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Significance of Polyphenol Metabolism in Explaining the Bioactivity of Polyphenol-rich Foods

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ABSTRACT

Dietary polyphenols are non-essential phytonutrients that are extensively metabolized upon consumption. Based on our previous studies, we hypothesized that the bioavailability of polyphenol metabolites is more important than unmetabolized polyphenols for the health-promoting effects of polyphenol-rich foods. We tested this hypothesis with three objectives. First we developed a more complete polyphenol profile for almond, by characterizing its tannins and stilbenes. Tannins and stilbenes are extensively metabolized prior to or upon absorption. In almond, hydrolysable tannin content was 54.7 ± 2.3 mg ellagic acid and 27.4 ± 7.3 mg gallic acid per 100 g almond among varieties. The stilbene resveratrol-3-*O*-glucoside was identified in almond extracts, with 7.19–8.52 µg/100 g almond. These results suggested that almonds have a large pool of polyphenols that would be extensively metabolized in the colon prior to absorption. Second, we determined the efficiency by which aronia berry polyphenols are metabolized in an acute pharmacokinetic study in humans. In this experiment, polyphenol metabolites constituted at least 99% of the total polyphenol pool measured in plasma and urine. Third, we determined the association between aronia polyphenol metabolites and changes in key biomarkers of CVD

risk in an intervention study. Former smokers, which were at greater risk for CVD, consumed aronia berry extract for 12 wk. Aronia consumption reduced fasting plasma total cholesterol (TC) level by 8% during the 12-week treatment compared with placebo group, and reduced plasma LDL-C level by 7% and 11% at both 6 wks and 12 wks compared with placebo group. In addition, participants in the aronia group who showed the largest decreases in TC had stronger correlations of urinary peonidin-3-*O*-galactoside and cyanidin-3-*O*-galactoside than those who did not respond to the intervention. These results suggested that increases in polyphenol metabolites were more highly correlated with reduced biomarkers of chronic disease than parent polyphenols. Thus, this study demonstrated that the cholesterol-lowering activity of polyphenol-rich foods is associated in-part with its tissue metabolites.

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APPROVAL PAGE

Doctor of Philosophy Dissertation

Significance of Polyphenol Metabolism in Explaining the Bioactivity of Polyphenol-rich Foods

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Table of Content

APPROVAL PAGE iii
ACKNOWLEDGEMENTS iv
List of Tables ix
List of Figures x
List of Abbreviations xii
Chapter 1: Introduction 1
1.1 Overview 2
1.2 Central Hypothesis and Specific Aims 2
1.2.1 Objective 1: Characterize the tannin and stilbene composition of almonds. 3
1.2.2 Objective 2: Determine the pharmacokinetics of aronia berry polyphenols and its metabolites. 5
1.2.3 Objective 3: Determine the extent to which polyphenol metabolites are associated with changes in plasma cholesterol in former smokers after 12 wk of chronic consumption of aronia berry extract. 6
1.3 References 8
Chapter 2: Literature Review 11
2.1 Introduction 12
2.2 Polyphenol variety and consumption 12
2.3 Polyphenol absorption 16
2.4 Polyphenol distribution 20
2.5 Polyphenol metabolism 21
2.6 Polyphenol excretion 23
2.7 Polyphenols and CVD Risk 24
2.7.1 Anthocyanins 27
2.7.2 Proanthocyanidins 31
2.8 Conclusion 34
2.9 References 35
Chapter 3: Characterize the tannin and stilbene composition of almonds 47
3.1 Abstract 49
3.2 Introduction 50
3.3 Materials and Methods 51
3.3.1 Reagents and Materials 51
3.3.2 Extraction of Proanthocyanidins 51
3.3.3 Acid and Alkaline Hydrolysis 52
3.3.4 Isolation of Tannins 53
3.3.5 HPLC Analysis of Proanthocyanidins 53
3.3.6 Colorimetric Determination of Total Phenols and Proanthocyanidins 54
3.3.7 Thin-layer Chromatography (TLC) of Hydrolyzable Tannins 54
3.3.8 Gallotannin and Ellagitanin Hydrolysis 55

3.3.9 HPLC Analysis of Gallic Acid and Ellagic Acid 55
3.3.10 Statistics and Data Analysis 56
3.4 Results 56
3.4.1 Extractable Proanthocyanidins 57
3.4.2 Bound Proanthocyanidins 57
3.4.3 Total Proanthocyanidins 58
3.4.4 Hydrolysable tannins 59
3.5 Discussion 59
3.5.1 Extractable Proanthocyanidins 59
3.5.2 Bound Proanthocyanidins 61
3.5.3 Total Proanthocyanidins 62
3.5.4 Hydrolyzable Tannins 62
3.6 Conclusion 63
3.7 Chapter 3 Tables and Figures 65
3.8 References 74
Chapter 4: Characterize the tannin and stilbene composition of almonds 78
4.1 Abstract 80
4.2 Introduction 81
4.3 Materials and methods 82
4.3.1 Chemicals and plant materials 82
4.3.2 Extraction of almond stilbenes 83
4.3.3 Clean-up of extract 83
4.3.4 UHPLC-MS analysis of stilbenes 84
4.3.5 Enzymatic hydrolysis of stilbene glucosides 85
4.3.6 Statistical analysis 85
4.4 Results 86
4.4.1 Method validation 86
4.4.2 Identification of almond stilbenes 87
4.4.3 Polydatin identification and quantitation in almond. 88
4.4.4 Compartmentalization of almond stilbenes 88
4.5 Discussion 89
4.6 Conclusions 92
4.7 Chapter 4 Tables and Figures 93
4.8 References 99
Chapter 5: Bioavailability of anthocyanins and colonic polyphenol metabolites following consumption of aronia berry extract 102
5.1 Abstract 103
5.2 Introduction 104
5.3 Materials and methods 105
5.3.1 Reagents and materials 105
5.3.2 Study participants and institutional approval 105
5.3.3 Inclusion criteria for study participants 106
5.3.4 Study design 106
5.3.5 Analysis and composition of aronia extract 107
5.3.6 Sample clean-up 107
5.3.7 UHPLC-MS analysis 108

5.3.8 Determination of recovery 109
5.3.9 Creatinine analysis 109
5.3.10 Pharmacokinetic and Statistical Analysis 110
5.4 Results 110
5.4.1 Analysis of supplement 110
5.4.2 UHPLC-MS conditions for polyphenols in plasma and urine 110
5.4.3 Determination of anthocyanins and metabolites in plasma samples 111
5.4.4 Determination of anthocyanins and metabolites in urine samples 112
5.4.5 Between-subjects differences 113
5.4.6 Correlation Analysis 113
5.5 Discussion 114
5.6 Conclusions 118
5.7 Chapter 5 Tables and Figures 119
5.8 References 132
Chapter 6: Aronia berry polyphenol consumption reduces plasma LDL and total cholesterol in former smokers without lowering biomarkers of inflammation or oxidative stress 136
6.1 Abstract 137
6.2 Introduction 139
6.3 Materials and methods 140
6.3.1 Participants 140
6.3.2 Study design 141
6.3.3 Preparation of biological samples 142
6.3.4 Anthropometric measurements 143
6.3.5 Plasma lipid analysis 144
6.3.6 Analysis of inflammatory biomarkers 144
6.3.7 Analysis of biomarkers related to oxidative status 144
6.3.8 Analysis of plasma biomarkers of vascular reactivity 145
6.3.9 Gene expression analysis by quantitative realtime PCR (qRT-PCR) 145
6.3.10 Western blot analysis 146
6.3.11 Urinary creatinine analysis 146
6.3.12 Plasma and urinary polyphenol analysis 146
6.3.13 Statistics 147
6.4 Results 147
6.4.1 Participant characteristics 147
6.4.2 Anthropometric measurements 148
6.4.3 Plasma lipids 148
6.4.4 Expression of hepatic genes involved in lipid metabolism 149
6.4.5 Other biomarkers relevant to CVD risk 149
6.4.6 Urinary and plasma polyphenols 149
6.4.7 Association of urinary polyphenols and lipids changes in aronia group 150
6.4.8 Analysis by respondent to total cholesterol 151
6.5 Discussion 152
6.6 Conclusions 157
6.7 Chapter 6 Tables and Figures 158
6.8 Reference 175
Chapter 7: Conclusion 181

7.1 References 190
Appendix 193
A.1 Appendix Materials for Chapter 3 194
A.2 Appendix Materials for Chapter 6 196

List of Tables

Table 2.1 Main classes of dietary polyphenols and their sources. 14
Table 2.2 Estimates of dietary polyphenol intake. 15
Table 3.1 Proanthocyanidin content and the extractable proanthocyanidin proportion of California almond cultivars.....65
Table 3.2 Hydrolyzable tannin content of California almond cultivars determined by methanolysis and HPLC analysis.....66
Table 3.3 Two dimensional TLC profiles of almond tannins. 67
Table 4.1 UHPLC-MS chromatography of various stilbene standards including instrument settings, limits of detection (LOD), and limits of quantification (LOQ). 93
Table 4.2 Stilbene content of whole almonds and recovery of spiked standards determined by UHPLC-MS analysis. 94
Table 4.3 Stilbene content and proportion of California almond cultivars. 95
Table 5.1 Characteristics of participant population. 119
Table 5.2 Optimized MS instrument parameters for UHPLC analysis of polyphenols from plasma and urine. 120
Table 5.3 Polyphenol retention time (RT), m/z ratio, LOD, LOQ and recovery rates of standards of optimized UHPLC-MS methods. 121
Table 5.4 Polyphenol composition of aronia supplement. 122
Table 5.5 Pharmacokinetic parameters of anthocyanins and metabolites in plasma and urine samples in n = 6 individuals consuming 500 mg aronia extract. 124
Table 6.1 Non-polyphenol contents analysis in aronia berry supplement 158
Table 6.2 Baseline characteristics of study population 159
Table 6.3 Changes in plasma lipids in former smokers after daily consumption of 500 mg aronia extract or a placebo 161
Table 6.4 Changes in anthropometric measures following of consumption of placebo or 500 mg aronia extract/d for 12 wk 162
Table 6.5 Changes in peripheral blood mononuclear cell gene and protein expression, and biomarkers of inflammation and antioxidant function following of consumption of placebo or 500 mg aronia extract per day for 12 wk 163
Table 6.6 Overnight urinary polyphenols and metabolites at baseline and 12 weeks following daily consumption of 500 mg aronia berry extract or placebo. 167
Table 6.7 Fasting plasma polyphenols and metabolites at baseline, 6 weeks, and 12 weeks following daily consumption of 500 mg aronia berry extract or placebo. 169
Table 7.1 Metabolic fates of polyphenols 182

List of Figures

Figure 1.1 The overall thesis hypothesis. 3
Figure 1.2 Objective 1 hypothesis 4
Figure 1.3 Objective 2 hypothesis 5
Figure 2.1 Chemical structure of representative polyphenol. 13
Figure 2.2 Potential mechanisms of polyphenol glycoside absorption in small intestine. 17
Figure 2.3 Pathway of polyphenol distribution. 21
Figure 2.4 The mechanism of polyphenol against CVD in mRNA level 27
Figure 3.1 A. Representative HPLC fluorescence chromatogram of solvent-extractable almond proanthocyanidin fraction. B. Representative HPLC fluorescence chromatogram of base hydrolyzed almond proanthocyanidin fraction. 69
Figure 3.2 A. Relative extractable proanthocyanidin profile of California almond genotypes. 70
Figure 3.3 Proanthocyanidin content of extracted and hydrolyzed residues from California almond genotypes determined by the DMAC (4-(dimethylamino)cinnamaldehyde) assay. 71
Figure 3.4 A. Representative HPLC fluorescence chromatogram of almond gallic acid fraction. B. Representative HPLC fluorescence chromatogram of almond ellagic acid fraction. 72
Figure 3.5 Recovery of proanthocyanidins following A. acidic and B. alkaline hydrolysis of defatted almond powder. 73
Figure 4.1 Representative UHPLC-MS chromatograms of whole almond extract following solid phase extraction clean-up or standard compounds following a 1 µL injection. 96
Figure 4.2 Representative UHPLC-MS chromatogram of a 1 µL injection of almond extract following solid phase extraction clean-up and before or after enzymatic hydrolysis with β-glucosidase 97
Figure 4.3 Representative UHPLC-MS chromatograms at m/z 389 (-) and 227 (-) of 0.4 ng polydatin on column at increasing Q-array DC voltages. 98
Figure 5.1 Proposed metabolic pathway of aronia polyphenols determined in the present study. Red lines represent colonic catabolic steps, while blue lines represent tissue metabolism. 126
Figure 5.2 Plasma concentrations of (A) cyanidin-3-*O*-glucoside, (B) peonidin-3-*O*-galactoside, (C) protocatechuic acid, (D) 3-(4-hydroxyphenyl)propionic acid, and (E) Hippuric acid, following consumption of 500 mg of a polyphenol-rich aronia supplement. 127
Figure 5.3 Cumulative urinary excretion of aronia polyphenols and catabolites following consumption of 500 mg of a polyphenol-rich aronia supplement. 129
Figure 5.4 Chromatograms of (A) polyphenol standards and (B) representative plasma samples. 130
Figure 5.5 Chromatograms of (A) polyphenol standards and (B) representative urine samples. 131
Figure 6.1 Flowchart of enrollment. 171
Figure 6.2 Principle component analysis correlation of urinary polyphenol and lipid changes A) loading plot and B) correlation coefficients of participants in the aronia extract supplemented group. 172
Figure 6.3 Principle component analysis correlation of urinary polyphenol and lipid changes A) loading plot and B) correlation coefficients of participants in the placebo group. 173

Figure 6.4 Principle component analysis of urinary polyphenol change in aronia supplement group A) responder and B) non-responder. 174

List of Abbreviations

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid

ACE: Angiotensin converting enzyme

ARE: Antioxidant response element

AUC: Area under the curve

BCRP: Breast cancer resistance protein

BP: Blood pressure

CAT: Catalase

CBG: Cytosolic β-glucosidase

CE: (+)-catechin equivalents

COMT: Catechol-O-methyltransferase

COX-2: Cyclooxygenase

CRP: C-reactive protein

CVD: Cardiovascular disease

CYPs: Cytochrome P450 monooxygenases

DMAC: 4-(dimethylamino)cinnamaldehyde

DP: Degree of polymerization

ECG: (-)-epicatechin-3-gallate

eNOS: Endothelial nitric oxide synthase

EROD: Ethoxyresorufin-O-deethylase

FMD: Flow-mediated vasodilation

GAE: Gallic acid equivalents

GSH-Px: Glutathione peroxidase

GST: Glutathione S-transferase

HDL-C: High-density lipoprotein cholesterol

HMGR: 3-hydroxy-3-methyl-glutaryl coenzyme A reductase

hsCRP: High-sensitivity C reactive protein

ICAM-1: Intercellular adhesion molecule-1

IKK: IκB kinase

IL-1β: Interleukin-1β

IL-6: Interleukin-6

iNOS: Inducible nitric oxide synthase

JNK: Jun amino-terminal kinases

LDL: low-density lipoprotein

LDL-C: Low-density lipoprotein cholesterol

LDLR: LDL receptor

LOD: Limit of detection

LOQ: Limit of quantification

LOX-1: Lipoprotein receptor-1

LPH: Lactase phlorizin hydrolase

MAPKs: p38 mitogen-activated protein kinases

MCP-1: Monocyte chemotactic protein-1

MCT: Monocarboxylic acid transporter

MMP: Matrix metalloproteinase

MRP: Multidrug-associated protein

MROD: Methoxyresorufin-O-demethylase

NF-κB: Nuclear factor-κB

NO: Nitric oxide

NQO1: NAD(P)H:quinone oxidoreductase

ox-LDL: Oxidized LDL

PACs: Proanthocyanidins

PBMCs: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PCSK9: Proprotein convertase subtilisin/kexin type 9

PNPH: P-nitrophenol hydroxylase

RE: Response elements

RSD: Relative standard deviation

RT: Retention time

SGLT: Sodium–glucose co-transporters

SMCs: Smooth muscle cells

SOD: Superoxide dismutase

SPE: Solid phase extraction

SULT: Sulfotransferase

TC: Total cholesterol

TNF-α: Tumor necrosis factor-α

TRAF-2: TNF receptor-associated factor 2

UDP-GT: UDP-glucuronosyltransferase

VCAM-1: Vascular cell adhesion molecule-1

VEGF: Vascular endothelial growth factor

VLA-4: Very late activation antigen-4

Chapter 1: Introduction

1.1 Overview

Increasing polyphenol consumption can improve dyslipidemia, inhibit inflammation, and reduce oxidative stress in individuals with increased cardiovascular disease (CVD) risk (Vita 2005, Rahman, Biswas et al. 2006, Chiva-Blanch, Urpi-Sarda et al. 2012, Annuzzi, Bozzetto et al. 2014). Polyphenols are extensively metabolized upon consumption, leading to a greater proportion of metabolites than parent compounds in tissues and circulation (Manach, Williamson et al. 2005). Even minor changes to a polyphenol structure can lead to a significant change in its biological activity (Heim, Tagliaferro et al. 2002). Thus, it is important to determine the polyphenol profile of foods and their metabolic fate to explain the mechanisms by which polyphenol consumption reduces CVD risk.

1.2 Central Hypothesis and Specific Aims

The prevailing approach to determining polyphenol efficacy has been to screen polyphenols *in vitro* (e.g. antioxidant activity, enzyme inhibition) or in cell culture to determine it relative potency or mechanism(s) of action. However, this approach may need to be re-evaluated for many dietary polyphenols. Polyphenols are extensively metabolized (e.g. flavonoids, phenolic acids) or have limited bioavailability (e.g. tannins). Thus, the overall hypothesis for this study was that the sum of polyphenol metabolites is more responsible than polyphenol parental compounds for exerting bioactivity after consumption (**Figure 1.1**). In order to test this hypothesis, we pursued three objectives: 1) characterize the polyphenol profile (e.g. metabolic availability) of almonds; 2) define polyphenol pharmacokinetics (e.g. metabolic efficiency) of aronia berry polyphenols; 3) determine the extent efficacy is associated with metabolism after chronic supplementation of aronia polyphenols.

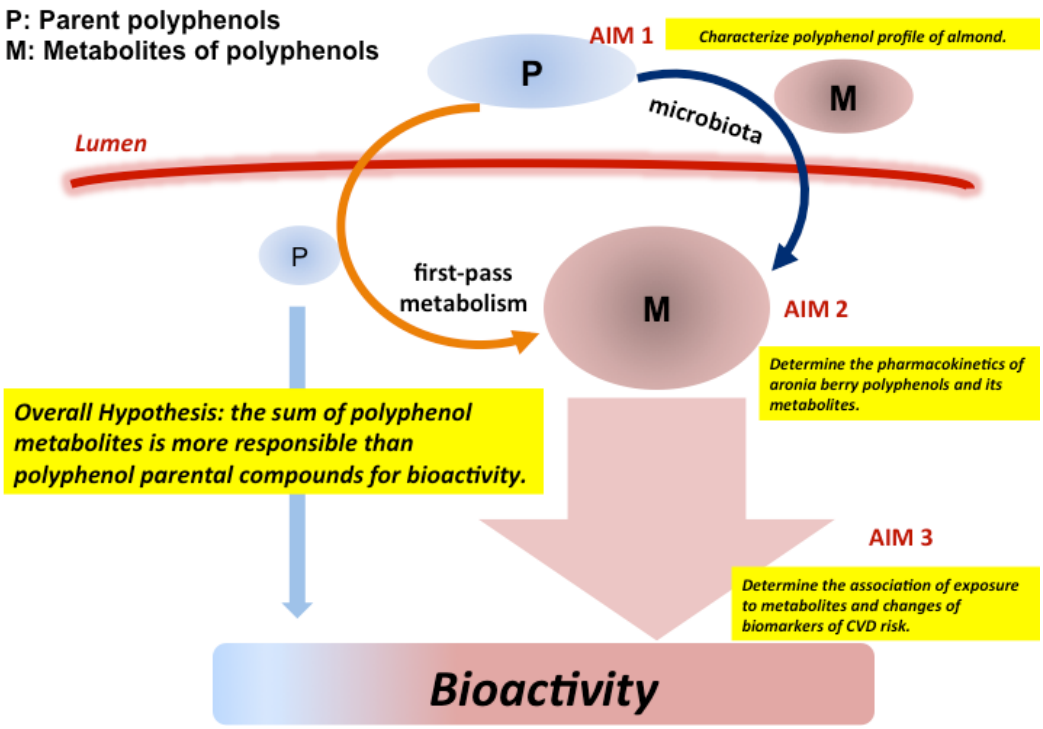

Figure 1.1 The overall thesis hypothesis.

1.2.1 Objective 1: Characterize the tannin and stilbene composition of almonds.

Almond, among other nuts, is a rich source of dietary polyphenols containing phenolic acids, flavonoids, and lignans (Smeds, Eklund et al. 2007, Bolling, Dolnikowski et al. 2010). Despite extensive research on almond flavonoids, little is known about its other polyphenols, especially tannins and stilbenes. Proanthocyanidins (PACs) may be the most abundant class of polyphenols in almonds (Bolling, Chen et al. 2011). The PAC content of foods might be underestimated because solvent extraction does not completely liberate polyphenols covalently bound to cell walls (Arranz, Saura-Calixto et al. 2009). Flavonoids, phenolic acids, and PACs could be released by acid or alkaline hydrolysis from other plant materials (Arranz, Saura-Calixto et al. 2009, White, Howard et al. 2010). Thus, more work is needed to determine the relative proportion of free and bound PACs in almonds. A previous study reported almond

contained little to no gallotannins and ellagitannins following hydrolysis (Molyneux, Mahoney et al. 2008). However, this study employed a moderate hydrolysis condition, which might not liberate these tannins. Furthermore, the content and distribution of stilbenes in almonds was unknown. Stilbenes have been reported to have a cardioprotective potential in *in vivo* studies (Baur and Sinclair 2006). The unknown content of tannins and stilbenes might comprise a potentially large pool of polyphenols that are extensively metabolized upon consumption.

