vinegar (5 mL) was mixed with methanol (5mL) and centrifuged for 5 min at 3000 rpm. The supernatant was discarded (consist of mainly water and methanol). The remaining pellet was re-suspended in 20 mL of acetone: methanol: hexane 1:1:2 (v/v/v) and continuously shaken for 15 min at 180 rpm at room temperature. Then, 3 mL of deionized water was added to the shaken mixture and again shaken for an additional 5 min (180 rpm). Samples were kept at room temperature overnight to encourage phase separation. Then, 6 mL of hexane top layer was separated and was dried under nitrogen flow. The dried pellet from acetone: methanol mixture was re-dissolved again in 500 µL of the acetone: ethanol: hexane 1:1:2 (v/v/v) mixtures and was used for HPLC analysis.

4.3.7.2 HPLC Analysis

Lycopene and beta-carotene were quantified, using the method described by Sandmann (2010), with some modifications. HPLC analysis was done using Waters Alliance 2695 separation module with a Phenomenex Luna C_{18} column (150mm x 2.0 mm, 5 µm; Phenomenex, Torrence, CA, USA) and photodiode array detector (PDA) at 440 nm. The mobile phase of the system was acetonitrile: methanol: 2-propanol (85: 10: 5) with an isocratic flow rate of 0.7 mL/min, column temperature was set at 40 °C and the detector temperature was 30 ^{o}C. Sample run time was 12 minutes per each sample.

Calibration curves were obtained using standard solutions of lycopene and beta-carotene with a concentration range of 10-200 mg/L.

4.4 Statistical Analysis

The design for the fermentation study was a randomized block design with time as the blocking factor and bio-active composition, antioxidant capacity, pH and titratable acidity as the factors of interest. Data was analyzed using the analysis of variance (ANOVA) using the general linear model (GLM). Assumptions of normality of error terms and assumptions of constant variance were checked (Montgomery 2005). When the data was not normally distributed necessary transformations were done accordingly to obtain the normality of data. Differences among means were tested by the least square of difference (LSD) range test at the level of $p < 0.05$ (SAS Version 9.2 SAS Institute Inc., Cary, NC, USA).

4.5 RESULTS

4.5.1 pH and Titratable Acidity

The pH of all the four products decreased after each stage of fermentation process (**Table 4.1**). The lowest pH levels were obtained after the acetic acid fermentation. Titratable acidity of the four fruit vinegars were increased after each stage of fermentation. Cranberry vinegar beverage resulted in the highest level of titratable acidity while tomato vinegar gave the lowest (**Table 4.1**).

4.5.2 Assessment of Antioxidant Capacity During Each Stage of Fermentation

4.5.2.1 Oxygen Radical Absorbance Capacity (ORAC) Assay

Blueberry vinegar beverage exhibited an increase in antioxidant capacity after the both fermentation process. However, cranberry and tomato vinegar beverages exhibited a decrease in antioxidant capacity after both fermentation process (**Table 4.2**). In addition, apple and blueberry exhibited an increase of ORAC values after both the alcoholic and acetic acid fermentation processes. ORAC value of tomato vinegar beverage remained constant after the alcoholic fermentation and thus, increased after the acetic acid fermentation (**Table 4.2**). However, with cranberry vinegar beverage, the ORAC value was reduced after the alcoholic fermentation and acetic acid fermentation processes (**Table 4.2**).

4.5.3 Ultra Performance Liquid Chromatography Analysis of Bio-active Compounds

The samples were analyzed for the changes of bio-active composition according to the steps of vinegar processing. All the compounds showed a reduction in bio-active composition after the alcoholic fermentation process except in apple vinegar beverage. Tomato vinegar beverage did not contain any flavonols (**Table 4.3**). Cranberry vinegar beverages contained only quercetin glucoside as flavonols and apple vinegar beverage contained only quercetin rhamnoside (**Table 4.3**). However, blueberry vinegar beverage contained all four types of flavonols (**Table 4.3**). There was a significant reduction in quercetin glucoside content in cranberry after the fermentation.

Epicatechin, catechin, phloridzin, phloritin and chlorogenic acids were present in both apple and blueberry vinegar beverages. However, the above five compounds were not observed in cranberry and tomato vinegar beverages (**Table 4.4**). In addition, isoferulic acid occurred in both cranberry and tomato vinegar beverages but not in apple and blueberry vinegar beverages (**Table 4.4**). Thus, all four types of vinegar beverages contained caffeic and ferulic acids (**Table 4.4**). However, phloritin concentration was constant throughout the processing in both apple and blueberry vinegar beverages (**Table 4.4**).

Glucosides and galactosides of cyanidin, petunidin, peonidin, malvidin and delphinidin were quantified in blueberry and cranberry vinegar beverages to understand the changes in concentration after the fermentation process. Except for petunidin, all the

other anthocyanin compounds showed a reduction after the alcoholic fermentation in blueberry vinegar beverage (**Table 4.5**). Cranberry, except cyanidin glucoside, four types of galactocides were present and both fermentation steps reduced the concentration of anthocyanins (**Table 4.5**).

The three unique bio-active compounds (myricitin, p-coumaric acid and protocatechuric acid) present in cranberry vinegar beverage were further quantified during the fermentation process (**Table 4.6**). Both myricitin and coumaric acid showed a reduction in concentration after the alcoholic fermentation (**Table 4.6**). Except for protocatechuric and p-coumaric acid; myricitin showed further reduction after the acetic acid fermentation (**Table 4.6**).

4.5.4 HPLC Analysis of Lycopene and Beta-Carotene

Two major bio-active compounds (lycopene and beta-carotene) present in tomato vinegar beverage were quantified to understand the changes in composition after the fermentation process (**Table 4.7**). Both compounds showed a reduction in concentration after the alcoholic fermentation (**Table 4.7**).

4.6 DISCUSSION

Both pH and temperature are two factors that could influence the degradation of the color of juices during the fermentation process (Su and Chien 2007). Usually, the pH values start to decrease after the alcoholic fermentation process and get further reduced after the acetic acid fermentation and finally stabilize when complete conversion of alcohol to acetic acid occurs (Othman et al. 2009). Similar differences among pH were observed in the current study after both fermentation processes. The titratable acidity showed an increase after alcoholic fermentation and further increased after the acetic acid fermentation processes and then stabilized. In the production process of blueberry and cranberry vinegar beverages, juice was used instead of raw fruit. Incorporation of fruit skins during the fermentation process could increase aroma and phenolic compounds (Su and Chien 2010). The composition of the fruit, enzymes and microorganisms living on the fruit could account for these phenolic, aromatic and flavor compounds during the fermentation process (Su and Chien 2010).

Further, higher pigment content in the product could result in a higher antioxidant capacity. For example, red wines contain a higher antioxidant capacity than white wines (Su and Chien 2007). In some studies, a significant decrease in antioxidant capacities of the fruit vinegar beverages has been observed during the acetification process (Su and Silva 2006; Su and Chien 2007; Wang et al. 2012). Thus, a decrease of the antioxidant activity after the fermentation process could be due to the loss of flavonoids during the fermentation (Wang et al 2012). This was evident from the results of the loss of quercetin composition after the fermentation. However, Bastante et al. (2010) found in their study

that vinegars macerated with fruits resulted in a higher total antioxidant capacity. Apple and blueberry vinegar beverages have a high antioxidant capacity due to their composition of bio-active compounds (Budak et al. 2011), confirming the results of the ORAC study. In addition, the researchers suggests that the formation of malanoidins after the fermentation process could also result in an increase of antioxidant capacity (Wang et al 2012), and further, the antioxidant capacity could be increased during the aging process (Verzelloni et al. 2010). The composition of phenolic acids, monomeric catechins, flavonols and tannins content could also be increased during the aging process of the vinegar beverages (Verzelloni et al. 2010).

All the juice samples, including the vinegar beverage samples, contain a very high amount of sugars; therefore, it is important to remove the sugars before the UPLC MS/MS analysis because sugar can interfere with the ionization of phenolics and their quantification. To overcome the interfering effect of sugars, sample preparation technique of SPE has been used. The UPLC is an advanced form of liquid chromatography (LC) which operates the mobile phase delivery system under very high pressures. The major advantages of the UPLC over the conventional HPLC are: improved resolution, shorter retention time and higher sensitivity (Yu et al. 2006). Coupling the UPLC with electro-spray ionization (ESI) tandem mass spectrometry (MS/MS) offers advantages of specifications and sensitivities. MS/MS is unique in its ability to give accurate identification of analytes, based on not only the precursor ion, but also the product ions, assuring the correct identification of target compounds (Ferrer and Thurman 2009). SPE

method using the C_{18} column could be used for sample preparation to analyze the bio-active compounds present in the juices using the UPLC.

Generally, the compositional analysis of the bio-active compounds from the current research study concludes that there was a significant loss ($p < 0.05$) of bio-active compounds after the alcoholic fermentation. However, the bio-active composition after acetic acid fermentation was mostly constant. Similar patterns of degradation of polyphenols were observed in another two fermentation studies done by Su and Silva (2006) and Su and Chien (2007). In their studies, it was observed that there was a significant difference in all blueberry by-products; juice had the highest amount of anthocyanins (11.9 ± 0.03 mg/g cyanidin-3-glucocide equivalents) followed by alcoholic fermentation (10.9 ± 0.03 mg/g cyanidin-3-glucocide equivalents) and acetic acid fermentation (2.3 ± 0.01 mg/g cyanidin-3-glucocide equivalents). Furthermore, the results of this study indicated that the apple vinegar beverages contained very low amounts of catechin, epicatechin, ferulic acid, phloridizin, phloritin and caffeic acid. However, cholrogenic acid content was higher in apple vinegar beverage, compared to the other bio-active compounds, in agreement with the results of the studies conducted by Andlauer et al. (2000) and Budak et al. (2011). Furthermore, most of the flavonols showed a reduction in their composition after the fermentation process and the results were in agreement with a study conducted by Kim et al. (2011). Kim et al. (2011) observed a 38% reduction in total flavonol glycosides after the fermentation process. This was in agreement with another study conducted by Wang et al. (2012), indicating a significant reduction of flavonoids after the alcoholic fermentation (154.2 ± 4.1 mg/g)

and acetic fermentation (107.8 ± 6.1 mg/g), in agreement with the results of the current study. Authors suggest that this could be due to oxidative degradation or de-glycosylation of the bio-active compounds which takes place during the alcoholic fermentation process (Kim et al. 2011).

Furthermore, in another study conducted by Andlauer et al. (2000), it was found that there was an ~ 40% decrease in the total phenol content after the acetification process, indicating a degradation or transformation of phenols (Andlauer et al. 2000). Furthermore, the results of the current study indicated an increase in caffeic acid content after the alcoholic fermentation in apple and blueberry vinegar beverages, in agreement with the results of Nogueira et al. (2008). Thus, the extraction of cyanidins was significantly lower in apple and cranberry vinegar beverages and this could be due to the fact that cyanidins could associate with the solid part of the fruit, particularly the cell wall or it may be due to their interaction with the yeast cell wall (Nogueira et al. 2008). Moreover, in another two studies conducted by Verzelloni et al. (2010) and Wang et al. (2012) it was observed that there was a progressive loss of flavonols and phenolic acids during the aging of vinegar beverages and the authors suggested that the loss of phenolic acids during aging could be a result of polymerization reactions (Verzelloni et al. 2010).

As similar pattern of compositional and concentration changes were observed in both tomato and cranberry bio-active compounds. Lycopene and beta-carotene composition was reduced after the alcoholic fermentation. However, there was no significant difference observed in the lycopene content after acetic acid fermentation in tomato vinegar beverage. Thus, the beta-carotene content was reduced after the acetic

acid fermentation. Moreover, all three major bio-active compounds present in cranberry vinegar beverage showed a decrease in composition after the alcoholic fermentation. However, the composition of myricitin was further reduced after the acetic acid fermentation. Thus, the composition of protocatechuric acid increased after the acetic acid fermentation (from 3.5 mg/L to 4.8 mg/L). The metabolism of yeast might convert the bio-active composition of juices into different forms. However, minor differences ($p > 0.05$) in the loss of bio-active compounds under acetic acid fermentation suggested that the acetic acid bacteria had the ability to reduce the metabolic activity of yeast. Thus, the acidity produced by the acetic acid bacteria might retard the growth of yeast, which has the ability to reduce the bio-active composition.

The preservation of fruit components makes fermentation one of the more environmentally friendly processes. Furthermore, transformation by fermentation can add some value to a food due to the presence some microorganisms which produce vitamins, organic acids and other compounds that can improve the nutritional components of fruits (Hidalgo et al. 2010). During fruit vinegar processing, oxidation and condensation of the polyphenols takes place due to the activity of the polyphenoloxidase (PPO) (Nogueira et al. 2008). This could lead to a reduction of phenolic acids, flavanols, and cyanidin compounds after the fermentation (Nogueira et al. 2008).

Further investigation of this phenomenon is required to investigate whether yeast has the ability to convert the composition of bio-actives present in fruit juice in the production process of fruit vinegar beverages. Moreover, the maceration of fruits with vinegars could also help to increase the nutritional and bio-active composition of the final

product as well as to overcome the bio-active losses which took place in the fermentation process (Bastante et al. 2010). Through common processing by fermentation, only an inconsequential part of the phenolic components could be present in the final vinegar beverage product and its quality is difficult to control, choosing an improved processing method during fermentation could result in better value-added product.

Table 4.1: pH and Titratable Acidity During the Fruit Vinegar Beverage Manufacturing Process[p]

Type of Vinegar	Processing Step	pH	TA[q]
Apple	Juice	3.94 ± 0.18[a]	0.88 ± 0.17[a]
	AF[r]	3.72 ± 0.09[b]	1.42 ± 0.29[b]
	AAF[s]	3.15 ± 0.02[c]	2.81 ± 0.52[c]
Blueberry	Juice	3.47 ± 0.01[a]	0.53 ± 0.14[a]
	AF	3.16 ± 0.15[b]	1.13 ± 0.35[b]
	AAF	2.91 ± 0.07[c]	3.11 ± 1.39[c]
Cranberry	Juice	2.56 ± 0.01[a]	2.99 ± 0.08[a]
	AF	2.64 ± 0.01[b]	2.97 ± 0.14[a]
	AAF	2.56 ± 0.03[a]	3.94 ± 0.87[b]
Tomato	Juice	4.34 ± 0.01[a]	0.62 ± 0.22[a]
	AF	4.06 ± 0.06[b]	0.84 ± 0.27[a]
	AAF	3.95 ± 0.10[c]	1.33 ± 0.22[b]

[p]Mean values ± standard deviation (n=3) (3 blocks, each block with 3 replicates for each step of fermentation)
[q] TA- Titratable Acidity (mg acetic acid equivalents/g)
[r] AF- After Alcoholic Fermentation
[s]AAF- After Acetic Acid Fermentation
[a-c]Means followed by a different letter within each type of vinegar within each column are significantly different (LSD range test [P<0.05])

Table 4.2: Antioxidant Capacities During the Fruit Vinegar Beverage Manufacturing Process[p]

Type of Vinegar	Processing Step	ORAC[q] (mmol TE[r] /L)
Apple	Juice	1.35 ± 0.22[a]
	AF[r]	1.48 ± 0.14[ab]
	AAF[s]	1.57 ± 0.07[b]
Blueberry	Juice	7.65 ± 0.79[a]
	AF	8.32 ± 1.04[a]
	AAF	9.76 ± 0.47[b]
Cranberry	Juice	1.85 ± 0.57[a]
	AF	1.26 ± 0.17[b]
	AAF	1.02 ± 0.20[a]
Tomato	Juice	0.07 ± 0.02[a]
	AF	0.07 ± 0.01[a]
	AAF	0.08 ± 0.01[a]

[p]Mean values ± standard deviation (n=3) (3 blocks, each block with 3 replicates for each step of fermentation)
[r] AF- After Alcoholic Fermentation
[s]AAF- After Acetic Acid Fermentation
[q] ORAC - Oxygen Radical Absorbance Capacity
[r]TE - Trolox equivalents
[a-c]Means followed by a different letter within each type of vinegar is significantly different (LSD range test [P<0.05])

Table 4.3: Changes of the Flavonol Concentration During the Processing Step of Fruit Vinegar Beverage Production (mg/L)[p]

Type of Vinegar	Processing Step	Quercetin Galactoside	Quercetin Glucoside	Quercetin Rhamnoside	Quercetin Rutinoside
Apple	Juice	nd	nd	1.32 ± 0.05[a]	nd
	AF[r]	nd	nd	1.36 ± 0.11[a]	nd
	AAF[s]	nd	nd	1.54 ± 0.19[b]	nd
Blueberry	Juice	6.34 ± 5.35[a]	2.59 ± 0.79[a]	2.43 ± 1.27[a]	1.42 ± 1.82[a]
	AF	3.56 ± 2.09[a]	2.38 ± 0.82[a]	1.92 ± 0.50[a]	1.38 ± 1.16[a]
	AAF	4.78 ± 2.96[a]	2.25 ± 0.44[a]	2.16 ± 1.11[a]	1.22 ± 1.35[a]
Cranberry	Juice	nd	2.40 ± 0.68[a]	nd	nd
	AF	nd	1.59 ± 0.52[b]	nd	nd
	AAF	nd	1.29 ± 0.17[b]	nd	nd
Tomato	Juice	nd	nd	nd	nd
	AF	nd	nd	nd	nd
	AAF	nd	nd	nd	nd

[p]Mean values ± standard deviation (n=3) (3 blocks, each block with 3 replicates for each step of fermentation)
[r]AF- After Alcoholic Fermentation
[s]AAF- After Acetic Acid Fermentation
nd - Not Detected
[a-c]Means followed by a different letter within each type of vinegar within each column are significantly different (LSD range test [P<0.05])

Table 4.4: Changes of the Flavan-3-ols, Dihydrochalcones, and Phenolic Acid Concentration During the Processing Steps of Fruit Vinegar Beverage Production (mg/L)[p]

		Flavan-3-ol		Chalcone	Phenolic Acid			
Type of Vinegar	Type of Processing Step	Epicatechin	Catechin	Phloridzin	Chlorogenic Acid	Caffeic Acid	Ferulic Acid	Iso Ferulic Acid
Apple	Juice	1.04 ± 0.90[a]	1.61 ± 0.27[a]	1.51 ± 0.09[a]	11.41 ± 14.09[a]	1.29 ± 0.00[a]	0.99 ± 0.01[a]	nd
	AF[r]	0.97 ± 0.99[a]	3.88 ± 0.99[a]	1.50 ± 0.16[a]	14.09 ± 24.45[a]	1.31 ± 0.02[a]	1.05 ± 0.05[a]	nd
	AAF[s]	1.01 ± 0.82[a]	2.43 ± 2.04[a]	1.68 ± 0.21[a]	12.84 ± 21.25[a]	1.40 ± 0.23[a]	1.33 ± 0.51[a]	nd
Blueberry	Juice	1.04 ± 0.79[a]	13.07 ± 11.26[a]	1.64 ± 0.29[a]	33.02 ± 41.78[a]	2.49 ± 2.36[a]	1.14 ± 0.18[a]	nd
	AF	1.08 ± 0.47[a]	6.86 ± 5.40[a]	1.47 ± 0.10[a]	32.13 ± 33.41[a]	2.12 ± 1.08[a]	1.27 ± 0.34[a]	nd
	AAF	1.05 ± 0.68[a]	7.24 ± 6.51[a]	1.51 ± 0.14[a]	17.58 ± 12.87[a]	2.10 ± 1.11[a]	1.32 ± 0.51[a]	nd
Cranberry	Juice	nd	nd	nd	nd	1.32 ± 0.01[a]	1.11 ± 0.10[a]	1.51 ± 0.46[a]
	AF	nd	nd	nd	nd	1.30 ± 0.01[b]	1.27 ± 0.16[b]	1.78 ± 0.49[a]
	AAF	nd	nd	nd	nd	1.31 ± 0.02[ab]	1.14 ± 0.10[ab]	1.39 ± 0.24[a]
Tomato	Juice	nd	nd	nd	nd	1.29 ± 0.01[a]	1.14 ± 0.09[ab]	1.32 ± 0.25[a]
	AF	nd	nd	nd	nd	1.31 ± 0.03[b]	1.17 ± 0.07[a]	1.34 ± 0.32[a]
	AAF	nd	nd	nd	nd	1.31 ± 0.02[ab]	1.07 ± 0.03[b]	1.27 ± 0.27[a]

[p]Mean values ± standard deviation (n=3) (3 blocks, each block with 3 replicates for each step of fermentation)

[r]AF- After Alcoholic Fermentation

[s]AAF- After Acetic Acid Fermentation

nd - Not Detected

[a-c]Means followed by a different letter within each type of vinegar within each column are significantly different (LSD range test [P<0.05])

Table 4.5: Changes of the Anthocyanin Concentration During the Processing Steps of Fruit Vinegar Beverage Production (mg/L)[p]

Type of Vinegar	Type of Processing Step	Glucosides				Galactosides			
		Cyanidin[p]	Petunidin[q]	Peonidin[r]	Malvidin[s]	Cyanidin	Petunidin	Peonidin	Malvidin
Blueberry	Juice	9.66 ± 6.51^{a}	4.10 ± 2.54^{b}	6.37 ± 5.17^{a}	34.45 ± 23.76^{a}	5.90 ± 2.48^{a}	2.86 ± 2.43^{a}	2.99 ± 1.55^{a}	22.07 ± 17.44^{a}
	AF	4.70 ± 2.68^{b}	8.17 ± 4.19^{a}	2.80 ± 1.25^{b}	20.09 ± 6.13^{b}	5.00 ± 1.41^{a}	3.33 ± 0.95^{a}	2.27 ± 0.78^{a}	13.37 ± 5.74^{ab}
	AAF	2.03 ± 1.36^{b}	4.09 ± 2.50^{b}	0.84 ± 0.40^{b}	11.31 ± 3.17^{b}	2.76 ± 0.59^{b}	2.10 ± 0.84^{a}	0.98 ± 0.12^{b}	6.21 ± 1.53^{b}
Cranberry	Juice	0.49 ± 0.41	nd	3.73 ± 1.10	nd	31.29 ± 9.02^{a}	0.38 ± 0.13^{a}	36.35 ± 7.78^{a}	2.65 ± 0.85^{a}
	AF	nd	nd	nd	nd	5.86 ± 0.87^{b}	0.04 ± 0.02^{b}	9.53 ± 0.66^{b}	0.32 ± 0.09^{b}
	AAF	nd	nd	nd	nd	3.50 ± 0.91^{b}	0.03 ± 0.02^{b}	6.30 ± 2.39^{b}	0.18 ± 0.08^{b}

[p]Mean values ± standard deviation (n=3) (3 blocks, each block with 3 replicates for each step of fermentation)
[p] Transformations were done to obtain normality of data ([p-s]log transformation)
[r] AF- After Alcoholic Fermentation
[s]AAF- After Acetic Acid Fermentation
[a-c]Means followed by a different letter within each type of vinegar within each column are significantly different (LSD range test [P<0.05])

Table 4.6: Changes of the Myricitin, P-coumaric acid and Protocatechuric acid Composition during the Processing Step of Fruit Vinegar Beverage Production (mg/L)[p]

Type of Vinegar	Type of Processing Step	Myricitin[p]	P-coumaric acid	Protocatechuric acid
Cranberry	Juice	6.47 ± 1.10^{a}	10.70 ± 1.24^{a}	3.79 ± 0.58^{a}
	AF[r]	3.33 ± 0.52^{b}	6.93 ± 0.56^{b}	3.54 ± 0.47^{a}
	AAF[s]	2.07 ± 0.36^{c}	6.91 ± 0.59^{b}	4.77 ± 0.60^{b}

[p]Mean values ± standard deviation (n=3) (3 blocks, each block with 3 replicates for each step of fermentation)
[p] Transformations were done to obtain normality of data (square transformation)
[r] AF- After Alcoholic Fermentation
[s]AAF- After Acetic Acid Fermentation
[a-c]Means followed by a different letter within each column are significantly different (LSD range test [P<0.05])

Table 4.7: Changes of the Lycopene and beta-carotene Composition during the Processing Step of Fruit Vinegar Beverage Production (mg/L)[p]

Type of Vinegar	Type of Processing Step	Lycopene	Beta-Carotene
Tomato	Juice	6.84 ± 8.14[a]	2.04 ± 2.49[a]
	AF[r]	1.63 ± 1.91[b]	0.77 ± 1.56[b]
	AAF[s]	1.88 ± 0.69[ab]	0.10 ± 0.05[b]

[p]Mean values ± standard deviation (n=3) (3 blocks, each block with 3 replicates for each step of fermentation)

[p] Transformations were done to obtain normality of data (square transformation)

[r] AF- After Alcoholic Fermentation

[s]AAF- After Acetic Acid Fermentation

[a-c]Means followed by a different letter within each column are significantly different (LSD range test [$P<0.05$])

CHAPTER 5.0 PHYSICO-CHEMICAL AND SENSORY QUALITY OF FOUR DIFFERENT FRUIT VINEGAR BEVERAGES

5.1 ABSTRACT

The current study was designed to evaluate the sensory and physico-chemical properties of four different fruit vinegar beverages (apple, blueberry, cranberry and tomato), each prepared by acetic acid fermentation to have three different acetic acid concentrations (0.5%, 1.0%, and 1.5%). Odor, acidity, aftertaste and overall acceptability were evaluated by trained panelists, using descriptive sensory methods. The panelists observed that the odor of the 0.5% concentration was lower ($p < 0.05$), compared to the higher concentrations, in each fruit vinegar type (1.0% and 1.5%). The overall acceptability of the 0.5% level had a higher mean score value ($p < 0.05$), indicating panelists' preference for the lower acid fruit vinegar, compared to the other two levels of acetic acid concentrations. As for the physico-chemical properties, pH, TA and color were measured on all 12 samples (three different acetic acid concentrations of the four fruit vinegar beverages). The pH ranged from 2.64 to 3.51 and values of TA ranged from 0.54 to 1.60 mg acetic acid equivalents/g. Sensory attributes of the final fruit vinegar products were improved by blending them with respective fruit juices(apple, blueberry, cranberry and tomato), resulting in a higher sweetness and lower aftertaste of the final beverage.

Keywords: functional beverage, fruit vinegar beverages, acetic acid, sensory evaluation, acidity

5.2 INTRODUCTION

CVD (Chu et al. 2004) and hypertension (Tanaka et al. 2009) have become the major causes of mortality in the world. Increasing health care concerns, due to the above diseases, have led to the development of bioactive and antioxidant-rich functional foods and beverages. Recent statistics (2010) indicate that the average North American consumer would spend approximately US $90 per year on functional foods and beverages (Granato et al. 2010). Further, vinegar beverages are the latest group of functional beverages that have sparked the North American market (Berry 2011). Generally, fruit vinegar beverages are manufactured through the conversion of sugars to alcohol by yeast fermentation and the subsequent addition of acetic acid bacteria to induce acetic acid fermentation (Kato et al. 1998). It has been reported that the phytochemicals present include: phenolic acids, anthocyanins, and carotenoids which are plant secondary metabolites found in fruits. They have been shown to exhibit the antioxidant properties that are beneficial to health (Pinsirodom et al. 2008). However, it has been also reported that fruit vinegars have higher antioxidant capacities and higher total phenolic indices (TPI) (Ubeda et al. 2011; Su and Chien 2007). Researchers also suggest that higher antioxidant capacities and TPI in fruit vinegar beverages may be due to yeast's metabolism of phenolics in the fruit juice, as well as the yeasts ability to release antioxidant compounds differently from the polyphenols (Ubeda et al. 2011).

Although there are a number of imported commercial fruit vinegar beverages available in the North American market at present, there is still no fruit vinegar beverage produced within the North America from fruits such as apple (*Malus domestica*),

blueberry (*Vaccinium angustifolium*), cranberry (*Vaccinium oxycoccus*) and tomato (*Solanum lycopersicum*), which are familiar tastes to the North American consumers. Therefore, the focus of this study was on the sensory evaluation of fruit vinegar beverages, prepared using apples, wild blueberries, cranberries and tomatoes. The specific objectives of the current study were to: (i) evaluate the sensory properties (odor, acidity, aftertaste and overall acceptability) of four different fruit vinegar beverages which had been fermented using *Acetobacter* and later blends with respective juice to produce three different acetic acid concentrations and (ii) characterize the physico-chemical properties of these resulting 12 fruit vinegar beverage blends.

5.3 MATERIALS AND METHODS

5.3.1 Samples and Chemical Reagents

Fresh apples and fresh tomatoes were purchased from the local grocery store (Sobeys, Truro, NS, Canada) and cranberry juice (concentrated ~ 35° Brix) was acquired from a commercial cranberry juice manufacturer (Cranberry Acres, Berwick, NS, Canada) and blueberry juice (100% juice) was obtained from a commercial blueberry juice manufacturer (Van Dyke, Caledonia, Queens County, NS, Canada). Sodium hydroxide solution was purchased from Sigma Aldrich Canada. Sulfuric acid (HPLC grade > 99.8%) was bought from Fisher Scientific, Ottawa, ON, Canada. The liquid chromatography standards (glucose, fructose, sucrose and ethanol) used for the study were purchased from Sigma Aldrich (Oakville, ON, Canada) (HPLC grade > 99%).

5.3.1.1 Sample Preparation

All the fruit vinegar beverages were prepared using the standardized procedure explained in **Chapter 4.0**. The final three levels of acetic acid concentrations (0.5%, 1.0% and 1.5%) were obtained by blending the fermented beverage with the respective fruit juices (apple, blueberry, cranberry or tomato) until they reached the final three levels of acetic acid concentrations. After the fermentation, juice samples were again filtered through four layers of cheesecloth to remove sediments (Su and Chien 2010). Filtered fruit vinegar samples were then pasteurized using a batch type pasteurizer (Model SK-620X-BLT, Advantage, Greenwood, IN, USA) at 90^oC for 5 minutes. Soon after

pasteurization, the samples were bottled hot into sterilized containers and stored at -20 ^{o}C until the sensory analysis.

5.3.2 Screening and Training of the Sensory Panel

Approval of the Research Ethics Board of Nova Scotia Agricultural College (NSAC) was obtained before conducting the sensory evaluation study. The descriptive sensory evaluation used 18 trained sensory panelists to describe the odor, acidity, aftertaste and overall acceptability of the four different fruit vinegar beverages at all three acetic acid levels. An unstructured scaling technique was utilized in the descriptive sensory analysis (Poste et al. 1991). A screening session was done to identify the potential panelists who were able to sense and measure taste and flavor characteristics (Meilgaard et al. 1991). Training of the selected sensory panelists was done in order to standardize and develop the sensory skills. Two training sessions were conducted where panelists participated in focus group sessions to build their self-confidence (Meilgaard et al. 1991).

5.3.3 Descriptive Sensory Analysis of Fruit Vinegar Beverages

The sensory panel was conducted in the Product Quality Evaluation Laboratory, Haley Institute of NSAC during the period of December 2011 to January 2012. Fruit vinegar beverage bottles were transferred from the freezer (-20 ^{o}C) to the fridge (4 ^{o}C) 48 hours before the sensory evaluation and the sealed bottles were opened 2 to 5 minutes prior to the testing. Samples were presented to panelists in a balanced fashion and randomized to the number of panelists, where each sample appeared in a given position

on the tray an equal number of times. Three digit numbers were used to identify samples in order to avoid the expectation bias (Meilgaard et al. 1991). Each panelist was asked to evaluate/describe fruit vinegar beverages with different acidity levels. Three different acidity levels (0.5, 1.0, and 1.5%) of fruit vinegar beverages from each type of fruit (apple, tomato, cranberry and blueberry) were served in transparent polyethylene cups (food grade) using one tray (four products, each with three acidity levels on the tray). A 30 mL portion of each sample was provided to the panelist. Panelists were asked to scale the perceived level of odor, acidity, aftertaste and overall acceptability on a horizontal 15 cm long line with two anchor points of 1.5 cm from each end on the score sheet provided. Potable water, salt free crackers and apple were used as palate cleansing agents.

5.3.4 Assessment of Physico-Chemical Properties of Four Different Fruit Vinegar Beverages

5.3.4.1 pH and Titratable Acidity

All the fruit vinegar beverages were evaluated for pH and titratable acidity, as explained in **Chapter 4.0**.

5.3.4.2 Color

The color of the samples was measured using a colorimeter (0.01 - 160% reflectance range) (Model CR-300, Minolta Camera Co. Ltd, Osaka, Japan) and the L*, a* and b* values. The colorimeter was calibrated using a white plate with the reference values of X=92.30, Y=0.3137 and Z=0.3195.

5.3.5 Determination of Sugars and Ethanol using High Performance Liquid Chromatography (HPLC)

Sugars (glucose, fructose and sucrose) were quantified using a HPLC using Waters Alliance 2695 separation module with an ROA-organic acid column (300 x 7.8 mm; Phenomenex, Torrence, CA, USA) and refractive index (RI) detector. All the samples were filtered using 0.45 micron filters (Chromaspec, Chicago, IL, USA) before being applied to the system. Solvent conditions for the HPLC system were as follows: column temperature was set at 65 °C; the detector temperature was at 30 ^{o}C; the mobile phase of the system was 0.005 N sulfuric acid, with a flow rate of 0.6 mL/min and the limit of detection was 5 mg/L. The sample run time was 30 minutes per each sample. Calibration curves were obtained using standard solutions of glucose, fructose, sucrose and ethanol using a concentration range of 50-200 mg/L.

5.4 Statistical Analysis

The design for the sensory evaluation study was a randomized block design with panelists as the blocking factor and four different beverages at three different acid levels (fruit-acid combination) as the factor of interest. Data was analyzed using the analysis of variance (ANOVA) and the general linear model (GLM). Assumptions of normality of error terms and assumptions of constant variance were checked (Montgomery, 2005). When the data was not normally distributed necessary transformations were done accordingly to obtain the normality of data. Differences among means were tested by the

Tukey's studentized range test at the level of $p < 0.05$ (SAS Version 9.2 SAS Institute Inc., Cary, NC, USA).

5.5 RESULTS

5.5.1 Sensory Evaluation of Four Different Fruit Vinegar Beverages

The sensory evaluation of the four different fruit vinegar beverages (apple, blueberry, cranberry, and tomato) with three different levels of acetic acid concentrations (0.5%, 1.0%, and 1.5%) was carried out to study the level of acceptable acidity, odor, aftertaste and overall acceptability of each different fruit vinegar beverage (**Table 5.1**). Furthermore, ANOVA p-values were obtained to understand the impact of the sensory panelists on the score values. All four products have shown a similar trend for the odor, acidity, and aftertaste, expect for overall acceptability in cranberry vinegar beverage. With regard to the odor levels of the vinegar beverages, sensory panelists have observed 0.5% acetic acid concentration having the lowest level of odor (**Table 5.1**) and panelists preferred this to the higher concentration levels of acetic acid (1.0% and 1.5%).

Similarly, 0.5% acetic acid concentration level had the highest overall acceptability value (**Table 5.1**) which panelists preferred compared to the other two levels of acetic acid concentrations. However, the sensory attribute of overall acceptability of cranberry vinegar beverage was a bit differed, resulting in all three levels of acetic acid concentration having no significant difference in overall acceptability (**Table 5.1**). Furthermore, tomato vinegar beverages exhibited a similar result for the

overall acceptability, resulting in no significant difference between 0.5% and 1.0% acetic acid concentrations (**Table 5.1**).

In addition, the aftertaste of the vinegar beverages showed the same pattern for the apple and blueberry vinegar beverages, resulting in 0.5% having the lowest level of aftertaste (**Table 5.1**). However, cranberry vinegar beverage demonstrated that there was no significant difference between the 0.5% and 1.0% levels of acetic acid concentration for the aftertaste. In addition, tomato vinegar beverage showed no significant difference between the 1.0% and 1.5% levels of acetic acid concentration for the aftertaste (**Table 5.1**).

5.5.2 Physico-Chemical Properties of Four Different Fruit Vinegar Beverages

Physico-chemical characteristics of four different fruit vinegar beverages with three different acetic acid concentrations were assessed **(Table 5.2)**. In the four different vinegar beverages, pH ranged from 2.6 to 3.5, TA ranged from 0.5 to 1.6, acetic acid equivalence/g. The results indicated that TA increased with the level of acetic acid concentration.

5.5.3 Sugars and Residual Ethanol Determination of Four Different Fruit Vinegar Beverages

Three types of sugars (glucose, fructose and sucrose) commonly present in fruit juices were quantified using HPLC with respective standards (**Table 5.3**). Residual ethanol was quantified as a quality parameter. All the vinegar beverages had fructose as

the main type of sugar, followed by glucose. However, glucose was not present in tomato vinegar beverage (**Table 5.3**). Both blueberry and tomato vinegar beverages did not have any sucrose, although apple vinegar beverage had some higher levels of residual sucrose (**Table 5.3**). All four vinegar beverages had no ethanol; this indicated that during the fermentation, ethanol was fully converted to acetic acid (**Table 5.3**), confirming the final quality of the vinegar beverages.

5.6 DISCUSSION

The above result indicated that the lower the level of acetic acid concentration, the higher the level of consumer preference for all four different beverage products. This preference for lower acidic product could be attributed to the high level of sweet taste, as well as to the lower level of acidic odor of the four different beverage products.

Furthermore, the color of the vinegar beverages is an important factor in consumer preference; thus, the color of four different vinegar beverages measured. Value of L* ranged from 0 to 100, where 0 was considered as completely opaque and 100 as completely transparent. Furthermore, positive values of a* were given for reddish colors and negative values were given to greenish colors. Positive values of b* are associated given to yellowish color and negative values were given to bluish color. There was no significant ($p < 0.05$) variation in the color of the four different vinegar beverages with the level of acetic acid concentration. This indicates that the level of acetic acid concentration may have no effect on the color of the final product.

Sugars are the most abundant carbohydrate found in the fruit juice samples and followed the order fructose > glucose > sucrose (Pinela et al. 2012; Viljakainen et al. 2010). Inversion of sucrose has been observed to occur during thawing of the berries and also as a result of the juicing process (Viljakainen et al. 2010). This was in agreement with our results in the sugar analysis (**Table 5.3)**. Pinela et al. (2012), found that the total sugar content of tomato was 6.6 g/100 g fresh weight, with the highest levels of fructose (3.4 g/100 g fresh weight), glucose (3.2 g/100 g fresh weight) and sucrose (0.02 g/100 g

fresh weight), which was in agreement with our results (**Table 5.3)**. However, in the study conducted by Viljakainen et al. (2010), the total sugar content (48.7 g/L) of cranberry juice slightly deviated from our results (**Table 5.3)**.

Overall, considering sensory and physico-chemical parameters, the fruit vinegar beverages with lower levels of acetic acid concentration (0.5%) has more potential to be used in developing a functional beverage. However, further studies are necessary for the understanding of how antioxidant properties are related to the blood pressure and lipid lowering ability of the fruit vinegar beverages. Thus, the product could then be introduced as health enhancing functional beverage.

Table 5.1: Descriptive Analysis of Fruit Vinegar Beverages^{x}

Sensory Attributes^{y}	Acetic Acid Concentration(mg acetic acid equivalents/g)			P- Value^{z}	
	0.5%	1.0%	1.5%	Acid Levels	Panelists
		Apple			
Odor	3.61 ± 1.49^{a}	6.22 ± 2.85^{b}	7.93 ± 3.43^{b}	0.00	0.20
Acidity^{p}	5.35 ± 2.76^{a}	8.96 ± 2.81^{b}	12.27 ± 1.38^{c}	0.00	0.01
Aftertaste	5.33 ± 2.25^{a}	7.56 ± 2.55^{b}	11.36 ± 2.04^{c}	0.00	0.13
Overall Acceptability	11.56 ± 2.07^{a}	5.97 ± 2.93^{b}	2.87 ± 1.54^{c}	0.00	0.02
		Blueberry			
Odor^{q}	3.63 ± 1.90^{a}	5.76 ± 2.39^{b}	6.91 ± 3.17^{b}	0.00	0.21
Acidity	3.14 ± 1.92^{a}	7.76 ± 2.30^{b}	10.94 ± 1.44^{c}	0.00	0.00
Aftertaste	4.02 ± 3.09^{a}	6.76 ± 2.14^{b}	10.02 ± 2.45^{c}	0.00	0.01
Overall Acceptability	9.20 ± 3.53^{a}	6.54 ± 3.03^{b}	4.69 ± 2.97^{b}	0.00	0.49
		Cranberry			
Odor^{q}	2.78 ± 1.74^{a}	4.00 ± 2.31^{b}	4.57 ± 2.76^{b}	0.00	0.02
Acidity^{r}	7.85 ± 3.21^{a}	9.18 ± 3.49^{ab}	9.78 ± 2.78^{b}	0.01	0.00
Aftertaste	7.75 ± 3.34^{a}	8.41 ± 3.28^{a}	9.96 ± 2.67^{b}	0.00	0.00
Overall Acceptability	6.00 ± 3.32^{a}	5.24 ± 2.23^{a}	5.81 ± 3.18^{a}	0.51	0.00
		Tomato			
Odor	4.20 ± 1.92^{a}	8.26 ± 2.60^{b}	10.04 ± 2.10^{c}	0.00	0.01
Acidity	4.80 ± 2.16^{a}	8.66 ± 3.14^{b}	10.58 ± 2.24^{c}	0.00	0.00
Aftertaste	5.30 ± 3.17^{a}	8.59 ± 3.22^{b}	9.81 ± 3.27^{b}	0.00	0.00
Overall Acceptability	6.90 ± 2.91^{a}	5.34 ± 3.56^{ab}	3.78 ± 2.68^{b}	0.01	0.09

^{x}Mean value ± standard deviation (n=18 trained panelists)

^{y} Mean score values from the unstructured scale ranged from 1 to 15, where higher numbers indicates greater intensity of the identified attribute.

^{z}P-values from the ANOVA table showing the effect of blocking factor panelists and the treatment effect.

$^{p-r}$ Transformations were done to obtain normality of data (^{p}-square root, ^{q}-log, ^{r}-square value)

$^{a-c}$ Means followed by a different letter within each row are significantly different (Tukey's Studentized Ranged test [$p < 0.05$])

Table 5.2: Physico-Chemical Properties of Fruit Vinegar Beverages[p]

Type of Vinegar Beverage	Acetic acid concentration[u]	pH	TA[q]	Color		
				L*	a*	b*
Apple	0.5%	3.40 ± 0.01^{a}	0.62 ± 0.00^{a}	68.8	-2.5	12.3
	1.0%	3.25 ± 0.03^{b}	1.06 ± 0.01^{b}	59.4	-2.5	14.9
	1.5%	3.12 ± 0.02^{c}	1.56 ± 0.03^{c}	60.1	-1.8	13.0
Blueberry	0.5%	2.74 ± 0.01^{a}	0.67 ± 0.00^{a}	27.7	37.0	25.8
	1.0%	2.66 ± 0.01^{b}	1.03 ± 0.02^{b}	28.8	40.3	26.9
	1.5%	2.64 ± 0.01^{c}	1.60 ± 0.02^{c}	22.9	34.2	18.3
Cranberry	0.5%	3.36 ± 0.01^{a}	0.54 ± 0.02^{a}	15.1	11.6	5.9
	1.0%	3.22 ± 0.01^{b}	1.05 ± 0.02^{b}	14.8	8.6	5.4
	1.5%	3.16 ± 0.02^{c}	1.49 ± 0.00^{c}	15.2	9.4	5.3
Tomato	0.5%	3.51 ± 0.01^{a}	0.56 ± 0.01^{a}	65.2	-0.4	3.7
	1.0%	3.45 ± 0.01^{b}	1.06 ± 0.00^{b}	64.2	-0.8	5.5
	1.5%	3.43 ± 0.01^{c}	1.53 ± 0.01^{c}	61.3	-1.1	7.5

[u]mg acetic acid equivalents/g

[p]Mean values ± standard deviation (n=3)

[q]TA – Titratable acidity (mg acetic acid equivalence/g)

[a-c]Means followed by a different letter within each column for each fruit beverage(apple, blueberry, cranberry, tomato)are significantly different (Tukey's Studentized Ranged test [P<0.05])

Table 5.3: Sugars and Residual Ethanol Concentration of Four Different Fruit Vinegar Beverages[p]

Fruit Vinegar Beverage		Sugar Concentration (g/L)			
Type of Fruit	Acetic Acid Concentration[q]	Fructose	Glucose	Sucrose	Ethanol
Apple	0.5	37.63 ± 3.54[a]	11.02 ± 1.04[a]	21.63 ± 2.14[a]	nd
	1.0	37.69 ± 4.73[a]	14.74 ± 1.79[b]	23.02 ± 2.84[a]	nd
	1.5	37.74 ± 1.09[a]	16.60 ± 0.64[b]	12.75 ± 0.43[b]	nd
Blueberry	0.5	25.32 ± 0.47[a]	21.99 ± 0.38[a]	nd	nd
	1.0	30.17 ± 7.44[a]	25.52 ± 6.36[a]	nd	nd
	1.5	44.99 ± 1.45[b]	37.60 ± 1.19[b]	nd	nd
Cranberry	0.5	4.40 ± 0.32[a]	9.11 ± 0.74[a]	2.68 ± 0.11[a]	nd
	1.0	6.05 ± 0.24[b]	11.58 ± 0.55[b]	2.20 ± 0.18[b]	nd
	1.5	9.63 ± 0.06[c]	19.31 ± 0.11[c]	2.79 ± 0.13[a]	nd
Tomato	0.5	0.36 ± 0.10[a]	nd	nd	nd
	1.0	1.24 ± 0.09[b]	nd	nd	nd
	1.5	2.12 ± 0.03[c]	nd	nd	nd

[p]Mean values ± standard deviation (n=3)
[q]mg acetic acid equivalents/g
nd - not detected
[a-c]Means followed by a different letter within each column for each type of fruit beverage is significantly different (Tukey's studentized range test [$P<0.05$])

CHAPTER 6.0 ANTIOXIDANT AND ANTIHYPERTENSIVE PROPERTIES *in vitro* OF FOUR DIFFERENT FRUIT VINEGAR BEVERAGES

6.1 ABSTRACT

Fruits and vegetables are known to be a good source of antioxidants and biologically active polyphenol compounds. Even though there have been many studies carried out investigating the effects of apples, berries and tomato *in vitro*, a research related to functional beverages produced from apples, berries and tomatoes has not yet been reported. Fruit vinegar beverages gained the attention of the North American market as one of the emerging functional beverages. However, the antioxidant effects of fruit vinegar beverages on vascular function and blood pressure are largely unknown. An assessment of antioxidant capacities, oxidation of LDL inhibition and ACE inhibition are required to understand the ability of fruit vinegar beverages on regulation of vascular function and blood pressure. The current study was designed to evaluate the antioxidant and antihypertensive properties of four different fruit vinegar beverages: apple, blueberry, cranberry and tomato at 0.5%, 1.0%, and 1.5% acetic acid concentrations. Tomato and apple vinegar beverages showed a significant difference ($p < 0.05$) in antioxidant capacity, measured by FRAP assay in relation to acidity. Except for tomato, the other vinegar beverages demonstrated higher antioxidant capacities in both antioxidant assays and blueberry being the highest. Over 50% inhibition results in LDL oxidation *in vitro* are further in agreement with the functional properties of cranberry (68% ± 1.7) and blueberry (85% ± 1.5) vinegar beverages. Both cranberry (92% ± 0.2) and blueberry (60% ± 0.7) vinegar beverages had higher levels of enzyme inhibition, while tomato demonstrated the lowest enzyme inhibition, confirming the antioxidant and antihypertensive properties of the fruit vinegar beverages.

Keywords: FRAP, ORAC, ACE inhibition, LDL oxidation, acetic acid concentration, fruit vinegar beverages

6.2 INTRODUCTION

Fruits and vegetables are known to be a good source of antioxidants and biologically active polyphenol compounds and plant secondary metabolites (Hurst et al. 2010). The complex mixture of phytochemicals present in fruits and vegetables provides protective health benefits, mainly through an additive and/or synergistic effect (Sun et al. 2002). Furthermore, these polyphenol compounds have been found to exhibit strong antioxidant properties, both *in vitro* and *in vivo* (Hurst et al. 2010). Since fruits and vegetables are high in antioxidants, a diet containing fruits and vegetables could help prevent oxidative stress, prevent chronic disease and slow the aging process (Boyer and Liu 2004). Furthermore, phytochemicals which exist in fruits and vegetables could demonstrate mechanisms of oxidative agents, regulation of gene expression, hormone metabolism, etc. (Sun et al. 2002). The basic feature of all polyphenols is the presence of one or more hydroxylated aromatic rings, which are radical scavengers (Mendiola et al. 2008). Overproductions of oxidants, including H_2O_2, superoxide and hydroxyl radicals, are associated with chronic diseases (Sun et al. 2002; Vuong et al. 2010). Therefore, increased consumption of fruits and vegetables containing high levels of antioxidants has been recommended to prevent the oxidative stress (Sun et al. 2002).

Traditionally, apples have been regarded as a healthy fruit in many cultures, as seen from the popular proverb “an apple a day keeps the doctor away” (Lam et al. 2008). The amount of total phenolic content and the amount of dietary fibre was found to be higher in apple peels than in apple pulp (Leontowicz et al. 2007). Furthermore, apples are one of the major sources of dietary flavonoids in the North America and Europe (Boyer

and Liu 2004). Compared to other commonly consumed fruits in the US, apples had the second highest level of antioxidant activity, (~ 83 μmol vitamin C equivalents) (Boyer and Liu 2004). Pearson et al. (1999) examined the effects of six commercial apple juices and 'Red Delicious' apples on human LDL oxidation *in vitro* and found LDL oxidation inhibition ranged from 9 to 34%. Furthermore, it has been found that the protective effects of apples on LDL oxidation reached maximum at three hours following the apple consumption (Breinholt et al. 2003). In addition, researchers found from a clinical trial that women who ingested apples had a 13 - 22% decrease in cardiovascular disease risk (Boyer and Liu 2004), confirming the inverse relationship between the flavonoid intake and the risk of cardiovascular diseases (Boyer and Liu 2004).