The hypothesis for Objective 1 was that most almond polyphenols are extensively metabolized prior to absorption (**Figure 1.2**). We developed qualitative and quantitative methods to more completely characterize almond tannins and stilbenes. Furthermore, we determined the compartmentalization of almond stilbenes, as almonds are consumed whole or blanched. Almond genotype also affects polyphenol profile (Milbury, Chen et al. 2006, Bolling, Dolnikowski et al. 2010). Thus, we characterized tannins and stilbenes in the top three cultivated California almond genotypes. This work is potentially applicable for other types of foods that contain a large portion of bound polyphenols.

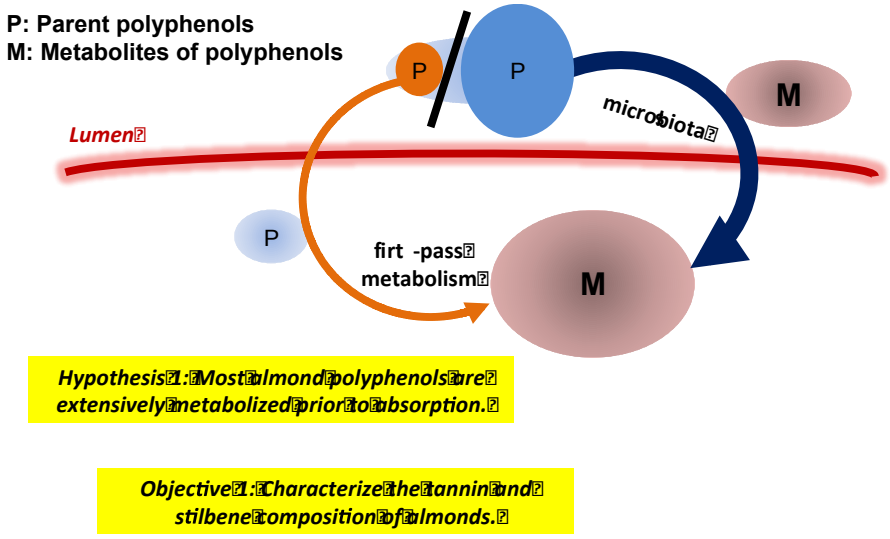

Figure 1.2 Objective 1 hypothesis

1.2.2 Objective 2: Determine the pharmacokinetics of aronia berry polyphenols and its metabolites.

Previous studies on the pharmacokinetics of anthocyanin-rich extracts have provided limited information about the extent of polyphenol colonic catabolism. Chokeberry (aronia berry) is rich in polyphenol antioxidants, such as PACs and anthocyanins (Taheri, Connolly et al. 2013). PACs and anthocyanins may be extensively metabolized by phase II conjugation reactions (e.g. glucuronidation, sulfation, methylation) or catabolized to smaller phenolic compounds by gut microbiota. The hypothesis for Objective 2 was that the rate of first-pass metabolism and microbiota catabolism creates a larger pool of metabolites than bioavailable parent compounds from aronia berry extract *in vivo* (**Figure 1.3**). To test this hypothesis, we quantitated the major aronia berry polyphenols and metabolites in plasma and urine following consumption of aronia berry extract. We then determined pharmacokinetic parameters for metabolites and parent compounds.

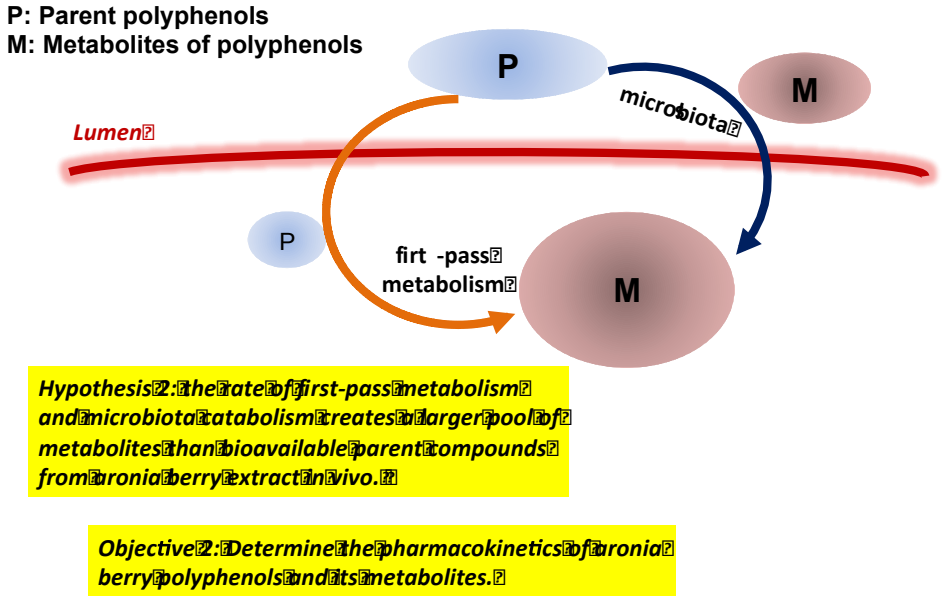

Figure 1.3 Objective 2 hypothesis

1.2.3 Objective 3: Determine the extent to which polyphenol metabolites are associated with changes in plasma cholesterol in former smokers after 12 wk of chronic consumption of aronia berry extract.

Smoking increases CVD risk (Ambrose and Barua 2004). Although smoking cessation may curve the risk, CVD risk remains elevated for 10-15 years in former smokers compared with never-smokers (Kawachi, Colditz et al. 1993, Iso, Date et al. 2005). Consumption of a polyphenol-rich aronia berry extract might be a promising intervention for reducing CVD risk in former smokers. Prior human intervention studies indicated aronia berry consumption lowers cholesterol and reduces oxidative stress in other populations with increased CVD risk (Naruszewicz, Łaniewska et al. 2007, Broncel M 2010). Despite these studies, the compounds responsible for these effects remain uncharacterized.

We hypothesized that following consumption of aronia berry polyphenols, increases in polyphenol metabolites will more strongly correlate to reductions in plasma total cholesterol than increases in unmetabolized polyphenols. Thus, the metabolites would be more responsible for reduction in CVD risk in former smoker than parent compounds. Protocatechuic acid, a principal metabolite of anthocyanins could reduce serum total cholesterol level in diabetic mice (Lin, Huang et al. 2009). Protocatechuic acid was suggested to reduce serum total cholesterol level by increasing the hepatic LDL receptor, apo E, lecithin-cholesterol acyltransferase and hepatic triglyceride lipase expression in rats (Tamura, Fukushima et al. 2004). Thus, our working hypothesis for this objective was that plasma and urinary protocatechuic acid will be highly correlated to reductions in total cholesterol after aronia berry supplementation. To test this hypothesis, we determined metabolites and polyphenols in plasma and urine of former smokers consuming aronia berry extract or a placebo. We used the methods developed in Objective 2 to determine metabolites of PACs and anthocyanins in urine and plasma of participants consuming

aronia berry extract. With these data, we determined the association between metabolites of PACs and anthocyanins and biomarkers of lipid metabolism.

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Chapter 2: Literature Review

2.1 Introduction

Polyphenols are a class of organic compounds characterized by phenol structural units. Polyphenols are abundant in tea, fruits, vegetables, and grains. Increased polyphenol consumption may reduce chronic inflammation, improve antioxidant defenses, modulate lipid metabolism, and inhibit cell-signaling pathways. Epidemiological and intervention studies have associated increased consumption of polyphenol-rich foods or beverages with lower risk of chronic disease, such as cancer, cardiovascular diseases (CVD), Alzheimer's, diabetes and metabolic syndrome. Polyphenol bioactivity is limited by their absorption, distribution, metabolism and excretion (ADME). In this chapter, dietary polyphenols and their ADME will be presented with a focus on linking these to their putative ability to reduce biomarkers of CVD risk.

2.2 Polyphenol variety and consumption

More than 8000 naturally occurring polyphenols have been identified, many which are present in foods. The main classes of dietary polyphenols include phenolic acids, flavonoids, stilbenes, lignans, and tannins, among others (**Figure 2.1**). Phenolic acids can be divided into hydroxybenzoic acids and hydroxycinnamic acids. Flavonoids can be divided into 6 classes, which are anthocyanins, flavonols, flavones, flavanones, isoflavones, and flavan-3-ols. Tannins include both hydrolysable and condensed tannins which are also known as proanthocyanidins (PACs).

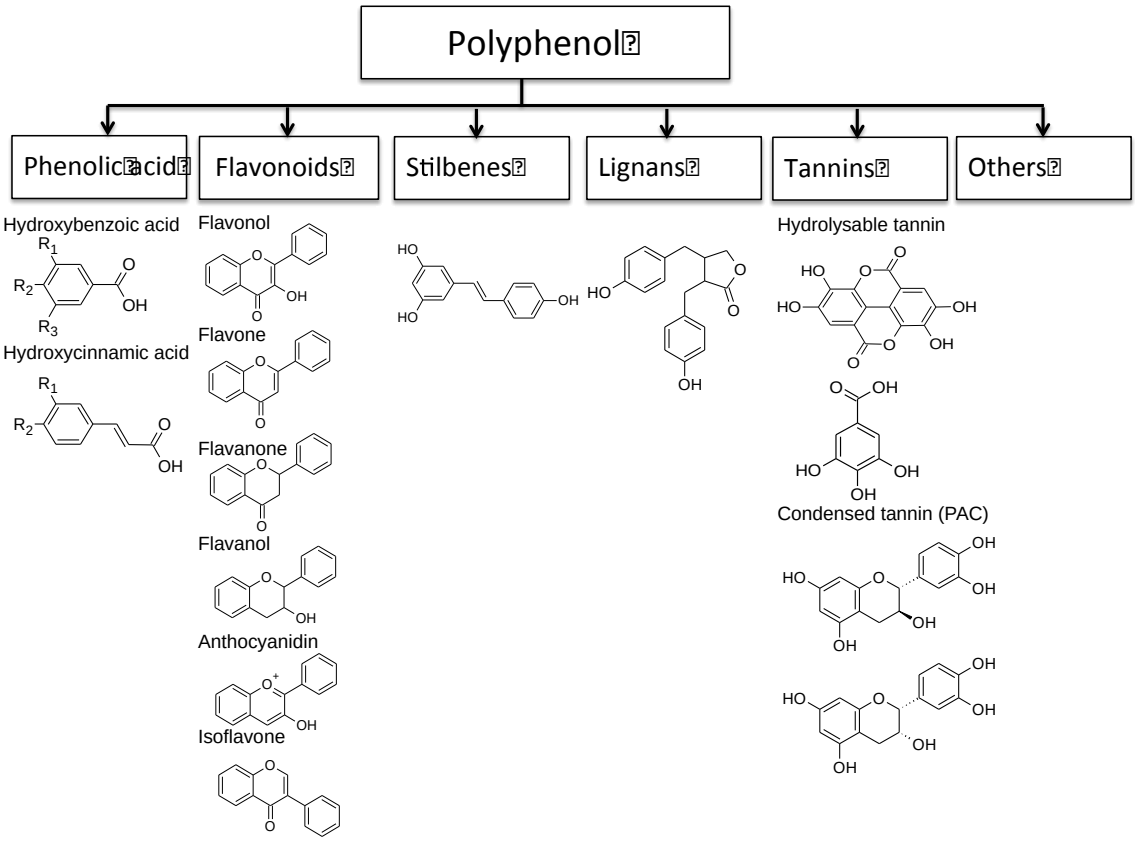

Figure 2.1 Chemical structure of representative polyphenol.

Dietary polyphenols are widely distributed in plant foods, such as fruits and vegetables, and beverages, such as coffee and tea (**Table 2.1**). For example, black chokeberry ranks 2 of the top 100 polyphenol rich food per serving, and almonds ranks 62 (Perez-Jimenez, Neveu et al. 2010). The average intake of total polyphenols in the adult diet is ~1 g/day (**Table 2.2)** (Hertog, Hollman et al. 1993, Kimira M 1998, Kirk, Patterson et al. 1999, Wu, Beecher et al. 2006, Chun, Chung et al. 2007, Ovaskainen, Törrönen et al. 2008, Wang, Chung et al. 2011, McCullough, Peterson et al. 2012). Phenolic acids may be the most abundant dietary polyphenols (**Table 2.2**). Flavonoids, are second largest contributor to polyphenol intake followed by condensed tannins, hydrolyzable tannins and lignans. Among flavonoids, flavones and isoflavones contribute the least to daily polyphenol intake in western diets. The quantity of polyphenol consumption in

Table 2.2 is estimated based on the polyphenols reported in fruits and vegetables, which include non-extractable and non-digestible polyphenols. For example, among the total of 95 mg/day condensed tannins intake, only monomers (22%) and dimers (16%) are absorbed (Wang, Chung et al. 2011). Thus, the amount of total polyphenols absorbed after consumption is likely less than the estimated intake. A significant portion of unabsorbed metabolites would be catabolized by gut microbiota. Dietary polyphenol intake is also influenced by cultural dietary habits. For example, Japanese have the largest consumption of isoflavones, 40 mg/day, because soy food products are more popular in Japan than in western countries (Kimira M 1998).

Table 2.1 Main classes of dietary polyphenols and their sources (Manach, Scalbert et al. 2004).

Classes	Subclass	Food sources
Phenolic acids	Derivatives of benzoic acid	red fruits, black radish, onions, and tea
	Derivatives of cinnamic acid	most fruits, coffee, cereals, and grains
Flavonoids	Anthocyanins	fruits, red wine, certain cereal, and certain leafy and root vegetables
	Flavonols	most foods and beverages
	Flavones	parsley, celery, and citrus
	Flavanones	tomatoes, aromatic plants, and citrus
	Isoflavones	leguminous plants and products
	Flavan-3-ols	many types of fruits, red wine, tea, and chocolates
Stilbenes		wine, nuts, certain fruits
Lignans		certain cereal, grains, fruits and vegetables
Tannins	Hydrolyzable tannins	red fruits
	Condensed tannins	astringent fruits and beverages, and chocolates

Table 2.2 Estimates of dietary polyphenol intake (Hertog, Hollman et al. 1993, Kimira M 1998, Kirk, Patterson et al. 1999, Wu, Beecher et al. 2006, Chun, Chung et al. 2007, Ovaskainen, Törrönen et al. 2008, Wang, Chung et al. 2011, McCullough, Peterson et al. 2012).

Classes	Subclass	Intake (Country)
Phenolic acids	Derivatives of benzoic acid	33 mg/day (Finnish)[1]
	Derivatives of cinnamic acid	608 mg/day (Finnish)
	Total	23 mg/day (Netherland) 6-987 mg/day (Germany) 641 mg/day (Finnish)
Flavonoids	Anthocyanins	47 mg/day (Finnish) 12.5 mg/day (USA) 3.8-22.2 mg/day (USA)
	Flavonols	5.4 mg/day (Finnish) 21 mg/day (Netherland) 6.9-27.2 mg/day (USA)
	Flavones	2 mg/day (Netherland) 0.4-3.0 mg/day (USA)
	Flavanones	27 mg/day (Finnish) 3.5-49.9 mg/day (USA)
	Isoflavones	0.9 mg/day (Finnish) 0.024-0.713 mg/day (USA)
	Flavan-3-ols	7.0-63.7 mg/day (USA)
	Total	189.7 mg/day (USA) 94.5-512.5 mg/day (USA)
Stilbenes	--	--
Lignans	--	0.9 mg/day (Finnish)
Tannins	Hydrolyzable tannins	12 mg/day (Finnish)

	Condensed tannins	74 mg/day (USA)
	(≥2 degree of polymerization)	53.1-379.4 mg/day (USA)
Total	--	863 mg/day (Finnish)
		1 g/day (USA)

[1]The results are presented as average daily intake or range of the daily intake

2.3 Polyphenol absorption

The absorption of polyphenols occurs in two major locations, the small intestine and colon. The small intestine mainly absorbs polyphenol aglycones or glycosides with low-molecular-weights (<800 Daltons) (e.g. anthocyanins, phenolic acids, and flavonoids) through passive transport or active transport (Tamai, Sai et al. 1999, Day, Cañada et al. 2000, Kay 2006, Crozier, Del Rio et al. 2010). In the colon, gut microbes catabolize unabsorbed small (e.g. curcumin) and large molecular-weight polyphenols (e.g. proanthocyanidins, ellagitannins and flavonoid rutinosides) into simple phenolics (Rios, Gonthier et al. 2003, Crozier, Del Rio et al. 2010, González-Barrio, Edwards et al. 2011). A portion of quercetin, anthocyanins and phenolic acid may also be absorbed through stomach, although this pathway has been poorly characterized (Crespy, Morand et al. 2002, Konishi, Zhao et al. 2006, Fernandes, de Freitas et al. 2012).

In the small intestine, polyphenol glycosides can be transported from the lumen by sodium–glucose co-transporters (SGLT) into small intestine cells, remaining intact (**Figure 2.2**) (Day, Cañada et al. 2000, Kay 2006). Alternatively, polyphenol glycosides can be hydrolyzed at the brush-border of the small intestine epithelial cells, and the aglycone transported to the small intestine cell. Once inside small intestine epithelial cells, polyphenol glycosides are transpoported to the portal circulation or hydrolyzed by broad-specificity cytosolic β-glucosidase (CBG) within the epithelial cell to the polyphenol aglycone (Németh, Plumb et al. 2003). Then,

polyphenol aglycones are glucuronidated, methylated or sulfated by phase II enzymes, such as UDP-glucuronosyltransferase (UDP-GT), catechol-*O*-methyltransferase (COMT), or sulfotransferase (SULT), and transported into the portal circulation. For other low-molecular-weight polyphenols, such as flavan-3-ol, are transported into the small intestine epithelial cells intact by active transport, subjected to phase II metabolism, and then transported into portal circulation (Kuhnle, Spencer et al. 2000, Spencer 2003, Crozier, Del Rio et al. 2010). Phenolic acids can be absorbed into the small intestine epithial cells through the monocarboxylic acid transporter (MCT) by active transport (Tamai, Sai et al. 1999).

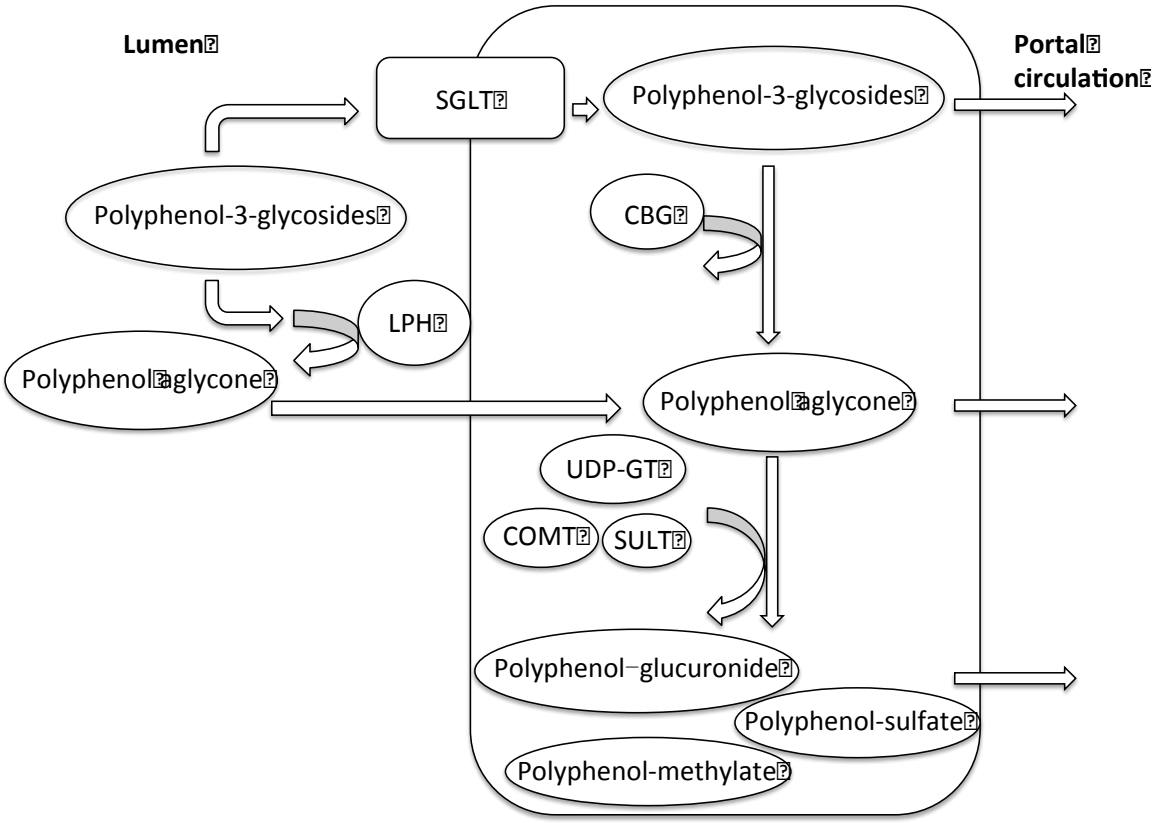

Figure 2.2 Potential mechanisms of polyphenol glycoside absorption in small intestine.

High-molecular-weight polyphenols or substituted with irregular glycosides (e.g. rutinosides, quinic acid) cannot be absorbed intact and may be catabolized before absorption. Proanthocyanidins are oligomers or larger polymers of flavan-3-ols. Previous studies have suggested that proanthocyanidin oligomers having degrees of polymerization ≤ 4 can be rapidly absorbed across differentiated Caco-2 cells, a model of intestinal absorption (Deprez, Mila et al. 2001, Ou, Percival et al. 2012). In the colon, proanthocyanidins with degrees of polymerization > 4 are degraded by microbes into low-molecular-weight phenolic acid compounds, and subsequently absorbed into the large intestine cells (Déprez, Brezillon et al. 2000, Ou and Gu 2013). Human colonic MCT may be largely responsible for microbial-derived phenolic acid absorption (Iwanaga, Takebe et al. 2006). The major proanthocyanidin metabolites observed in human urine or plasma after consumption are 3-(3'-hydroxyphenyl) propionic acid, 3'-hydroxyphenylacetic acid, 3',4'-hydroxyphenylacetic acid, 3-hydroxybenzoic acid, hippuric acid, ferulic acid and phenolic acid (Rios, Gonthier et al. 2003, Urpi-Sarda, Monagas et al. 2009).

Ellagitannins are glucose-bound hexahydroxydiphenic acids. In the colon, ellagitannins are catabolized by colonic microflora to ellagic acid (González-Barrio, Edwards et al. 2011). Subsequently, ellagic acids are transformed to urolithins by microbes and then aborbed into large interstine cells.

The stomach also has a limited role in absoption of dietary polyphenols. Low molecular weight aglycone compounds, such as quercetin or phenolic acid, can be absorbed directly by gastric cells (McCullough, Peterson et al. 2012). Quercetin, but not quercetin-glycoside was absorbed across the gastric lining of rats with ligated sphincters to prevent gastro-esophagus reflux (Crespy, Morand et al. 2002). Similary, quercetin can quickly appear (15 min) in human plasma after consumption, suggesting stomach absorption (Kaushik, O'Fallon et al. 2012).

However, the transporters responsible for gastric polyphenol absorption are not well-defined. Tamai, et al. suggested that stomach MCT might contribute to the gastric absorption of phenolic acids in rats, similar to the small intestinal mechanism (Crespy, Morand et al. 2002). However, MCT has not yet been reported in the human stomach. Further, it is unknown if pheolic acids can be absorbed through the human stomach. The earliest appearance of phenolic acids in human plasma is 1 h after consumption, which suggests this is plausible (Caccetta, Croft et al. 2000).

In contrast to other polyphenols, glycosylated anthocyanidins (e.g. anthocyanins) can be absorbed by the human stomach. Anthocyanins are absorbed intact by human cultured stomach cells in a time dependent manner, likely via a saturable transport mechanism (Fernandes, de Freitas et al. 2012). Passamonti, et al proposed that bilitranslocase could transport anthocyanins across gastric epithelial cells (Passamonti, Vrhovsek et al. 2002). In this study, the anthocyanin-bilitranslocase interactions were modeled using rat plasma membrane vesicles. Anthocyanins, including cyanidin-3-glucoside, behaved as competitive inhibitors of bilitranslocase transport activity, which suggested anthocyanins have high affinities for bilitranslocase. Similar results were also reported by using human plasma membrane vesicles (Ziberna, Tramer et al. 2012). However, bilitranslocase has only been characterized in the rat stomach (Battiston, Macagno et al. 1999), and not been confirmed in the human stomach. Previous study indicated that cyanidin-3-glucoside can be detected by 30 min in human plasma after consumption (Miyazawa, Nakagawa et al. 1999). Thus, a limited number of low molecular-weight polyphenols including anthocyanins and aglycone phenolic acids or flavonols may be absorbed through the human stomach.

2.4 Polyphenol distribution

After absorption from the gastrointestinal tract, polyphenols are transported to the liver through portal circulation (**Figure 2.3**), then distributed to tissues or excreted out of the body (Scalbert, Morand et al. 2002). Polyphenols have been detected in the blood, urine, feces, and tissue *in vivo* (Morton, Chan et al. 1997, Pan, Huang et al. 1999, Zhao and Agarwal 1999, Hong, Kim et al. 2002, Maubach, Bracke et al. 2003, de Boer, Dihal et al. 2005, Garcea, Berry et al. 2005, Hoh, Boocock et al. 2006, Czank, Cassidy et al. 2013). Isoflavones and lignans have been detected in human urine, plasma, prostate gland and breast tissue (Morton, Chan et al. 1997, Hong, Kim et al. 2002, Maubach, Bracke et al. 2003). Curcumin and its metabolites, curcumin sulfate and curcumin glucuronide, were detected in the colorectal tissue of colorectal cancer patients supplemented with 450 to 3600 mg curcumin daily for 7 days (Garcea, Berry et al. 2005). Silibinin, a flavonolignan from milk thistle, was detected in plasma, colorectal tissue and liver tissue of patients with colorectal adenocarcinoma receiving silibinin at dosages of 360, 720, or 1,440 mg silibinin daily for 7 days (Hoh, Boocock et al. 2006). Resveratrol and resveratrol-3-*O*-glucuronide were also recovered from colorectal tissue in colorectal cancer patients consuming supplemental resveratrol (Patel, Brown et al. 2010). In animals, polyphenols can be found in heart, brain, skin, lung, prostate, pancreas, liver, intestine, spleen, kidney and adipose tissues, which suggest polyphenol may be transported to a broad range of tissues (Pan, Huang et al. 1999, Zhao and Agarwal 1999, de Boer, Dihal et al. 2005, Suresh and Srinivasan 2010).

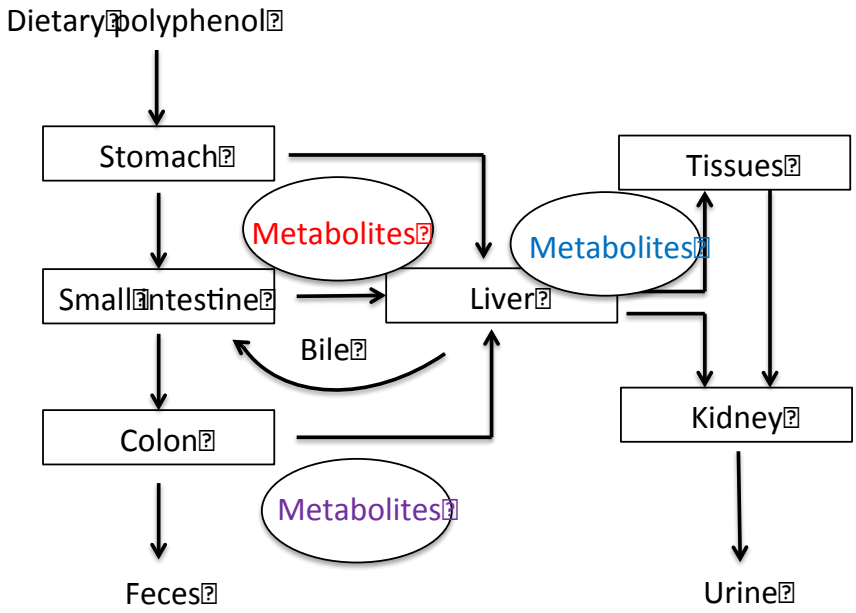

Figure 2.3 Pathway of polyphenol distribution (Scalbert, Morand et al. 2002).

2.5 Polyphenol metabolism

Polyphenols are extensively metabolized after absorption. For example, 70% to 80% of a single cyanidin-3-glucoside dose was recovered as metabolites after consumption (Czank, Cassidy et al. 2013). The human intestine and liver are the two major sites of polyphenol metabolism, although other tissues may also contribute to this process. In the small intestine, lactase phlorizin hydrolase (LPH) at the brush-border can hydrolyze polyphenol β-glycosides of glucose, arabinose, and galactose to its aglycone form. Inside small intestine epithelial cells, polyphenol β-glycosides can be hydrolyzed to aglycones by CBG. Subsequently, polyphenol aglycone hydroxyl groups can be sulfated, methylated, and/or glucuronidated by UDP-GT, COMT, and/or SULT.

In the liver, polyphenols can be metabolized by both phase I and II enzymes. Phase I enzymes are a family of cytochrome P450 monooxygenases (CYPs) that catalyze oxidation, reduction, hydroxylation or demethylation reactions (Hodek, Trefil et al. 2002). For example,

galangin and kaempferide are oxidized to kaempferol by both CYP1A2 and CYP2C9 by human liver microsomes (Otake and Walle 2002). Isoflavones, curcumin, and hesperetin are also readily metabolized by phase I enzymes (Kulling, Honig et al. 2001, Duarte Silva, Rodrigues et al. 1997, Silva, Rodrigues et al. 1997, Ireson, Orr et al. 2001, Ireson, Jones et al. 2002, Garcea, Jones et al. 2004, Roberts-Kirchhoff, Crowley et al. 1999, Doostdar, Burke et al. 2000). The phase I isoflavone metabolites, 6,7,4'-trihydroxyisoflavone, 7,3',4'-trihydroxyisoflavone, 7,8,4'-trihydroxyisoflavone, 7,8,3',4'-tetrahydroxyisoflavone, and 6,7,3',4'-tetrahydroxyisoflavone, were present in the urine of human consumed ~70 mg of soy isoflavones (Kulling, Honig et al. 2001).

Polyphenols and/or their phase I metabolites can be further metabolized by hepatic phase II glucuronidation, sulfation, and/or methylation. Curcumin glucuronides and sulftates were identified from a human hepatocyte suspension from livers of patients with hepatic metastases (Ireson, Orr et al. 2001, Ireson, Jones et al. 2002, Garcea, Jones et al. 2004). Anthocyanins are primarily methylated or glucuronidated after consumption (Wu, Cao et al. 2002, Czank, Cassidy et al. 2013).

Other tissues have phase I and II enzymes, and potential capacity for polyphenol metabolism. Rat kidney microsomes have ethoxyresorufin-O-deethylase (EROD), methoxyresorufin-O-demethylase (MROD), p-nitrophenol hydroxylase (PNPH), glutathione S-transferase (GST), UDP-glucuronosyltransferase (UDP-GT) and NAD(P)H:quinone oxidoreductase (NQO1) activity (Krajka-Kuźniak and Baer-Dubowska 2003). The extent to which non-hepatic and GI tissue contribute to polyphenol metabolism is an ongoing area of interest.

2.6 Polyphenol excretion

Polyphenols and their metabolites are mainly excreted through breath, urine or feces (Scalbert, Morand et al. 2002, Czank, Cassidy et al. 2013). Fecal polyphenols include absorbed polyphenols secreted back to the intestine from bile and unabsorbed dietary polyphenols (Scalbert, Morand et al. 2002). In healthy subjects that ingested a single dose of 500 mg ^{13}C cyanidin-3-*O*-glucoside, ^{13}C cyanidin-3-*O*-glucoside and ^{13}C cyanidin-3-*O*-glucoside metabolites were found in the feces, which suggested unabsorbed cyanidin-3-*O*-glucoside remain intact through the feces (Czank, Cassidy et al. 2013). A fraction of polyphenols is excreted in bile. After infusion of ^{14}C genistein into the duodenum of rats for 1 h, cumulative ^{14}C genistein as its 7-O-β-glucuronide conjugate was found in the bile (Sfakianos, Coward et al. 1997). Many polyphenols have enterohepatic recirculation, including isoflavones (Setchell, Brown et al. 2001, Zubik and Meydani 2003). Many transport proteins contribute to polyphenol excretion. In the small intestine, ATP-binding cassette (ABC) transporters contribute to polyphenol efflux (Vaidyanathan and Walle 2003, Juan, González-Pons et al. 2010). For example, (-)-epicatechin-3-gallate (ECG) is transported by multidrug-associated protein 1 (MRP1, ABCC1), multidrug-associated protein 2 (MPR2, ABCC2), and P-glycoprotein (ABCB1) (Vaidyanathan and Walle 2003). MRP1 and MRP2, but not P-glycoprotein contribute to EGCG efflux (Hong, Lambert et al. 2003). In addition, MRP2 and breast cancer resistance protein (BCRP, ABCG2) have been associated with the efflux of *trans*-resveratrol glucuronide and *trans*-resveratrol sulfate (Juan, González-Pons et al. 2010). However, P-glycoprotein does not efflux of *trans*-resveratrol and its conjugates. The fraction of polyphenols excreted varies by route. In a tracer study, $5.37 \pm 0.67\%$ of the ^{13}C of cyanidin-3-glucoside was excreted through urine, $6.91 \pm 1.59\%$ was excreted through breath, and $32.13 \pm 6.3\%$ was excreted through feces (Czank, Cassidy et al. 2013). In

the breath, ^{13}C was present as CO_2. In the urine, ^{13}C was present as cyanidin-3-glucoside (11%) and its metabolites (89%).

2.7 Polyphenols and CVD Risk

Epidemiological studies, animal studies and human intervention studies suggest that polyphenol consumption may reduce CVD risk (Arts and Hollman 2005, Chong, Macdonald et al. 2010). According to WHO, CVD is defined as a group of disorders of the heart and blood vessels, which include coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism (WHO 2015). In 2012, CVD led to about 17.5 million deaths globally, and was the primary cause of non-communicable disease deaths world world-wide (WHO 2014). Atherosclerosis is the major cause of CVD. Endothelial dysfunction is a characteristic feature throughout the pathogenesis of atherosclerosis, and it is an independent predictor for future cardiovascular risk (Gonzalez and Selwyn 2003). Endothelial dysfunction is initiated by plaque formation (Libby, Ridker et al. 2002). At the beginning of plaque formation, low-density lipoprotein (LDL) penetrates into the intima, binds to the proteoglycan matrix, and undergoes oxidative modification to form oxidized LDL (ox-LDL) (Berliner J 1997). Monocytes and macrophages then will uptake ox-LDL and develop into foam cells, which leads to intimal thickening, plaque formation, and vessel narrowing (Gonzalez and Selwyn 2003). Cytokines and growth factors secreted by activated monocytes, macrophages, endothelial cells and T cells can promote the migration and proliferation of smooth muscle cells (SMCs). SMC enzymes can digest the arterial extracellular matrix by hydrolyzing elastin and collagen (Libby, Ridker et al. 2002). The degradation of the arterial extracellular matrix permits the penetration of the SMCs and a fibrous cap develops to cover the plaque (Libby, Ridker et al. 2002). Subsequently,

thrombosis is triggered as a result of increased accumulation of lipid and foam cells, and fibrous cap rupture leads to infarction (Fuster V 1990).

During the pathogenesis of atherosclerosis, ox-LDL can activate nuclear factor-κB (NF-κB) in monocyte and macrophage (Brand, Eisele et al. 1997). NF-κB activation promotes the endothelial cell monolayer to secret adhesion molecules, such as L-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (Takei, Huang et al. 2001). NF-κB activation can also induce migration of blood monocytes, macrophages, dendritic cells, T cells and some B-cells into the intima (Frostegård, Nilsson et al. 1990, J A Berliner 1990, Frostegård, Wu et al. 1992, Atout R 2012). NF-κB can also active the expression of pro-inflammatory cytokines and growth factors (Hoesel and Schmid 2013). Pro-inflammatory cytokines and growth factors, such as IL-1, IL-6, monocyte chemotactic protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) accelerate atherosclerosis. For example, IL-6 can stimulate the liver to produce C-reactive protein (CRP) which can further stimulate inflammation (Saito, Ishimitsu et al. 2003). Patients with CVD have increased plasma levels of VCAM–1, ICAM–1, IL-6, oxidative stress, NF-κB, and CRP (Gonzalez and Selwyn 2003). Thus, the pathogenesis of CVD is partially a consequence of chronic cardiovascular inflammation.

Free radicals also contribute to the pathogenesis of atherosclerosis. Nitric oxide (NO) is synthesized by the endothelial nitric oxide synthase (eNOS), which is important for maintaining vasodilation (Cannon 1998). Ox-LDL and reactive oxygen species stimulate the expression of inducible nitric oxide synthase (iNOS) in aortic endothelial cells (Hirata, Miki et al. 1995, Cai and Harrison 2000). iNOS overproduction promotes the formation and activation of peroxynitrite (Buttery LD 1996). Peroxynitrite can down-regulate the eNOS production, ultimately reducing endothelial NO (Buttery LD 1996).

Furthermore, oxidative stress and inflammation activate protein kinase intracellular signaling molecules such as extracellular signal-regulated kinases (ERK), jun amino-terminal kinases (JNK) and p38 mitogen-activated protein kinases (MAPKs) (González-Gallego, Sánchez-Campos et al. 2007) (**Figure 2.4**). These will activate Nrf2 and NF-κB, key transcription factors modulating antioxidant and inflammatory responses. Activated protein kinase intracellular signaling molecules disrupt the bonding between Nrf2 and keap1, leading to nuclear translocation of Nrf2. In the nucleus, Nrf2 binds to the specific DNA sequence (antioxidant response element, ARE) in the upstream promoter region of many antioxidative genes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), and initiates their transcription and expression. While in an inactivated state, NF-κB is located in the cytosol complexed with the inhibitory protein IκBα. Activated protein kinase intracellular signaling molecules can activate the enzyme IκB kinase (IKK). IKK, in turn, phosphorylates the IκBα protein, which results in dissociation of IκBα from NF-κB. The activated NF-κB is then translocated into the nucleus where it binds to specific sequences of DNA (response elements, RE) and initiate transcription and expression of gene coding cyclooxygenase (COX-2), iNOS, CRP, cytokines and adhesion molecules, which increase inflammation.

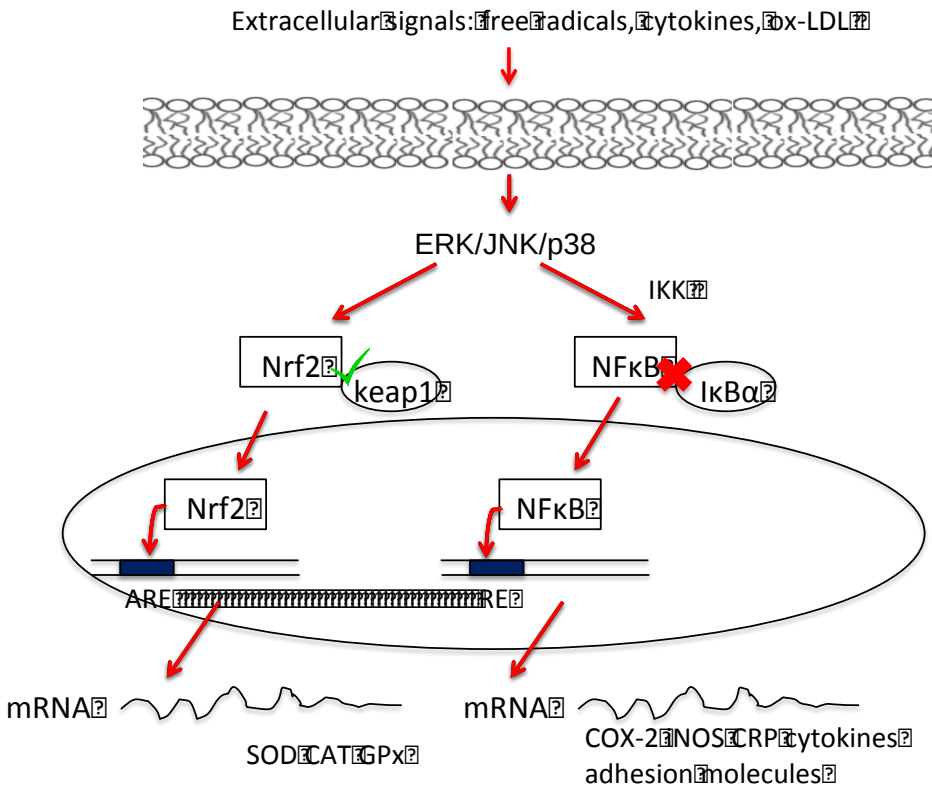

Figure 2.4 The mechanism of polyphenol against CVD in mRNA level (González-Gallego, Sánchez-Campos et al. 2007)

Previous observational cohort studies and clinical trial suggested diet rich in polyphenols, such as anthocyanins and proanthocyanidins, could prevent the risk of CVD (Rasmussen, Frederiksen et al. 2005, Wallace 2011, Estruch, Ros et al. 2013).

2.7.1 Anthocyanins

Preventing chronic inflammation is a promising approach to reduce CVD risk. During the pathogenesis of atherosclerosis, NF-κB was responsible for initiating the inflammatory response (Brand, Eisele et al. 1997). Deactivation of NF-κB could suppress the expression of pro-inflammatory chemokines, growth factors, and adhesion molecules in the treatment of

inflammatory and chronic disease (Yamamoto and Gaynor 2001). In *in vitro* studies, anthocyanins were observed to directly inhibit the LPS-induced NF-κB transactivation in human monocytes (Karlsen, Retterstøl et al. 2007). In human studies, reduced plasma level of NF-κB and IL-8 were found in human subjects with anthocyanins-rich red wine intake (Blanco-Colio, Valderrama et al. 2000). It has been suggested that ox-LDL can stimulate CD40 production through the function of lipoprotein receptor-1 (LOX-1), a major receptor of ox-LDL (Li, Liu et al. 2003). Up-regulation of CD40 was associated with the pathogenesis of atherosclerosis (Mach, Schonbeck et al. 1998, Lutgens, Cleutjens et al. 2000, Schönbeck, Sukhova et al. 2000). The function of CD40 was to activate NF-κB and induce the release of pro-inflammatory chemokines and cytokines through up-regulating the expression of TNF receptor-associated factor 2 (TRAF-2). TRAF-2 could induce the distribution of cholesterol in lipid rafts of membrane in endothelial cells. Anthocyanin could reduce CD40-mediated activation of NF-κB by disrupting the function of TRAF-2 in human endothelial cells. (Xia, Ling et al. 2007).