Vaccinium oxycoccus (small cranberry) is native to Europe, North America and North Asia (Andersen 1989) and closely related to low-bush blueberry (*Vaccinium angustifolium*) and high-bush blueberry (*Vaccinium corymbosum*) (Yan et al. 2002). Increased consumption of berries has been shown to improve cognitive function, as well as lower the risk of cardiovascular disease and cancer (Ahmet et al. 2009). The claimed health benefits of berries have been attributed to their phenolic bioactive compounds such as anthocyanins, polyphenols and flavonoids which have antioxidant properties (Ahmet et al. 2009; Stull et al. 2010). Phenolic compounds in cranberries are a diverse group that includes anthocyanins, flavonoids, proanthocyanidins, condensed tannins, and low molecular weight phenolic acids. They were reported to exhibit various health benefits, including prevention of bacterial adhesion in urinary tract infections (UTI) (Yan et al. 2002). In addition, blueberries have shown the highest antioxidant capacity among all

fruits and vegetables (Mizuno and Rimando 2009; Vuong et al. 2010), with total phenolic content of 994 mg gallic acid equivalents per liter (Araujo et al. 2010), suggesting the relationship between biological effects of phenolic compounds and consumption of berries (Yan et al. 2002). Furthermore, blueberries and their phenolic compounds have been identified as potential contributors of amelioration of neuronal cell dysfunction (Vuong et al. 2010).

Tomatoes (*Solanum lycopersicum*) are one of the richest sources of lycopene and over 80% of lycopene consumed in the North America and USA is derived from tomato products (Clinton 1998). Researchers have found that daily oral supplementation of tomato extract could significantly reduce both systolic blood pressure (SBP) and diastolic blood pressure (DBP) and further reduce the levels of lipid peroxidation products (Engelhard et al. 2006). Lycopene, the major bio-active compound present in tomatoes has demonstrated higher antioxidant capacities (Clinton 1998), and contributes to the reduction of CVD risk (Kohlmeier et al. 1997).

Even though there are many studies carried out investigating the antioxidant properties, inhibition of LDL oxidation effects of apples, berries and tomato *in vitro*, a study related to functional beverages produced from the apples, berries and tomato has not been reported. Fruit vinegar beverages are one of the emerging functional beverages in the North American market. Fruit vinegar beverage is defined as a beverage that has been fermented from at least one kind of fruit, with each litre of beverage must contain more than 300 g of fruit juice (Chang et al. 2005). Total sugar content of fruit vinegar beverage is normally less than 3%, the acidity level is around 5 to 7% and the key organic

acid found in fruit vinegar beverages is acetic acid (Chang et al. 2005; Natera et al. 2003). Furthermore, fruit vinegars consists of high concentrations of polyphenolic compounds (Bastante et al. 2010). However, to produce a consumer acceptable fruit vinegar beverage, the choice of raw materials and the method of acetification are major important factors to be considered (Su and Chien 2010). Blended fruit vinegar-juice beverages (mixtures of different juices or juice vinegar) could be implemented for the North American market as a functional beverage which has higher consumer acceptability (due to low level of acidity) as well as higher antioxidant properties.

The antioxidant effects of fruit vinegar beverages on vascular function and blood pressure are largely unknown; this is an initial step to assess the antioxidant capacities, LDL inhibition and ACE inhibition to understand the ability of fruit vinegar beverages to regulate vascular function and blood pressure. Therefore, the present study has aimed to investigate the effect of fruit vinegar beverages on: (i) the total antioxidant capacities of four different fruit vinegar beverages; (ii) the LDL oxidation inhibition of fruit vinegar beverages using thiobarbituric acid reactive substances (LDL TBARS) assay; and (iii) the *in-vitro* antihypertensive properties of the fruit vinegar beverages using angiotensin converting enzyme (ACE) assay.

6.3 MATERIALS AND METHODS

6.3.1 Samples and Chemical Reagents

Apples and tomato were purchased from the local grocery store (Sobeys, Canada) and cranberry juice was acquired from a commercial cranberry juice manufacturer (Cranberry Acres, Berwick, NS, Canada) and blueberry juice (100% juice) was purchased from a commercial blueberry juice manufacturer (Van Dyke, Caledonia, Queens Co., NS, Canada). Iron (III) hexahydrate, potassium phosphate, sodium phosphate, sodium acetate trihydrate, sodium carbonate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-Tris (2-Pyridyl)-S-Triazine (TPTZ), 2,2′-Azobis(2-amidinopropane)dihydrocholride (AAPH) and fluorescein were obtained from Sigma-Aldrich Ltd., Oakville, ON. Sulfuric acid (HPLC grade) was bought from Fisher Scientific, Ottawa, ON, Canada. The liquid chromatography standards (glucose, fructose, sucrose and ethanol) used for the study were purchased from Sigma Aldrich (Oakville, ON, Canada). For the ACE inhibition assay, ACE extracted from rabbit lung, histidine-L-hippuryl-L-leucine-chloride (HHL), NaOH, HCl, and ethanol anhydrous were obtained from Sigma Aldrich Canada Ltd. (Oakville, ON, Canada). For the LDL TBARS assay, LDL isolated from human plasma (150 mM NaCl, 0.01% EDTA, pH 7.4) was procured from EMD Chemicals Inc. (Gibbstown, NJ, USA). PBS buffer and all the other chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

6.3.2 Sample Preparation

All the fruit vinegar beverages were prepared using the standardized procedure explained in **Chapter 4.0** and **Chapter 5.0**.

6.3.3 Assessment of Antioxidant Properties of Four Different Fruit Vinegar Beverages

6.3.3.1 Ferric Reducing Antioxidant Power (FRAP) Assay

The principle of FRAP assay is the reduction of the ferric (Fe^{3+}) ion to ferrous (Fe^{2+}) ion and it takes place at low pH conditions (Benzie and Strain 1996). Low pH conditions cause the non-colored ferric-tripyridyltriazine complex to change to a bright blue color ferrous-tripyridyltriazine complex. Absorbance value of the colored complex was measured at the 593 nm wavelength. This assay was carried out using a standardized modified method for a 96-well FLUOstar OPTIMA micro-plate reader with an injection port system (BMG Labtech Inc., Offenburg, Germany). FRAP working reagent consists of 300mM acetate buffer (pH 3.6), 1mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution, and 20 mM ferric chloride solution and was prepared fresh daily. All three solutions were mixed in a ratio of 10:1:1 to make the working solution. Trolox was used as the standard material for the FRAP assay with concentrations of 50, 100, 300, 500, 700, and 900 µM. Twenty microliters of each sample or standard was placed in the wells of the 96-well micro-plate (COSTAR 9017, Fisher Scientific, Ottawa, ON, Canada) and 180 µL of the working solution was injected using the injection port system.

6.3.4.2 Oxygen Radical Absorbance Capacity (ORAC) Assay

All the fruit vinegar beverages were assessed for antioxidant activity using the ORAC assay, as explained in **Chapter 4.0**.

6.3.4 Low Density Lipoprotein Thiobarbituric Acid Reactive Substances (LDL TBARS) Assay

6.3.4.1 LDL Preparation

LDL was dialyzed using cellulose dialysis tubing (type T3 membrane, Thermo Fisher Scientific Inc., Ottawa, ON, Canada) against phosphate buffer solution (PBS) (0.138 M NaCl and 0.0027 M KCl / pH 7.4, at 25 ^{o}C) to remove all the antioxidants. Dialysis was carried out for 24 hours at 4 ^{o}C and the buffer was changed at six hour time intervals. The dialyzed LDL was immediately stored at -80 ^{o}C and was used within two weeks. Protein content of the LDL was measured using the Lowry method (Lowry et al. 1951), using bovine serum albumin as the standard.

6.3.4.2 Oxidation of LDL

Copper-and peroxyl radical-induced oxidation of human LDL was carried out by the method described by Xu et al. (2007). LDL was oxidized in the presence of 10 µM of Cu^{2+}or 5 mM of peroxyl radical generator, AAPH separately at 37 ^{o}C, for 4 hours in the dark. The experiment consisted of a positive control (with the induction but without the antioxidant treatment), negative control (without either induction or antioxidant treatment), blank and the samples. Further oxidation was terminated by adding 1:1

mixture of 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM butylatedhydroxytoluene (BHT).

6.3.4.3 Thiobarbituric Acid Reactive Substances (LDL TBARS) Assay

TBARS assay was conducted using the method described by Xu et al. (2007), with slight modifications. After completion of the oxidation, 0.67% thiobarbituric acid (TBA) reagent and 20% trichloroacetic acid (TCA) in 0.2 M NaOH were added to the reaction mixture and it was mixed thoroughly. The mixture was incubated for 30 minutes at 95 ^{o}C to develop the pink color chromogen. Samples were kept at room temperature to cool down. Since all the fruit vinegar samples were colored, 2 mL of butanol was added to each sample to partition the pink color chromogen from the fruit pigments. After adding butanol, samples were centrifuged at 2000 g for 15 minutes. Fluorescence of an aliquot of butanol fraction was measured using the 96-well FLUOstar OPTIMA micro-plate reader (BMG Labtech Inc., Offenburg, Germany) at the excitation (λ_{ex} 532 nm) and the emission (λ_{em} 590 nm) wave lengths. TBARS activity was expressed as the percent inhibition of LDL oxidation, compared to the positive control.

$$\textbf{Percent Inhibition (\%)} = 100 \left\{ \frac{(\text{Fluorescence}_{\text{positive control}} - \text{Fluorescence}_{\text{sample}})}{(\text{Fluorescence}_{\text{positive control}})} \right\}$$

6.3.5 Angiotensin Converting Enzyme (ACE) Inhibition Assay

The ACE inhibitory activity of fruit vinegar beverages was performed according to the methods of Cinq-Mars et al. (2007) and Santos et al. (1985), with some modifications. First, 21 μL of samples were taken into the 2 mL Eppendorf tubes and then 150 μL the substrate HHL was added and mixed slowly by tapping the Eppendorf tubes. Next, 30 μL ACE was added to each tube and it was mixed using a pipette in and out several times. After adding the enzyme, all the experimental units were incubated at 37 ^{o}C in an oven (Model: HP 50, Apollo Instrumentation for Molecular Biology, CA, USA) for a one hour period. After one hour, 150 μL of 0.35 M NaOH solution was added to each tube to stop the enzymatic reaction. For each experiment, a positive control and a blank was used. Thirty micro liters of buffer solution was utilized in the blanks instead of the enzyme solution and in the positive control all the reagents were added except the ACE inhibitors. After the addition of NaOH solution, 100 μL of *O*-phaldialdehyde was added to each tube to make the fluorescent adduct. Solutions were kept at room temperature for 15 minutes. After 15 minutes, 50 μL of 3 M HCl solution was added to terminate the reaction. One hundred microliters from each sample, blank and positive control were loaded into the 96-well plate and fluorescence was measured using the FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany) at excitation (λ_{ex} 360 nm) and emission (λ_{em} 500 nm) wavelengths, respectively. Percent inhibition was expressed in comparison to the positive control. The positive control contained all the reagents except the ACE inhibitors. The blank contained all the reagents except the enzyme and the inhibitors.

$$\textbf{Percent enzyme inhibition (\%)} = \left\{ \frac{\textbf{1 - (Fluorescence}_{\textbf{sample}} - \textbf{Fluorescence}_{\textbf{blank}}\textbf{)}}{\textbf{(Fluorescence}_{\textbf{positive control}} - \textbf{Fluorescence}_{\textbf{blank}}\textbf{)}} \right\} *100$$

6.4 Statistical Analysis

The design for the antioxidant capacities and the ACE inhibition study was a completely randomized design. Data was analyzed using the analysis of variance (ANOVA) using the general linear model (GLM). Assumptions of normality of error terms were tested using the Anderson-Darling test. Assumptions of constant variance were checked by plotting residuals versus fitted scatter diagram (Montgomery 2005). Differences among means were tested by the Tukey's studentized range test at the level of $p < 0.05$ (SAS Version 9.2 SAS Institute Inc., Cary, NC, USA). Each analysis was performed in triplicates.

6.5 RESULTS

6.5.1 Total Antioxidant Capacity of Four Different Vinegar Beverages

Total antioxidant capacity of all four different fruit vinegar beverages with three different levels of acidity was tested (**Table 6.1**). The antioxidant capacity was estimated using both FRAP and ORAC assays. Apple and tomato vinegar beverages showed a significant difference ($p < 0.05$) in antioxidant capacity measured by the FRAP assay in relation to acidity (**Table 6.1**). However, in the ORAC assay, all the other vinegar beverages except tomato showed a significant difference ($p < 0.05$) in antioxidant capacity with respect to acid concentration levels (**Table 6.1**). Furthermore, among four different vinegar beverages, tomato showed the lowest antioxidant capacity in both antioxidant assays and blueberry demonstrated the highest (**Table 6.1**).

6.5.2 Low Density Lipoprotein-Thiobarbituric Acid Reactive Substances (LDL-TBARS) Assay

All four fruit vinegar beverages with three acetic acid concentrations (0.5%, 1.0%, and 1.5%) were incubated with LDL reaction mixture with peroxyl radical generator AAPH to determine the level of LDL oxidation *in vitro,* using the TBARS assay. Results indicated that blueberry and cranberry vinegar beverages have higher antioxidant capacities than the apple and tomato vinegar beverages (**Table 6.2**). This was evident from the percentage inhibition results. Furthermore, all the vinegar beverages at 0.5% and 1.5% acetic acid concentration demonstrated over 50% inhibition in LDL

oxidation (**Table 6.2**) in the TBARS assay confirming the antioxidant properties of the fruit vinegar beverages. However, the interesting finding of the current study was that both blueberry (~ 80%) and cranberry (~ 60%) showed similar percentages of inhibitions in all three levels of acidity (**Table 6.2**). Thus, apple and tomato exhibited acetic acid concentration dependent relationships for the antioxidant capacities (the higher the level of fruit juice ratio, the higher the antioxidant properties).

6.5.4 Angiotensin Converting Enzyme (ACE) Inhibition Assay

All the fruit vinegar beverages (above 1.0%) exhibited a concentration dependent enzyme inhibition for the ACE assay. When the concentration of the acetic acid increased, the level of inhibition also increased (from 1.0% to 1.5%) (**Table 6.3**). Tomato vinegar beverage exhibited the lowest level of inhibition (16% to 29%) where cranberry vinegar beverage exhibited the highest (54% to 92%). Furthermore, both blueberry and apple at 1.5% acetic acid concentrations also demonstrated over 50% inhibition in the ACE inhibition assay (**Table 6.3**). Results of the current study confirmed that the fruit vinegar beverages contain potential antihypertensive properties *in vitro*.

6.6 DISCUSSION

Anthocyanins are the major bio-active group present in cranberries and blueberries and have been well documented for their antioxidant properties. The above statement was in agreement with the results of the current study which demonstrated the highest antioxidant capacities for both ORAC and FRAP assay (**Table 6.1**). In addition, Stull et al. (2010) demonstrated that the blueberries have relatively higher antioxidant capacities. In their study they found that 45 g of blueberry powder exhibited an antioxidant capacity of 16.0 mmol TE (ORAC). Furthermore, Sablani et al. (2010) demonstrated that the total antioxidant activity of blueberries ranged between 9.1 and 16.9 mmol Trolox equivalents per kg fresh weight. The above results are in agreement with the current study which ranks blueberry the highest antioxidant capacity as measured by ORAC (**Table 6.1**). However, Sablani et al. (2010) demonstrated that blanching prior to the processing of blueberries could help to retain higher levels of total anthocyanins, phenolics and antioxidant activity. This indicates that blanching could be used prior to the processing to enhance the antioxidant properties. This was further in agreement with the results of the antioxidant studies for blueberry vinegar beverage where the juice was brought from Van Dyke (Caledonia, Queens Co., NS, Canada) (**Table 6.1**).

Cranberries also contain high anthocyanin content. This was evident from the results of Andersen (1989), confirming the total anthocyanin content of cranberries (78 mg/100 g fresh fruit). Anderson (1989) reported that the major anthocyanin pigments in cranberry were peonidin-3-glucoside and cyanidin-3-glucoside (80.2% of total

anthocyanin content). In addition, cranberries have been reported for higher antioxidant activity (0.177 mmol vitamin C equivalents /g of fresh weight) than apples (0.098 mmol vitamin C equivalents / g of fresh weight) Sun et al. (2002), in agreement with the results of the total antioxidant activity of the cranberry and apple vinegar beverages (**Table 6.1**). This has been further in agreement with the results of Yan et al. (2002), indicating the dose dependent radical-scavenging activity of cranberry flavonoids.

Apples have high polyphenol content, and antioxidant capacity. Total polyphenols in fresh peels amounted to 107 mg/100 g and was lower by over 36% in pulp (Leontowicz et al. 2007). Furthermore, the content of flavonoids was 45 mg/100 g for peels and 14 mg/100 g for pulp (Leontowicz et al. 2007). In addition, present results of the antioxidant capacities (**Table 6.1**) of FRAP and ORAC, of the apple vinegar beverage, were comparable with the results obtained by Lotito and Frei (2004) for antioxidant capacities of aqueous apple extracts prepared from three different varieties (‘Red Delicious’, ‘Granny Smith’ and ‘Fuji’). Antioxidant properties of the apples could be due to their high composition of bio-actives. Apple peel composition of chlorogenic acid (16.4%), phloretin (6.2%), proanthocyanidin B_2 (4.3%), epicatechin (2.6%), catechin (1.2%), phloretin (0.3%), rutin (0.2%), and quercetin (0.1%) could account for the high level of antioxidant properties of apples (Lam et al. 2008).

In addition, in a very recent study carried out by Pinela et al. (2012), it was found that tomatoes contain only 0.5 mg/100 g fresh weight β-carotene and 9.5 mg/100 g fresh weight of lycopene, respectively. The above results were in agreement with the current study, indicating the lower levels of antioxidant capacities of tomato vinegar beverage

(**Table 6.1**). Furthermore, Pinela et al. (2012) found that the DPPH scavenging activity of the tomatoes was ≤ 1.6 mg/mL. These results were further in agreement with a study conducted by Kohlmeier et al. (1997), who suggested that lycopene could be the only carotenoid with a significant independent association with lower risk of myocardial infarction. Thus, Kohlmeier et al. (1997) have also found that lycopene interacted with polyunsaturated fats; one of the major sources of oxidative stress suggesting that lycopene may be operating under a tissue-specific antioxidant mechanism.

Chu and Liu (2005) demonstrate the inhibition of LDL oxidation of cranberry extracts. Inhibition of LDL oxidation values of 5, 2.5, and 1 mg cranberry extract/mL showed inhibition of 94.7%, 71.4% and 50.7%, respectively. The above results were further in agreement with the results of the *in vitro* LDL-TBARS assay (**Table 6.2**). In another study, researchers measured the copper-induced LDL oxidation *in vitro* for commercial apple juices, whole apple, apple peel and apple flesh. However, the results varied greatly between commercial brands of fruit juice, ranging from 9 to 34% inhibition, whole apples and apple peels by 34% inhibition, while the flesh alone showed only 21% inhibition (Pearson et al. 1999), demonstrating the inhibition of LDL-oxidation ability of the apple fruit and its derivatives. A human clinical study, after 24 weeks, it was found that the consumption of apples/apple juice is negatively correlated with TG, the total cholesterol/HDL cholesterol ratio and positively with HDL cholesterol (Jenkins et al. 2011). These results were further in agreement with our current study demonstrating ~ 60% inhibition of LDL *in vitro* by the apple vinegar beverage (**Table 6.2**).

Furthermore, the proanthocyanidins from cranberry have been observed to increase the lag time of copper-induced LDL oxidation, probably due to their ability to bind to LDL and remain associated (Yan et al. 2002; Chu and Liu 2005). Thus, Basu et al. (2010) were able to demonstrate the cardio-protective properties of blueberries/blueberry juice. Significant decreases in systolic and diastolic blood pressures and plasma ox-LDL and lipid peroxidation were observed in their study. In addition, Jenkins and colleagues (2011) noted that after 24 weeks, there was a negative relationship with the berry intake and blood glucose and blood pressure in humans. The above results were in agreement with the current study demonstrating ~ 80% inhibition of LDL *in vitro* in blueberry vinegar beverage (**Table 6.2**).

A further study found that the dietary supplementation of 60 mg lycopene per day in six males for 3 months was associated with a 14% reduction in plasma LDL cholesterol concentrations (Clinton 1998) which was in agreement with the results of *in vitro* LDL oxidation (**Table 6.2)**. Thus, scientists also suggest that the cholesterol lowering effect of lycopene may be due to the inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme-A reductase (HMGCoA reductase), the rate-limiting enzyme in cholesterol biosynthesis (Clinton 1998) or the oxidative ability of LDL which, leads to its uptake by the macrophages inside the arterial wall and the formation of foam cells and atherosclerotic plaque (Riccioni et al. 2008).

In the literature, the ACE inhibition ability of plant extracts, berries and apple has been recorded (Balasuriya and Rupasinghe 2011). Thus, blueberries demonstrated an IC_{50} value of 46 µg/mL for ACE inhibition (Ojeda et al. 2010; Sakaida et al. 2007). This was

evident from the results of the current study for both cranberry and blueberry vinegar beverages, indicating higher inhibition percentages *in vitro* (**Table 6.3)**. However, in a recent study, researchers were able to demonstrate a concentration responsive enzyme inhibition for apple peel extracts, with the IC_{50} value of 49 µg/mL (Balasuriya and Rupasinghe 2011), confirming the current results for *in vitro* inhibition activity of ACE (**Table 6.3)**. Furthermore, researchers have found that anthocyanins and plant extracts rich in anthocyanins exhibited ACE inhibition in both *in vitro* and *in vivo,* in model systems of cells (Ojeda et al. 2010). It was further observed that the structure of the cyanidin molecule plays a major role in ACE inhibition. This was supported through the results of the current study, which demonstrated higher ACE inhibitions of both blueberry and cranberry (**Table 6.3)** which were high in concentrations of 1.0% and 1.5%.

Precise knowledge of the antioxidant capacities and antihypertensive activities of berries, apples and tomatoes are important to assess the effect of bio-active compounds on human health and disease. However, to support the research finding of the *in vitro* results, further research should include *in vivo* studies using an animal model or a human clinical trial. The current results have identified that fruit vinegar beverages demonstrate antioxidant and antihypertensive properties *in vitro*. Both blueberry and cranberry vinegar beverages were more efficacious functional beverages in vitro when compared with apple and tomato vinegar beverages.