Activated monocytes, macrophages, endothelial cells and T cells could secrete cytokines and growth factors, such as IL-1, IL-6, MCP-1 and TNF-α. The secretion of cytokines and growth factors may increase the inflammatory response in the pathogenesis of atherosclerosis (Libby, Ridker et al. 2002). MCP-1 was secreted by activated macrophages and endothelial cells. MCP-1 overproduction can induce macrophages to form plaque. Anthocyanins have the capacity to inhibit the production of MCP-1 induced by TNF-α. In an *in vitro* study, TNF-α-mediated MCP-1 production by primary human endothelial cells was inhibited by anthocyanin treatment (García-Alonso, Rimbach et al. 2004). Anthocyanin-rich blueberry and cranberry extracts can reduce the levels of IL-8, MCP-1 and ICAM-1 induced by TNF-α when applied to human endothelial cells (Youdim, McDonald et al. 2002). In healthy humans, plasma MCP-1 was

reduced by a single 12 g dose of red wine anthocyanins (Garcia-Alonso, Minihane et al. 2009). Red-wine consumption also reduced MCP-1 in high cholesterol-fed rabbits (Feng, Chen et al. 1999). Anthocyanins can also down-regulate TNF-α expression directly in other *in vivo* CVD models (Mao, van de Water et al. 2002, Kim, Tsoy et al. 2006).

Anthocyanins can also inhibit the production of adhesion molecules and CRP which stimulate the inflammatory response during the pathogenesis of atherosclerosis (Takei, Huang et al. 2001, Saito, Ishimitsu et al. 2003). TNF-α increased human umbilical vein endothelial ICAM-1 and VCAM-1 expression and increased nuclear GATA-4, GATA-6, and IRF-1 level (Nizamutdinova, Kim et al. 2009). Pretreatment of endothelial cells with anthocyanins inhibited VCAM-1, GATA-4, GATA-6 and IRF-1 expression, but not ICAM-1 expression. Also, pretreatment of cells with a specific Jak/STAT inhibitor elicited a similar response, suggesting anthocyanins reduce inflammation by down-regulating the adhesion molecules activated by Jak/STAT pathway. Anthocyanins can also inhibit expression of CD40-mediated adhesion molecules. Pretreatment of endothelial cells with CD40 ligand (CD40L) significantly increased cellular VCAM-1 and ICAM-1 (Xia, Ling et al. 2009). However, pretreatment of endothelial cells with anthocyanins inhibited the production of VCAM-1 and ICAM-1 (Xia, Ling et al. 2009). According to previous observational cohort studies, serum CRP was significantly inversely associated with anthocyanin intakes by adults in the U.S. (Chun, Chung et al. 2008). In a clinical trial, consumption of anthocyanin-rich cherries in healthy people reduced plasma CRP level but not plasma IL-6 level after 4 weeks (Kelley, Rasooly et al. 2006). Thus, anthocyanins might reduce the inflammatory level during the pathogenesis of CVD by inhibition the production of adhesion molecules and CRP.

SMCs are activated by inflammatory mediators, which stimulate the production of matrix metalloproteinase (MMP) which can hydrolyze the extracellular matrix during atherosclerosis (Libby, Ridker et al. 2002). Direct application of anthocyanins reduces CD40L-mediated MMP-1 and MMP-9 expression in human endothelial cells (Xia, Ling et al. 2009). Vascular endothelial growth factor (VEGF), a pro-atherosclerotic factor, is increased in SMCs during CVD development. Anthocyanins also inhibit VEGF release in vascular SMCs by inhibiting p38-MAPK and JNK pathways (Oak, Bedoui et al. 2006).

Thus, anthocyanins might prevent the CVD risk by reducing chronic inflammation in the vascular system. The anti-inflammatory mechanisms involve inhibiting the initiation of inflammatory response and inflammation-induced extracellular matrix break down.

Anthocyanins may also reduce CVD risk through antioxidant mechanisms. NO is a key molecule in the pathogenesis of CVD, because it has anti-hypertension, anti-thrombosis, anti-atherosclerosis, and anti-smooth muscle proliferation functions (Förstermann and Sessa 2012). Anthocyanins increase NO and improve endothelial function in models of CVD. Aronia and bilberry anthocyanins increased NO-mediated vasorelaxation when applied to pig coronary artery endothelial cells (Bell and Gochenaur 2006). Black currant concentrate also had a vasorelaxing effect when applied to rat coronary artery endothelial cells, which was specific to NO (Nakamura, Matsumoto et al. 2002). Delphinidin, but not malvidin or cyanidin, improved endothelium-dependent vasorelaxation in the rat artery endothelial cells (Andriambeloson, Magnier et al. 1998). In contrast, cyanidin inducing eNOS expression and NO production via a Src-ERK1/2-Sp1 signaling pathway in bovine artery endothelial cells (Xu, Ikeda et al. 2004). Thus, the ability of anthocyanins to mediate NO pathways appear to depend on its chemical structure. Anthocyanins also modulate NO in vivo. Healthy rat fed wild blueberries had better

endothelium-dependent vasorelaxation in the arterial endothelium after 7 weeks (Kalea, Clark et al. 2009).

Anthocyanin consumption can also increase serum antioxidant capacity. Following consumption of 12 g of anthocyanins from freeze-dried blueberries, a serum anthocyanins and *ex vivo* antioxidant capacity were correlated (Mazza, Kay et al. 2002). Previous studies have suggested that anthocyanin consumption may indirectly increase antioxidant function (Youdim, McDonald et al. 2002, Acquaviva, Russo et al. 2003, Broncel M 2010). Adults with metabolic syndrome that consumed aronia extract had reduced CAT, increased SOD, and increased GSH-Px in the blood, which suggested improved antioxidative enzyme function (Broncel M 2010). Anthocyanins can also protect membrane fatty acids of endothelial cells from oxidation and DNA cleavage induced by elevated inflammatory level (Youdim, McDonald et al. 2002, Acquaviva, Russo et al. 2003). Similarly aronia berry extract consumption reduced ox-LDL in patients that previously suffered a myocardial infarction (Naruszewicz, Łaniewska et al. 2007).

In conclusion, anthocyanins might have the potential in CVD prevention by reducing chronic inflammation and improving antioxidant capacity in cardiovascular. However, mechanistic data were mainly from cell studies.

2.7.2 Proanthocyanidins

Other than anthocyanins, clinical evidence for the anti-CVD effect of proanthocyanidins has also been suggested. However, the mechanism might be different from other polyphenols (Kruger, Davies et al. 2014). In *in vitro* studies, proanthocyanidins inhibited IL-1β-induced NF-κB transactivation in human monocytes (Gentile, Allegra et al. 2012). In patients with diabetes mellitus or had ≥3 of cardiovascular disease risk factors, the intake of 40 g proanthocyanidin-rich cocoa powder for 4 wks decreased the expression of very late activation antigen-4 (VLA-4,

CD49d), CD40, and CD36 in monocytes and the serum levels of P-selectin and ICAM-1 (Monagas, Khan et al. 2009). Also, proanthocyanidins can directly down-regulate TNF-α expression in *in vitro* CVD models (Mao, van de Water et al. 2002).

Other than preventing the initiation of chronic inflammation, proanthocyanidins can also prevent the expression of adhesion molecules and CRP which are responsible for stimulating inflammatory response during the pathogenesis of atherosclerosis. A grape seed proanthocyanidin extract inhibited TNF-α-induced expression of VCAM-1 and ICAM-1 in human umbilical vein endothelial cells, independent of a NF-κB-dependent pathway (Sen and Bagchi 2001). (+)-Catechin metabolites also inhibited U937 cell adhesion to IL-1 beta-stimulated human aortic endothelial cells and ROS generation in U937 cells (Koga and Meydani 2001). Since (+)-catechin is one of the building blocks of proanthocyanidins, it is possible that proanthocyanidin metabolites have similar bioactivity as (+)-catechin metabolites. Healthy adults that consumed 30 g/d of a proanthocyanidin-rich red wine for 28 d had reduced plasma VLA-4 compared with gin (low polyphenol) consumption (Badía, Sacanella et al. 2004). Also, in *ex vivo* analysis, red wine consumption decreased TNF-α-induced adhesion of human monocytes to endothelial cells (Badía, Sacanella et al. 2004). In a prospective randomized crossover trial, healthy adults that consumed 30 g/d proanthocyanidin-rich red wine consumption for 28 d had reduced expression of VLA-4 and MCP-1, as well as reduced serum concentrations of hs-CRP, VCAM-1 and ICAM-1 (Estruch, Sacanella et al. 2004). In patients with systemic sclerosis that consumed of 100 g grape seed-derived proanthocyanidins extract for a month had reduced plasma malondialdehyde formation and adhesion molecules (ICAM-1, VCAM-1 and E-selectin) (Kalfin, Righi et al. 2002).

Also, consumption of proanthocyanidins also may improve antioxidant function. In an *ex vivo* study, the plasma from rats fed a proanthocyanidins-rich extract from longan flower had increased the resistance to Cu^{2+}-induced LDL oxidation (Tsai, Wu et al. 2008). The direct anti-oxidative mechanisms of proanthocyanidins were explained by da Silva Porto, et at., which suggested the antioxidant activity increases with degree of polymerization (da Silva Porto, Laranjinha et al. 2003).

Animal and human studies have demonstrated that proanthocyanidins may reverse vascular endothelial function by increasing NO (Duffy and Vita 2003). Healthy rats that consumed a proanthocyanidin-rich red wine for 7 days had progressively lower systolic blood pressure after 4 days relative to the control (Diebolt, Bucher et al. 2001). NO appeared to mediate these effects (Diebolt, Bucher et al. 2001). Adults with impaired endothelial function that consumed a proanthocyanidin-rich purple grape juice had improved flow-mediated vasodilation (FMD), which might because a reduction in ox-LDL (Stein, Keevil et al. 1999).

Also, the protective effects of proanthocyanidin-rich food, such as red wine, cranberry products, grape skin extracts, cocoa, and chocolate, have been observed in human intervention studies, which suggests its potential capacity for reducing CVD risk (Blanco-Colio, Valderrama et al. 2000, Garcia-Alonso, Minihane et al. 2009). However, most studies have not used pure proanthocyanidins to investigate their mechanisms of CVD risk reduction. Thus, it is not possible to directly attribute these effects to proanthocyanidins alone. Also, proanthocyanidins have a low bioavailability, which might limit the bioactivity of proanthocyanidins (Manach, Scalbert et al. 2004).

2.8 Conclusion

Polyphenols have promise for CVD prevention. Polyphenols appear to reduce CVD risk by anti-inflammatory or antioxidant mechanism. For anti-inflammatory effects, polyphenols may reduce the level of many pro-inflammatory mediators, such as adhesion molecules, cytokines and growth factors. It is unclear whether the function of polyphenols is to inhibit the initiation of inflammatory response, the aggravation of inflammatory response or both. For antioxidant function, polyphenols can promote the function of NO, and elevate the serum antioxidant capacity. At the mRNA level, polyphenols can promote the Nrf2 translocation and expression of antioxidative genes, as well as inhibit the NF-κB translocation and expression of gene amplify inflammatory response (González-Gallego, Sánchez-Campos et al. 2007).

A limitation of the previous studies is that most evidence found were based on cell or animal studies. Cell or animials models treated with polyphenols might not refelct the metabolic profile of polyphenols in human. It is essential to determine whether polyphenol metabolites is responsible for the changes in biomarker of CVD risk in human.

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Chapter 3: Characterize the tannin and stilbene composition of almonds

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3.1 Abstract

Background: Almond, among other nuts, is a rich source of dietary polyphenols containing phenolic acids, flavonoids, and lignans. However, less is known about the content of bound proanthocyanidins and hydrolysable tannins in almonds.

Objectives: To quantify the extractable and bound proanthocyanidins and hydrolysable tannins content in almond.

Design: Extractable and bound proanthocyanidins and hydrolysable tannins in almond were characterized in Nonpareil, Carmel, and Butte almond varieties from California, with n = 3 samples/variety.

Results: Bound proanthocyanidins were recovered from extracted defatted almond residue by hydrolysis with 4 N sodium hydroxide and represented 3− 21% of the total proanthocyanidin content among varieties. The bound proanthocyanidins were recovered primarily as monomers and dimers. In contrast, acid hydrolysis of extracted almond residue did not yield bound proanthocyanidins. Hydrolysable tannins were characterized in aqueous acetone extracts of defatted almond using two-dimensional TLC and further quantitated by HPLC following acid hydrolysis. Almond hydrolysable tannin content was 54.7 ± 2.3 mg ellagic acid and 27.4 ± 7.3 mg gallic acid per 100 g almond among varieties. The tannin contents of Nonpareil, Carmel, and Butte almond varieties were not significantly different.

Conclusion: Bound proanthocyanidins and hydrolysable tannins significantly contribute to almond polyphenol content.

3.2 Introduction

Almonds (*Prunus dulcis*) are a rich dietary source of polyphenols (Pérez-Jiménez, Neveu et al. 2010). The most abundant class of polyphenols in almonds is proanthocyanidins, followed by flavonoids and phenolic acids (Bolling, Chen et al. 2011). Almond flavonoids include flavonols, flavanones, and flavanols, while almond proanthocyanidins are composed of flavan-3-ols with varying degrees of polymerization (Gu, Kelm et al. 2003, Gu, Kelm et al. 2004, Prior and Gu 2005, Garrido, Monagas et al. 2008, Urpi-Sarda, Monagas et al. 2009). However, while proanthocyanidins have been characterized in almond, data about its distribution among varieties is lacking (Gu, Kelm et al. 2004). Less is known about the content of hydrolysable tannins in almonds.

Tannins and other polyphenols may contribute to the health-promoting potential of almonds. Almond flavonoids and proanthocyanidins are bioavailable and extensively metabolized *in vivo* by phase II enzymes or intestinal microbiota (Urpi-Sarda, Garrido et al. 2009). These polyphenols and their metabolites may contribute to the antioxidant and anti-inflammatory effects of almonds observed in animal studies (Chen, Milbury et al. 2005, Mandalari, Bisignano et al. 2011).

A growing body of research suggests that the polyphenol content of foods could be underestimated because solvent extraction does not completely liberate polyphenols covalently bound to cell walls (Arranz, Saura-Calixto et al. 2009, Arranz, Silván et al. 2010). Previously, non-covalently bound phenolic acids and flavonoids (e.g. free or extractable) from almond skin extracts were quantified by HPLC-MS (Bolling, Dolnikowski et al. 2009, Bolling, Blumberg et al. 2010, Bolling, Dolnikowski et al. 2010). However, Mandalari and colleagues reported a portion of almond phenolic acids, mainly *p*-hydroxybenzoic acid, vanillic acid and ferulic acid,

was covalently bound and released by acid hydrolysis (Mandalari, Tomaino et al. 2010). Flavonoids, phenolic acids, and proanthocyanidins can also be released by acid or alkaline hydrolysis from other plant materials (Arranz, Saura-Calixto et al. 2009, White, Howard et al. 2010). Thus, more work is needed to determine the relative proportion of free and bound polyphenols in almonds. The objectives of this study were to further characterize almond tannins by 1) quantifying extractable and bound proanthocyanidins, and 2) screening for hydrolysable tannins. We utilized Nonpareil, Carmel and Butte almonds, as these are the top three cultivated California almond varieties.

3.3 Materials and Methods

3.3.1 Reagents and Materials

Methanol, dichloromethane, and acetic acid were HPLC grade from Fischer Scientific (Fair Lawn, NJ). Water was ultrapure grade. All other chemicals and reagents were acquired from Sigma-Aldrich (St. Louis, MO). Samples of whole, unpasteurized Butte, Carmel, and Nonpareil California almonds from harvest year 2011 were provided by the Almond Board of California. Each variety had 3 unique samples representing different orchards, for a total of n = 9 samples. Almonds were stored at -20°C until analysis.

3.3.2 Extraction of Proanthocyanidins

Frozen almonds were finely ground using an IKA A11 basic grinder (Wilmington, NC). Almond powder (4 g) was homogenized in 40 mL hexane using an IKA Ultra-Turrax T18 homogenizer for 2 min at a power setting of 4. The homogenate was centrifuged and the supernatant was removed. The residue was similarly homogenized twice, and the resulting supernatants were combined and dried under a nitrogen gas stream. The mass of the extracted,

dried lipids were recorded to determine almond lipid content. The defatted almond residue was then extracted with acidified aqueous acetone (70:29.5:0.5 mixture of acetone, distilled water, and acetic acid), according to methods described by Prior et al. with modifications (Prior and Gu 2005). Acidified aqueous acetone (40 mL) was added to the defatted nut residue and vortexed for 30 to 40 s, and sonicated for 10 minutes at 37°C (Fisher Scientific, Utrasonic cleaner, FS30). The sample was then agitated on a tube rocker for 50 min at 23 °C, centrifuged, and the supernatant was filtered through a 0.2 µm nylon filter. The pelleted almond residue was dried under a nitrogen gas stream and stored at -20 °C until hydrolysis. The filtered supernatant was dried under a nitrogen gas stream to remove acetone, frozen at -80 °C, lyophilized to a powder, and stored at -80 °C.

3.3.3 Acid and Alkaline Hydrolysis

Residual defatted or defatted, extracted almond powder was subjected to acid or alkaline hydrolysis. Acid hydrolysis was based on previously described methods (Singleton, Orthofer et al. 1998, Prior and Gu 2005). Almond residue was resuspended in 20 mL of 1.2 N hydrochloric acid in methanol:water (80:20, v/v) and 0.5 g/L tert-butylhydroquinone and heated at 75 °C in a dry bath for 3 h. Incubates were then cooled over ice brought to pH 5.5 with 3.7 M ammonium acetate. The hydrolysis solution was centrifuged, and the supernatant was dried of residual methanol under nitrogen gas at 40 °C. The resulting aqueous solution was stored at -80°C until further analysis.

Base hydrolysis was performed according to a previously described method (White, Howard et al. 2010). Defatted and extracted almond residue was resuspended in 20 mL of 4 N aqueous sodium hydroxide. Resuspended residue was and incubated in a water bath at 60 °C for 15 min with 30 s vortexing every 3 min. The hydrolyzed sample was cooled over ice and

neutralized with 4 N hydrochloric acid. The neutralized sample was centrifuged, and the supernatant was frozen at -80 °C, lyophilized to a powder, and stored at -80 °C until further testing.

3.3.4 Isolation of Tannins

Tannins were isolated by stepwise elution from Sephadex LH-20 columns, as described previously (Prior and Gu 2005). Neutralized aqueous hydrolysis solutions or dried almond extracts were reconstituted in 2 to 4 mL of 30% methanol in water, and were loaded on 1 cm diameter column containing ~4 g Sephadex LH-20. Sugars and salts were eluted with 50 mL methanol:water (30:70 v/v), and discarded. Proanthocyanidins were eluted with 100 mL acetone:water (70:30 v/v). The proanthocyanidin fraction was dried of residual acetone by a nitrogen gas stream at 37 °C. The resulting aqueous residue was frozen at -80 °C and lyophilized to a powder prior to HPLC analysis.

3.3.5 HPLC Analysis of Proanthocyanidins

Proanthocyanidins were quantified based a modified method of Prior and colleagues using a Dionex Ultimate 3000 HPLC equipped with a refrigerated autosampler, column oven, diode array detector, and fluorescence detector (Sunnyvale, CA) (Prior and Gu 2005). Proanthocyanidin isolates were reconstituted in 100 µL methanol:dichloromethane (1:1) and 20 µL was injected on a 260 × 4.60 mm Hypersil silica column (ThermoFisher, Bellefonte, PA). Samples were resolved by a 1 mL/min gradient of 100% dichloromethane (A) and 100% methanol (B) with a constant 4% of acetic acid:water (50:50, v/v). The gradient increased linearly from 14% B at 0 min to 28.4% B at 30 min, to 39.6% B at 45 min, to 86 % B at 55 min and held until 60 min, then decreased to 14% B until 75 min. Proanthocyanidins were quantified

by fluorescence, with excitation at 276 nm and emission at 316 nm, using (+)-catechin, (-)-epicatechin, and proanthocyanidin B2 as standards. A check standard of cocoa proanthocyanidins purified in-house was used to control for intraday variation.

3.3.6 Colorimetric Determination of Total Phenols and Proanthocyanidins

The total phenol content of almond extract and hydrolyzed residue was determined according to the method of Singleton et al., adapted to a microtiter plate (Singleton, Orthofer et al. 1998). Results were expressed as mg gallic acid equivalents (GAE) per 100 g whole almonds. Proanthocyanidin content was determined by the 4-(dimethylamino)cinnamaldehyde (DMAC) method.(Payne, Hurst et al. 2010) Samples or standards were reconstituted in 70 μL 50% aqueous methanol and mixed with 210 μL of 0.1% DMAC (w/v) in 1: 1: 6 (v/v/v) 37% hydrochloric acid:water:ethanol in a microtiter plate. The plate was incubated at 25 °C in a spectrophotometer (Synergy HT Multi-Mode Microplate Reader, BioTek, Winooski, VT) and absorbance was monitored at 640 nm in 1 min intervals for 30 min. Results were expressed as mg (+)-catechin equivalents (CE) per 100 g whole almonds.

3.3.7 Thin-layer Chromatography (TLC) of Hydrolyzable Tannins

Almond hydrolyzable tannins were characterized by two-dimensional TLC (White, Howard et al. 2010). Duplicate cellulose plates (20 × 20 cm, 100 μm, Selecto Scientific Inc., Suwanee, GA) were spotted with 50 μL of the almond tannin fraction reconstituted in 100% methanol. Plates were developed in glass chambers saturated with 100 to 150 mL of a 14:1:5 (v/v/v) mixture of butan-2-ol/acetic acid/water until the solvent front was at least 18 cm. Plates were then removed from the chambers and dried at ambient temperature. Plates were then rotated 90° and placed into a glass chamber saturated with 100 to 150 mL of a 2:98 (v/v) mixture of

acetic acid/water. The plates were dried at ambient temperature. Plates were sprayed with a saturated potassium iodate solution to detect gallotannins or 4% sodium nitrite (w/v) in 50% aqueous acetic acid (v/v) to detect ellagitannins.