Table 6.1: Total Antioxidant Capacity of the Four Different Fruit Vinegar Beverages[p]

Type of Vinegar Beverage	Acetic acid concentration[q]	Antioxidant Capacity FRAP[r] (mmol TE[t] /L)	Antioxidant Capacity ORAC[s] (mmol TE /L)
Apple	0.5	1.20 ± 0.01[a]	0.62 ± 0.10[ab]
	1.0	0.97 ± 0.01[b]	0.72 ± 0.11[a]
	1.5	0.78 ± 0.03[c]	0.59 ± 0.08[b]
Blueberry	0.5	5.40 ± 0.04[a]	6.73 ± 1.23[a]
	1.0	5.33 ± 0.08[a]	3.56 ± 0.68[b]
	1.5	5.58 ± 0.05[a]	2.05 ± 0.28[c]
Cranberry	0.5	2.08 ± 0.04[a]	0.03 ± 0.02[a]
	1.0	2.11 ± 0.08[a]	1.15 ± 0.34[b]
	1.5	2.20 ± 0.05[a]	1.41 ± 0.17[b]
Tomato	0.5	0.14 ± 0.00[a]	0.53 ± 0.09[a]
	1.0	0.21 ± 0.01[b]	0.58 ± 0.03[a]
	1.5	0.29 ± 0.01[c]	0.59 ± 0.03[a]

[p]Mean values ± standard deviation (n=3)
[q]mg acetic acid equivalents/g
[r]FRAP -Ferric Reducing Antioxidant Power
[s]ORAC - Oxygen Radical Absorbance Capacity
[t]TE - Trolox equivalents
[a-c]Means followed by a different letter within each column for each type of fruit beverage is significantly different (Tukey's studentized range test [P<0.05])

Table 6.2: Inhibition of LDL Oxidation *in vitro* by Four Different Fruit Vinegar Beverages[p]

Fruit Vinegar Beverage		% Inhibition of LDL oxidation
Type of Fruit	Acetic acid concentration[q]	
Apple	0.5	67.46 ± 1.88[a]
	1.0	56.93 ± 2.07[b]
	1.5	46.64 ± 1.94[c]
Blueberry	0.5	80.72 ± 8.71[a]
	1.0	84.33 ± 1.03[a]
	1.5	84.76 ± 1.46[a]
Cranberry	0.5	66.66 ± 4.76[a]
	1.0	67.05 ± 4.09[a]
	1.5	67.94 ± 1.71[a]
Tomato	0.5	61.79 ± 3.17[a]
	1.0	50.48 ± 1.89[b]
	1.5	35.23 ± 3.47[c]

[p]Mean values ± standard deviation (n=3)
[q]mg acetic acid equivalents/g
[a-c]Means followed by a different letter within each column for each type of fruit beverage is significantly different (Tukey's studentized range test [P<0.05])

Table 6.3: ACE Inhibition of Four Different Fruit Vinegar Beverages *in vitro*[p]

Fruit Vinegar Beverage		% Inhibition of ACE activity
Type of Fruit	Acetic acid concentration[q]	
Apple	0.5	36.11 ± 1.79[a]
	1.0	42.04 ± 0.44[b]
	1.5	50.50 ± 1.01[c]
Blueberry	0.5	50.94 ± 0.85[a]
	1.0	50.90 ± 0.89[a]
	1.5	60.17 ± 0.66[b]
Cranberry	0.5	53.93 ± 1.35[a]
	1.0	66.51 ± 0.62[b]
	1.5	91.62 ± 0.18[c]
Tomato	0.5	15.83 ± 0.28[a]
	1.0	25.63 ± 2.94[b]
	1.5	29.35 ± 4.22[b]

[p]Mean values ± standard deviation (n=3)
[q]mg acetic acid equivalents/g
[a-c]Means followed by a different within each column for each type of fruit beverage is significantly different (Tukey's studentized range test [P<0.05])

CHAPTER 7.0 REGULATIONS OF BLOOD PRESSURE AND CHOLESTEROL METABOLISM IN SPONTANEOUSLY HYPERTENSIVE RATS BY FRUIT VINEGAR BEVERAGES

7.1 ABSTRACT

Elevated levels of blood cholesterol are positively correlated with hypertension and cardiovascular diseases. Though there are prescribed drugs available to control hypertension and hypercholesterolemia, there is a demand for functional foods and natural health products. The fruit vinegar beverages introduced to the North American market as functional beverages have shown the ability to reduce both the blood pressure levels and lipid levels. The present study was carried out to investigate the effects of four vinegar beverages on the regulation of blood pressure and cholesterol metabolism in spontaneously hypertensive rats (SHR). After seven days of adaptation in individual cages with 12:12 hour light: dark cycle and free access regular rodent chow and water, SHR rats were randomly divided into six groups and fed with an atherogenic diet. One group was used as the normal control and the other five were treated with vinegar beverages of apple, blueberry, cranberry, tomato, and acetic acid. Dietary treatments were administrated by gavage feeding twice a day for 5 weeks. Results indicated that after four weeks of treatment, the diastolic blood pressure was lower ($p < 0.05$) in rats fed with blueberry, apple and tomato vinegar beverage of than the normal control group. Further, serum total cholesterol and triacylglycerol levels of the all dietary supplemented treatment groups were lower ($p < 0.05$) than the normal control group. All treatment groups demonstrated higher ($p < 0.05$) serum HDL-cholesterol, compared to the normal control group. In conclusion, fruit vinegar beverages reduced the blood pressure levels and regulated the serum cholesterol levels of SHR. Thus, findings of the current study suggest that functional beverages, such as fruit vinegar beverages, could be an alternative to drugs in controlling hypertension and hypercholesterolemia upon the confirmation of the results, using a human clinical trial.

Keywords: Fruit Vinegar Beverages, SHR**,** Blood Pressure, Total Cholesterol, HDL-cholesterol, Triacylglycerol

7.2 INTRODUCTION

Cardiovascular disease (CVD) (Krista et al. 2003; Kodavanthi et al. 2000) is one of the major causes of death worldwide. Systemic hypertension is generally chronic, developing throughout the entire life span and often leads to heart failure or stroke (Kodavanthi et al. 2000). Atherosclerosis is one of the major cause for the development of CVD, and a high level of low density lipoprotein (LDL) and oxidative modification of LDL in the vasculature is believed to be a significant factor contributing to atherosclerosis, and ischemic stroke (Lotito and Frei 2004; Iizuka et al. 2010; Setorki et al. 2011). Thus, a reduction in both systolic and diastolic blood pressure may be helpful in the prevention of stroke and other adverse vascular events (Tanaka et al. 2009). Meals containing high carbohydrates and fat could lead to exaggerated levels in blood TG levels, which may cause an immediate increase in oxidative stress, which could subsequently result in endothelial dysfunction, vasoconstriction, and systolic blood pressure (O'Keefe et al. 2008; Potter et al. 2011).

Spontaneously hypertensive rats (SHR) are a genetic strain obtained by selective in-breeding of Wistar rats (Kundu and Rao 2008). The blood pressure of SHR starts to increase at around 5-6 weeks of age and the systolic pressures may reach 200 mmHg between 40-50 weeks, where SHR start to develop the characteristics of CVD (Kundu and Rao 2008). These SHR have been shown to develop increased vascular resistance, systemic blood pressure, and overall activation of the renin-angiotensin system early in their lives (Kodavanthi et al. 2000). Some antihypertensive drugs such as Captopril® have a more significant lowering effect on the blood pressure of SHR than on normal

Wistar rats due to the altered sensitivity of their cardiovascular systems (Vainionpaa et al. 1974; Triantafyllidi et al. 2004).

Flavonoids are polyphenolic compounds that occur ubiquitously in plants and are consumed in the form of fruits (apple, blueberry, cranberry); they have shown an inverse correlation with coronary heart diseases (Potter et al. 2011; Wiseman et al. 2011; Chong et al. 2010; Galindo et al. 2012). Like other flavonoids, anthocyanins are powerful free radical scavengers, and are known to exhibit antioxidant properties *in vivo* in lipid environments (Kahkonen et al. 2003; Dillard and German 2000). Anthocyanins, quercetin and catechin have demonstrated a better dose-dependent activity against platelet aggregation and activation in a form of mixture than individuals (Shivashankara and Acharya 2010; Chong et al. 2010), indicating the improved benefit of a mixture of dietary polyphenols.

General interest in functional food products, especially functional beverages that may be consumed as a part of a normal diet to prevent or treat cardiovascular diseases are becoming more fashionable (Berry 2011). Fruit vinegar beverages are well known for their medicinal properties from ancient times (Dogaru et al. 2009; Fushimi and Sato 2005). They have been reported to possess antihypertensive (Kondo et al. 2001; Nakamura et al. 2010) and hypolipidemic properties (Setorki et al. 2010; Setorki et al. 2011; Ubeda et al. 2011). Other bio-active constituents, such as organic acids, vitamins and minerals, present in fruit vinegar beverages could also act as antioxidants, antihypertensive agents and LDL oxidation inhibitors (Kris-Etherton 2004; Iizuka et al. 2010 Setorki et al. 2010; Zhang et al. 2011).

Although few studies on fruit vinegar beverages have been conducted in Europe and South East Asia, less scientific data has been reported in North America. In the previous chapter, blueberry and cranberry vinegar beverages have shown over 50% inhibition (**Table 6.3**) on the angiotensin converting enzyme (ACE) activity *in vitro*. However, there were no direct scientific evidence related to the effects of fruit vinegar beverages on lipid and blood pressure lowering effects which comprised the objective of the present study. It is important to investigate the *in vivo* effects of these vinegar beverages on hypertension and cholesterol metabolism. Therefore, the current study was carried out to determine the effects of fruit vinegar beverages on (i) systolic and diastolic blood pressure levels and (ii) cholesterol metabolism in an SHR model with diet-induced hypercholesterolemia.

7.3 MATERIALS AND METHODS

7.3.1 Experimental Materials and Chemical Reagents

Fresh apples and fresh tomato were purchased from the local grocery store (Sobeys, Canada) and, cranberry juice (concentrated ~ 35° Brix) was purchased from a commercial cranberry juice manufacturer (Cranberry Acres, Berwick, NS, Canada) and blueberry juice (100% juice) was purchased from a commercial blueberry juice manufacturer (Van Dyke, Caledonia, Queens Co., NS, Canada). Serum lipid profiles of the rats were analyzed using commercial kits purchased from Pointe Scientific Inc., Canton, MI, USA and the liver lipids were analyzed using commercial kits purchased from Biovison Inc., Milpitas, CA, USA. All the animals were gavage-fed using 15" MED-RX feeding tubes (Benlan Inc., Oakville, ON, Canada). Iron (III) hexahydrate, potassium phosphate, sodium phosphate, sodium acetate trihydrate, sodium carbonate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-Tris (2-Pyridyl)-S-Triazine (TPTZ), and fluorescein were obtained from Sigma-Aldrich Ltd., Oakville, ON. For the ACE inhibition assay, ACE extracted from rabbit lung, histidine-L-hippuryl-L-leucine-chloride (HHL), NaOH, HCl, and ethanol anhydrous were purchased from Sigma Aldrich Canada Ltd. (Oakville, ON, Canada).

7.3.2 Experimental Materials

All fruit vinegar beverages were prepared using the standardized procedure explained in **Chapter 4.0** and **Chapter 5.0**.

7.3.3 Evaluation of Antioxidant and Antihypertensive Properties of Fruit Vinegar Beverages

All fruit vinegar beverages were assessed for antioxidant activity using the FRAP assay explained in **Chapter 6.0** and ORAC assays as described in **Chapter 4.0** and antihypertensive properties using the inhibition of ACE as shown in **Chapter 6.0**.

7.3.4 Animals and Diets

The experiment was conducted in the animal facility of the Atlantic Veterinarian College, University of Prince Edward Island (UPEI), Charlottetown, PE, Canada, subjected to the guidelines of the Canadian Council for Animal Care. Ethical approval was obtained from the Animal Care and Use Committee (ACUC) at UPEI prior to the experiment. Seventy-two male spontaneously hypertensive rats (SHR) weighing 175 g - 200 g were purchased from Charles River Laboratories Inc. (Quebec, QC, Canada). Animals were housed individually in cages and were subjected to 12-hour light/dark cycle with free access to regular rodent chow and water. After 7 days of adaptation, rats were weighed and randomly assigned to six groups of 12 animals per group. The animals were subjected to a five-week dietary intervention period. All rats were fed with a casein-cornstarch-sucrose-based diet, prepared according to the American Institute of Nutrition

93 Growth (AIN 93-G) formulation and modified to contain 2.0% cholesterol and 0.5% cholic acid to induce hypercholesterolemia, (**Table 7.1**). The test diet for the animals was prepared weekly and stored at -20°C.

Table 7.1 Composition of the AIN 93-G Diet[a]

Ingredients	Amount (%)
Casein	20.0
Corn starch	28.0
Sucrose	36.3
Oil[x]	5.0
Cellulose	4.9
DL-methionine	0.5
Mineral mixture[y]	4.0
Vitamin mixture[z]	1.0
Choline bitartrate	0.2
Cholic acid	0.5
Butylated hydroxytoluene (BHT)	0.02
Total	100.4

[a]2% cholesterol was added to the standard AIN 93-Gdiet to induce hypercholesterolemia in rats

[x]96% of the oil was lard and 4% was sunflower oil

[y]The composition of the AIN 93-G mineral mix: 5000 mg Ca, 1561 mg P, 3600 mg K, 1019 mg Na, 1571 mg Cl, 300 mg S 507 mg Mg, 35 mg Fe, 6 mg Cu, 10 mg Mn, 30 mg Zn, 1 mg Cr, 0.2 mg I, 0.15 mg Se, 1.0 mg F, 0.5 mg B, 0.15 mg Mo, 5 mg Si, 0.5 mg Ni, 0.1 mg Li and 0.1 mg V per kilogram of the mix

[z]The composition of the AIN 93-G vitamin mix: 20 mg Thiamin HCl, 15 mg riboflavin, 7 mg pyridoxine HCl, 90 mg niacin, 40 mg calcium pentothenate, 2 mg folic acid, 0.6 mg biotin, 10 mg cyanocobalamin (B12, 0.1%), 4 mg menadione sodium bisulfite, 5000 IU vitamin A palmitate, 50 IU vitamin E acetate, 2400 IU vitamin D3, 100 mg inositol per kilogram of the mix.

All five beverage treatments (apple, blueberry, cranberry, tomato and acetic acid) were prepared once a week and stored at 4 °C until used. Treatments were orally administrated to the rats by twice daily gavage, based on their body weights. Total daily dosages were calculated per rat of 200 g; 2.0 mL/kg BW per day. The normal control

group received water via oral gavage as the control vehicle whereas the acetic acid control group received acetic acid via the control vehicle of acetic acid. Rationale behind the gavage volume calculation was based on an average human (body weight - 70 kg); recommended daily allowance (RDA) of beverage consumption (350 mL twice a day). The gavage was administrated using soft plastic gavage tubing to minimize the stress and potential damage to animals. The behavior of the animals and their feed intake were observed and recorded on a daily basis. Furthermore, 12 hour fasting body weights of the animals were recorded at weekly intervals.

7.3.5 Collection and Storage of Blood and Tissue Samples

At the end of the dietary intervention period, rats were anesthetized by isoflurane inhalation and sacrificed. Blood (6 - 8 mL) was collected into the serum tubes via cardiac puncture, allowed to clot at room temperature for one hour and then placed on ice. Serum of the blood was separated by centrifugation and was stored at -80 ^{o}C. Liver and kidneys were dissected, cleaned by rinsing with phosphate buffer solution (1x PBS), weighed and immediately frozen in liquid nitrogen and then stored at -80°C until the analysis.

7.3.6 Measurement of Blood Pressure of SHR

Blood pressures of SHR were measured with a CODA System (Kent Scientific, Torrington, CT, USA) (Daugherty et al. 2009). Both systolic and diastolic blood pressures were measured fifteen times, with 30 second intervals. All the readings were taken after the rats were put on a warming platform (maintained at 37 ^{o}C) for 10 minutes. The average of five readings was used for each animal, excluding the highest and lowest

values using a scatter plot to minimize the measurement errors. Both systolic and diastolic blood pressures were measured at the end of two weeks and four weeks of the study, respectively.

7.3.7 Analysis of Serum Lipids of SHR

7.3.7.1 Analysis of Serum TC

Serum TC was measured using cholesterol (liquid) reagents (Pointe Scientific Inc. Canton, MI, USA). The principle of the study was as follows (Allain et al. 1974; Tinder 1969):

- Cholesterol Esters --------------------------------> Cholesterol + Fatty Acids (Cholesterol Esterase)
- Cholesterol -------------------------------------> Cholestero-3-one + H_2O_2 (Cholesterol Oxidase)
- $2H_2O_2$ + 4-aminoantipyrine + Phenol ---------------------> Quinoneimine (Red) + $4H_2O$ (Peroxidase)

The intensity of the red color was read on a chemistry analyzer (Model Pointe 180, Pointe Scientific Inc., Canton, MI, USA) at 500 nm (standard concentration of 200 mg/dL). If the intensity was too high, samples were diluted 1:1 with saline. The intensity of color is directly proportional to TC.

7.3.7.2 Analysis of Serum TG

Serum TG was measured using triglyceride (liquid) reagents (Pointe Scientific Inc. Canton, MI, USA). This single reagent procedure quantified the total glycerides in

serum including the mono- and di-glycerides with free glycerol fractions. The principle of the study is as follows (Fossati and Prencipe 1982; Tinder 1969):

- triacylglycerols ------------------> Glycerol + Fatty Acids (Lipase)
- Glycerol + ATP ------------------------> Glycerol-1-phospate + ADP (Glycerol kinase)
- Glycerol-1-phospate + O_2 ---------------------------------------> diaminophenazone + H_2O_2 (Glycerol phosphate oxidase)
- 4-aminophenazone + H_2O_2+ Chlorophenol -------------------> Quinoneimine (Red) + HCl + $2H_2O$ (Peroxidase)

The intensity of the red color was measured as in the serum TG (standard concentration of 200 mg/dL).

7.3.8 Analysis of Serum HDL

Serum HDL was measured using HDL cholesterol reagents (Pointe Scientific Inc. Canton, MI, USA). When serum was combined with the cholesterol precipitating reagent (Pointe Scientific Inc. Canton, MI, USA), dextrin sulfate and magnesium ions precipitated the LDL and VLDL fractions, leaving HDL fraction in the solution. The HDL cholesterol was determined using the same method for cholesterol. The intensity of the red color was measured as for serum TC (standard concentration of 50 mg/dL).

7.3.9 Analysis of Liver Cholesterol/Cholesterol Esters and TG of SHR

7.3.9.1 Analysis of Liver TG

Liver TG was estimated using a triglyceride quantification kit (Biovison Inc. Milpitas, CA, USA). Approximately 100 mg of liver was weighed and homogenized in 1 mL of 5% nonyl-phenoxypolyethoxylethanol-40 (NP-40) in water. Homogenized samples were heated to 80 - 100 ^{o}C in a water bath for 2 - 5 minutes until the solution became cloudy, and then cooled down to room temperature. The heating step was repeated one more time to solubilize all TG. All the insoluble materials were removed using micro-centrifugation (Microlite, Harlow Scientific, Arlington, MA, USA) for 2 minutes. Supernatant was separated and diluted 20-fold before the assay. Five microliters of the diluted sample was used in the assay. Test samples were prepared to a final volume of 50 μL/well with triglyceride assay buffer in a 96-well plate (COSTAR 9017, Fisher Scientific, Ottawa, ON, Canada). Samples were analyzed using a plate reader (Varioskan Flash, Thermo Fisher Scientific, Nepean, ON, Canada) at an excitation (λ_{ex} 535 nm) and emission (λ_{em} 590 nm) wave lengths.

7.3.9.2 Analysis of Liver Cholesterol/Cholesterol Esters

Liver cholesterol/cholesterol esters were quantified using a cholesterol/cholesterol ester quantification kit (Biovison Inc. Milpitas, CA, USA). Approximately 50 mg of liver was weighed and homogenized (PowerGen 125, Fischer Scientific, Pittsburgh, PA, USA) in 200 μL of chloroform: Isopropanol: NP-40 (7:11:0.1). Homogenized samples were centrifuged (Microlite, Harlow Scientific, Arlington, MA, USA) at 15,000 g for 5 - 10

minutes. The organic phase was transferred to a glass tube and air dried at 50 °C to remove chloroform. The remaining trace amount of organic solvents was removed using vacuum drying for 30 minutes. Dried lipids were dissolved with 1 mL of cholesterol assay buffer (Biovison Inc. Milpitas, CA, USA) by vortexing (VWR Mini Vortexer, Henry Troemner Ltd. Thorofare, NJ, USA) until homogeneous. Five microliters of extracted sample was used in the assay. Test samples were prepared to final volume of 50 µL/well with a cholesterol assay buffer in a 96-well plate (COSTAR 9017, Fisher Scientific, Ottawa, ON, Canada) and samples were analyzed using a plate reader (Varioskan Flash, Thermo Fisher Scientific, Nepean, ON, Canada) at excitation (λ_{ex} 535 nm) and emission (λ_{ex} 590 nm) wave lengths. Liver total cholesterol (TC) and free cholesterol (FC) was measured directly and cholesterol esters (CE) were determined by subtracting the free cholesterol from the total cholesterol (TC = FC + CE).

7.4 Statistical Analysis

All the data were expressed as mean ± standard deviation (n = 12). Data was analyzed with ANOVA using the general linear model (GLM). Assumptions of normality of error terms and assumptions of constant variance were checked (Montgomery 2005). When the data was not normally distributed necessary transformations were done accordingly to obtain the normality of data. Differences among means were tested by the Tukey's Studentized Ranged test (*in vitro*) and the least square difference (LSD) test (*in vivo*) at a significance level of $p < 0.05$ (SAS Version 9.2 SAS Institute Inc., Cary, NC, USA).

7.5 RESULTS

7.5.1 *In vitro* Tests

Both FRAP and ORAC values of blueberry vinegar beverage were higher ($p < 0.05$) than all other vinegar beverages (**Table 7.2**). The blueberry, cranberry and apple vinegar beverages had stronger inhibitory effects for ACE activity, even at a lower level of acetic acid concentration (**Table 7.2**). Blueberry and cranberry vinegars showed higher ($p < 0.05$) percentage inhibition of ACE as compared with the other two vinegar beverages (**Table 7.2**).

7.5.2 Effect of Fruit Vinegar Beverages on Feed Intake and Body Weight of SHR

Average body weights of the SHR were 237.3 ± 0.2 g before their assignment to the treatment groups. After five weeks of treatment, feed intake was not different among the treatment groups ($p < 0.05$) (**Table 7.3**). The average feed intakes of the SHR were 13.7 ± 1.9 g per day in all groups at the end of the five week dietary intervention period. Similarly, there was no treatment effect ($p < 0.05$) on the body weights of the SHR (**Table 7.4**).

7.5.3 Effect of Fruit Vinegar Beverages on Systolic and Diastolic Blood Pressure of SHR

The systolic and diastolic blood pressures were not different ($p = 0.42$ and $p = 0.28$) in all the treatment groups after two weeks of treatments (**Table 7.5**). However, after 4 weeks of treatment, the diastolic blood pressures of rats treated with vinegar containing blueberry (90.9 ± 8.7 mmHg), apple (90.7 ± 12.4 mmHg), cranberry (97.8 ± 10.4mmHg) or tomato (93.6 ± 16.3 mmHg) were significantly lower ($p < 0.05$) than the normal control group (113.3 ± 15.9 mmHg) (**Table 7.5**). In addition, the diastolic blood pressure of rats treated with vinegar containing blueberry (90.9 ± 8.7 mmHg) and apple (90.7 ± 12.4 mmHg) were lower ($p < 0.05$) than the acetic acid control group (102.4 ± 10.2 mmHg) (**Table 7.5**). There was no difference ($p = 0.23$) in systolic blood pressure level after four weeks of the treatment. Thus, there were no differences in the blood pressure between the acetic acid control group and the treatment groups as well as normal control group and the treatment groups (**Table 7.5**).

The results also indicate that fruit vinegar beverages have reduced the blood pressure of the SHR by ~ 20% after four weeks of study, compared to the normal control (**Table 7.5**). Furthermore, with comparison to the average increase of diastolic and systolic blood pressure over two weeks of study, there was an increase ($p < 0.05$) of average diastolic blood pressure in the control (22.7 mmHg), compared with the apple (-1.1 mmHg) and blueberry (2.3 mmHg) vinegar beverages (**Table 7.5**). As well there was an increase ($p < 0.05$) of average systolic blood pressure in the control (20.7 mmHg)

compared with the apple (1.6 mmHg) and blueberry (-1.2 mmHg) vinegar beverages (**Table 7.5**).

7.5.4 Serum Lipid profiles of SHR

The serum TC of all treatment groups were lower ($p < 0.05$) than the normal control group (130.3 ± 26.6 mg/dL) similar with the acetic acid control group (89.3 ± 29.5 mg/dL) (**Table 7.6**). Furthermore, the treatment groups of blueberry, cranberry, tomato and apple vinegar beverages showed higher ($p < 0.05$) serum HDL-cholesterol levels compared to the normal control group (39.5 ± 12.3 mg/dL) similar with the acetic acid control group (50.4 ± 10.6 mg/dL) (**Table 7.6**). The cranberry and tomato vinegar beverages demonstrated the highest values for the HDL-cholesterol (62.6 ± 6.4 mg/dL and 63.2 ± 9.1 mg/dL, respectively) higher than the acetic acid control group (**Table 7.6**). The level of serum LDL-cholesterol depicted a similar pattern as per the serum TC where all treatment groups were lower ($p < 0.05$) than both the normal control group and the acetic acid control group (**Table 7.6**). The acetic acid control demonstrated a lower value for serum non HDL-cholesterol (43.1 ± 23.7 mg/dL) and the value was similar to those of the other treatment groups (**Table 7.6**). The, TG levels of all four fruit vinegar beverages and acetic acid group were lower ($p < 0.05$) than the normal control (105.7 ± 13.7 mg/dL) (**Table 7.6**). Moreover, the apple and cranberry vinegar beverages had the lower TG (59.4 ± 9.3 mg/dL and 61.5 ± 18.0 mg/dL, respectively) than the acetic acid control group (**Table 7.6**).

7.5.5 Liver Lipid profiles of SHR

There were no significant difference among the six treatment groups for liver TC ($p = 0.95$), FC ($p = 0.66$) and CE ($p = 0.97$) levels (**Table 7.7**). However, liver TG was lower ($p < 0.05$) in all the treatment groups compared to the normal control (6.0 ± 0.9 mM) and the acetic acid control (6.4 ± 0.8 mM) groups (**Table 7.7**). Furthermore, blueberry (4.3 ± 0.8 mM) and cranberry (4.0 ± 0.7 mM) vinegar beverage groups had the lowest levels of the liver TG, which was lower than the tomato and apple vinegar beverages ($p < 0.05$).

7.6 DISCUSSION

Bioactive composition of apples and berries contribute to their high antioxidant capacities (Basu et al. 2010; Neto 2007; Stone et al. 2007). The FRAP and ORAC values confirmed the higher antioxidant capacity of the fruit vinegar beverages which demonstrated the higher bioactive composition among the fruit vinegar beverages. A study conducted by Sablani et al. (2010) demonstrated that the total antioxidant activity of blueberries ranged between 9.1 and 16.9 mol TE /kg fresh weight, further in agreement with the results of the ORAC assay (**Table 7.2**). Dogaru et al. (2009) found that the antioxidant capacities of fruit vinegar beverages depend on the type of fruits and the highest antioxidant capacity was found in the order of raspberry > blackberry > bilberry > apple. The above results were in agreement with the results of the current study (**Table 7.2**).

In addition, recent research has revealed that antihypertensive properties of fruit bio-actives *in vitro* (Balasuriya and Rupasinghe 2011). This was evident from the *in vitro* results for ACE inhibition in the fruit vinegar beverages which all illustrated a relatively higher enzyme inhibition for the ACE inhibition assay (**Table 7.2**). Thus, cranberry and blueberry vinegar beverages demonstrated a higher level of enzyme inhibition compared to the other two types of vinegar beverages, confirming the in vitro antihypertensive properties of the fruit vinegar beverages (**Table 7.2**).

After five weeks of treatment, feed intake (**Table 7.3**) and body weight (**Table 7.4**) of SHR were not different among the treatment groups ($p < 0.05$). Similar results

were obtained by Wiseman et al. (2011), Elks et al. (2011), Decorde et al. (2008), and Kondo et al. (2001).

SHR are a genetic strain of hypertensive rats which develop increased vascular resistance, blood pressure, and overall activation of the renin-angiotensin system early in their lives (Kodavanthi et al. 2000; Kundu and Rao 2008). Blood pressure of SHR starts to increase around 5-6 weeks of age and the systolic pressures may reach values between 180 and 200 mmHg in the adult. Furthermore, between 40- 50 weeks, SHR develops characteristics of cardiovascular disease (Kundu and Rao 2008). Furthermore, SHR develop not only moderate to severe hypertension, but also typical complications of hypertension such as stroke (Badyal et al. 2003). Thus, SHR were used in the current study to evaluate the effect of blood pressure on treatments. The above statements were further in agreement with a study conducted by Luo et al. (2008) confirming that there was an increase in the systolic blood pressure of male SHR, consistently at a rate of 3.5 mmHg per week from 164 mmHg (at 9 weeks) to 185 mmHg (at 15 weeks) and then the pressure would get plateaued at an average of 187 mmHg (during 16 - 28 weeks).

It was found that 8 weeks of blueberry supplementation (~ 350 g fresh blueberries) could significantly reduce both the systolic and diastolic blood pressures (Gavrilova et al. 2011). In addition, in another comparative study, researchers have found that the SHR fed with blueberry-enriched diet (2% w/w) for 6 or 12 weeks compared to control Wistar-Kyoto rats, exhibited lower blood pressure levels at the end of the study (Elks et al. 2011). The above comparative research has demonstrated the SHR ability towards the changes of systolic and diastolic blood pressures. The results of systolic and

diastolic blood pressures of the current study was in agreement with the results of Odahara et al. (2008) and Kondo et al. (2001), demonstrating the blood pressure lowering effect of fruit vinegar beverages in SHR (**Table 7.5**). One of the suggested mechanisms for hypotensive properties of fruit bio-actives is by improving the endothelium-dependent vasodilation by acetylcholine (Kivimaki et al. 2011; Wiseman et al. 2011) of blood vessels which helps in the reduction of blood pressure. Thus, the reduction of blood pressure in SHR after four weeks of study might be due to the above mechanism, link to *in vitro* results of ACE inhibition.

A major disadvantage of using the rats as an animal model is due to their resistance towards atherogenesis. Further, ~ 80% of the total plasma cholesterol in rats is composed of HDL particles and generally, the rats are hypo-responsive to dietary cholesterol intake (Li et al. 2010). Due to the resistance ability of rats towards atherogenesis, scientists used high cholesterol diets containing cholic acid (Li et al. 2010) to induce hyperlipidemia. Thus, cholic acid, together with cholesterol, was used in this study to induce the hypercholesterolemia in SHR. A previous experiment by Leontowicz et al. (2007) demonstrated that in normal rats, apple peels and pulp slightly changed the lipid profile in their plasma.

Alterations in blood cholesterol levels are associated with atherosclerotic lesions. Briefly, high plasma LDL cholesterol concentrations, especially oxidized LDL (ox-LDL), initiate the formation of the atherosclerotic lesions (Li et al. 2010) which leads to the development of CVD. Therefore, LDL could be used as a biomarker to investigate the effect of fruit vinegar beverages on plasma and liver lipid profiles. In **Chapter 6.0**, it was

clearly demonstrated that fruit vinegar beverages possess both strong antioxidant activity against inhibition of LDL oxidation, and antihypertensive properties *in vitro*. Moreover, apples, berries and tomato have also been shown to lower lipid profiles in many animal models. In 2008, Lee et al. reported a significant decrease in TC and LDL-cholesterol (LDL-C) following a 12week administration of cranberry extracts (1500 mg/day) in a human clinical trial. Furthermore, in a recent study, it has been reported that an 8week feeding trial with low-caloric cranberry juice (27% juice, 480 mL/day) caused a significant increase in plasma antioxidant capacity, measured by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay and decreased plasma oxidized LDL in a human clinical trial (Basu et al. 2011). Furthermore, Basu and colleagues have found that 8 weeks supplementation (~350 g) of fresh blueberries caused a significant reduction in oxidized LDL in humans (Basu and Lyons 2012). Furthermore, in another animal model of SHR, 8% wild blueberry supplementation had significant improvement against vasoconstriction and endothelial dysfunction (Kristo et al. 2010).

These findings provide evidence of the role of berries, tomato and apple products in attenuating dyslipidemia and they are biomarkers of atherosclerosis. In addition, Decorde et al. (2008) and Boyer and Liu (2004) have shown that supplements of apple and grape extracts prevent diet-induced atherosclerosis in hamsters and significantly lower the plasma TC in treatment groups, compared to the control. This was evident from the results of the current study where there was a higher level ($p < 0.05$) of TC in the normal control and the acetic acid control, compared to the other treatment groups (**Table 7.6**). In another study, Sertoki et al. (2010) indicates that the use of high dose vinegar

with cholesterolemic diet (1% cholesterol with 10 mL vinegar) significantly reduced the TC and LDL-C levels in rabbits, compared to the control diet group. Furthermore, another study conducted by Lam et al. (2008) found an increase (~ 15%) in the plasma HDL-C in apple peel fed hamsters, compared to the control group. In a similar kind of study conducted using rabbits, 1% cholesterol with 5 mL or 10 mL vinegar induced a significant decrease in TC, compared to the control group (Sertoki et al. 2011). The above results were in agreement with our findings, indicating higher ($p < 0.05$) results of HDL-C level in treatment groups compared to the normal control group and the acetic acid control group (**Table 7.6)**. Even though some researchers have confirmed that a diet containing acetic acid enhanced glycogen repletion (Fushimi and Sato 2005) and helped in reduction in body weight, in this study there was no significant reduction of the body weights of the rats after a 5-week treatment period in the acetic acid group and all the other vinegar treated groups (**Table 7.4**). Although there was no significant difference in the TC, CE and FC levels of the liver lipid profiles of SHR, there was a significant difference in TG levels in treatment groups, compared to the control group. All the treatment groups exhibited a lower ($p < 0.05$) TG level, compared to the both control groups (**Table 7.7**). The above results confirm that the bioactive composition of apple, blueberry, cranberry and tomato may regulate the serum and liver lipid profiles in SHR.

Consistent with epidemiological investigations and animal studies, the clinical researches suggest that polyphenol-rich natural sources may also have a beneficial effect on vascular function in humans (Kerth, et al. 2011). For the past two decades, cholesterol-lowering and blood pressure lowering drugs have been the first-line

therapeutics for the treatment of hypercholesterolemia and hypertension. Findings of the current study demonstrated that the fruit vinegar beverages contain very high antioxidant capacities, have antihypertensive properties and lipid lowering properties. This suggests that functional beverages, like fruit vinegar beverages, could be an alternative to the drugs in controlling hypertension and hypercholesterolemia, upon confirmation of results using a human clinical trial. Furthermore, the mechanisms of the lipid and blood pressure lowering effects of the fruit vinegar beverages await further investigation.

Table 7.2: Antioxidant Capacity and Percent Inhibition of ACE[s] Activity of the Fruit Vinegar Beverages[x]

Type of vinegar beverage[y]	Antioxidant capacity		
	FRAP[p] (mmol TE[q] /L)	ORAC[r] (mmol TE/L)	% Inhibition of ACE
Apple	1.20 ± 0.01^{c}	0.62 ± 0.10^{b}	36.11 ± 1.79^{b}
Blueberry	5.40 ± 0.04^{a}	6.73 ± 1.23^{a}	50.94 ± 0.85^{a}
Cranberry	2.08 ± 0.04^{b}	0.03 ± 0.02^{d}	53.93 ± 1.35^{a}
Tomato	0.14 ± 0.00^{d}	0.53 ± 0.09^{c}	15.83 ± 0.28^{c}

[x]Antioxidant and antihypertensive properties are expressed as mean ± SD (n=3)
[y]All the vinegar beverages were containing the same concentration level of acetic acid (0.5%)
[p]FRAP - Ferric Reducing Antioxidant Power
[r]ORAC - Oxygen Radical Absorbance Capacity
[q]TE - Trolox equivalents
[s]ACE - Angiotensin Converting Enzyme
[a-d] Means followed by a different letter within each column for each type of fruit beverage are significantly different (Tukey's Studentized Ranged test [$P<0.05$])

Table 7.3: Feed Intakes of SHR During 5-Week Dietary Intervention Period[x]

Treatment Group[y]	Dietary Intervention Period (weeks)				
	1	2	3	4	5
Atherogenic Diet (AD) + Water (Control)	16.24 ± 1.37	13.76 ± 1.83	11.51 ± 0.83	12.24 ± 0.86	15.31 ± 0.86
AD + Acetic acid	16.18 ± 0.83	13.14 ± 1.61	11.33 ± 0.84	12.16 ± 0.60	15.57 ± 0.78
AD + Blueberry Vinegar Beverage	15.90 ± 0.73	15.02 ± 4.33	11.51 ± 0.98	11.54 ± 0.80	14.43 ± 1.32
AD + Cranberry Vinegar Beverage	16.60 ± 1.20	13.43 ± 0.87	11.43 ± 0.66	11.94 ± 0.88	15.23 ± 0.90
AD + Tomato Vinegar Beverage	16.65 ± 1.30	13.61 ± 0.88	11.66 ± 1.53	12.48 ± 0.76	15.56 ± 1.13
AD + Apple Vinegar Beverage	16.30 ± 1.08	12.15 ± 4.85	10.66 ± 0.94	12.17 ± 0.89	13.99 ± 4.48

[x]Feed intake is expressed as mean ± SD (n=12)
[y]All the treatment groups were containing the same concentration level of acetic acid (0.5%)
Means within each column are not significantly different ($p = 0.05$)

Table 7.4: Fasting Body Weight of SHR During 5-Week Dietary Intervention Period[x]

Treatment Group[y]	Dietary Intervention Period (weeks)					
	0	1	2	3	4	5
	Average body weight in grams (g)					
Atherogenic Diet(AD) + Water (Control)	237.56 ± 10.63	262.79 ± 11.27	280.45 ± 12.56	290.68 ± 11.92	300.47 ± 11.32	311.48 ± 11.79
AD + Acetic acid	237.10 ± 10.00	260.90 ± 11.00	277.20 ± 11.90	285.78 ± 12.29	295.98 ± 12.16	311.23 ± 14.33
AD + Blueberry Vinegar Beverage	237.47 ± 10.52	259.86 ± 10.98	277.37 ± 11.43	284.96 ± 14.07	294.50 ± 13.03	306.61 ± 13.19
AD + Cranberry Vinegar Beverage	237.32 ± 10.37	261.13 ± 8.38	277.98 ± 9.00	288.42 ± 11.42	297.64 ± 11.22	310.72 ± 11.52
AD + Tomato Vinegar Beverage	237.20 ± 10.20	263.30 ± 11.10	279.90 ± 10.70	289.10 ± 11.42	296.83 ± 12.72	310.55 ± 12.79
AD + Apple Vinegar Beverage	237.20 ± 10.00	259.80 ± 10.50	276.40 ± 12.80	285.08 ± 13.23	294.01 ± 14.82	307.95 ± 17.80

[x]Body weight of SHR are expressed as mean ± SD (n=12)
[y]All the treatment groups were containing the same concentration level of acetic acid (0.5%)
Means within each column are not significantly different (p = 0.05)

Table 7.5: Systolic and Diastolic Blood Pressure of SHR During 5-Week Dietary Intervention Period[x]

Treatment Group[y]	Diastolic Blood Pressure (mmHg)		Systolic Blood Pressure (mmHg)	
	Week 2	Week 4	Week 2	Week 4
Atherogenic Diet (AD) + water(Control)	90.58 ± 12.36[ab]	113.30 ± 15.86[a]	142.36 ± 11.72[a]	163.01 ± 19.00[a]
AD + Acetic Acid	103.17 ± 25.88[a]	102.43 ± 10.24[b]	155.73 ± 21.85[a]	156.78 ± 15.96[ab]
AD + Blueberry Vinegar Beverage	88.61 ± 20.11[ab]	90.92 ± 8.71[c]	149.69 ± 18.13[a]	148.48 ± 11.37[b]
AD + Cranberry Vinegar Beverage	90.34 ± 11.65[ab]	97.82 ± 10.43[bc]	148.13 ± 18.18[a]	150.71 ± 9.58[b]
AD + Tomato Vinegar Beverage	81.16 ± 17.15[b]	93.58 ± 16.25[bc]	157.23 ± 23.25[a]	154.99 ± 13.41[ab]
AD + Apple Vinegar Beverage	91.75 ± 18.23[ab]	90.68 ± 12.38[c]	154.28 ± 20.79[a]	155.91 ± 16.21[ab]

[x]Blood pressure of SHR are expressed as mean ± SD (n=12)
[y]All the treatment groups were containing the same concentration level of acetic acid (0.5%)
[a-b] Means followed by a different letter within each column for each type of fruit beverage are significantly different (least square difference test [$P<0.05$])

Table 7.6: Serum Lipid Profiles of SHR During 5-Week Dietary Intervention Period[x]

Treatment Group[y]	Serum Lipid Profile (mg/dL)			
	TC[z]	HDL-C	Non HDL-C	TG
Atherogenic Diet (AD) + water (Control)	130.31 ± 26.58[a]	39.46 ± 12.26[c]	90.85 ± 25.00[a]	105.72 ± 13.73[a]
AD + Acetic Acid	89.30 ± 29.50[b]	50.35 ± 10.60[b]	43.11 ± 23.72[b]	67.06 ± 34.77[bc]
AD + Blueberry Vinegar Beverage	101.91 ± 30.95[b]	58.57 ± 13.53[ab]	43.34 ± 26.82[b]	79.63 ± 19.59[b]
AD + Cranberry Vinegar Beverage	85.93 ± 14.24[b]	62.56 ± 6.36[a]	23.26 ± 13.34[b]	61.51 ± 18.03[c]
AD + Tomato Vinegar Beverage	102.58 ± 25.05[b]	63.15 ± 9.08[a]	39.44 ± 19.78[b]	74.10 ± 14.23[bc]
AD + Apple Vinegar Beverage	95.40 ± 30.66[b]	58.25 ± 13.68[ab]	37.15 ± 30.97[b]	59.35 ± 9.25[c]

[x]Serum lipid profiles of SHR are expressed as mean ± SD (n=12)

[y]All the treatment groups were containing the same concentration level of acetic acid (0.5%)

[z]TC: Total Cholesterol, HDL-C: HDL Cholesterol, Non HDL-C: Non HDL Cholesterol, TG: triacylglycerols

[a-c] Means followed by a different letter within each column for each type of fruit beverage are significantly different (least square difference test [P<0.05])

Table 7.7: Liver Lipid Profiles of SHR During 5-Week Dietary Intervention Period[x]

Treatment Group	Liver Lipid Profile (mM)			
	TC[zp]	FC[q]	CE	TG
Atherogenic Diet (AD) + water (Control)	11.70 ± 3.39[a]	3.42 ± 1.24[a]	8.24 ± 2.48[a]	5.99 ± 0.92[ab]
AD + Acetic Acid	11.80 ± 2.67[a]	3.24 ± 1.19[a]	8.56 ± 1.94[a]	6.39 ± 0.79[a]
AD + Blueberry Vinegar Beverage	12.15 ± 2.66[a]	3.42 ± 0.97[a]	8.54 ± 2.08[a]	4.30 ± 0.77[d]
AD + Cranberry Vinegar Beverage	12.09 ± 3.29[a]	3.71 ± 1.21[a]	8.44 ± 2.76[a]	4.00 ± 0.73[d]
AD + Tomato Vinegar Beverage	12.55 ± 3.12[a]	3.96 ± 0.87[a]	8.51 ± 2.42[a]	5.22 ± 0.59[c]
AD + Apple Vinegar Beverage	11.74 ± 2.53[a]	3.16 ± 1.34[a]	8.09 ± 2.64[a]	5.51 ± 1.28[bc]

[x]Serum lipid profiles of SHR are expressed as mean ± SD (n=12)

[y]All the treatment groups were containing the same concentration level of acetic acid (0.5%)

[z]TC: Total Cholesterol, FC: Free Cholesterol, CE: Cholesterol esters, TG: triacylglycerols

[p-q] Transformations were done to obtain normality of data ([p-q]-square root)

[a-d] Means followed by a different letter within each column for each type of fruit beverage are significantly different (least square difference test [P<0.05])

CHAPTER 8.0 CONCLUSION

8.1 ANTIOXIDANT, ANTIHYPERTENSIVE, AND LIPID LOWERING PROPERTIES OF FRUIT VINEGAR BEVERAGES

Currently, hypertension and CVD are among the leading causes of death in the world. Intake of high-caloric foods could elevate the blood glucose levels and triglyceride levels, leading to CVD and hypertension. The scavenger receptor-facilitated mechanism of oxidized LDL, by macrophages, leads to foam cell formation and this plays an important role in the initiation and progression of atherosclerosis (Iizuka et al. 2010), while narrowing of blood vessels would lead to progression of hypertension. Fruit vinegar beverages have become a great interest due to their positive effects on reducing hypertension and regulation of blood cholesterol levels (Honsho et al. 2005; Budak et al. 2011).

In **Chapter 4.0** of this study, four different fruit vinegar beverages were produced, using a standardized procedure, to investigate the changes which take place in bio-active concentrations, during the fermentation process. Strong correlations on reduction of bio-active concentrations were observed during the alcoholic fermentation process. Most of the bio-active compounds decreased during the alcoholic fermentation, resulting in an approximate 10% - 30% reduction in bio-active concentrations in fruit vinegar beverages. However, blending of fruit juices with vinegars could help to increase the sensory, antioxidant and bio-active composition of the final product (Bastante et al. 2010). This was evident from the results of the sensory study, which is in agreement with

that the fruit vinegar blended with higher content of corresponding fruit juices has better sensory quality.

Fruit vinegar beverages are known to have higher antioxidant capacities (Bastante et al. 2010; Su and Chien 2010). All four vinegar beverages demonstrated higher antioxidant capacities in both FRAP and ORAC assays. LDL-TBARS assay was done to investigate the human LDL oxidation ability of the fruit vinegar beverages. All the vinegar beverages resulted in higher LDL oxidation *in vitro* for the TBARS assay, confirming the strong antioxidant properties of the fruit vinegar beverages. Furthermore, to investigate the ability of fruit vinegar beverages to reduce blood pressure, ACE inhibition assay *in vitro* was conducted. The inhibition assay revealed the potential antihypertensive properties of the fruit vinegar beverages *in vitro*. Apple, blueberry and cranberry vinegar beverages exhibited stronger antioxidant properties and antihypertensive properties than the tomato vinegar beverage.

To further evaluate the effect of fruit vinegar beverages on lowering blood pressure under *in vivo* conditions, an experimental animal model of SHR were used. The results of the current study demonstrated that reduction of high blood pressure and hypocholesterolemia were successfully archived in the SHR model. All four vinegar beverages exhibited strong antihypertensive properties after four weeks of the study. Even though fruit vinegar beverages have exhibited strong antioxidant properties *in vitro*, it has demonstrated very low biological activity *in vivo* since only a very low percentage of the bio-active compounds reached the target tissues. The poor bioavailability may be the reason for the low effectiveness of fruit vinegar beverages *in vivo*. Furthermore, all

the four vinegar beverages demonstrated a reduction in serum TG levels, non HDL-C levels and an increase in the HDL-C levels, after five weeks of treatments over the two controls. Even though there was no significant difference in the liver, TC, FC, and CE liver TG levels showed significantly lower values in all four vinegar beverage groups than the both control groups. The literature suggests that the intakes of fruit vinegar beverages are associated with lower risk of hypertension and hypercholesterolemia. This was supported through the results of the current study. However, the antioxidant, antihypertensive and lipid lowering properties of the fruit vinegar beverages merit further evaluation in the development of new functional beverages.

8.2 RECOMMENDATIONS FOR FUTURE RESEARCH

As a result of the current research project, many further questions evolved which remained to be explored. All four fruit vinegar beverages have demonstrated relatively higher antioxidant capacities in all the antioxidant assays used in this study. However, during the alcoholic fermentation process a reduction in the concentration of the phenolic compounds were observed. This could be either due to the ability of yeast to convert the phenolic compounds into different forms, or may be due to the fact that metabolites of phenolic compounds during the alcoholic fermentation could possess reducing power and free radical scavenging ability. However, to have a better understanding of the different phenolic compounds and their potency during fermentation, further research using pure phenolic compounds are needed.

In the animal model study, certain findings with fruit vinegar beverages were not consistent compared to the acetic acid control. Acetic acid control also demonstrated a reduction in serum TC, non HDL-C and TG and an increase in the serum HDL-C concentration compared to the normal control group. However, similar results were observed with acetic acid and its ability to lower the plasma lipid concentrations in another study (Setorki et al. 2010; Setorki et al. 2011). There were significant difference in plasma lipid profiles among the treatment groups compared to the control groups; the variations between the groups were relatively high. This might be due to the variations of the bodyweight between the animals within each group and this could be avoided by further extending the duration of the animal study.

Therefore, it is necessary to discover the mechanism and the mode of action of these functional beverages on the lowering of blood pressure and serum and liver lipid profiles. However, these results indicated some interesting biological properties of functional fruit vinegar beverages, and further research on mode of action of fruit vinegar beverages *in vivo* in comparison to acetic acid is required to understand unique effects of bio-actives.

It could be assumed that the effects of lipid lowering and blood pressure regulating might be manifested through multiple mechanisms and mode of actions. It is important to evaluate the absorption mechanism involved for bio-active compounds (flavan-3-ol, chalcone, phenolic acids, anthocyanins, lycopene and carotene) present in fruit vinegar beverages and acetic acid; the bio-transformation of bio-active compounds and acetic acid in the digestive track; metabolism in liver, and; plasma concentrations of bio-active compounds and their metabolites and acetic acid after consumptions. As all the fruit vinegar beverages and acetic acid control markedly increased serum HDL-C and reduced serum triacylglycerols in SHR, it is also worth to investigate mechanism of action of fruit vinegar beverages on cholesterol metabolism.

In conclusion, results of the present study have indicated that functional fruit vinegar beverages have potential antioxidant properties as demonstrated by the *in vitro* assays of FRAP, ORAC and LDL-oxidation, as well as antihypertensive properties and hypolipidemic properties as demonstrated using the animal study. The functional fruit vinegar beverages might have future implication for treatment of hypertension and atherosclerosis.

REFERENCES

Ahmet, I.; Spangler, E.; Hale, B. S. Blueberry-Enriched Diet Protects Rat Heart from Ischemic Damage. *PLoS ONE*, **2009**, 4, (6).

Allain, C. C.; Poon, L. S.; Chan, C. S. G.; Richmond, W.; Fu, P. C. Enzymatic Determination of Total Serum Cholesterol. *Clinical Chemistry*, **1974**, 20, (4), 470-475.

Andersen, O. M. Anthocyanins in Fruits of *Vaccinium oxycoccus* L. (Small Cranberry). *Journal of Food Science*, **1989**, 54, (2), 383-387.

Andlauer, W.; Stumpf, C.; Furst, P. Influence of the Acetification Process on Phenolic Compounds. *Journal of Agricultural Food Chemistry*, **2000**, 48, 3533-3536.

Araujo, L. V.; Chambers, E.; Adhikari, K.; Barrachina, A. A. C. Sensory and Physicochemical Characterization of Juices Made with Pomegranate and Blueberries, Blackberries, or Raspberries. *Journal of Food Science*, **2010**, 75, (7), S398-S404.

Atanasova, V.; Fulcrand, H.; Cheynier, V.; Moutounet, M. Effect of Oxygenation on Polyphenol Changes Occurring in the Course of Wine Making. *Analytica Chimica Acta*, **2002**, 458, 15–27.

Badyal, D. K.; Lata, H.; Dadhich, A. P. Animal Models of Hypertension and Effect of Drugs. *Indian Journal of Pharmacology*, **2003**, 35, 349-362.

Bastante, M. J. C.; Guerrero, E.; Meji´as, R. C.; Mari´n, R. N.; Dodero, M. C. R.; Barroso, C. G. Study of the Polyphenolic Composition and Antioxidant Activity of New Sherry Vinegar-Derived Products by Maceration with Fruits. *Journal of Agriculture Food Chemistry,* **2010**, 58, 11814–11820.

Basu, A.; Rhone, M.; Lyons, T. J. Berries: Emerging Impact on Cardiovascular Health. *Nutrition Reviews,* **2010**, 68, (3), 168-177.