3.3.8 Gallotannin and Ellagitanin Hydrolysis

Defatted almond was hydrolyzed according to a previously described method (Lei, Jervis et al. 2001). Defatted almond powder (20 mg) was reconstituted in 2 mL methanol in 50 mL glass screw-top test tubes. Sulfuric acid (18% in water, 100 µL) was slowly added to samples in two aliquots, manually swirling the solution between additions. Samples were then incubated in a dry bath at 85 °C for 20 h, cooled over ice, and neutralized by carefully adding 4 aliquots of 50 µL ethanolamine to each sample. Caffeic acid or trans-stilbene (5 µg/mL) were used as internal standards for analysis of gallic acid or ellagic acid, respectively. Solutions were brought to 2 mL with water and centrifuged. Supernatants were passed through a 0.2 µm nylon syringe filter if cloudy or used directly for HPLC analysis.

3.3.9 HPLC Analysis of Gallic Acid and Ellagic Acid

HPLC was performed using the previously described Dionex Ultimate 3000 system. Diluted, neutralized hydrolysis solutions or standards (20 µL) were injected onto a 50 × 2.10 mm Dionex 2.2 µm 120Å Acclaim RSLC PolarAdvantage column (ThermoScientific, Sunnyvale, CA). Gradients of 1% aqueous acetic acid (A) and methanol (B) and were used to resolve samples at 0.2 mL/min. For gallic acid, 10% B was held to 3 min, followed by a linear gradient to 40% B at 7 min, returning to 10% B at 12 min and held for 5 min. For ellagic acid, 10% A was held for 3 min, followed by a linear gradient to 100% B at 10 min, returning to 10% B at 12 min and held for 8 min. Gallic acid and ellagic acid were used as external standards for

quantification. Caffeic acid and trans-stilbene were internal standards for gallic acid and ellagic acid, respectively. Gallic acid and ellagic acid were identified in hydrolysis solution by comparison of UV spectra analysis and retention time (RT) to authentic standards. Methyl gallate eluted at 4.4 min but was not detected below 0.12 ng on-column in hydrolysis solutions. Gallic acid and caffeic acid were quantified at 280 nm, and ellagic acid and trans-stilbene were quantified at 320 nm and 250 nm, respectively. Inter- and intra-assay RSD for gallic acid analysis were 14.55 and 7.36 %, respectively. Inter- and intra-assay RSD for ellagic acid analysis were 1.06 and 2.98 %, respectively. Gallic acid, caffeic acid, ellagic acid and trans-stilbene eluted at 1.9, 8.7, 12.4, and 15.2 min, respectively.

3.3.10 Statistics and Data Analysis

Data are mean ± standard deviation of at least duplicate samples. Statistical significance was determined by two or one-way ANOVA as indicated, followed by Tukey's multiple comparison test using GraphPad Prism 5.01 software (GraphPad Software, Inc., La Jolla, CA). Differences were considered significant at $P < 0.05$.

3.4 Results

Tannins are the most abundant class of polyphenols in almonds (Bolling, Chen et al. 2011). Little is known about the abundance of bound proanthocyanindins and hydrolyzable tannins in almonds or their distribution between varieties. Therefore, we characterized and quantified extractable and bound proanthocyanidins and hydrolyzable tannins in Nonpareil, Carmel, and Butte almond varieties.

3.4.1 Extractable Proanthocyanidins

The extractable proanthocyanidins from almond consisted of (+)-catechin and (-)-epicatechin, three dimer peaks, including proanthocyanidin B2, as well as trimers and greater oligomers (**Figure 3.1A**). Among the three almond varieties, Nonpareil had ~2-fold more extractable proanthocyanidins (107.27 mg/100 g almond) than Butte (25.35 mg/100 g almond) and Carmel (28.09 mg/100 g almond) because of increased oligomer content (**Table 3.1**). Nonpareil had 2-fold the monomers of Butte and 3-fold the monomers than Carmel. Nonpareil had 3-fold the dimers of Butte and Carmel, and 4-fold the trimers of Butte and Carmel. Besides, Nonpareil also had ~3 fold the oligomers (4-7) and 8mers and greater polymers than Butte and Carmel almonds. The extractable proanthocyanidin profiles, e.g. proportion to the sum of proanthocyanidin, varied among the three almond genotypes (**Figure 3.3A**). 8 mers and greater polymers were the most abundant proanthocyanidins among the three almond genotypes, ranging from 27.2 to 33.3 % of extractable proanthocyanidins, with the greatest polymer proportion in Carmel. Nonpareil had the greatest relative proportion of dimer 2, trimers and 4mers. Butte had the highest proportion of (+)-catechin (0.9%), (-)-epicatechin (6.8%), dimer 1(2.4%) and dimer 3 (15.9%). Carmel had the highest proportion of 5mers, 6mers and 7mers, which were 10.8%, 7.5% and 7.4%, respectively.

3.4.2 Bound Proanthocyanidins

The recovery of proanthocyanidins from defatted almond residue was greatest at 3 h, as quantified by the DMAC assay (**Figure 3.5A**). Alkaline hydrolysis of unextracted was maximized at 4 N sodium hydroxide by the DMAC assay (**Figure 3.5B**). Alkaline hydrolysis yielded 23 to 98% of the extractable proanthocyanidins, with the highest recovery in Carmel (**Figure 3.2**) ($P = 0.0002$ for extraction method, $P = 0.4105$ for genotype, and $P = 0.4004$ for

their interaction). In contrast, acid hydrolysis yielded minimal proanthocyanidin content, with < 0.2 mg catechin equivalents (CE)/100 g almond.

Following almond extraction with acidic aqueous acetone, acid hydrolysis of the residue did not yield additional proanthocyanidins by HPLC fluorescence analysis (<0.02 mg/100 g almond). Proanthocyanidins recovered from alkaline hydrolysis of extracted almond residue were quantified by HPLC (**Figure 3.1B**). Only proanthocyanidin monomers, dimers, and oligomers with polymerization of 8 and greater were recovered after hydrolysis (**Table 3.1**). Alkaline hydrolysis of extracted almond residue yielded an additional 3.4, 6.8, and 6.4 mg proanthocyanidin B2 equivalents (PE)/100 g almond in Nonpareil, Butte, and Carmel almonds, respectively (**Table 3.1**). These yields added 22 to 66% toward monomer content and 45 to 81% to dimer 1 content. Nonpareil almonds had the lowest recovery of bound proanthocyanidins, despite having the highest yield of extractable proanthocyanidins among almond varieties.

3.4.3 Total Proanthocyanidins

The sum of extractable and bound proanthocyanidins almonds reflects the total proanthocyanidin content and profile (**Table 3.1, Figure 3.2B**) of almonds. Nonpareil almonds had the most proanthocyanidins with 110.6 mg/100 g, representing 3.4- and 3.2-fold of Butte and Carmel varieties. Butte almonds had greatest relative proportion of monomers and dimers contributing ~50% to the total proanthocyanidin content. In contrast, trimers and greater oligomers contributed more than 70% of the proanthocyanidin content of Nonpareil and Carmel almonds. Base hydrolysis yielded an additional 3 to 27% of the sum of extractable proanthocyanidins (**Table 3.1**).

3.4.4 Hydrolysable tannins

Extractable almond tannin fractions were resolved by 2-dimensional TLC to screen for the presence of gallotannins and ellagitannins (**Table 3.3**). In each almond variety, at least 5 to 6 unique spots reacted with potassium iodate, indicating potential gallotannin content. Nonpareil and Butte almonds each had 4 orange-brown spots after a sodium nitrate spray, indicating the presence of ellagitannins (Mueller-Harvey 2001). Carmel almonds had a unique ellagitannin profile, with 3 orange-brown and 3 pink spots after the sodium nitrate spray. Low retention factors in the second TLC dimension tentatively indicated the presence of large molecular-weight ellagitannin and gallotannins (Mueller-Harvey 2001).

Gallic acid and ellagic acid were present in hydrolysis solutions of defatted almonds (**Figure 3.4**). Nonpareil, Butte, and Carmel almond contained 19.64 to 34.11 mg gallic acid/100 g, and 43.28 to 57.36 mg ellagic acid/100 g with no significant differences between varieties (n = 3/variety) (**Table 3.2**).

3.5 Discussion

3.5.1 Extractable Proanthocyanidins

Normal-phase HPLC resolution of proanthocyanidins in California almond varieties was similar to Spanish almonds (Prodanov, Garrido et al. 2008). Almond proanthocyanidins have (epi)afzelechin, (epi)catechin and (epi)gallocatechin as constituent units, with both A-type and B-type linkages and an average degree of polymerization of 12.7 (Gu, Kelm et al. 2003, Monagas, Garrido et al. 2007, Prodanov, Garrido et al. 2008). Almond proanthocyanidins with polymerization of 6 or more have only B-type linkages (Prodanov, Garrido et al. 2008).

Proanthocyanidins and hydrolyzable tannins contribute a significant portion of the polyphenol content of almonds and other tree nuts. Almonds have more extractable proanthocyanidins than walnuts, but less than hazelnuts, pecans and pistachios (Gu, Kelm et al. 2004). Gu and colleagues reported 184.1 mg extractable proanthocyanidins/100 g almond in a commercial almond sample using similar extraction conditions (Gu, Kelm et al. 2004). While the distribution of proanthocyanidin polymers is similar to this study, the content was higher than the 25 to 107 mg PE/100 g obtained in the present study. The use of proanthocyanidin B2 equivalents may contribute to this difference. Also, pre-harvest factors such as climate and environment and post-harvest factors, such as storage and processing may contribute to differences among almond samples (Bolling, Chen et al. 2011). However, our values appear greater than a previous study by Garrido and colleagues, adjusted by the relative proportion of almond skins to 100 g almonds (Garrido, Monagas et al. 2008).

DMAC values of extractable proanthocyanidins were 75.1% to 89.9% less than HPLC. DMAC reacts with terminal flavan-3-ols, so this method underestimates polymeric proanthocyanidins (Prior, Fan et al. 2010). The present work is also limited by a lack of suitable analytical standards for HPLC analysis. Further work is needed to purify proanthocyanidins of similar composition, linkages, and with varying polymerization to validate methods for quantification of almond proanthocyanidins.

The differences in extractable proanthocyanidins between Nonpareil, Carmel, and Butte almonds are greater than other reported polyphenols. In the present study, Nonpareil had more extractable proanthocyanidins than Butte and Carmel, while there were no differences in flavonoid and phenolic acid between these varieties (Bolling, Dolnikowski et al. 2010). Nonpareil almonds had significantly greater (-)-epicatechin content than the same study, with 3.4

mg/100 g almonds.(Bolling, Dolnikowski et al. 2010) It is unclear if this difference is due to yearly variation in polyphenol content or due to different extraction conditions.

3.5.2 Bound Proanthocyanidins

Acid hydrolysis was insufficient to release additional bound proanthocyanidins in almond, despite previous studies that have used acid hydrolysis prior to analysis of catechins (Merken and Beecher 2000, Harnly, Doherty et al. 2006). Alkaline hydrolysis yielded additional bound proanthocyanidins from extracted almond residue, as monomer and dimers. This is consistent with a previous study in cranberry pomace, where alkaline hydrolysis liberated 4-fold the monomer to hexamer proanthocyanidins than conventional extraction (White, Howard et al. 2010). Alkaline hydrolysis of cranberry polymeric proanthocyanidins yielded primarily monomers and dimers (White, Howard et al. 2010).

According to our results, bound proanthocyanidin yielded 3.1 to 26.8% of the extractable proanthocyanidins (**Table 3.1**). This proportion was less than that of apple, peach and nectarine, which bound proanthocyanidin were 6 to 20-fold of the extractable proanthocyanidins measured by HPLC (Arranz, Saura-Calixto et al. 2009). Other bound polyphenols were 2 to 6-fold of the extractable polyphenols in apple, peach and nectarine measured by LC-MS (Arranz, Saura-Calixto et al. 2009). However, the bound polyphenol content of tree nuts may be less than other fruits and vegetables. In walnuts and heartnuts, bound total phenolic contents were for 20 to 77 % of extractable total phenolic contents measured by Folin-Ciocalteu assay (Li, Tsao et al. 2006). In a study of 29 kinds of food, bound phenolic compounds accounted nearly the same or more antioxidant capacity than free phenolic compounds measured (Pellegrini, Serafini et al. 2006). Thus, the proportion of bound polyphenols may depend on plant varieties, genotypes, and

polyphenol composition. Further work is needed to define the contribution of bound polyphenols to almond bioactivity.

3.5.3 Total Proanthocyanidins

The proanthocyanidin profile of Butte and Carmel almonds changed after summing extractable and bound proanthocyanidins. This was due to the high content of monomers, dimers and trimers in Butte and Carmel almonds following alkaline hydrolysis of their extracted residue. For oligomers (>3) and polymers, the total proanthocyanidins profile reflected the extractable proanthocyanidin profile.

3.5.4 Hydrolyzable Tannins

Hydrolyzable tannins vary according to their polymeric level of gallic acid and ellagic acid (Chung, Wong et al. 1998). TLC analysis indicated the presence several different gallotannins and ellagitannins in almonds. A number of ellagitannins have been identified in walnut, including pedunculagin, glansrins, casuarictin and others (Fukuda, Ito et al. 2003, Cerdá, Tomás-Barberán et al. 2005). Thus, almonds may also contain a diverse ellagitannin profile. Precaution should be taken when selecting almond tannin TLC bands for further analysis, since potassium iodate also reacts with catechins (**Table 3.3**). Further work is needed to isolate and identify these compounds from almonds.

Walnuts and pecans contain hydrolyzable tannins (Daniel, Krupnick et al. 1989). In comparison to previous studies, almond ellagitannins are 3 to 10-fold less than walnut, which have 150 to 546 mg of ellagic acid/100 g (Cerdá, Tomás-Barberán et al. 2005, Li, Tsao et al. 2006). A previous study reported almond contain little to no gallotannins and ellagitannins following hydrolysis (Molyneux 2008). However, this study employed a more moderate

hydrolysis condition than the present study. Thus, almond hydrolysable tannin content may have been previously underestimated. Ellagic acid consumption in the United States is estimated to be 3.4 to 15.1 mg/1000 kcal energy in adult females, varying by whether they meet fruit and vegetable intake recommendations (Murphy, Barraj et al. 2012). Therefore, consuming a 43 g (1.5 oz) serving of almond could more than double the estimated ellagic acid intake.

While originally recognized as anti-nutrients, a number of putative health effects are attributed to tannins. Gallic acid is antioxidant and apoptotic to SH-SY5Y and U 937 cancer cells *in vitro* (Saeki, Yuo et al. 2000, Lu, Nie et al. 2006). Likewise, ellagic acid has *in vitro* antioxidant potential, anti-inflammatory, estrogenic and/or anti-estrogenic roles and antimicrobial and prebiotic effects (Landete 2011). However, ellagic acid is not bioavailable from ellagitannins, and is metabolized to the bioavailable urolithins, which may be a marker for nut intake (Tulipani, Urpi-Sarda et al. 2012). Almond proanthocyanidins are bioavailable, and tend to be metabolized to (epi)catechin conjugated metabolites by phase II enzymes after 2 to 6 h consumption in healthy adults (Garrido, Urpi-Sarda et al. 2010). There is a need to characterize the bioaccessability of bound phenolics in almonds and other polyphenol-rich foods. Thus, future studies characterizing the effects of almond polyphenol consumption, should consider of hydrolyzable tannins and bound proanthocyanidins.

3.6 Conclusion

In conclusion, almonds are a source of diverse polyphenols, including phenolic acids, flavonoids, proanthocyanidins, ellagitannins, gallotannins, and likely other uncharacterized constituents. Base hydrolysis yielded additional polyphenols from extracted almond residue, increasing total proanthocyanidin content 3 to 21 % in California almonds. Almonds were also found to contain 27.43 mg gallic acid and 54.69 mg ellagic acid/100 g following hydrolysis.

Database values and future analytical research efforts should consider the presence of “bound” polyphenols, ellagitannins, and gallotannins in California almonds.

3.7 Chapter 3 Tables and Figures

Table 3.1 Proanthocyanidin content and the extractable proanthocyanidin proportion of California almond cultivars

	Nonpareil			**Butte**			**Carmel**		
PACs	**Extractable**	**Base hydrolysis**	**Total**	**Extractable**	**Base hydrolysis**	**Total**	**Extractable**	**Base hydrolysis**	**Total**
(+)-catechin	0.33 ± 0.10	0.10 ± 0.05	0.43 ± 0.13	0.14 ± 0.13	0.18 ± 0.06	0.32 ± 0.10	0.10 ± 0.03	0.22 ± 0.10	0.31 ± 0.08
(-)-epicatechin	3.44 ± 0.50	1.17 ± 0.89	4.61 ± 1.20	1.24 ± 1.25	2.64 ± 1.14	3.88 ± 1.08	0.76 ± 0.31	1.80 ± 1.03	2.56 ± 1.16
PAC dimer 1	1.99 ±1.60	1.45 ± 0.93	3.43 ± 1.38	0.43 ± 0.40	1.80 ± 0.54	2.23 ± 0.14	0.37 ± 0.06	1.89 ± 1.22	2.26 ± 1.29
PAC B2	8.29 ± 2.88	0.03 ± 0.03	8.31 ± 2.90	1.53 ± 1.83	0.79 ± 1.32	2.32 ± 1.52	1.17 ± 0.46	0.05 ± 0.03	1.22 ± 0.47
PAC dimer-3	8.39 ± 2.17	0.02 ± 0.01	8.41 ± 2.18	2.49 ± 2.01	0.21 ± 0.25	2.69 ± 1.83	2.43 ± 0.18	0.11 ± 0.06	2.55 ± 0.20
trimers	14.03 ± 3.26		14.03 ± 3.26	2.97 ± 3.84		2.97 ± 3.84	2.68 ± 1.46		2.68 ± 1.46
4-mers	15.10 ± 3.53		15.10 ± 3.53	3.15 ± 4.00		3.15 ± 4.00	3.34 ± 2.09		3.34 ± 2.09
5-mers	10.87 ± 4.10		10.87 ± 4.10	2.23 ± 2.86		2.23 ± 2.86	3.08 ± 1.88		3.08 ± 1.88
6-mers	7.23 ± 2.33		7.23 ± 2.33	1.61 ± 2.09		1.61 ± 2.09	2.22 ± 1.57		2.22 ± 1.57
7-mers	6.33 ± 2.11		6.33 ± 2.11	1.65 ±2.14		1.65 ± 2.14	2.21 ± 1.63		2.21 ± 1.63
8-mers and greater	31.28 ± 8.87	0.61 ± 0.07	31.89 ± 8.81	7.91 ±10.45	1.18 ± 0.98	9.09 ± 10.12	9.74 ± 6.72	2.29 ± 0.33	12.03 ± 7.04
Sum	107.27	3.37	110.6	25.35	6.80	32.15	28.09	6.37	34.46

Table 3.2 Hydrolyzable tannin content of California almond cultivars determined by methanolysis and HPLC analysis.

Hydrolyzable Tannin	**Value**	**Almond Cultivar**		
		Nonpareil	Butte	Carmel
mg gallic acid/100 g	mean	34.11± 2.36	28.54 ± 10.45	19.64 ± 5.00
	range	32.6-32.8	22.4-40.6	14.1-23.8
mg ellagic acid /100 g	mean	57.36 ± 6.44	53.28 ± 1.45	53.44 ± 5.36
	range	50.5-63.2	54.6-54.3	48.7-59.3

Data are mean ± standard deviation, n = 3/cultivar. Statistical significances among almond cultivars by ANOVA were $P = 0.1016$ for gallotannins, and $P = 0.5490$ for ellagitannins.

Table 3.3 Two dimensional TLC profiles of almond tannins.

Genotype	Sample	Spray Reagent					
		Gallotannins (KIO_3)			**Ellagitannins ($NaNO_2$)**		
		Rf_1	Rf_2	Color[b]	Rf_1	Rf_2	Color
Nonpareil	2079	0.234 (B [a])	0	brown	0.435 (B)	0	orange-brown
		0.208 (B)	0.138	brown	--	--	--
		0.734	0.225	brown	--	--	--
	2080	0.833 (B)	0	brown	0.723 (B)	0	orange-brown
		0.303 (B)	0.180	brown	0.730	0.222	orange
		0.877	0.066	brown	--	--	--
		0.809	0.216	brown	--	--	--
	2081	0.950 (B)	0	brown	0.712 (B)	0	orange-brown
		0.256 (B)	0.129	brown	0.600	0.216	orange
		0.844	0.082	brown	0.689	0.252	orange
		0.750	0.224	brown	--	--	--
Butte	2082	0.862 (B)	0	brown	0.700 (B)	0	orange-brown
		0.745	0.083	brown	0.680	0.222	orange
		0.655	0.232	brown	--	--	--
	2083	0.258 (B)	0	brown	0.323 (B)	0	orange-brown
		0	0.136 (B)	brown	0.194 (B)	0.205	orange-brown
		0.182	0.178	brown	0.684	0.211	orange-brown
		0.711	0.201	brown	--	--	--

	2084	0.312 (B)	0	brown	0.388 (B)	0	orange-brown
		0.243 (B)	0.139	brown	0.273 (B)	0.151	orange-brown
		0.832	0.208	brown	0.792	0.235	orange
Carmel	2085	0.205 (B)	0	brown	0.440 (B)	0	pink
		0.325 (B)	0	brown	0.217 (B)	0	orange-brown
		0.723	0.224	brown	0.464	0.135	pink
		--	--	--	0.512	0	pink
		--	--	--	0.602	0	pink
	2086	0.823 (B)	0	brown	0.351 (B)	0	orange-brown
		0.253	0.194	brown	0	0.136	orange-brown
		--	--	--	0.225 (B)	0.183	orange-brown
	2087	0.477 (B)	0	brown	0.433 (B)	0	orange-brown
		0	0.151 (B)	brown	0	0.128 (B)	orange-brown
		0.305(B)	0.205	brown	0.293 (B)	0.151	orange-brown

For standard compounds, potassium iodate spray gave an orange spot for (+)-catechin (0.739, 0.269), a brown spot for gallic acid (0.816, 0.313), and brown-pink spots for tannic acid (0.365-0.825, 0), (0.745, 0.176), (0.650, 0.364), (0.380, 0.339) and (0.277, 0.461). Sodium nitrite spray gave a brown-pink spot with ellagic acid (0.5347, 0).