Basu, A.; Betts, N. M.; Ortiz, J.; Simmons, B.; Wu, M.; Lyons, T. J. Low-calorie Cranberry Juice Decreases Lipid Oxidation and Increases Plasma Antioxidant Capacity in Women with Metabolic Syndrome. *Nutrition Research*, **2011**, 31, (3), 190–196.

Basu, A.; Du, M.; Leyva, M. J.; Sanchez, K.; Betts, N. M.; Wu, M.; Aston, C. E.; Lyons, T. J. Blueberries Decrease Cardiovascular Risk Factors in Obese Men and Women with Metabolic Syndrome1–3. *American Society for Nutrition,* **2010**, 1582-1587.

Basu, A.; Lyons, T. J. Strawberries, Blueberries, and Cranberries in the Metabolic Syndrome: Clinical Perspectives. *Journal of Agriculture Food Chemistry*, **2012**, 60, 5687–5692.

Balasuriya, N. B. W.; Rupasinghe, H. P. V. Plant Flavonoids as Angiotensin Converting Enzyme Inhibitors in Regulation of Hypertension. *Functional Foods in Health and Diseases*, **2011**, 5, 172-188.

Benzie, I.; Strain, J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of Antioxidant Power: the FRAP Assay. *Analytical Biochemistry*, **1996**, 239, 70-76.

Berry, B. The Global Vinegar Market: Opportunities for Canadian Vinegar Exporters. **2011**, available online-http://www.ats-sea.agr.gc.ca/inter/4344-eng.htm. (accessed on 25.01.2011).

Boyer, J.; Liu, R. H., Apple Phytochemicals and Their Health Benefits. *Nutrition Journal,* **2004**, 3, (5), 1475-1490.

Breinholt, V.; Nielson, S.; Knuthsen, P.; Lauridsen, S.; Daneshvar, B.; Sorensen, A. Effects of Commonly Consumed Fruit Juices and Carbohydrates on Redox Status and Anticancer Biomarkers in Female Rats. *Nutrition Cancer*, **2003**, 45, 46-52.

Buchert, J.; Koponen, J. M.; Suutarinen, M.; Mustranta, A.; Lille, M.; Törrönen, R.; Poutanen, K. Effect of Enzyme-Aided Pressing on Anthocyanin Yield and Profiles in Bilberry and Blackcurrant Juices. *Journal of the Science of Food and Agriculture*, **2005**, 85, (15), 2548-2556.

Budak, N. H.; Doguc, D. K.; Savas, C. M.; Seydim, A. C.; Tas, T. K.; Ciris, M. I.; Seydim, Z. B. G. Effects of Apple Cider Vinegars Produced with Different Techniques on Blood Lipids in High-Cholesterol-Fed Rats. *Journal of Agriculture Food Chemistry,* **2011,** 59, 6638–6644.

Cao, G.; Alessio, H.; Cutler, R. Oxygen Radical Absorbance Capacity Assay for Antioxidants. *Free Radical Biology & Medicine*, **1993**, 14, 303-311.

Clinton, S. K. Lycopene: Chemistry, Biology, and Implications for Human Health and Disease. *Nutrition Reviews,* **1998**, 56, (2), 35-51.

Chaovanalikit, A.; Wrolstad, R. E. Total Anthocyanins and Total Phenolics of Fresh and Processed Cherries and Their Antioxidant Properties. *Journal of Food Science*, **2004**, 69 (1), FCT67-FCT72.

Chang, R. C.; Lee, H. C.; Ou, S. M. Investigation of the Physicochemical Properties of Concentrated Fruit Vinegar. *Journal of Food and Drug Analysis,* **2005**, 13, (4), 348-356.

Chong, M. F. F.; Macdonald, R.; Lovegrove, J. A. Fruit Polyphenols and CVD Risk: A Review of Human Intervention Studies. *British Journal of Nutrition*, **2010**, 104, S28–S39.

Chu, Y. F.; Liu, R. H. Cranberries Inhibit LDL Oxidation and Induce LDL Receptor Expression in Hepatocytes. *Life Sciences*, **2005**, 77, 1892–1901.

Chu, Y.; Liu, R. H. Novel Low-Density Lipoprotein (LDL) Oxidation Model: Antioxidant Capacity for the Inhibition of LDL Oxidation. *Journal of Agriculture and Food Chemistry,* **2004**, 52, (22), 6818-6823.

Cinq-Mars, C. D.; Li-Chan, E. C. Optimizing Angiotensin 1-Converting Enzyme Inhibitory Activity of Pacific Hake (*Merluccius productus*) Fillet Hydrolysate Using Response Surface Methodology and Ultrafiltration. *Journal of Agriculture Food Chemistry*, **2007**, 55, 9380-9388.

Corrales, M.; García, A. F.; Butz, P.; Tauscher, B. Extraction of Anthocyanins from Grape Skins Assisted by High Hydrostatic Pressure. *Journal of Food Engineering*, **2009**, 90 (4), 415-421.

Daugherty, A.; Rateri, D.; Hong, L.; Balakrishnan, A. Measuring Blood Pressure in Mice using Volume Pressure Recording, a Tail-cuff Method. Journal of Visualized Experiments, **2009**, 27, available online-http://www.jove.com/index/Details.stp?ID=1291, doi: 10.3791/1291 (accessed on 25.01.2012).

Decorde, K.; Teissedre, P. L.; Auger, C.; Cristol, J. P.; Rouanet, J. M. Phenolics from Purple Grape, Apple, Purple Grape Juice and Apple Juice Prevent Early Atherosclerosis Induced by an Atherogenic Diet in Hamsters. *Molecular Nutrition Food Research*, **2008**, 52, 400-407.

Diaz, D. E. R.; Santos, A.; Francis, D. M.; Saona, L. E. R. Carotenoid Stability during Production and Storage of Tomato Juice Made from Tomatoes with Diverse Pigment Profiles Measured by Infrared Spectroscopy. *Journal of Agricultural Food Chemistry*, **2010**, 58, 8692–8698.

Dillard, C. J.; German, J. B. Phytochemicals: Nutraceuticals and Human Health. *Journal of the Science of Food and Agriculture*, **2000**, 80, (12), 1744-1756.

Dogaru, D. V.; Hădărugă, N.; Traşcă, T.; Jianu, C.; Jianu, I. Researches Regarding the Antioxidant Capacity of Some Fruits Vinegar. *Journal of Agroalimentary Processes and Technologies,* **2009**, 15 (4), 506-510.

Elks, C. M.; Reed, S. D.; Mariappan, N.; Hale, B. S.; Joseph, J. A.; Ingram, D. K.; Francis, J. A. Blueberry-Enriched Diet Attenuates Nephropathy in a Rat Model of Hypertension via Reduction in Oxidative Stress. *PLoS ONE*, **2011**, 6, (9), e24028.

Engelhard, Y. N.; Gazer, B.; Paran, E.; Sheva, B. Natural Antioxidants from Tomato Extract Reduce Blood Pressure in Patients with Grade-1 Hypertension: A Double-Blind, Placebo-Controlled Pilot Study. *American Heart Journal,* **2006**, 151, (1), 100.e1-100.e6.

Ferrer, I.; Thurman, E. M. The Mass Detect, Isotope Clusters, and Accurate Mass for Elemental Determination. In: *Liquid Chromatography Time-of-Flight Mass Spectrometry- Principles, Tools, and Applications for Accurate Mass Analysis,* (Ed.) I. Ferrer, and E. M. Thurman, **2009**, 17-35.

Fischer, U. A.; Dettmann, J. S.; Carle, R.; Kammerer, D. R. Impact of Processing and Storage on the Phenolic Profiles and Contents of Pomegranate (*Punica granatum L.*) Juices. *European Food Research Technology*, **2011**, 1560-1563.

Fossati, P.; Prencipe, L. Serum Triglycerides Determined Colorimetrically with an Enzyme That Produces Hydrogen Peroxide. *Clinical Chemistry*, **1982**, 28, (10), 2077-2080.

Fushimi, T.; Sato, Y. Effect of Acetic Acid Feeding on the Circadian Changes in Glycogen and Metabolites of Glucose and Lipid in Liver and Skeletal Muscle of Rats. *British Journal of Nutrition,* **2005**, 94, 714–719.

Fushimi, T.; Suruga, K.; Oshima, Y.; Fukiharu, M.; Tsukamoto, Y.; Goda, T., Dietary Acetic Acid Reduces Serum Cholesterol and Triacylglycerols in Rats Fed a Cholesterol Rich Diet. *British Journal of Nutrition,* **2006**, 95, (5), 916-924.

Fushimi, T.; Tayama, K.; Fukaya, M.; Kitakoshi, K.; Nakai, N.; Tsukamoto, Y.; Acetic Acid Feeding Enhances Glycogen Repletion in Liver and Skeletal Muscle of Rats. *Journal of Nutrition,* **2001**, 131, (7), 1973-1977.

Galindo, P.; Gomez, I. R.; Manzano, S. G.; Duenas, M.; Jimenez1, R.; Menendez, C.; Vargas, F.; Tamargo, J.; Buelga, C. S.; Vizcaıno, F. P.; Duarte, J. Glucuronidated Quercetin Lowers Blood Pressure in Spontaneously Hypertensive Rats via Deconjugation, *PLoS ONE*, **2012**, 7, (3), e32673.

Gavrilova, V.; Kajdzanoska, M.; Gjamovski, V.; Stefova, M. Separation, Characterization and Quantification of Phenolic Compounds in Blueberries and Red and Black Currants by HPLC-DAD-ESI-MS. *Journal of Agriculture Food Chemistry*, **2011**, 59, 4009–4018.

Granato, D.; Branco, G. F.; Nazzaro, F.; Cruz, A. G.; Faria, A. F., Functional Foods and Non Dairy Probiotic Food Development Trends, Concepts and Products. *Food Science and Food Safety,* **2010**, 9, (3), 292-302.

Hakkinen, S. H.; Karenlampi, S. O.; Heinonen, I. M.; Mykkanen, H. M.; Torronen, A. R. Content of the Flavonols Quercetin, Myricetin, and Kaempferol in 25 Edible Berries. *Journal of Agriculture Food Chemistry*, **1999**, 47, 2274-2279.

Hidalgo, C.; Mateo, E.; Cerezo, A. B.; Torija, M. J.; Mas, A. Technological Process for Production of Persimmon and Strawberry Vinegars. *International Journal of Wine Research*, **2010**, 2, 55–61.

Honsho, S.; Sugiyama, A.; Takahara, A.; Satoh, Y.; Nakamura, Y.; Hashimoto, K.A Red Wine Vinegar Beverage can Inhibit the Renin-Angiotensin System: Experimental Evidence *in vivo*. *Biological and Pharmaceutical Bulletin*, **2005**, 28, (7), 1208-1210.

Huntley, A. L. The Health Benefits of Berry Flavonoids for Menopausal Women: Cardiovascular Disease, Cancer and Cognition. *Maturitas,* **2009**, 63, 297–301.

Hurst, R. D.; Wells, R. W.; Hurst, S. M. Blueberry Fruit Polyphenolics Suppress Oxidative Stress Induced Skeletal Muscle Cell Damage *in vitro*. *Molecular Nutrition Food Research*, **2010**, 54, (3), 353-363.

Iizuka, M.; Tani, M.; Kishimoto, Y.; Saita, E.; Toyozaki, M.; Kondo, K., Inhibitory Effects of Balsamic Vinegar on LDL Oxidation and Lipid Accumulation in THP-1 Macrophages. *Journal of Nutritional Science and Vitaminology*, **2010**, 56, 421-427.

Jenkins, D. J. A.; Ssrichaikul, K.; Kendall, C. W. C. The Relation of Low Glycaemic Index Fruit Consumption to Glycaemic Control and Risk Factors for Coronary Heart Disease in Type 2 Diabetes. *Diabetologia*, **2011**, 54, (2), 271-279.

Jepson, R. G.; Craig, J. C., Review: A Systematic Review of the Evidence for Cranberries and Blueberries in UTI Prevention. *Molecular Nutrition Food Research*, **2007**, 51, 738-745.

Johnston, C. S.; Buller, A. J. Vinegar and Peanut Products as Complementary Foods to Reduce Postprandial Glycemia. *Journal of American Dietetic Association*, **2005**, 105, (12), 1939-1942.

Kahkanen, M. P.; Heinamaki, J.; Ollilainen, V.; Heinonen M. Berry Anthocyanins: Isolation, Identification and Antioxidant Activities. *Journal of the Science of Food and Agriculture*, **2003**, 83 (14), 1403-1411.

Kato, Y.; Hirayama, N.; Omori, T.; Hoshino, M.; Fujii, Y. Fruit Vinegar From Raw Material Flavorful Acid Citrus Fruit Juice and Method for Producing the Same. U.S. Patent 7005149, **1998**.

Kathiirvel, P.; Rupasinghe, H. P. V. Plant-derived Antioxidants as Potential Omega-3 PUFA Stabilizers. In: *Fish Oil: Production, Consumption and Health Benefits* (Ed.) M. Van Dijk and J. Vitek, **2012**, 157-186.

Kerth, V. B. S.; Selloum, N. É.; Chataigneau, T.; Auger, C. Vascular Protection by Natural Product-Derived Polyphenols: *in vitro* and *in vivo* Evidence. *Planta Medicine*, **2011**, 77, 1161–1167.

Kim, Y.; Goodner, K. L.; Park, J. D.; Choi, J.; Talcott, S. T. Changes in Antioxidant Phytochemicals and Volatile Composition of *Camellia Sinensis* by Oxidation During Tea Fermentation. *Food Chemistry*, **2011**, 129, 1331–1342.

Kivimaki, A. S.; Ehlers, P. I.; Turpeinen, A. M.; Vapaatalo, H.; Korpela, R. Lingonberry Juice Improves Endothelium-dependent Vasodilatation of Mesenteric Arteries in Spontaneously Hypertensive Rats in a Long-term Intervention. *Journal of Functional Foods*, **2011**, 3, 267-274.

Kodavanti, U. P.; Schladweiler, M. C.; Ledbetter, A. D.; Watkinson, W. P.; Campen, M. J.; Winsett, D. W.; Richards, J. R.; Crissman, K. M.; Hatch, G. E.; Costa, D. L. The Spontaneously Hypertensive Rat as a Model of Human Cardiovascular Disease: Evidence of Exacerbated Cardiopulmonary Injury and Oxidative Stress from Inhaled Emission Particulate Matter. *Toxicology and Applied Pharmacology*, **2000**, 164, 250–263.

Kohlmeier, L.; Kark, J. D.; Gomez-Gracia, E.; Martin, B. C.; Steck, S. E.; Kardinaal, A. F. M; Ringstad, J.; Thamm, M.; Masaev, V.; Riemersma, R.; Martin-Moreno, J. M.; Huttunen, J. K.; Kok, F. J. Lycopene and Myocardial Infarction Risk in the EURAMIC Study. *American Journal of Epidemiology,* **1997**, 146, (8), 618-626.

Kondo, T; Kishi, M; Fushimi, T; Ugajin, S; Kaga, T. Vinegar Intake Reduces Body Weight, Body Fat Mass, and Serum Triglyceride Levels in Obese Japanese Subjects. *Bioscience, Biotechnology, and Biochemistry,* **2009**, 73, (8), 1837-1843.

Kondo, S.; Tayama, K.; Tsukamoto, Y.; Ikeda, K.; Yamori, Y. Antihypertensive Effects of Acetic Acid and Vinegar on Spontaneously Hypertensive Rats. *Bioscience, Biotechnology, and Biochemistry,* **2001**, 65, (12), 2690-2694.

Koponen, J. M.; Buchert, J.; Poutanen, K. S.; Torronen, A. R. Effect of Pectinolytic Juice Production on the Extractability and Fate of Bilberry and Black Currant Anthocyanins. *European Food Research Technology*, **2008**, 227, 485–494.

Kristo, A. S.; Kalea, A. Z.; Schuschke, D. A.; Klimis-Zacas, D. J. A. Wild Blueberry-Enriched Diet (*Vaccinium angustifolium*) Improves Vascular Tone in the Adult Spontaneously Hypertensive Rat. *Journal of Agriculture Food Chemistry*, **2010**, 58, 11600–11605.

Kundu, S.; Rao, J. P. The Story of Spontaneously Hypertensive Rat (SHR): A Review. *Al Ameen Journal of Medical Science*, **2008**, 1, (1), 65-66.

Lam, C. K.; Zhang, Z.; Yu, H.; Tsang, S. Y.; Huang, Y.; Chen, Z. Y. Apple Polyphenols Inhibit Plasma CETP Activity and Reduce the Ratio of non-HDL to HDL Cholesterol. *Molecular Nutrition Food Research*, **2008**, 52, 950–958.

Larrauri, J. A.; Rupe´rez, P.; Calixto, F. S. Effect of Drying Temperature on the Stability of Polyphenols and Antioxidant Activity of Red Grape Pomace Peels. *Journal of Agricultural and Food Chemistry*, **1997**, 45, 1390-1393.

Lee, I. T.; Chan, Y. C.; Lin, C. W.; Lee, W. J.; Sheu, W. H. Effect of Cranberry Extracts on Lipid Profiles in Subjects with Type 2 Diabetes. *Diabetic Medicine*, **2008**, 25, 1473–1477.

Lee, J.; Durst, R. W.; Wrolstad, R. E. Impact of Juice Processing on Blueberry Anthocyanins and Polyphenolics: Comparison of Two Pretreatments. *Journal of Food Science,* **2002**, 67, (5), 1660–1667.

Leontowicz, H.; Leontowicz, M.; Gorinstein, S.; Martin-Belloso, O.; Trakhtenberg, S. Apple Peels and Pulp as a Source of Bioactive Compounds and their Influence on Digestibility and Lipid Profile in Normal and Atherogenic Rats. *Medycyna Weterynaryjna,* **2007**, 63, (11), 1434-1436.

Li, X.; Liu, Y.; Zhang, H.; Ren, L.; Li, Q.; Li, N. Animal Models for the Atherosclerosis Research: *A Review. Protein Cell*, **2011**, 2, (3), 189–201.

Lohachoompol, V.; Srzednicki, G.; Craske, J. The Change of Total Anthocyanins in Blueberries and Their Antioxidant Effect After Drying and Freezing. *Journal of Biomedicine & Biotechnology*, **2004**, 5, 248-252.

Lotito, S. B.; Frei, B. Relevance of Apple Polyphenols as Antioxidants in Human Plasma: Contrasting *in vitro* and *in vivo* Effects. *Free Radical Biology & Medicine,* **2004**, 36, (2), 201 – 211.

Luo, Y.; Owens, D.; Mulder, G.; McVey, A.; Fisher, T. Blood Pressure Characterization of Hypertensive and Control Rats for Cardiovascular Studies. Charles River Laboratories, Wilmington, MA, USA. **2008**.

Machado, M. M.; Montagner, G. F. F. S.; Boligon, A.; Athayde, M. L.; Rocha, M. I. U. M.; Lera, J. P. B.; Cruz, C. B. I. B. M., Determination of Polyphenol Contents and Antioxidant Capacity of No-Alcoholic Red Grape Products (*Vitis labrusca*) from Conventional and Organic Crops. *Química Nova,* **2011**, 34, (5), 798-803.

Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L., Polyphenols: Food Sources and Bioavailability. *American Journal of Clinical Nutrition*, **2004**, 79, (5), 727-747.

McCarron, D. A.; Lucas, P. A.; Shneidman, R. J.; LaCour, B.; Drueke, T. Blood Pressure Development of the Spontaneously Hypertensive Rat after Concurrent Manipulations of Dietary Ca^{2+} and Na^{+}. *Journal of Clinical Investigation*, **1985**, 76, 1147-1154.

McKay, D. L.; Blumberg, J. B. Cranberries (*Vaccinium macrocarpon*) and Cardiovascular Disease Risk Factors. *Nutrition Reviews,* **2007**, 65, (11), 490–502.

Meilgaard, M.; Civille, G. V.; Carr, B. Sensory Evaluation Techniques., 2nd edition, Boca Raton, Florida, USA. **1991**.

Mendiola, J. A.; Marin, F. R.; Senorans, F. J.; Reglero, G.; Martin, P. J.; Cifuentes, A.; Ibanez, E. Profiling of Different Bioactive Compounds in Functional Drinks by High-Performance Liquid Chromatography. *Journal of Chromatography*, **2008**, 1188, 234–241.

Mittal, B. V.; Singh, A. K. Hypertension in the Developing World: Challenges and Opportunities. *American Journal of Kidney Dis*eases, **2010**, 55, 590-598.

Mizuno, C. S.; Rimando, A. M. Blueberries and Metabolic Syndrome. *Silpakorn University Science and Technology Journal*, **2009**, 3, (2), 7-17.

Montgomery, D. C. *Design and analysis of experiments*. 6th ed. John Wiley and Sons, Hoboken, New Jersey. **2005**.

Nakamura, K.; Ogasawara, Y.; Endou, K.; Fujimori, S.; Koyama, M.; Akano, H. Phenolic Compounds Responsible for the Superoxide Dismutase-like Activity in High-Brix Apple Vinegar. *Journal of Agricultural Food Chemistry*, **2010**, 58, 10124–10132.

Nardo, T. D.; Kian, C. S.; Halim, Y.; Francis, D.; Saona, L. E. R. Rapid and Simultaneous Determination of Lycopene and β-Carotene Contents in Tomato Juice by Infrared Spectroscopy. *Journal of Agricultural Food Chemistry*, **2009**, 57, 1105–1112.

Natera, R.; Castro, R.; Moreno, M. D. V. G.; Hernandez, M. J.; Barroso, C. G. Chemometric Studies of Vinegars from Different Raw Materials and Processes of Production. *Journal of Agricultural and Food Chemistry*, **2003**, 51, 3345-3351.

Neto, C. C. Cranberry and Blueberry: Evidence for Protective Effects against Cancer and Vascular Diseases. *Molecular Nutrition & Food Research,* **2007**, 51, (6), 652-664.

Neto, C. C. Cranberry and Its Phytochemicals: A Review of *in vitro* Anticancer Studies. *The Journal of Nutrition,* **2007**, 186-193.

Nogueira, A.; Guyot, S.; Marnet, N.; Lequéré, J. M.; Drilleau, J. F.; Wosiacki, G. Effect of Alcoholic Fermentation in the Content of Phenolic Compounds in Cider Processing. *Brazilian Archives of Biology and Technology*, **2008**, 51, (5), 1025-1032.

Odahara, M.; Ogino, Y.; Takizawa, K.; Kimura, M.; Nakamura, N.; Kimoto, K., Hypotensive Effect of Black Malt Vinegar on Spontaneously Hypertensive Rats. *Nippon Shokuhin Kagaku Kogaku Kaishi,* **2008**, 55, (3), 81-86.

Ojeda, D.; Jiménez-Ferrer, E.; Zamilpa, A.; Herrera-Arellano, A.; Tortoriello, J.; Alvarez, L. Inhibition Of Angiotensin Converting Enzyme (ACE) Activity by the Anthocyanins Delphinidin- and Cyanidin-3-O-Sambubiosides from *Hibiscus Sabdariffa*. *Journal of Ethnopharmacology*, **2010**, 127, 7-10.

O'Keefe, J. H.; Gheewala, N. M.; O'Keefe, J. O. Dietary Strategies for Improving Post-prandial Glucose, Lipids, Inflammation, and Cardiovascular Health. *Journal of American College of Cardiology,* **2008**, 51, (3), 249-255.

Othman, N. B.; Roblain, D.; Chammen, N.; Thonart, P.; Hamdi, M. Antioxidant Phenolic Compounds Loss During the Fermentation of *Chetoui* Olives. *Food Chemistry*, **2009**, 116 662–669.

Ou, A. S.; Chang, R. C. *Vinegars of the world*. Springer: Milan, Italy, **2009**; p 222-242.

Paran, E.; Novack, V.; Engelhard, Y.N.; Halevy, I. H. The Effects of Natural Antioxidants from Tomato Extract in Treated but Uncontrolled Hypertensive Patients. *Cardiovascular Drugs Theraphy,* **2009**, 23, 145–151.

Pearson, D.; Tan, C.; German, B.; Davis, P.; Gershwin, M. Apple Juice Inhibits Low Density Lipoprotein Oxidation. *Life Science*, **1999**, 64, 1919-1920.

Pergola, C.; Rossi, A.; Dugo, P.; Cuzzocrea, S.; Sautebin, L. Inhibition of Nitric Oxide Biosynthesis by Anthocyanin Fraction of Blackberry Extract. *Nitric Oxide*, **2006**, 15, 30-39.

Pinela, J.; Barros, L.; Carvalho, A. M.; Ferreira, I. C. F. R. Nutritional Composition and Antioxidant Activity of Four Tomato (*Lycopersicone sculentum* L.) Farmer Varieties in Northeastern Portugal Home Gardens. *Food and Chemical Toxicology*, **2012**, 50, 829-834.

Pinsirodom, P.; Rungcharoen, J.; Liumminful, A. Quality of Commercial Wine Vinegars Evaluated on the Basis of Total Polyphenol Content and Antioxidant Properties. *Asian Journal of Food and Agro-Industry,* **2008**, 1, (4), 232-241.