[a]B: band observed

[b]Reactivity of spot with spray reagent

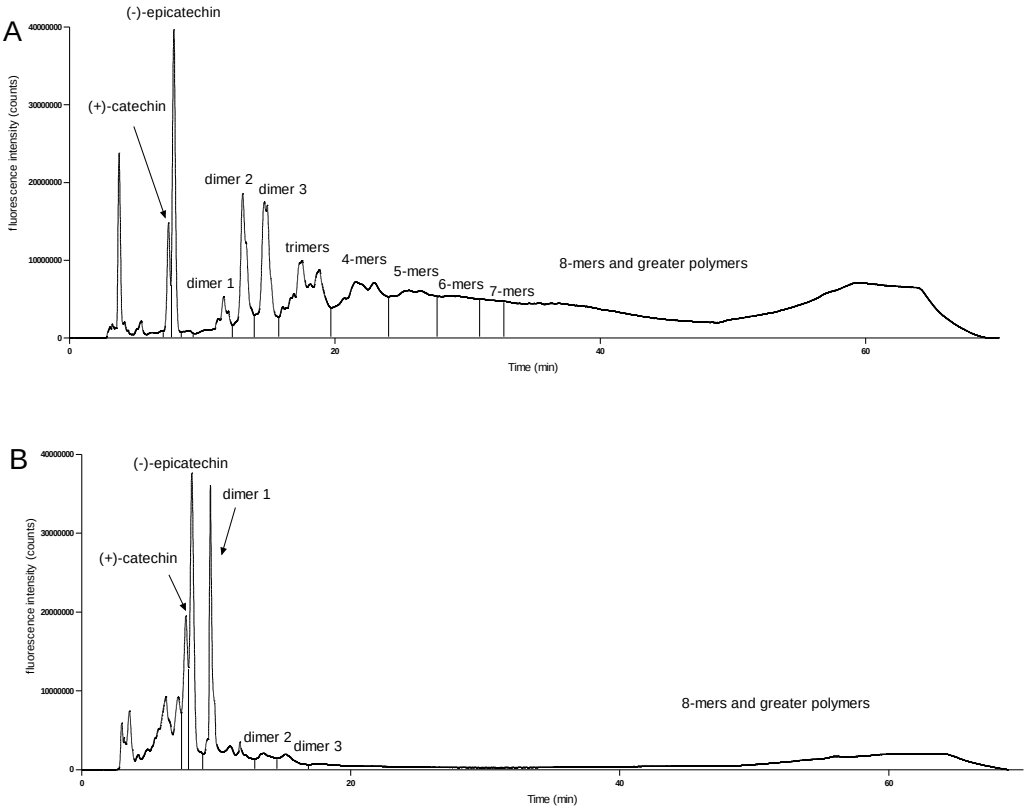

Figure 3.1 A. Representative HPLC fluorescence chromatogram of solvent-extractable almond proanthocyanidin fraction. B. Representative HPLC fluorescence chromatogram of base hydrolyzed almond proanthocyanidin fraction.

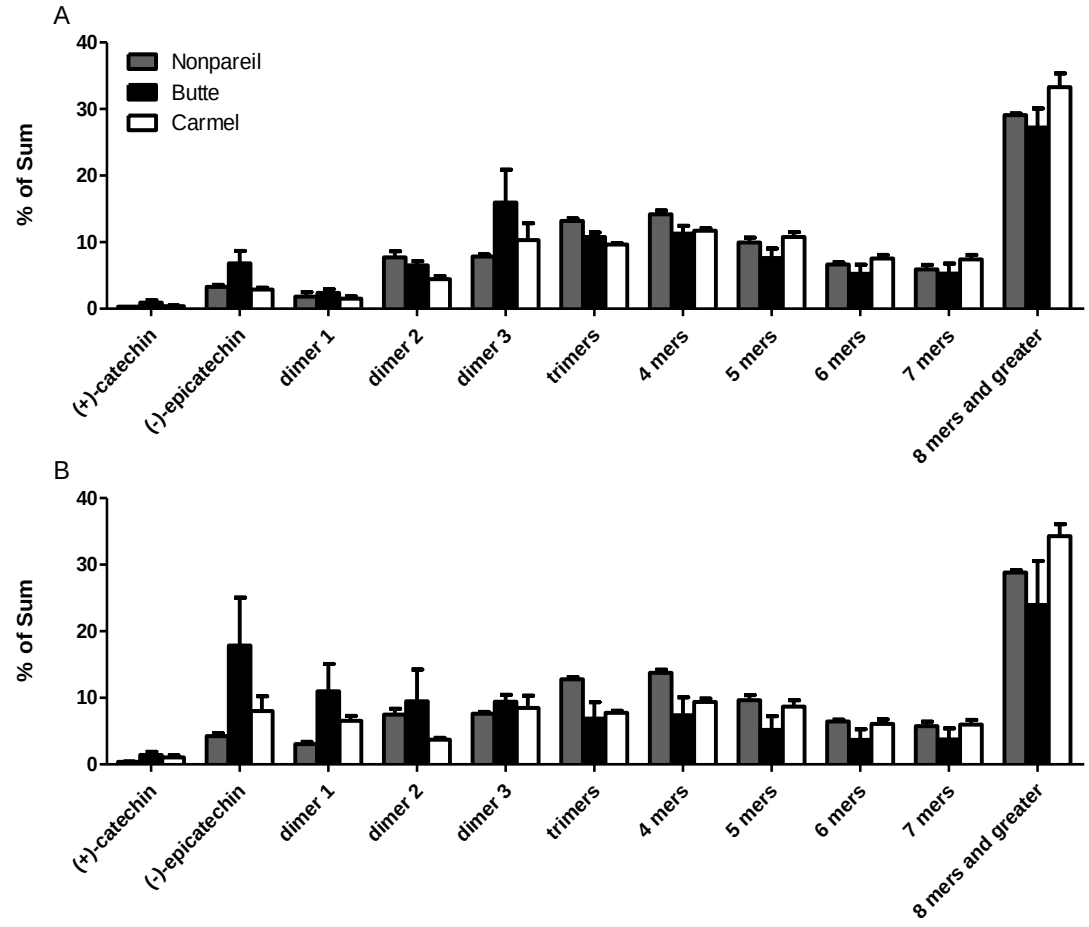

Figure 3.2 A. Relative extractable proanthocyanidin profile of California almond genotypes. Data are mean ± SEM of Nonpareil, Butte, and Carmel almonds (n=3/genotype). By 2-way ANOVA, P =0.2921 for genotype, P <0.0001 for proanthocyanidin type, with P =0.0087 for their interaction. B. Relative total proanthocyanidin profile of California almond genotypes. Data are mean ± SEM of Nonpareil, Butte, and Carmel almonds (n=3/genotype). By 2-way ANOVA, P =0.5959 for genotype, P <0.0001 for proanthocyanidin type, with P =0.0019 for their interaction.

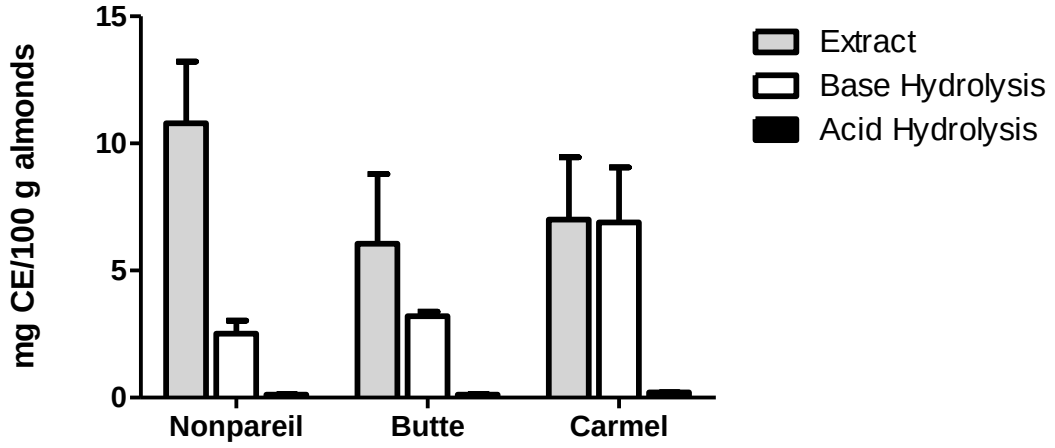

Figure 3.3 Proanthocyanidin content of extracted and hydrolyzed residues from California almond genotypes determined by the DMAC (4-(dimethylamino)cinnamaldehyde) assay. CE: catechin equivalents. By 2-way ANOVA, P = 0.0002 for extraction method, P = 0.4105 for genotype, and P = 0.4004 for their interaction.

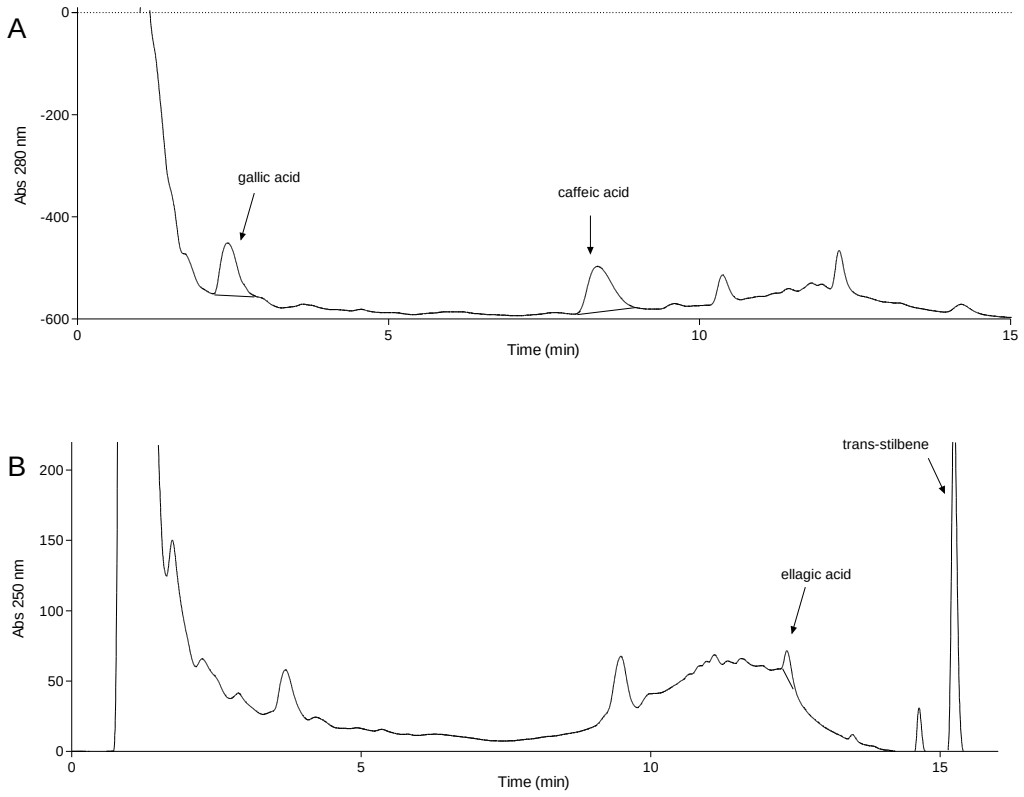

Figure 3.4 A. Representative HPLC fluorescence chromatogram of almond gallic acid fraction.

B. Representative HPLC fluorescence chromatogram of almond ellagic acid fraction.

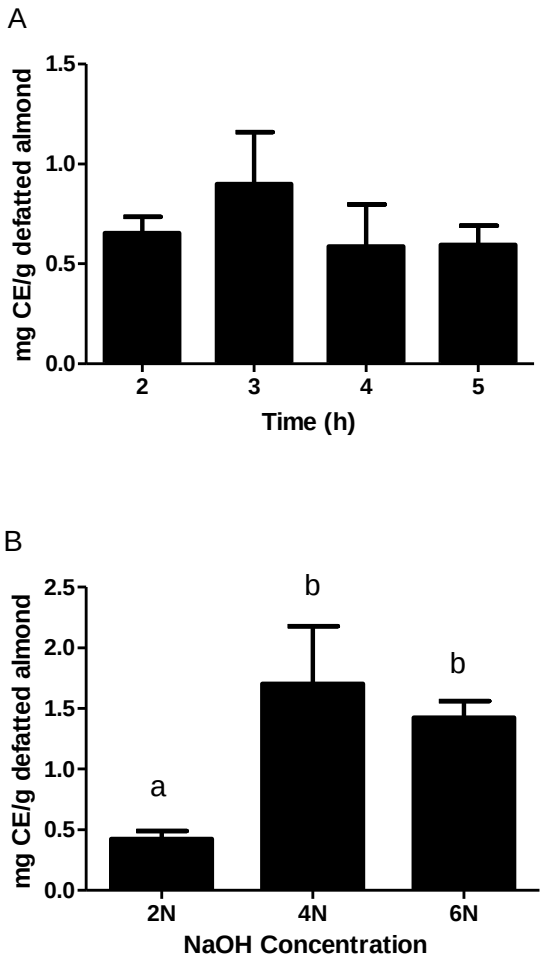

Figure 3.5 Recovery of proanthocyanidins following A. acidic and B. alkaline hydrolysis of defatted almond powder. Data are mean ± standard deviation of triplicate determinations, where P=0.6514 for acidic and P = 0.0189 for alkaline hydrolysis, by one-way ANOVA.

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Chapter 4: Characterize the tannin and stilbene composition of almonds

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4.1 Abstract

Background: Stilbene polyphenols are present in some fruits and nuts, but their abundance in many foods, such as almonds, is unknown.

Objectives: To develop UHPLC-MS methods to quantitate and characterize stilbenes content in almond.

Design: Stilbenes in almond were characterized in Nonpareil, Carmel, and Butte almond varieties from California, with n = 3 samples/variety.

Results: Stilbenes were isolated from ethanolic almond extracts by solid phase extraction and identified with UHPLC–MS by comparison of retention times (RTs), mass spectra, insource CID spectra, and enzymatic hydrolysis to authentic standards. Polydatin was identified in almond extracts, with 7.19–8.52 μg/100 g almond. Piceatannol + oxyresveratrol was tentatively identified in almond blanch water, at 0.19–2.55 μg/100 g almond. Polydatin was concentrated in almond skins, which contained 95.6–97.5% of the total almond content.

Conclusion: Almonds contain the stilbene class of polyphenols in addition to the previously identified proanthocyanidin, hydrolysable tannin, flavonoid, and phenolic acid classes.

4.2 Introduction

Almonds (*Prunus dulcis*) contribute to dietary polyphenol intake, and are ranked as one of the top 40 richest food sources of polyphenols (Perez-Jimenez, Neveu, Vos, & Scalbert, 2010). Clinical and preclinical studies have demonstrated that almonds have antioxidant, anti-diabetic, and hypocholesterolemic actions (Li, Jia et al. 2007, Li, Liu et al. 2011, Foster, Shantz et al. 2012).

The most abundant class of polyphenols in almonds is proanthocyanidins, followed by hydrolysable tannins, flavonoids and phenolic acids (Bolling, Chen et al. 2011, Xie, Roto et al. 2012). Almond phenolic acids and flavonoids have been well-characterized, and are enriched in almond skins (Milbury, Chen et al. 2006, Mandalari, Tomaino et al. 2010). Some almond polyphenols are readily extractable, while a smaller proportion requires hydrolysis of the extracted residue to be liberated from the food matrix (Xie, Roto et al. 2012).

The polyphenol diversity of many foods have been reported and indexed in databases such as Phenol-Explorer (Neveu, Perez-Jiménez et al. 2010). Despite these efforts, a significant portion of dietary polyphenols may be underestimated simply because previous analytical studies have not targeted particular polyphenol classes for certain foods. A more complete characterization almond polyphenols is desirable to relate almond bioactivities to its chemical composition. For example, we recently reported that almonds contain ellagitannins and gallotannins (Xie, Roto et al. 2012). Likewise, stilbenes are not well-characterized in nuts. Stilbenes have been reported in peanuts and pistachios, but have yet to be identified in almonds, Brazil nuts, hazelnuts, macadamias, pine nuts, or walnuts (Sobolev and Cole 1999, Tokuşoğlu, Ünal et al. 2005).

Stilbenes are synthesized in plants through the shikimate and phenylalanine/polymalonate pathways (Jeandet, Delaunois et al. 2010). The reported stilbene content of foods is generally less than other polyphenols, e.g. flavonoids and tannins. *Trans*-resveratrol or its glucoside is the most abundant stilbene in wine, grapes, peanuts and *Vaccinium* species (Cassidy, Hanley et al. 2000, Rimando, Kalt et al. 2004). Stilbenes may contribute to the health-promoting potential of polyphenol-rich foods, through antioxidant or phytoestrogen activities (Cassidy, Hanley et al. 2000).

The content and distribution of stilbenes in almonds is unknown. Therefore, the objectives of this study were to 1) develop a UHPLC-MS method for determining stilbenes in almonds; 2) identify and quantitate stilbenes in Nonpareil, Carmel, and Butte California almonds, as these are the top three cultivated varieties of California almonds; and 3) determine the compartmentalization of stilbenes in almonds.

4.3 Materials and methods

4.3.1 Chemicals and plant materials

Methanol and formic acid were LC-MS grade and acetyl acetate was HPLC grade from Fischer Scientific (Fair Lawn, NJ). Ethanol was absolute, anhydrous, ACS/USP grade from Pharmco-AAPER (Brookfield, CT). Water was Type II ultrapure grade produced by Picopure®2 (Hydro Service and Supplies, Inc). β-D-glucosidase (49290-1G), and all other chemicals and reagents were acquired from Sigma-Aldrich (St. Louis, MO) unless otherwise noted**.** Samples of whole, unpasteurized Butte, Carmel, and Nonpareil California almonds from harvest year 2011 were provided by the Almond Board of California. Each variety had 3 unique samples representing different orchards, for a total of n = 9 samples. Samples were not matched by orchard location or geography. Almond samples were stored at -20 °C in darkness until analysis.

4.3.2 Extraction of almond stilbenes.

Whole, frozen almonds were ground to a powder using an IKA A11 Basic Grinder (St. Louis, MO). Extraction was based on methods previously described, with modifications (Sanders, McMichael et al. 2000). First, 10 g of almond powder was homogenized in 20 mL of ethanol and water (80:20, v/v) at 4 °C for 5 min using an IKA T-18 Basic Ultra-Turrax homogenizer at power level 4 (Staufen, Germany). The homogenate was then centrifuged for 10 min at 4 °C and 3000 × g, and the supernatant was removed from the residue. The residue was homogenized and supernatant removed as above, for a total of three extractions. The supernatants were then combined and stored at -20 °C in darkness before clean-up. Before the clean-up step, supernatants were spiked with internal standard 1 (IS1, *trans*-resveratrol).

For compartmentalization studies, almond skins were separated by hot water blanching (Bolling, Dolnikowski et al. 2009). A sample of ~100 g whole almond was placed in a beaker filled with 175 mL boiling water for 2 min. The blanch water was decanted, and its volume was recorded. The blanched almond skins were manually removed from the seed, and the meat and skins were dried at room temperature overnight. Almond meat, skins, and blanch water were stored at -20 °C until analysis. For extraction, almond skins (1 g) or meat (10 g) was powdered by a lab mill and homogenized in 20 mL of extraction solvent as described above. Blanch water was filtered through a 2.0 µm nylon membrane, spiked with the appropriate internal standard 2 (IS2, pterostilbene), and analyzed directly by UHPLC-MS.

4.3.3 Clean-up of extract

The combined supernatant from whole-almond homogenate was cleaned-up by solid phase extraction (SPE) (Sanders, McMichael et al. 2000). First, a 1 cm diameter column was packed with 1 g of 1:1 (w/w) 50-200 µm neutral alumina and 40-63 µm, 60Å, endcapped C18 silica gel

(Sorbent Technologies, Norcross, GA). The column was successively preconditioned with 10 mL each of water, ethanol, and ethanol:water (80:20, v/v). The extract supernatant (3 mL) was applied to the column and collected by vacuum (2 to 3 drops per second). An additional 6 mL of 80% ethanol was applied to the column and the eluate was collected. Both elutes were combined, dried under nitrogen gas at 23 °C, then held at -80 °C until UHPLC-MS analysis.