Poste, M. L.; Deborah, A. M.; Butler, G.; Larmond, E. Laboratory Methods for Sensory Analysis of Food. Research Branch Agriculture Canada Publication, **1991**.availableonline-http://archive.org/details/laboratorymethod00otta, (accessed on-2012.07.18) ISBN 0-660-13807-7.

Potter, A. S.; Foroudi, S.; Stamatikos, A.; Patil, B. S.; Deyhim, F. Drinking Carrot Juice Increases Total Antioxidant Status and Decreases Lipid Peroxidation in Adults. *Nutrition Journal*, **2011**, 10, 96-102.

Raczkowska, J.; Mielcarz, G.; Howard, A.; Raczkowski, M. UPLC and Spectrophotometric Analysis of Polyphenols in Wines Available in the Polish Market. *International Journal of Food Properties*, **2011**, 14, 514–522.

Riccioni, G.; Mancini, B.; Di-Ilio, E.; Bucciarelli, T.; D'orazio, N. Protective Effect of Lycopene in Cardiovascular Disease. *European Review for Medical and Pharmacological Sciences,* **2008**, 12, (3), 183-190.

Rupasinghe, H. P. V.; Kean, C. Polyphenol Concentrations in Apple Processing By-products Determined Using Electrospray Ionization Mass Spectrometry. *Canadian Journal of Plant Science*, **2008**, 88, 759-762.

Rupasinghe, H. P. V.; Wang, L.; Huber, G. M.; Pitts. N. L. Effect of Baking on Dietary Fibre and Phenolics of Muffins Incorporated With Apple Skin Powder. *Food Chemistry,* **2008**, 107, 1217-1224.

Rupasinghe, H. P. V.; Ronalds, C. M.; Rathgeber, B.; Robinson, R. Absorption and Tissue Distribution of Dietary Quercetin in Broiler Chickens. *Journal of the Science of Food and Agriculture*, **2010**, 9, (7), 1172-1178.

Sablani, S. S.; Andrews, P. K.; Davies, N. M. Effect of Thermal Treatments on Phytochemicals in Conventionally and Organically Grown Berries. *Journal of Science Food Agriculture*, **2010**, 90, (5), 769-778.

Sakaida, H.; Nagao, K.; Higa, K.; Shirouchi, B.; Inoue, N.; Hidaka, F.; Kai, T. Effect of *Vaccinium Ashei* Reade Leaves on Angiotensin Converting Enzyme Activity *in vitro* and on Systolic Blood Pressure of Spontaneously Hypertensive Rats *in vivo*. *Bioscience Biotechnology and Biochemistry*, **2007**, 71, 2335-2337.

Santos, R. A.; Krieger, E. M.; Greene, L. J. An Improved Fluorometric Assay of Rat Serum and Plasma Converting Enzyme. *Hypertension*, **1985**, 7, 244-252.

SAS Institute, Inc. SAS User's Guide: Statistics. Version 9.2 SAS Institute Inc., Cary, NC, USA **2008**.

Saura-Calixto, F. Antioxidant Dietary Fiber Product: A New Concept and a Potential Food Ingredient. *Journal of Agricultural and Food Chemistry*, **1998**, 46, 4303–4306.

Sandmann, G. A. Nitromethane-Based HPLC System Alternative to Acetonitrile for Carotenoid Analysis of Fruit and Vegetables. *Phytochemical Analysis*, **2010**, 21, 434–437.

Sengun, I. Y.; Karabiyikli, S. Review: Importance of Acetic Acid Bacteria in Food Industry. *Food Control,* **2011,** 22 647-656.

Setorki, M.; Asgary, S.; Eidi, A.; Haeri rohani, A.; Majid, K. Acute Effects of Vinegar Intake on Some Biochemical Risk Factors of Atherosclerosis in Hypercholesterolemic Rabbits. *Lipids in Health and Disease,* **2010**, 9, (10), 1-8.

Setorki, M.; Asgary, S.; Haghjooyjavanmard, S.; Nazari, B. Reduces Cholesterol Induced Atherosclerotic Lesions in Aorta Artery in Hypercholesterolemic Rabbits. *Journal of Medicinal Plants Research*, **2011**, 5, (9), 1518-1525.

Shahidi, F.; McDonald, J.; Chandrasekara, A.; Zhong, Y. Phytochemicals of Foods, Beverages and Fruit Vinegars: Chemistry and Health Effects. *Asia Pacific Journal of Clinical Nutrition,* **2008**, 17, (S1), 380-382.

Shaughnessya, K. S.; Boswalla, I. A.; Scanlana, A. P.; Gottschall-Passb, K. T.; Sweeneya, M. I. Diets Containing Blueberry Extract Lower Blood Pressure in Spontaneously Hypertensive Stroke-Prone Rats. *Nutrition Research,* **2009**, 29, (2), 130-138.

Shibayama, Y.; Nagano, M.; Fuji, A.; Taguchi, M.; Takeda, Y.; Yamada, K. Safety Evaluation of Balck Rice Vinegar (Kurosu) from a Jar on Food-drug Interaction: 30-day Ingestion Study on Expressions of Drug Metabolism Enzymes and Transporters in Rats. *Journal of Health Science*, **2010**, 56, (6), 712-716.

Shivashankara, K. S.; Acharya, S. N. Bioavailability of Dietary Polyphenols and the Cardiovascular Diseases. *The Open Nutraceuticals Journal*, **2010**, 3, 227-241.

Shizuma, T.; Ishiwata, K.; Nagano, M.; Mori, H.; Fukuyama, N. Protective Effects of Fermented Rice Vinegar Sediment (*Kurozu moromimatsu*) in a Diethylnitrosamine-Induced Hepatocellular Carcinoma Animal Model. *Journal of Clinical Biochemistry and Nutrition,* **2011**, 49, (1), 31-35.

Shoji, T. Polyphenols as Natural Food Pigments: Changes during Food Processing. *American journal of Food Technology*, **2007**, 2, (7), 570-581.

Statistics Canada, Fruit and vegetable production February **2012**. available online-http://www.statcan.gc.ca/pub/22-003-x/22-003-x2011002-eng.pdf (accessed on 2012.09.09)

Stone, S. Z.; Yasmin, T.; Bagchi, M.; Chatterjee, A.; Vinson, J. A.; Bagchi, D. Berry Anthocyanins as Novel Antioxidants in Human Health and Disease Prevention. *Molecular Nutrition Food Research*, **2007**, 51, 675-683.

Stull, A. J.; Cash, K. C.; Johnson, W. D. Bioactives in Blueberries Improve Insulin Sensitivity in Obese, Insulin-Resistant Men and Women. *The Journal of Nutrition*, **2010**, 140, (10), 1764-1768.

Srivastava, A.; Akoh, C. C.; Yi, W.; Fischer, J.; Krewer, G., Effect of Storage Conditions on the Biological Activity of Phenolic Compounds of Blueberry Extract Packed in Glass Bottles. *Journal of Agricultural and Food Chemistry*, **2007**, 55, 2705-2713.

Su, M. S.; Chien, P. J. Antioxidant Activity, Anthocyanins, and Phenolics of Rabbiteye Blueberry (*Vaccinium ashei*) Fluid Products as Affected by Fermentation. *Food Chemistry,* **2007**, 104, 182–187.

Su, M. S.; Chien, P. J. Aroma Impact Components of Rabbiteye Blueberry (*Vaccinium ashei*) Vinegars. *Food Chemistry,* **2010**, 119, 923–928.

Su, M. S.; Silva, J. L. Antioxidant Activity, Anthocyanins, and Phenolics of Rabbiteye Blueberry (*Vaccinium ashei*) By-products as Affected by Fermentation. *Food Chemistry*, **2006**, 97, 447–451.

Sun, J.; Chu, Y. F.; Wu, X.; Liu, R. H. Antioxidant and Anti-proliferative Activities of Common Fruits. *Journal of Agriculture Food Chemistry*, **2002**, 50, 7449-7454.

Sugiyama, A.; Saitoh, M.; Takahara, A.; Satoh, Y.; Hashimoto, K., Acute Cardiovascular Effects of a New Beverage Made of Wine Vinegar and Grape Juice, Assessed Using an *in vivo* Rat. *Nutrition Research,* **2003**, 23, (9), 1291-1296.

Tanaka, H.; Watanabe, K.; Ma, M.; Hirayama, M.; Kobayashi, T.; Oyama, H.; Sakaguchi, Y.; Kanda, M.; Kodama, M.; Aizawa, Y. The Effects of Γ-Aminobutyric Acid, Vinegar, and Dried Bonito on Blood Pressure in Normotensive and Mildly or Moderately Hypertensive Volunteers. *Journal of Clinical Biochemistry and Nutrition,* **2009**, 45, (1), 93–100.

Terahara, N.; Matsui, T.; Minoda, K.; Nasu, K.; Kikuchi, R.; Fukui, K.; Ono, H.; Matsumoto, K. Functional New Acylated Sophoroses and Deglucosylated Anthocyanins in a Fermented Red Vinegar. *Journal of Agricultural Food Chemistry*, **2009**, 57, 8331–8338.

Triantafyllidi, E.; Baldwin, C.; Schwartz, F.; Gavras, H. Study of Hypertension in Spontaneous Hypertensive Rats by Sequencing the Genomic DNA of Alpha2B Receptors. *Hellenic Journal of Cardiology*, **2004**, 45, 65-70.

Trinder, P. Determination of Blood Glucose Using 4-Aminophenazone as Oxygen Acceptor. *Annals of Clinical Biochemistry*, **1969**, 6, (24).

Tsao, R.; Yang, R.; Xie, S.; Sockovie, E.; Khanizadeh, S. Which Polyphenolic Compounds Contribute to the Total Antioxidant Activities of Apple. *Journal of Agriculture and Food* Chemistry, **2005**, 53, (12), 4989-4995.

Ubeda, C.; Hidalgo, C.; Torija, M. J.; Mas, A.; Troncoso, A. M.; Morales, M. L. Evaluation of Antioxidant Activity and Total Phenols Index in Persimmon Vinegars Produced by Different Processes. *LWT - Food Science and Technology*, **2011**, 44, 1591-1596.

Ugliano, M.; Squillante, E.; Genovese, A.; Moio, L. Investigation on Aroma Compounds of Modern Balsamic Vinegars. Flavour Research at the Dawn of the Twenty-First Century. *Proceedings of the 10th Weurman Flavour Research Symposium*, Beaune, France, **2002**.

Vainionpaa, V.; Heikkinen, E. R.; Vapaatalo, H. Drug Metabolism in Spontaneously Hypertensive Rats. *Pharmacological Research Communications*, **1974**, 6, (4), 343-346.

Varady, K. A.; Wang, Y.; Jones, P. J. H. Role of Policosanols in the Prevention and Treatment of Cardiovascular Disease. *Nutrition Reviews*, **2003**, 61, (11), 376 –383.

Verbeyst, L.; Crombruggen, K. V.; Plancken, I. V.; Hendrickx, M.; Loey, A. V., Anthocyanin Degradation Kinetics During Thermal and High Pressure Treatments of Raspberries. *Journal of Food Engineering*, **2011**, 105, 513–521.

Verzelloni, E.; Tagliazucchi, D.; Conte, A. Changes in Major Antioxidant Compounds During Aging of Traditional Balsamic Vinegar. *Journal of Food Biochemistry*, **2010**, 34, 152–171.

Viljakainen, S.; Visti, A.; Laakso, S. Concentrations of Organic Acids and Soluble Sugars in Juices from Nordic Berries. *Acta Agriculturae Scandinavica, Section B - Soil & Plant Science*, **2010**, 52, (2), 101-109.

Vrhovsek, U.; Rigo, A.; Tonon, D.; Mattivi, F. Quantitation of Polyphenols in Different Apple Varieties. *Journal of Agriculture and Food Chemistry,* **2004**, 52, (21), 6532-6538.

Vuong, T.; Matar, C.; Ramassamy, C. Bio-transformed Blueberry Juice Protects Neurons from Hydrogen Peroxide-induced Oxidative Stress and Mitogen-activated Protein Kinase Pathway Alterations. *British Journal of Nutrition*, **2010**, 104, (5), 656-663.

Wach, A.; Pyrzyn´ska, K.; Biesaga, M. Quercetin Content in Some Food and Herbal Samples. *Food Chemistry,* **2007**, 100, (2), 699-704.

Wang, A.; Zhang, J.; Li, Z. Correlation of Volatile and Nonvolatile Components with the Total Antioxidant Capacity of Tartary Buckwheat Vinegar: Influence of the Thermal Processing. *Food Research International*, **2012**, doi:10.1016/j.foodres.2012.07.020 (accepted manuscript).

Wang, L.; Liu, S.; Manson, J. E.; Gaziano, J. M.; Buring, J. E.; Sesso, H. D. The Consumption of Lycopene and Tomato-Based Food Products is Not Associated With the Risk of Type 2 Diabetes in Women. *Journal of Nutrition,* **2006**, 136, (3), 620-625.

Wang, W.; Xu, S. Degradation Kinetics of Anthocyanins in Blackberry Juice and Concentrate. *Journal of Food Engineering*, **2007**, 82 (3), 271-275.

Williamson, G.; Manach, C. Bioavailability and Bioefficacy of Polyphenols in Humans. II. Review of 93 Intervention Studies. *American Journal of Clinical Nutrition,* **2005**, 81, (1), 243S-255S.

Wiseman, W.; Egan, J. M.; Slemmer, J. E.; Shaughnessy, K. S.; Ballem, K.; Gottschall-Pass, K. T.; Sweeney, M. I. Feeding Blueberry Diets Inhibits Angiotensin II Converting Enzyme (ACE) Activity in Spontaneously Hypertensive Stroke-Prone Rats. *Canadian Journal Physiology and Pharmacology*, **2011**, 89, 67–71.

Wu, X.; Kang, J.; Xie, C.; Burris, R.; Ferguson, M. E.; Badger, T. M.; Nagarajan S. Dietary Blueberry Attenuates Atherosclerosis in Apolipoprotein E-Deficient Mice by up-regulating Antioxidant Enzyme Expression. *Journal of Nutrition*, **2010**, 140, 1628-1632.

Xu, B. J.; Yuan, S. H.; Chang, S. K. C. Comparative Studies on the Antioxidant Activities of Nine Common Food Legumes Against Copper-Induced Human Low-Density Lipoprotein Oxidation in vitro. *Journal of Food Science*, **2007**, 72, S522-S527.

Yan, X.; Murphy, B. T.; Hammond, G. B.; Vinson, J. A.; Neto, C. C. Antioxidant Activities and Antitumor Screening of Extracts from Cranberry Fruit (*Vacciniumm acrocarpon*). *Journal of Agriculture Food Chemistry*, **2002**, 50, 5844-5849.

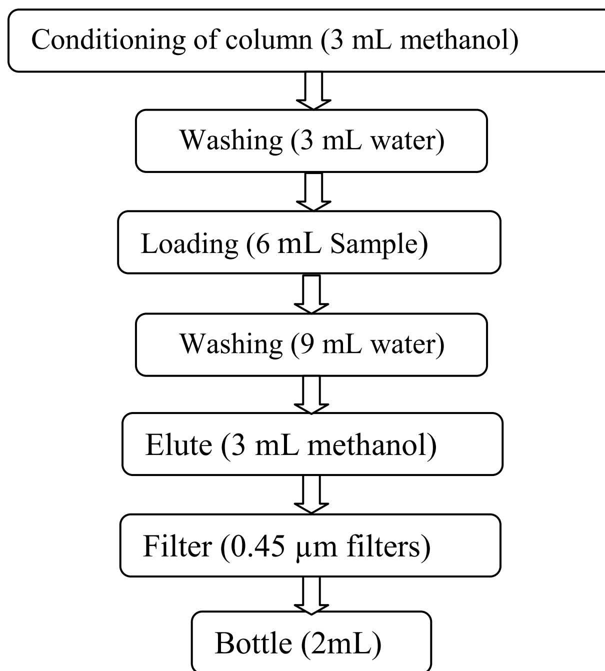
Yang, H. L.; Qi1, Z. L.; Xia, X. L.; Xin, Y.; Zhang, L.; Leng, Y. W.; Quan, W.; Wang, W. An Optimum Medium Designed and Verified for Alcohol Vinegar Fermentation. *African Journal of Biotechnology*, **2011**, 10, (42), 8421-8427.

Yu, K.; Little, D.; Plumb, R.; Smith, B. High-throughput Quantification for a Drug Mixture in Rat Plasma a Comparison of Ultra Performance TM Liquid Chromatography/Tandem Mass Spectrometry with High-performance Liquid Chromatography/Tandem Mass Spectrometry. *Rapid Communication in Mass Spectrometry*, **2006**, 20, 544–552.

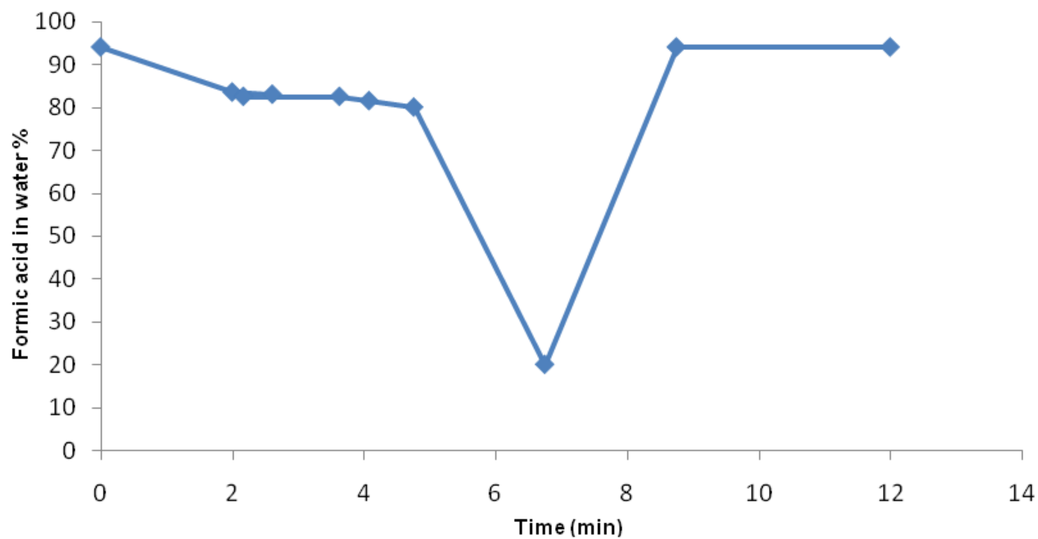
Zafra-Stone, S.; Yasmin, T.; Bagchil, M.; Chatterjee, A.; Vinson, J. A.; Bagchi1, D. Berry Anthocyanins as Novel Antioxidants in Human Health and Disease Prevention. *Molecular Nutrition & Food Research,* **2007**, 51, (6), 675-683.

Zhang, A; Fang, Y. L.; Meng, J. F.; Wang, H.; Chen, S. X.; Zhang, Z. W. Analysis of Low Molecular Weight Organic Acids in Several Complex Liquid Biological Systems via HPLC with Switching Detection Wavelength. *Journal of Food Composition and Analysis,* **2011**, 24, 449–455.

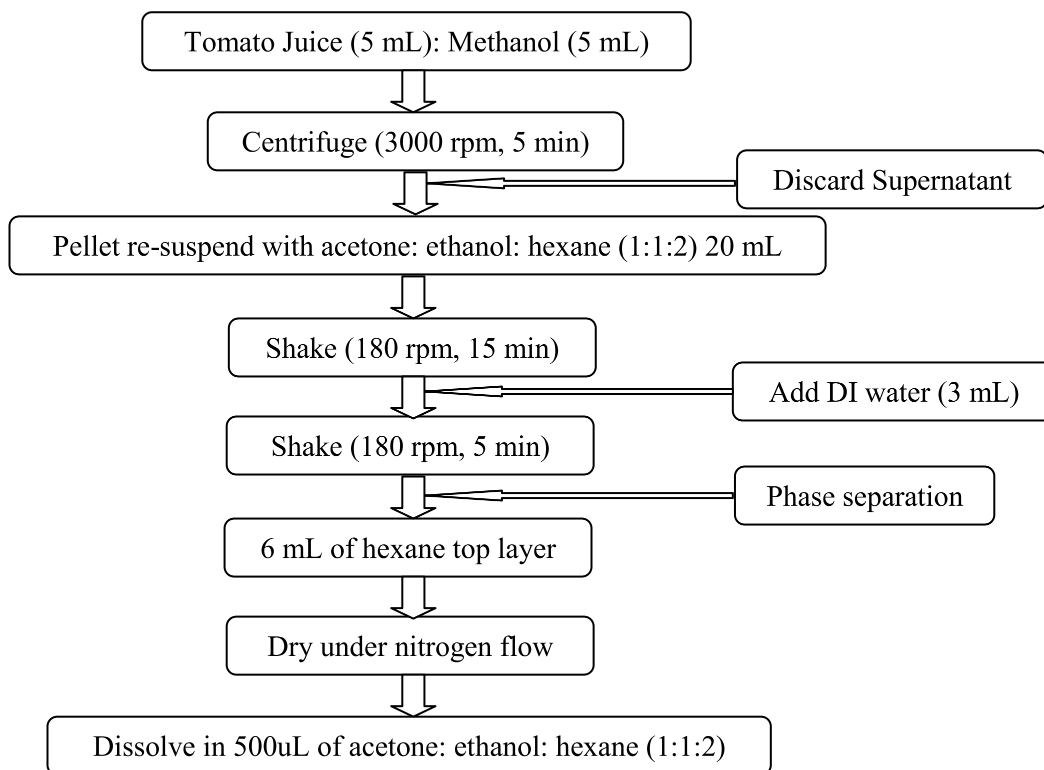
APPENDIX A: SPE SAMPLE PREPARATION METHOD FOR JUICE/VINEGAR SAMPLES



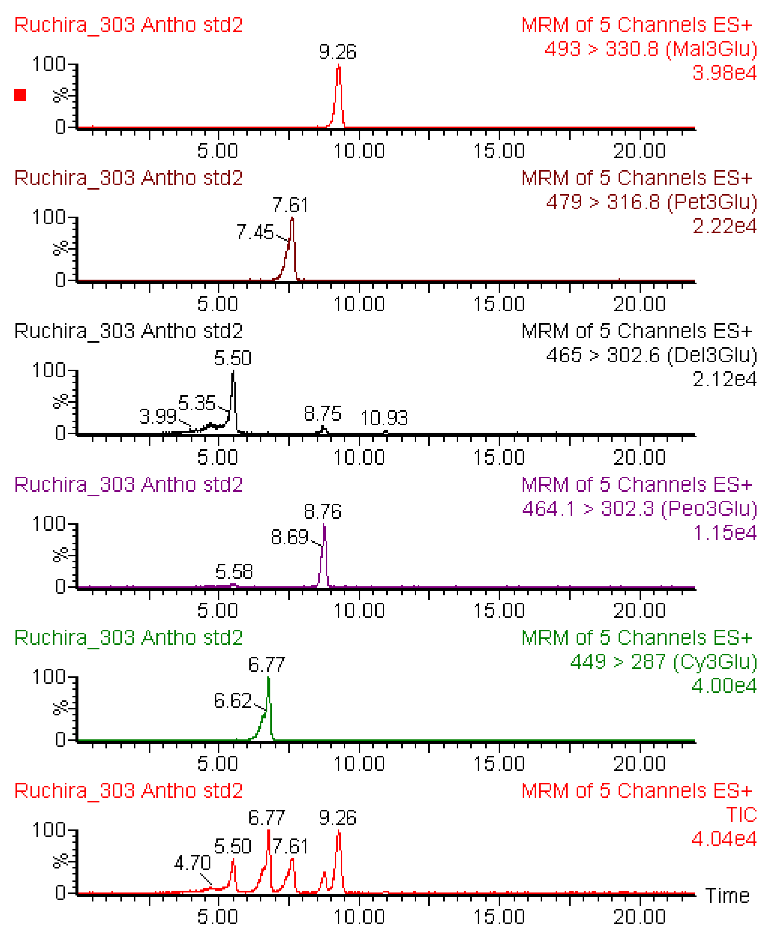
APPENDIX B: SOLVENT PROGRAM (FORMIC ACID IN WATER) FOR UPLC FOR QUANTIFICATION OF NON-ANTHOCYANIN PHENOLICS

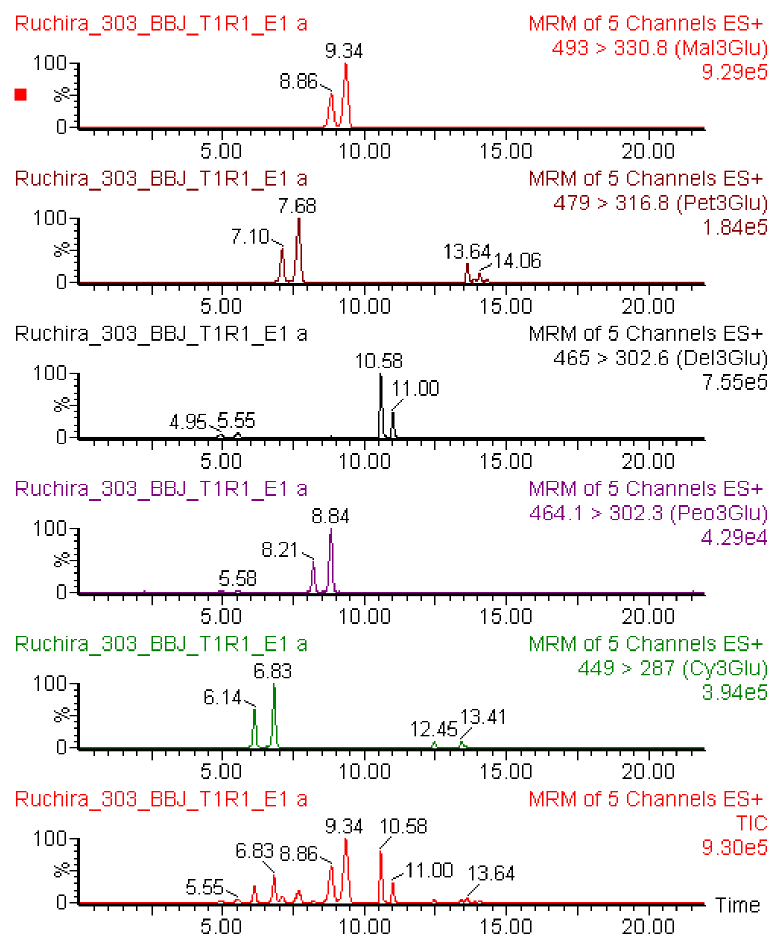


APPENDIX C: EXTRACTION METHODOLOGY FOR LYCOPENE AND BETA-CAROTENE

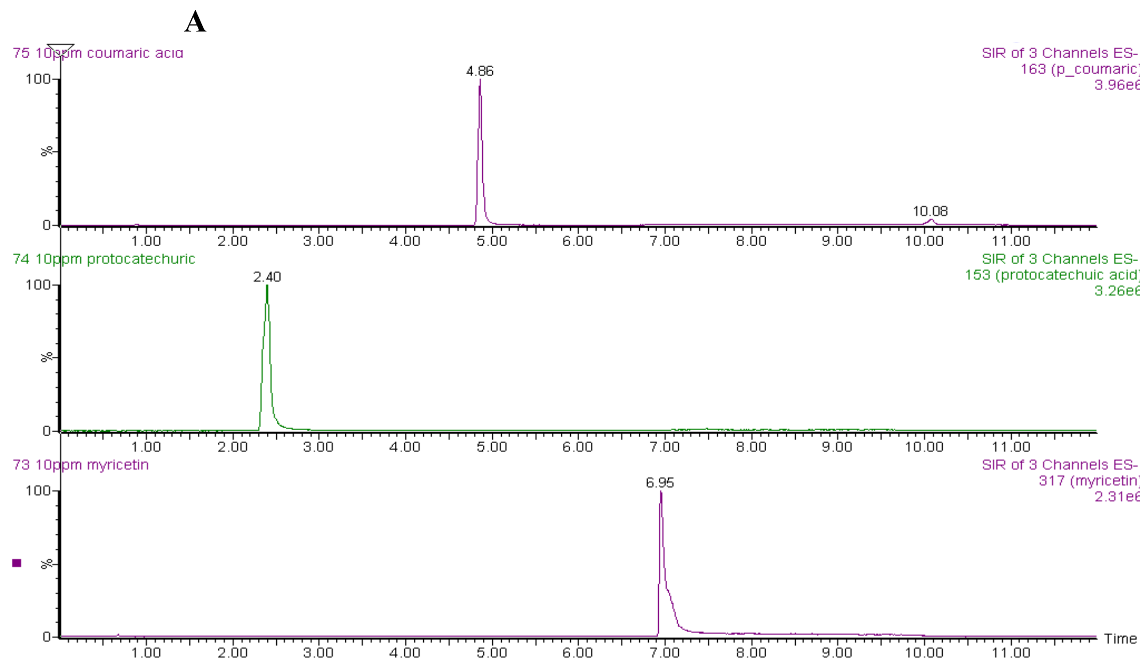


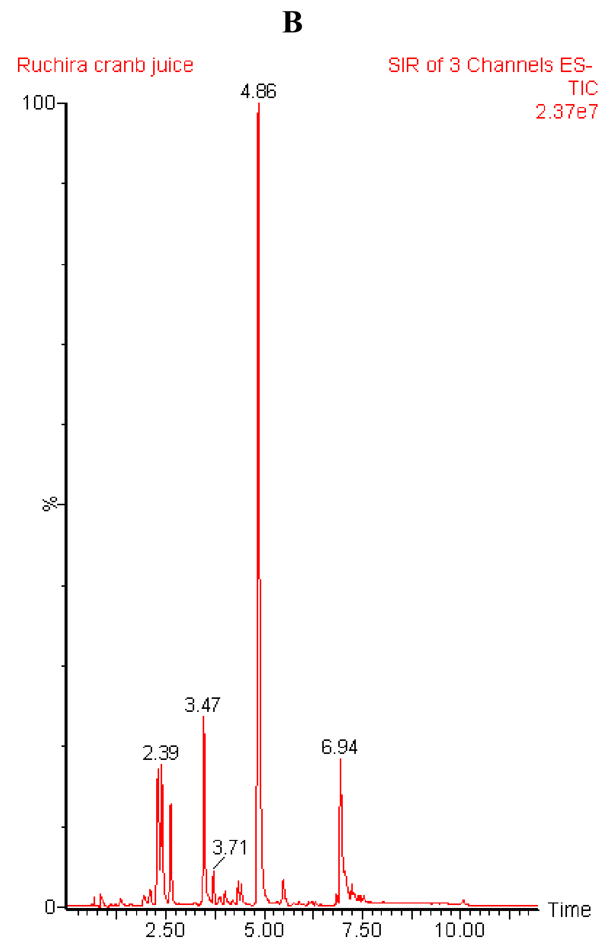
APPENDIX D: MRM CHANNELS OF SOME SELECTED ANTHOCYANIN COMPOUNDS (A) AND A BLUEBERRY JUICE SAMPLE (B)



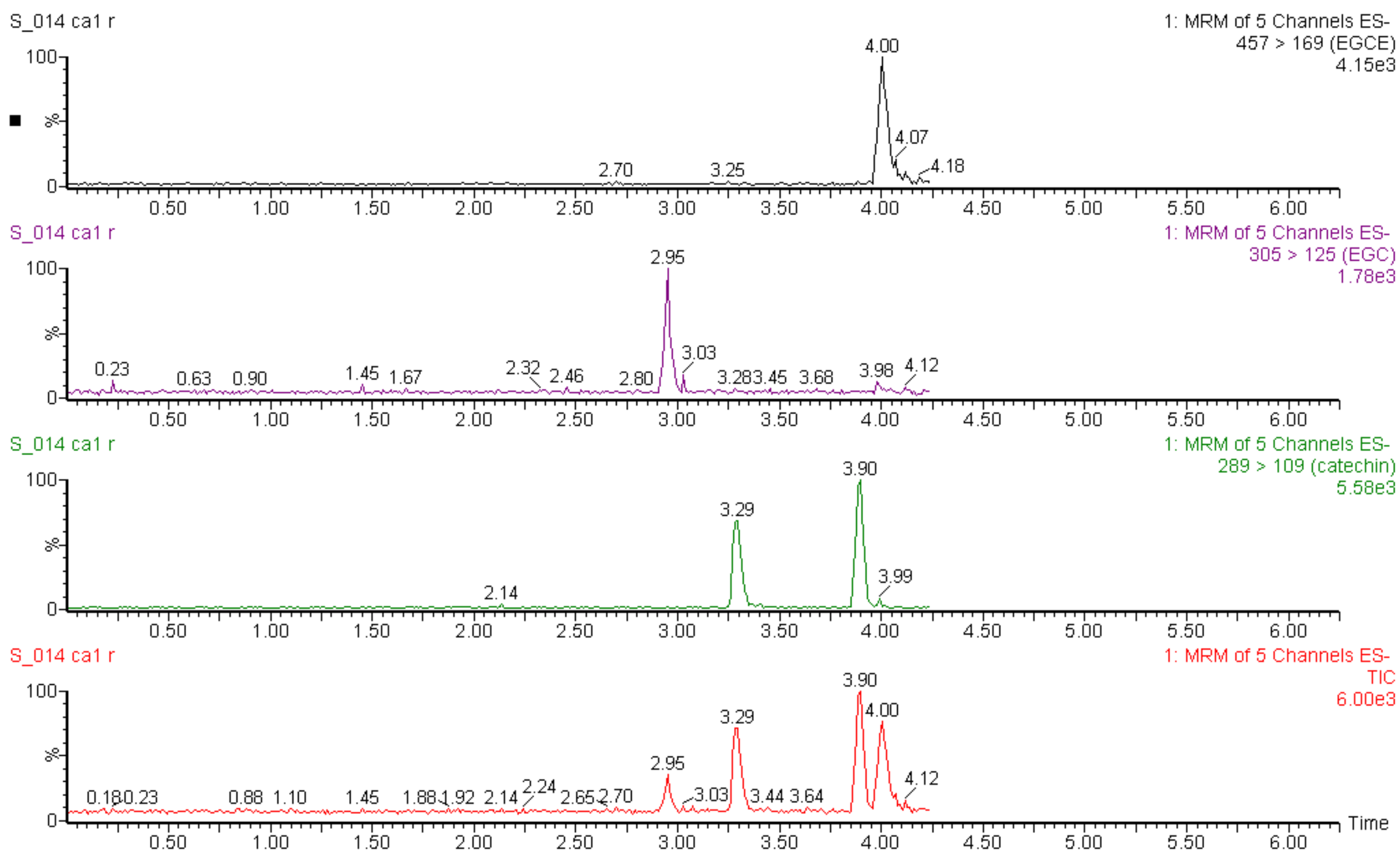


APPENDIX E: SIM CHANNELS OF THREE PHENOLIC COMPOUNDS (A) AND OF A CRANBERRY JUICE SAMPLE (B)

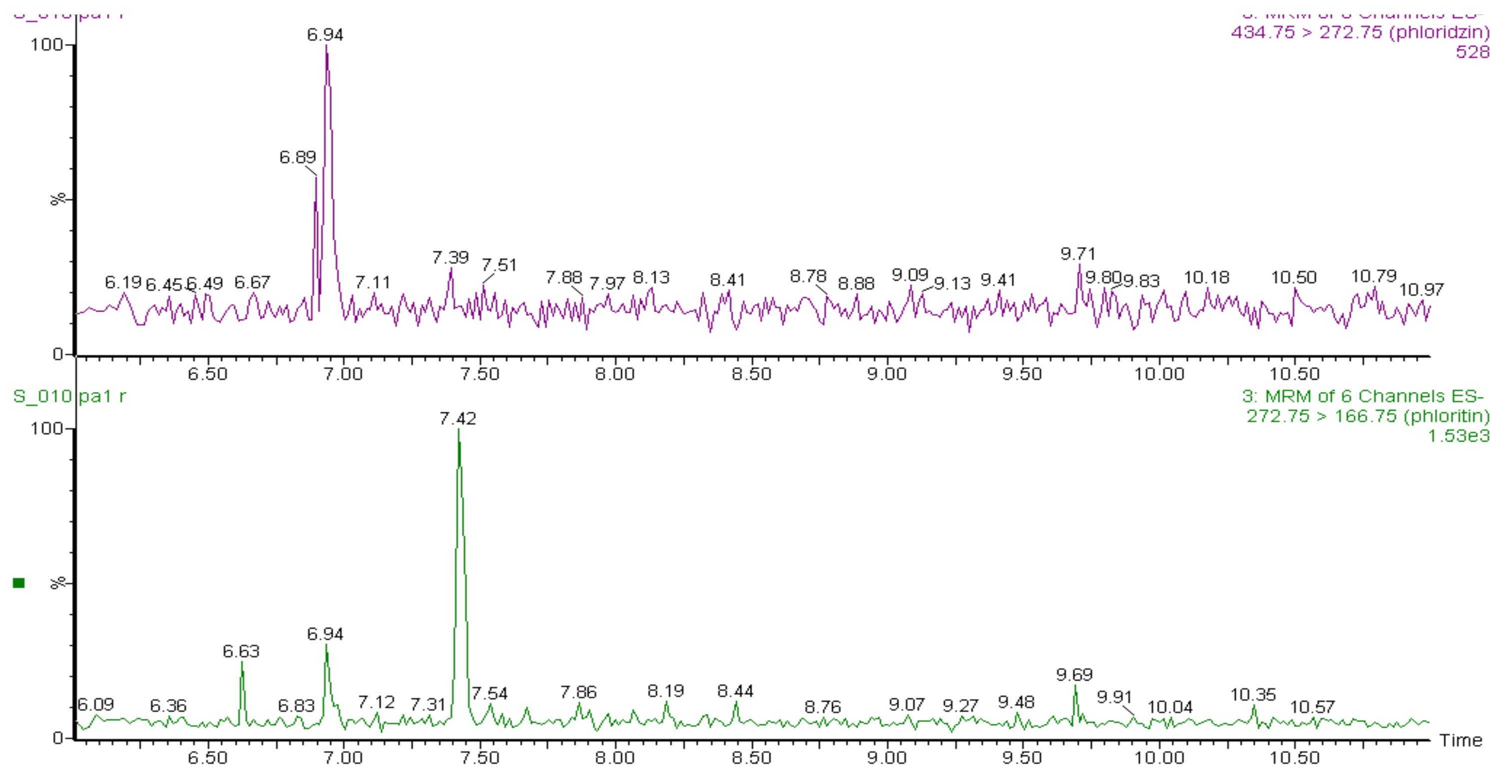




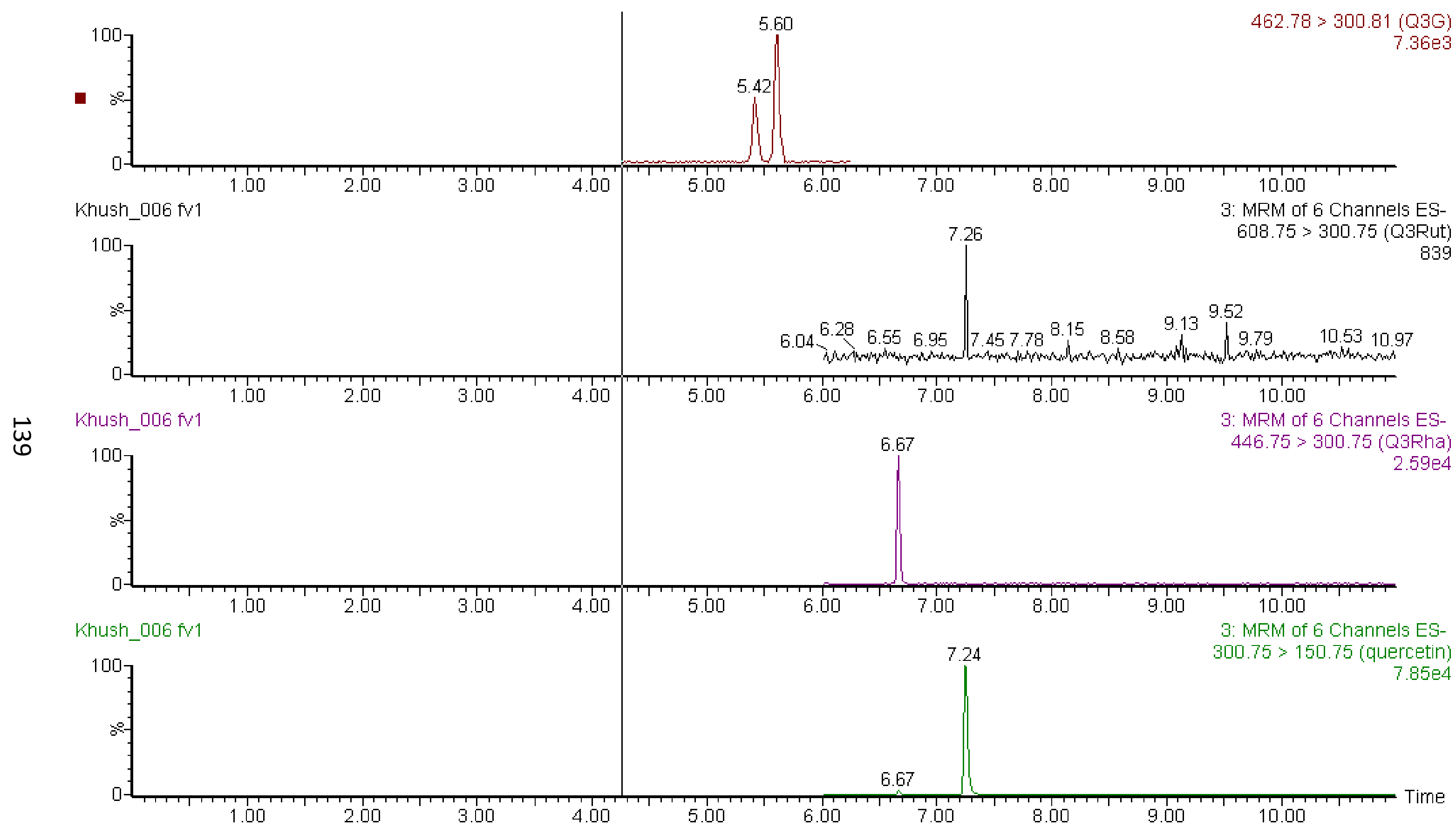
APPENDIX F: MRM CHANNELS AND TIC OF SOME FLAVANOL STANDARDS



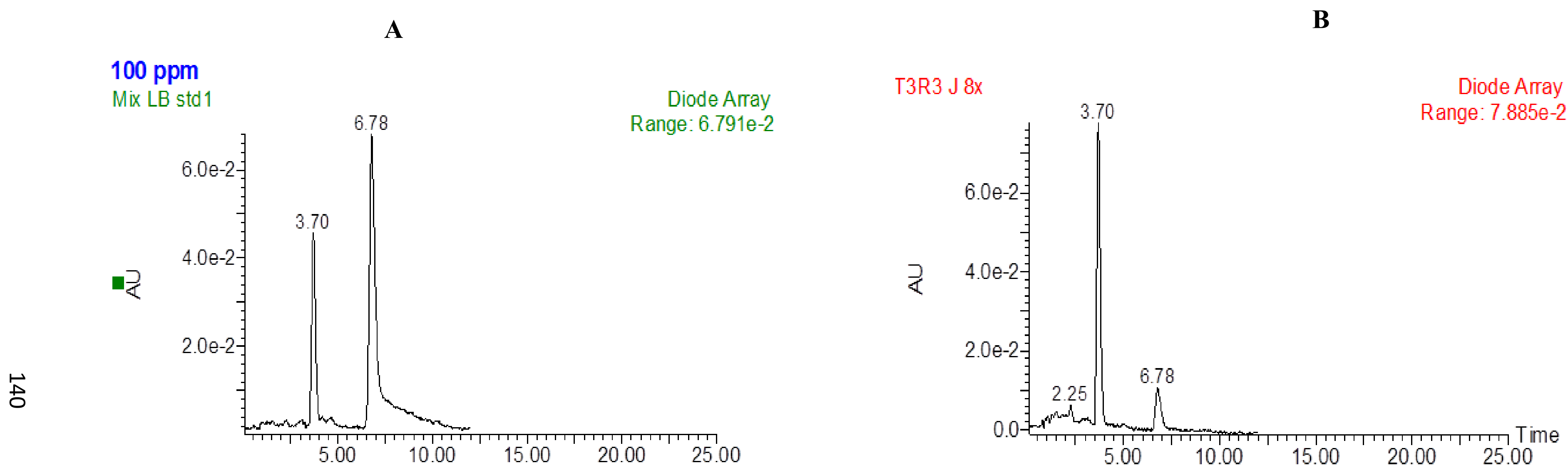
APPENDIX G: MRM CHANNELS OF TWO DIHYDROCHALCONES



APPENDIX H: MRM CHANNELS OF THE MAJOR FLAVONOL COMPOUNDS



APPENDIX I: PDA CHROMATOGRAMS OF LYCOPENE AND CAROTENE STANDARDS (A) AND A TOMATO JUICE SAMPLE



APPENDIX J: APPROVAL LETTER FROM THE RESEARCH ETHICS BOARD

Nova Scotia Agricultural College

Research Ethics Board

P.O. Box 550
Truro, Nova Scotia
Canada B2N 5E3
Ph: 902-893-4413
Fx: 902-893-3430
nsac.ca/reb

November 7, 2011

Dear Ruchira Nandasiri:

Your project entitled, "Antioxidant and Cardioprotective Properties of Fruit Vinegar Beverages" has been approved by the NSAC Research Ethics Board. The Board appreciated your response to its concerns.

There are a few administrative details that you should be aware of:

1) If the methodology or instruments of the study change, then please send the revisions to the Chair of the REB as soon as possible. The Chair will determine if the changes need to be reviewed and approved.

2) If an adverse event occurs such as a violation of privacy or a complaint by a respondent, please inform the REB within a week of the occurrence.

3) Please ensure that you keep a complete record of all material to this project in a secure location accessible for review by the REB or Tri-Council auditors including a copy of the submission, as well as correspondence with and from the REB such as those related to adverse events and amendments. Please also keep copies of the original signed consent forms and the data forms as outlined in the REB submission.

4) You are required to submit an annual and/or final report updating the REB about the progress of the research study. The form on which this report is completed can be found on the REB website.

Best of luck with your research.

Sincerely,

Steven Dukeshire
Chair, NSAC REB

cc Carolyn Terry

NSAC. Embrace Your World.

APPENDIX K: SCORE SHEET FOR SCREENING SESSION

Name (Print): …………………. E-mail: ……………….. Tel. no: …………………

1. Please mention if any, food flavors, smells, or taste that you do not like?

……………………………………………………………………………………………

2. Please evaluate the following products.

Instructions:

Taste samples from left to right in each row. Two of these three samples are identical, the third is different. Identify the different sample and indicate by placing X in the box of the sample. Code numbers are identified on the containers.

Odor

code # --------	**code # --------**	**code # --------**
☐	☐	☐

Bitterness

code # --------	**code # --------**	**code # --------**
☐	☐	☐

Sourness

code # --------	**code # --------**	**code # --------**
☐	☐	☐

Sweetness:

code # --------	**code # --------**	**code # --------**
☐	☐	☐

Comments:…………………………………………………………………………….
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APPENDIX L (1): SCORE SHEET FOR SENSORY ANALYSIS TEST FOR APPLE VINEGAR BEVERAGE

Please select your age range (please put X in the box):

20-30 ☐ 31-40 ☐ 41-50 ☐ above 50 ☐

Please evaluate and score the products using the code numbers identified on the containers for odor, appearance, fruit flavor, acidity level, after taste and overall acceptability.

1.Please rate the following for **odor**:

slight odor very high odor

2. Please rate the following for **acidity**:

slight acidity very high acidity

3. Please rate the following for **aftertaste**:

slight aftertaste very high aftertaste

4.Please rate the following for **overall acceptability**:

slight acceptability very high acceptability

Comments:……………………………………………………………………………………
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APPENDIX L (2): SCORE SHEET FOR SENSORY ANALYSIS TEST FOR BLUEBERRY VINEGAR BEVERAGE

Please select your age range (please put X in the box):

20-30 ☐ 31-40 ☐ 41-50 ☐ above 50 ☐

Please evaluate and score the products using the code numbers identified on the containers for odor, appearance, fruit flavor, acidity level, after taste and overall acceptability.

1.Please rate the following for **odor**:

slight odor — very high odor

2. Please rate the following for **acidity**:

slight acidity — very high acidity

3. Please rate the following for **aftertaste**:

slight aftertaste — very high aftertaste

4.Please rate the following for **overall acceptability**:

slight acceptability — very high acceptability

Comments:………………………………………………………………………………….
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APPENDIX L (3): SCORE SHEET FOR SENSORY ANALYSIS TEST FOR CRANBERRY VINEGAR BEVERAGE

Please select your age range (please put X in the box):

20-30 ☐ 31-40 ☐ 41-50 ☐ above 50 ☐

Please evaluate and score the products using the code numbers identified on the containers for odor, appearance, fruit flavor, acidity level, after taste and overall acceptability.

1.Please rate the following for **odor**:

slight odor — very high odor

2. Please rate the following for **acidity**:

slight acidity — very high acidity

3. Please rate the following for **aftertaste**:

slight aftertaste — very high aftertaste

4.Please rate the following for **overall acceptability**:

slight acceptability — very high acceptability

Comments:……………………………………………………………………………………….
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APPENDIX L (4): SCORE SHEET FOR SENSORY ANALYSIS TEST FOR TOMATO VINEGAR BEVERAGE

Please select your age range (please put X in the box):

20-30 ☐ 31-40 ☐ 41-50 ☐ above 50 ☐

Please evaluate and score the products using the code numbers identified on the containers for odor, appearance, fruit flavor, acidity level, after taste and overall acceptability.

1.Please rate the following for **odor**:

slight odor — very high odor

2. Please rate the following for **acidity**:

slight acidity — very high acidity

3. Please rate the following for **aftertaste**:

slight aftertaste — very high aftertaste

4.Please rate the following for **overall acceptability**:

slight acceptability — very high acceptability

Comments:……………………………………………………………………………………….
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