4.3.4 UHPLC-MS analysis of stilbenes

The dried residue from SPE was reconstituted with 50 µL of methanol and spiked with the appropriate internal standard (IS2) in 1 µL methanol. UHPLC-MS analysis was conducted using a Shimadzu Nexera UHPLC equipped with DGU-20A_5 Prominence Degasser, SIL-30AC Nexera Auto Sampler, SPD-M20A Prominence Diode Array Detector, CTD-30A Nexera column oven, and two LC-30AD pumps (Kyoto, Japan) equipped with a Phenomenex Kinetex PFP column (1.7 µm, 100 Å, 2.1 × 50 mm, Torrance, CA, USA). The following gradient of water/formic acid (99.05:0.5, v/v) (A) and methanol (B) was used: 0 min, 70% A and 30% B; 6 min, 10% A and 90% B; 7 min, 10% A and 90% B; 8 min, 70% A and 30% B and held until the stop time at 10 min. The flow was maintained at 0.2 mL/min and the sample injection volume was 1 µL. Absorbance was monitored at 320 nm. Mass spectrometry analyses were performed using a Shimadzu LCMS-2020 mass spectrometer equipped with ESI, APCI, and DUIS (dual ionization) modes. The data were acquired in full-scan DUIS mode (MS) from m/z 100 to 500 in both positive and negative polarity modes, with instrument settings as follows: detector voltage was 1.1 kV, interface voltage was 4.5 V, nebulizing gas flow was 1.5 L/min, drying gas flow was 15.0 L/min, desolvation line was 250°C, heat block was 500°C, and Q-array DC was varied according to compounds of interest (**Table 4.1**). Signal (S) to noise (N) ratios were determined experimentally using area under the curve (AUC) values of external standards. The limit of detection (LOD)

(S/N > 3) and limit of quantification (LOQ) (S/N > 10) for standard compounds were determined experimentally by at least 3 injections of 2-fold serial dilutions of the calibration curve, where S/N = AUC/standard deviation$_{AUC}$ (Long and Winefordner 1983). Recovery was determined using IS1, whereas IS2 was used to normalize AUC values for quantitation. Quantification was based on MS AUC responses.

4.3.5 Enzymatic hydrolysis of stilbene glucosides

Almond extract was hydrolyzed as previously described (Rimando and Barney 2005) with modification. Briefly, 10 mL of almond extract (Section 2.2) was dried to ~1 mL by a nitrogen gas stream at 23 °C to remove ethanol. The pH of the extract was adjusted to 6 by using 0.1 M sodium hydroxide. The solution was hydrolyzed by incubating 30 units of β-D-glucosidase for 18 h at 37 °C. The resulting solution was extracted three times with 6 mL ethyl acetate. The ethyl acetate extracts were combined and dried under a nitrogen gas stream at 23 °C. The dried ethyl acetate extract was resuspended in 3 mL ethanol:water (80:20, v/v) and subjected to SPE clean-up as above (Section 2.3).

4.3.6 Statistical analysis

Data are presented as mean ± standard deviation of triplicate analyses of n samples indicated in tables and figures. Statistical significance was determined by two or one-way ANOVA, followed by Tukey's multiple comparison test using GraphPad Prism v 5.01 software (GraphPad Software, Inc., La Jolla, CA). Differences were considered significant at $P \leq 0.05$.

4.4 Results

4.4.1 Method validation

As shown in **Table 4.1**, 11 standards of stilbenes and stilbenes analogous were screened. Among them, *trans*-stilbene and E-resveratrol trimethyl ether could not be ionized under the given condition. 5 of them, polydatin, piceatannol, *trans*-resveratrol, dienestrol and oxyresveratrol, were ionized under negative model. Piceatannol and oxyresveratrol were eluted under the same RT and m/z ratio. The other 4 standards, pterostilbene, *cis*-resveratrol, hexestrol and d4-resveratrol were ionized under positive model. The condition of Q-array DC was also optimized, which too high may breakdown the ionized structure, and too low may insufficient to ionize the molecular. The on column LOD and LOQ of 4 common stilbenes: polydatin, piceatannol, *trans*-resveratrol and pterostilbene were also determined. They were ranged from 0.38 pg/column to 24.41 pg/column, and from 1.53 pg/column to 146.48 pg/column. Pterostilbene, the most sensitive standard, had a 64-fold greater LOD sensitivity and a 96-fold greater LOQ sensitivity than *trans*-resveratrol. Comparing with its precursor, *trans*-resveratrol had a 16-fold lesser LOD sensitivity and a 24-fold lesser LOQ sensitivity than polydatin.

The recovery rates for polydatin, piceatannol, trans-resveratrol, and pterostilbene were determined by spiking homogenates with standards prior to SPE clean-up (**Table 4.2**). Stilbene recoveries from SPE were 61 to 73%, except for piceatannol, which was not recovered. Piceatannol is rarely reported in botanical analysis, because the alumina/C18 SPE methods typically employed for stilbene clean-up give inadequate recovery of piceatannol (Sanders, McMichael et al. 2000, Rudolf, Resurreccion et al. 2005). Piceatannol analysis requires either direct injection or use of a C18 SPE clean-up method (Lin, Lien et al. 2007).

The intraday and interday variation of IS1 and IS2 were 7.5% and 8.3%, and 3.6% and 1.4%, respectively.

Dienestrol, *cis*-resveratrol, hexesterol, and d4-resveratrol could not be used as internal standards because of their incomplete resolution from compounds with the same m/z in almond extracts. Oxyresveratrol was not a suitable internal standard, as it co-eluted with piceatannol at an identical m/z ratio, and may be present in almonds (Section 3.2). Thus, none of these five stilbenes was a suitable internal standard. *Trans*-resveratrol was not observed in any preparations or samples run while characterizing almond stilbene content, and pterostilbene was not recovered from almond extracts (**Table 4.2**). Therefore, we used *trans*-resveratrol (IS1), to determine variance introduced by SPE and drying, and pterostilbene (IS2) to determine variance introduced by UHPLC-MS.

4.4.2 Identification of almond stilbenes

Based on the mass spectra of almond extract (**Figure 4.1)**, only polydatin was identified based on the RT and m/z ratio. The other stilbene components were under the LOD.

Due to the limitation of the clean-up step, piceatannol might present in almonds. However, it could not be determined based on the method used in this paper.

In contrast to almond extracts, which required SPE cleanup prior to UHPLC-MS analysis, almond blanch water was analyzed directly after filtration. Therefore, we examined if piceatannol was present in blanch water. Indeed, a peak corresponding to piceatannol was identified at 3.5 min and $[M-H]^-$ m/z 243 (**Figure A.4.1**). Piceatannol and oxyresveratrol co-eluted in our UHPLC-MS method, so we could not exclude its presence in almonds. Therefore the piceatannol+oxyresveratrol content of almond varieties was 0.91-2.55 µg/100 g as quantitated by a piceatannol standard (**Table 4.3**). When accounting for the blanch water

recovery of piceatannol+oxyresveratrol, the total stilbenes content in almond were 8.33-9.60 μg/100 g almonds.

4.4.3 Polydatin identification and quantitation in almond.

As shown in **Figure 4.7.1**, both specific RT which equaled to 2.9 and m/z ratio at 389 can testify the presentation of polydatin. However, other evidence should also be provided to rule out the possibility of other similar components. Due to the content of polydatin in almonds were insufficient for visible absorbance detection under 320 nm. A hydrolysis method was developed for polydatin identification.

A β-D-glucosidase was chosen for hydrolyzing the β-D-glucosidic linkage between *trans*-resveratrol and glucoside on polydatin. As shown in **Figure 4.2**, an ionized peak at 4.0 min (RT) and 227 m/z was shown on the chromatography of almond after hydrolysis. The peak had the similar RT and m/z ratio as the ionized *trans*-resveratrol. In contrary, a smaller ionized polydatin peak was also found, which indicated the polydatin in almond was hydrolyzed to *trans*-resveratrol.

By quantitative analysis of whole-almond ethanolic extracts, the polydatin content of Nonpareil, Butte and Carmel almonds was 7.19-8.52 μg/100 g almond and not significantly different between varieties ($P = 0.8519$) (**Table 4.2**).

4.4.4 Compartmentalization of almond stilbenes

Following almond blanching, the blanch water contained 96 to 98% of the total polydatin content, which were markedly higher than what was recovered in the dried skin (2 to 3%) and meat (~1%) after extraction ($P < 0.0001$). When compartmentalized by blanching, polydatin content was highest in Butte and Carmel skins and meat and Nonpareil blanch water (**Table 4.3**).

4.5 Discussion

A UHPLC-MS method was developed to quantitate stilbenes in almonds. Commercially available stilbenes or potential internal standards were examined for suitability for UHPLC-MS analysis (**Table 4.1**). In our preliminary evaluation of standards by UHPLC-MS, pterostilbene, *cis*-resveratrol, hexestrol and d4-resveratrol had better S/N responses in positive ionization than negative ionization mode. In contrast, polydatin (resvatrol-3-*O*-β-glucoside), piceatannol, *trans*-resveratrol, dienestrol and oxyresveratrol had better S/N in negative ionization than positive ionization mode. Other LC-MS quantitative methods for stilbenes have employed solely positive or negative ionization of stilbenes by ESI analysis (Kammerer, Claus et al. 2004, Counet, Callemien et al. 2006, Burkon and Somoza 2008).

The Q-array DC voltage was also optimized for available commercial standard compounds (**Table 4.1**). In the Shimadzu LCMS2020, the Q-array is set after the ion source before the quadrupole and can apply a voltage that focuses ions entering the quadrupole. Increasing Q-array voltage may induce fragmentation of ions entering the quadrupole. The optimal Q-array voltages to maximize S/N varied between stilbenes. Both *cis*- and *trans*-resveratrol along with polydatin gave better responses at a Q-array of -30 V. Cycling Q-array voltage from -100 to 100 V did not significantly improve detection of other stilbenes. *Trans*-stilbene and E-resveratrol trimethyl ether were not ionized under a range of conditions tested (Q-array DC from -100 to 100 V). *Trans*-stilbene was previously ionized by atmospheric-pressure chemical-ionization mass spectrometry using benzene as a carrier solvent (Macha, McCarley et al. 1999). *E*-resveratrol trimethyl ether cations were ionized by ESI using a methanol-water mixture with 10 mM ammonium acetate and 0.2% formic acid (Ma, Liu et al. 2007).

In ethanolic extracts of whole almonds, the stilbenes piceatannol, *trans*-resveratrol, and pterostilbene were below the LOD (**Figure 4.1**). These stilbenes have previously been reported in foods. For example, piceatannol was 0.14−0.42 μg/g dry weight in two *Vaccinium* species (Rimando, Kalt et al. 2004, Rimando and Barney 2005). *Trans*-resveratrol was reported in pistachio (0.09-1.67 μg/g), cocoa products (0.4-0.5 μg/g), peanut products (0.06-5.14 μg/g), *Vaccinium* species (0.01-5.88 μg/g dry weight), and grape skins (11.1-123.0 μg/g dry weight) (Sobolev and Cole 1999, Kammerer, Claus et al. 2004, Rimando, Kalt et al. 2004, Rimando and Barney 2005, Tokuşoğlu, Ünal et al. 2005, Counet, Callemien et al. 2006). Pterostilbene was also reported in two *Vaccinium* species, at 0.1-0.5μg/g dry weight (Rimando, Kalt et al. 2004, Rimando and Barney 2005). Therefore, in most stilbene-containing foods, *trans*-resveratrol appears to be more abundant and widely distributed than other stilbenes.

Polydatin was identified in whole almond ethanolic extracts based on RT (2.9 min) and the presence of the pseudomolecular ion $[M-H]^-$ at m/z 389, together with a peak corresponding to the resveratrol daughter ion $[M-H\text{-glucose}]^-$ at m/z 227 matching with a reference standard (**Figure 4.1**). We performed Q-array experiments to further confirm this peak as polydatin, as MS scan spectra were not definitive due to the presence of co-eluting compounds. Based on the presence of the m/z 227 (-) daughter ion at the same RT as polydatin, we assessed the relationship of these peaks by increasing Q-array DC voltage. Increasing the Q-array DC reduced the polydatin, and increased $[M\text{-glucose}]^-$ at m/z 227 at the same RT (**Figure 4.2**). A similar result was obtained when increasing the Q-array voltage during injections of almond extracts (**Figure A.4.2**). Therefore Q-array fragmentation and enzymatic hydrolysis of whole almond extract corroborate peak the peak at 2.9 min as polydatin. A similar approach could be used to screen for other polyphenol glucoside-conjugates by UHPLC-MS.

We utilized a β-D-glucosidase hydrolysis method to identify other putative stilbene glucosides in almond extracts. Enzymatic hydrolysis of almond extract reduced polydatin and catalyzed the formation of *trans*-resveratrol, and eliminated putative resveratrol-containing peaks at 1.2, 3.4, 5.2 and 5.7 min (**Figure 4.2**). Due to the presence of co-eluting peaks, MS scan data did not provide further insight into the identity of these compounds. Further work utilizing MS/MS methods is necessary to determine if these unidentified peaks are composed of resveratrol glucosides. For example, grape cell suspension cultures produced resveratrol diglucoside (Decendit, Waffo-Teguo et al. 2002) and stilbene dimers and dimer diglucosides were isolated and characterized from wine (Baderschneider and Winterhalter 2000). Thus, almond stilbene content may be more abundant and diverse than reported in the present study.

Polydatin was also previously quantitated in peanut (7-38 µg/100 g), cocoa (100-120 µg/g), and pistachio (620 to 820 µg/100 g) (Counet, Callemien et al. 2006, Grippi, Crosta et al. 2008, Potrebko and Resurreccion 2009). In previous studies resveratrol content varied 31% among 12 pistachio samples and ~90-fold among 15 peanut cultivars (Sanders, McMichael et al. 2000, Grippi, Crosta et al. 2008). Further work is needed to determine the relationship of almond stilbene content to climactic, genotype, seasonal, and geographical variance, as these variables can influence polyphenol content of tree nuts (Bolling, Chen et al. 2011).

Polydatin was primarily present in almond skin, and readily extracted by hot water. Like almonds, stilbenes are also concentrated in grape and peanut skins (Sanders, McMichael et al. 2000, Kammerer, Claus et al. 2004). For example, grape skins contained 86 and 77% respectively, of total grape resveratrol and polydatin content (Kammerer, Claus et al. 2004).

In comparison to almond, the total stilbene content of peanuts and pistachios were 2.5-100 times more than almonds (Sanders, McMichael et al. 2000, Grippi, Crosta et al. 2008). In

almonds, stilbenes appear to be less abundant than flavonoids, phenolic acids, ellagitannins, and proanthocyanidins, which are present in milligram quantities (Bolling, Chen et al. 2011, Xie, Roto et al. 2012). It should be noted that stilbenes have synergistic bioactivities with other polyphenols present in almonds. Resveratrol was synergistic with ellagic acid and quercetin at inducing caspase-3 activity in cultured human leukemia cells (Mertens-Talcott and Percival 2005). Resveratrol was also synergistic with epigallocatechin gallate and γ-tocotrienol in the induction of quinone reductase activity in cultured MCF-7 breast cancer cells (Hsieh and Wu 2008). Almond extracts also induce quinone reductase activity in murine hepatocytes (Chen and Blumberg 2008). *In vivo*, polydatin is metabolized to resveratrol-sulfate, resveratrol-disulfate resveratrol glucuronide, and resveratrol diglucuronide (Burkon and Somoza 2008). Considering the extensive metabolism of stilbenes and other polyphenols *in vivo*, further studies are needed to determine relevant synergies between almond stilbenes and other polyphenols.

4.6 Conclusions

Polydatin was identified in almond extracts, and concentrated primarily in almond skins. Piceatannol + oxyresveratrol was tentatively identified in almond blanch water, and less abundant than polydatin. Further work is warranted to determine the identity of components in almond extract with putative resveratrol daughter ions. Stilbene content was not significantly different among extracts of Nonpareil, Butte, and Carmel California almond varieties. The diversity of polyphenols in almonds and other polyphenol-rich foods suggests that attention should be given to the contribution of less abundant constituents to the synergy or antagonism of polyphenols.

4.7 Chapter 4 Tables and Figures

Table 4.1 UHPLC-MS chromatography of various stilbene standards including instrument settings, limits of detection (LOD), and limits of quantification (LOQ).

Standard Type	**Stilbene**	**RT** (min)	**m/z** (ionization)	**Q-array DC** (V)	**LOD** (pg/column)	**LOQ** (pg/column)
external	polydatin	2.9	389 (-)	-30	1.53	6.10
external	piceatannol	3.4	243(-)	0	1.53	12.21
external	*trans*-resveratrol	4.0	227 (-)	-30	24.41	146.48
external	pterostilbene	6.2	257 (+)	0	0.38	1.53
external	*cis*-resveratrol	3.4	229 (+)	+30	--	--
internal	dienestrol	6.2	265 (-)	0	--	--
internal	hexestrol	5.4	271 (+)	0	--	--
internal	oxyresveratrol	3.4	243 (-)	0	--	--
internal	d4-resveratrol	4.0	233 (+)	+20	--	--

RT: Retention time

Table 4.2 Stilbene content of whole almonds and recovery of spiked standards determined by UHPLC-MS analysis.

Compound	**Content in Almond Variety** (μg/g)			**% recovery from clean-up**
	Butte	Carmel	Nonpareil	
polydatin	8.52 ± 3.47	7.44 ± 3.44	7.19 ± 2.19	73.0%
piceatannol+oxyresveratrol	ND[A]	ND	ND	0.0 %
trans-resveratrol	<LOD[B]	<LOD	<LOD	67.0%
pterostilbene	<LOD	<LOD	<LOD	60.5%

Data is mean ± standard deviation of three independent samples from each almond variety (n = 9), P = 0.8519 for polydatin content between varieties by one-way ANOVA analysis. Recovery of stilbenes was determined by spiking almond homogenate with internal standard prior to solid phase extraction clean-up. [A]ND, not determined as present method not suitable for quantitation; [B]content in almond extract below the limit of detection of 0.25 pg *trans*-resveratrol/g almond and 0.0039 pg pterostilbene/g almond.

Table 4.3 Stilbene content and proportion of California almond cultivars.

Almond compartment	**Compound**	**Content in almond variety** (µg/100 g almonds) Nonpareil	Butte	Carmel
skin	polydatin	0.15 ± 0.01^{b}	0.22 ± 0.01^{a}	0.22 ± 0.00^{a}
meat	polydatin	0.06 ± 0.00^{b}	0.08 ± 0.00^{a}	0.08 ± 0.00^{a}
blanch water	polydatin	8.43 ± 0.27^{a}	6.75 ± 0.07^{b}	6.33 ± 0.20^{b}
	piceatannol + oxyresveratrol[A]	0.91 ± 0.12^{c}	2.55 ± 0.22^{a}	1.71 ± 0.06^{b}
sum		*9.55 ± 0.37^{a}*	*9.60 ± 0.16^{a}*	*8.33 ± 0.19^{b}*

Data are mean ± standard deviation of a composite sample representing three orchards for each almond cultivar. Within rows, values bearing different letters are significantly different by ANOVA and Tukey's multiple comparison test ($P \leq 0.05$). Two-way ANOVA analysis of almond stilbene content by almond compartment and cultivar is $P < 0.0001$ for compartment, variety, and for their interaction. [A]Quantitated as piceatannol equivalents.

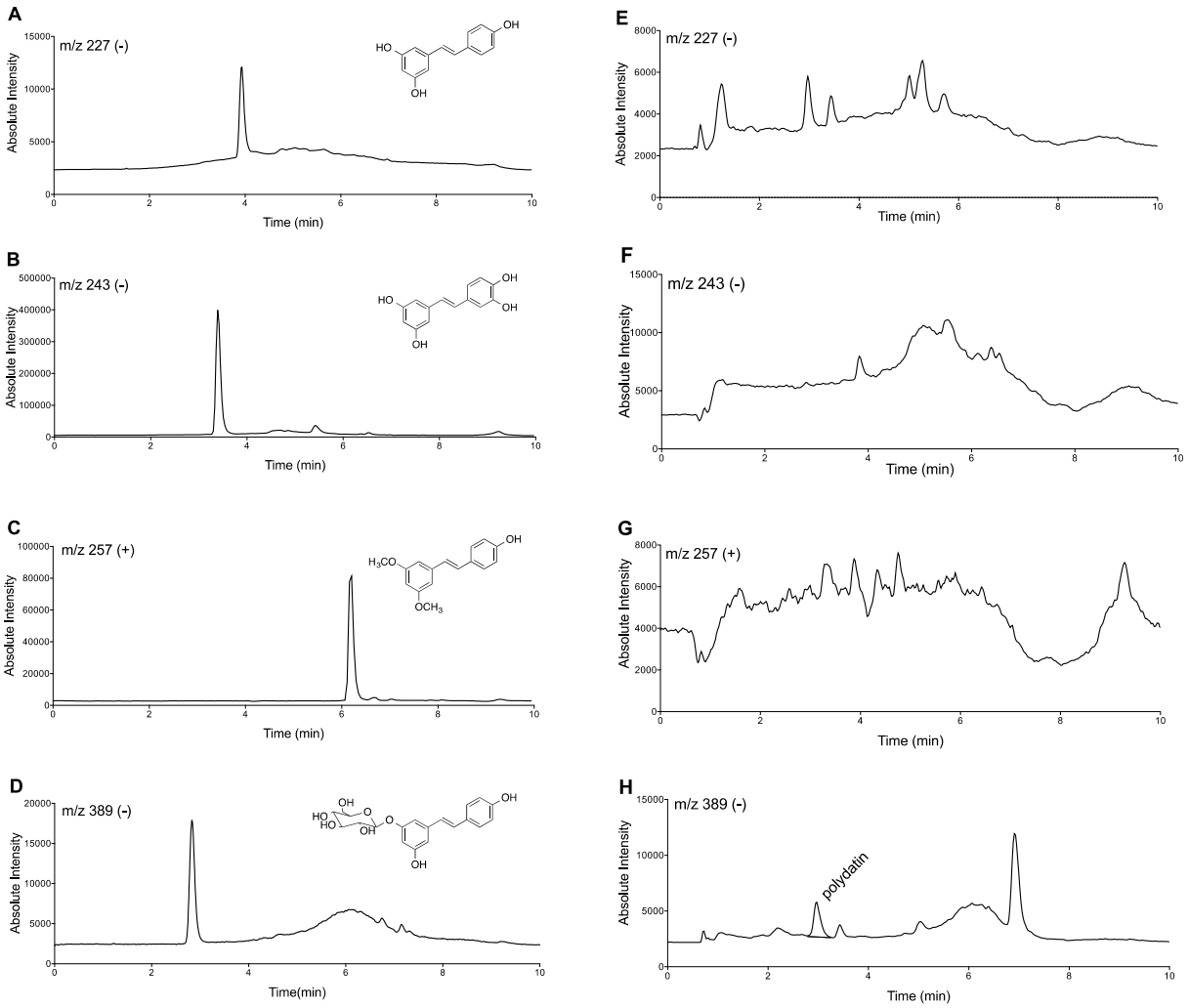

Figure 4.1 Representative UHPLC-MS chromatograms of whole almond extract following solid phase extraction clean-up or standard compounds following a 1 µL injection. A: resveratrol standard at m/z 227 (-); B: piceatannol standard at m/z 243 (-); C: pterostilbene standard at m/z 257 (+); D: polydatin standard at m/z 389 (-); E: almond sample at m/z 227 (-); F: almond sample at m/z 243 (-); H: almond sample at m/z 257 (+); G: almond sample at m/z 389 (-).

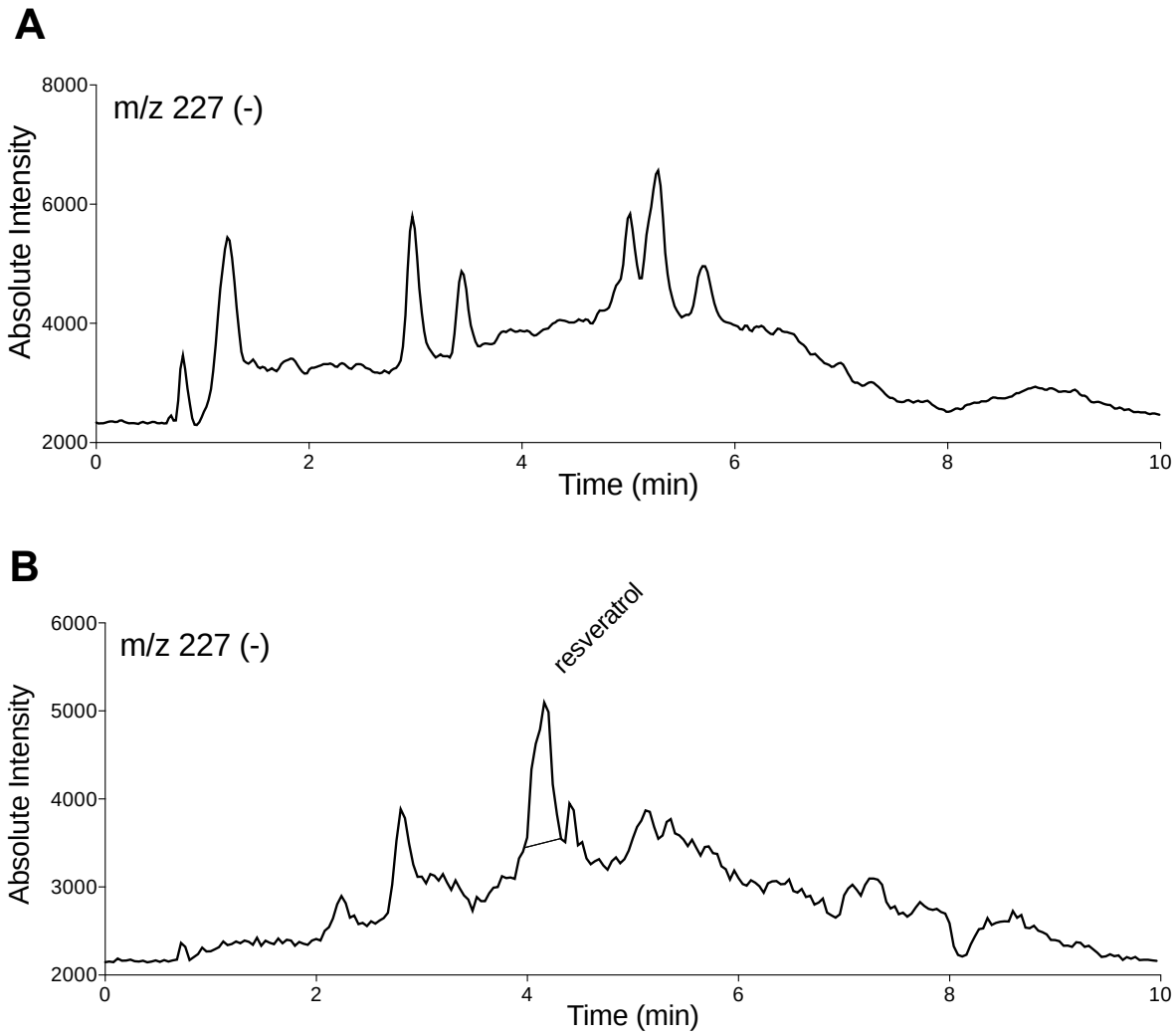

Figure 4.2 Representative UHPLC-MS chromatogram of a 1 µL injection of almond extract following solid phase extraction clean-up and before or after enzymatic hydrolysis with β-glucosidase: A. m/z 227 (-) prior to hydrolysis; B. resveratrol [M-H]- m/z 227 after hydrolysis.

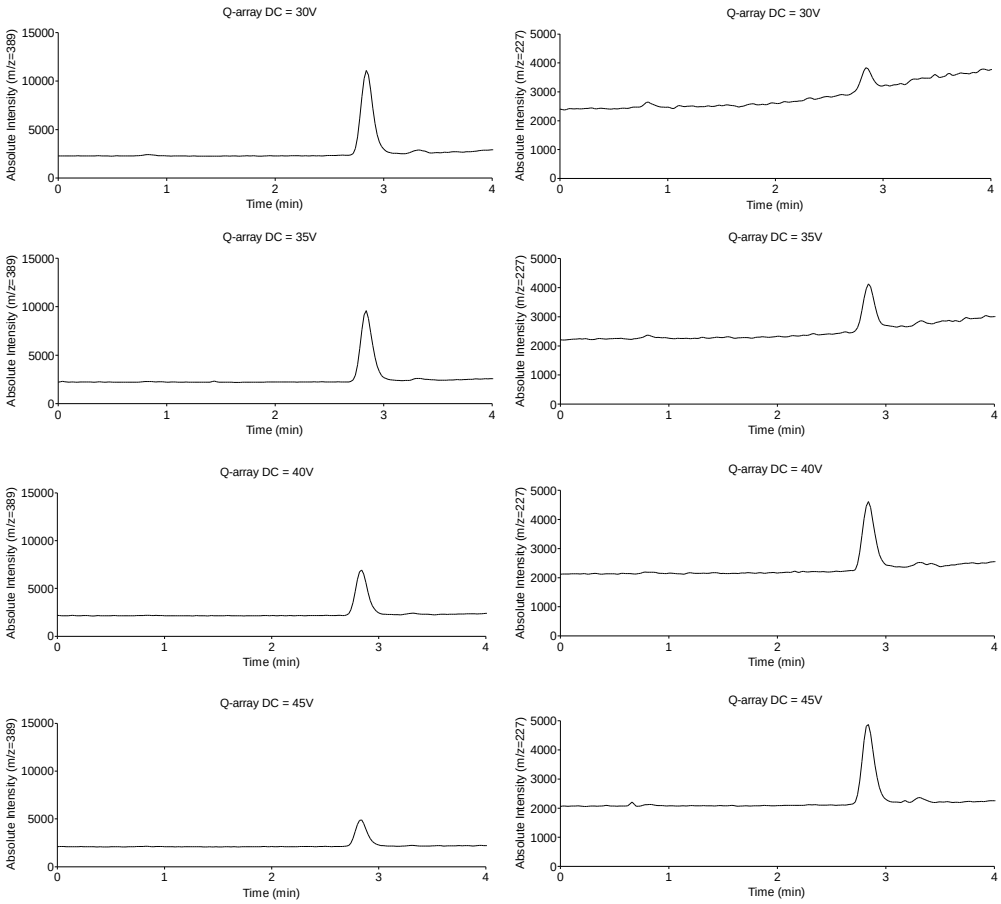

Figure 4.3 Representative UHPLC-MS chromatograms at m/z 389 (-) and 227 (-) of 0.4 ng polydatin on column at increasing Q-array DC voltages.

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Chapter 5: Bioavailability of anthocyanins and colonic polyphenol metabolites following consumption of aronia berry extract

5.1 Abstract

Background: Aronia berries are a rich food source of anthocyanins and other polyphenols, which likely contribute to their putative health benefits.

Objectives: To develop UHPLC-MS methods to quantitate aronia berry polyphenols and their metabolites in plasma and urine samples. To determine the bioavailability and pharmacokinetics of aronia berry polyphenols in human.

Design: A single-dose pharmacokinetic trial was conducted in 6 adults to evaluate the acute absorption and urinary excretion of anthocyanins and colonic polyphenol metabolites after consumption of 500 mg encapsulated aronia berry extract.

Results: While anthocyanins were bioavailable, catabolites of phenolic acid by microbiota, including phenylacetic acid, hippuric acid, ferulic acid, and benzoic acids, increased ~10-fold more than total anthocyanins in plasma and urine. Among the anthocyanins, cyanidin-3-*O*-galactoside was rapidly metabolized to peonidin-3-*O*-galactoside, and cyanidin-3-*O*-glucoside was present in plasma and urine at greater concentrations than other unmetabolized glycosides. Aronia polyphenols were absorbed and extensively metabolized with t_{max} of anthocyanins and other polyphenol catabolites from 1.0 h to 6.33 h in plasma and urine. Despite significant inter-individual variation in pharmacokinetic parameters, concentrations of polyphenol metabolites in plasma and urine at 24 h were positively correlated with total AUC in plasma and urine ($r = 0.9275$, and $r = 0.9828$, respectively).

Conclusion: The results suggested that blood and urine collections after supplemental aronia extract consumption could be used to estimate its polyphenol bioavailability and metabolism.

5.2 Introduction

Aronia berries are a rich source of dietary polyphenols, including anthocyanins, hydroxycinnamic acids, and proanthocyanidins (Taheri, Connolly et al. 2013). Studies have suggested that aronia berry consumption improves dyslipidemia, inhibits inflammation, and reduces oxidative stress associated with chronic diseases in humans (Naruszewicz, Łaniewska et al. 2007, Skoczyńska, Jedrychowska et al. 2007, Poręba, Skoczyńska et al. 2009, Broncel, Koziróg et al. 2010) and animals (Skoczyńska, Jedrychowska et al. 2007, Valcheva-Kuzmanova, Kuzmanov et al. 2007, Kujawska, Ignatowicz et al. 2011, Kim, Ku et al. 2013). Polyphenols are extensively metabolized and have apparently low bioavailability (Manach, Scalbert et al. 2004). Therefore, further data are needed to reconcile aronia polyphenol bioavailability with its apparent health effects.

The most abundant aronia polyphenols, i.e., anthocyanins and proanthocyanins, have less than 6% bioavailability of the initial dose ingested (Miyazawa, Nakagawa et al. 1999, Cao, Muccitelli et al. 2001, Wu, Cao et al. 2002). When considering colonic metabolites, the bioavailability of anthocyanin and proanthocyanidins may range from 12% to 18% (Gonthier, Donovan et al. 2003, Czank, Cassidy et al. 2013). Anthocyanins can be absorbed in the intestine, and further subjected to phase II metabolism in the gut or liver (Wu, Cao et al. 2002, Kay 2006). Also, spontaneous degradation or microbial catabolism of anthocyanins may lead to the formation of phenolic acids, such as protocatechuic acid (Czank, Cassidy et al. 2013). Aronia proanthocyanidins are primarily polymeric, which limits absorption to its microbial catabolites (Déprez, Brezillon et al. 2000, Ou and Gu 2013). These catabolites include valerolactones, hydroxyphenyl propionic acids, hydroxyphenyl acetic acids, and hydroxybenzoic acids that are absorbed from the colon (Rios, Gonthier et al. 2003, Urpi-Sarda, Monagas et al. 2009).

The bioavailability and excretion of aronia anthocyanins and its phase II metabolites following juice and extract consumption has been prevoiously described (Kay, Mazza et al. 2004, Kay, Mazza et al. 2005, Woodward, Kroon et al. 2009, Wiczkowski, Romaszko et al. 2010). However, the bioavailability of major colonic catabolites has not been well-defined. The catabolism of aronia polyphenols converges on several key intermediates, including hippuric acid, protocatechuic acid, and phenylproprionic acids (**Figure 5.1**) (Wu, Cao et al. 2002, Prior 2003, Wu, Pittman et al. 2004, El Mohsen, Marks et al. 2006, Monagas, Urpi-Sarda et al. 2010, Stalmach, Edwards et al. 2013). Therefore, the objectives of this study were to quantitate aronia anthocyanins and colonic polyphenol catabolites in humans following consumption of aronia extract. Furthermore, we describe the validation of analytical methods used to generate these data.

5.3 Materials and methods

5.3.1 Reagents and materials

Methanol was LC-MS grade from Fischer Scientific (Fair Lawn, NJ, USA). Anthocyanin standards with ≥98% purity were purchased from Wuxi App Tec Co. (Shanghai, China). All other chemicals and reagents, including LC-MS grade formic acid and tribluoroacetic acid, were acquired from Sigma-Aldrich (St. Louis, MO, USA). Aronia berry extract was purchased from Artemis International (Fort Wayne, IA, USA) and packaged into opaque cellulose capsules containing 250 mg extract ± 10% per capsule (Beehive Botanicals, Hayward, WI, USA).

5.3.2 Study participants and institutional approval

Baseline characteristics of healthy 18 to 60 year old adults participated in the study are described in **Table 5.1** Participants were part of a larger study evaluating the function of aronia

berry supplementation in reducing cardiovascular disease risk in former smokers. All participants consented to the study and the research protocol was approved by the University of Connecticut Institutional Review Board as protocol #H11-311, and registered at ClinicalTrials.gov as NCT01541826.

5.3.3 Inclusion criteria for study participants

Former smokers, defined as people whom previously smoked ≥10 cigarettes/day for at least 1 year, and cessation for at least 6 months. The exclusion criteria used to screen for their eligibility included: 1) previous diagnoses of CVD, diabetes, or arthritis (except for osteoarthritis), 2) currently being treated for cancer (i.e., chemotherapy, radiation therapy), 3) women with prescribed estrogen replacement therapy, 4) having slimming diets, 5) practicing vegetarian diet, 6) currently taking vitamin or mineral supplements or plant pills, 7) alcohol consumption exceeding the definition of moderate drinking (2 drinks/day or a total of 12/week for men or 1 drink/day or a total of 7/week for women).

5.3.4 Study design

The participants were advised to consume a low polyphenol diet for at least 3 days before the study began. Participants avoided polyphenol-rich foods and beverages (green or black tea, fruit or vegetable juice, grape products, berries, and certain fruits) and dietary supplements. After an overnight fast, baseline plasma and urine samples were collected from participants. The participants then consumed two capsules of aronia extract with water, providing ~500 mg of extract. Subsequent blood samples were taken at 0.5, 1, 2, 4, 6, 9, 12, and 24 h post-consumption of the extract. Urine samples were also collected at 2, 4, 6, 9, 12, and 24 h post-consumption of the extract. Over the course of the following 24 h, low polyphenol diets were provided to

participants. Breakfast was provided after the consumption of the aronia capsules. Lunch was provided between hours 4 to 6 and dinner was provided between hours 6 to 9 after consumption of the aronia extract. Snacks were provided between meals. The low polyphenol diet and snacks consisted of dairy products, tuna, chicken, banana, potato chips, white bread, and plain bagels. Blood samples (20 mL) were drawn from a brachial vein into EDTA vacutainer tubes (BD, Franklin Lakes, NJ, USA). The blood samples were immediately placed on ice, and then centrifuged at 1,500 ×g for 15 min at 4°C to recover plasma. The plasma and urine samples were then acidified to 0.5% HCl and centrifuged at 2,500 ×g for 15 min at 4 °C to recover polyphenols (Woodward, Kroon et al. 2009). The supernatant was collect and stored at -80°C until analysis.

5.3.5 Analysis and composition of aronia extract

The polyphenol content of the aronia supplement was analyzed using methods previously reported by our group (Taheri, Connolly et al. 2013). Briefly, anthocyanins, flavonols, and hydroxycinnamic acids were quantitated by UHPLC-MS, proanthocyanidins by normal-phase HPLC, and total phenols by the Folin-Ciocalteu assay.

5.3.6 Sample clean-up

The extraction of anthocyanins and polyphenol metabolites from plasma and urine was adapted from the methods reported by others (Kay, Mazza et al. 2004). Solid phase extraction (SPE) of biofluid was performed using HyperSep C18 cartridges (500 mg/3 mL, Thermo Scientific, Waltham, MS) attached to a vacuum manifold. The SPE cartridge was preconditioned with 6 mL each of 0.5% formic acid in methanol (v:v) (A), and then 0.5% formic acid in water (v:v) (B). Plasma (1.5 mL) or urine (4 mL) was then applied to the cartridge. An additional 6 mL of B was then applied to wash the cartridge. Subsequently, the elute was collected with 6 mL of

A and dried under nitrogen gas at 23°C. The dried residue was held at −80°C until UHPLC–MS analysis.

5.3.7 UHPLC-MS analysis

Dried residue from SPE clean-up of urine or plasma was reconstituted with 100 µL methanol. UHPLC–MS analysis was conducted using a Shimadzu Nexera UHPLC equipped with DGU-20A5 Prominence degasser, SIL-30AC Nexera autosampler, CTD-30A Nexera column oven, two LC-30AD pumps (Shimadzu, Kyoto, Japan), LCMS-2020 mass spectrometer, and a Kinetex PFP column (1.7 µm, 100 Å, 2.1 × 50 mm; Phenomenex, Torrance, CA). The following chromatographic methods were developed to optimize resolution and detection of aronia polyphenols and metabolites in the reconstituted samples:

3,4-Dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, hippuric acid, and ferulic acid: A gradient of water with formic acid (99.5:0.5, v/v) (A) and methanol (B) started at: 92% A and 8% B; increased to 10% B at 8 min; increased to 20% B at 9.6 min; increased to 30% B at 14 min; increased to 40% B at 18 min; increased to 100% B at 25 min; returned to 8% B at 39 min, and was held until the stop time at 40 min. The flow was maintained at 0.2 mL/min and the sample injection volume was 1 µL.

3-(4-Hydroxyphenyl)propionic acid: A gradient of water (A) and methanol (B) started at 95% A and 5% B; increased to 100% B at 12 min; and returned to 5% at 16 min, and held until the stop time at 20 min. The flow was maintained at 0.2 mL/min and the sample injection volume was 1 µL.

Cyanidin-3-O-glucoside, cyanidin-3-O-galactoside, cyanidin-3-O-arabinoside, and peonidin-3-O-galactoside: A gradient of water with trifluoroacetic acid (99.9:0.1, v/v) (A) and methanol (B) started at 80% A and 20% B; increased to 40% B at 9 min; increased to 100% B at

12 min; returned to 20% B at 18 min, and was held until the stop time at 20 min. The flow was maintained at 0.2 mL/min and the sample injection volume was 5 μL.

For all chromatographic methods, the mass spectrometer was set to a nebulizing gas flow of 1.5 L/min, a drying gas flow of 15.0 L/min, the desolvation line at 250 °C, the heat block at 500 °C, and the interface temperature at 350 °C. Other detector parameters were set to optimize the response of individual compounds of interest (**Table 5.7.2**).

Signal (S) to noise (N) ratios, limits of detection (LOD), limits of quantification (LOQ) were determined for each compound based on methods previously described (Xie and Bolling 2014). Quantitation was based on MS area under the curve (AUC) responses and comparison to authentic standards.

5.3.8 Determination of recovery

Recovery was determined according to the method of previous study (Wiczkowski, Romaszko et al. 2010). Briefly, blanks of plasma and urine samples were spiked with standard compounds to concentrations within the expected ranges of anthocyanins and metabolites (0.01-42.2 μmol/L) that have been previously observed in human samples following anthocyanin consumption (Kay, Mazza et al. 2004, Kay, Mazza et al. 2005, Wiczkowski, Romaszko et al. 2010, Czank, Cassidy et al. 2013). Polyphenol concentrations of spiked samples were determined by SPE clean-up and UHPLC-MS analysis as described above.

5.3.9 Creatinine analysis

The urine creatinine content was measured by an urinary creatinine colorimetric assay kit (Cayman Chemical, Ann Arbor, MI).

5.3.10 Pharmacokinetic and Statistical Analysis

C_{max}, t_{max}, and AUC of anthocyanins and metabolites in plasma and urine were determined as previously described (McKay, Chen et al. 2015). The results of plasma and urine variables included 6 subjects and are presented as means ± SEM, unless otherwise noted. Pearson correlation analysis with Fisher's z transformation was conducted by SAS (9.4, SAS Institute, Inc., Cary, NC). Other statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Statistical significance was defined as P-value <0.05.

5.4 Results

5.4.1 Analysis of supplement

The supplement constitute were reported in the **Table 5.4**. Anthocyanins and proanthocyanins were the most abundant polyphenol classes in aronia extract, accounting for 34% and 31% of the total polyphenol content, respectively. Anthocyanins were primarily cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-arabinoside, and cyanidn-3-*O*-xyloside. Proanthocyanidins were 0.2% monomers, 7.1% dimers, 32.2% trimers, and 60.4% degree of polymerization (DP) >4. In total, a 500 mg dose of aronia berry extract provided 45.07 mg anthocyanins, 41.9 mg proanthocyanidins as catechin equivalents, 9.9 mg flavonols, 36.9 mg hydroxycinnamic acids, and 306 mg total phenols as gallic acid equivalents.

5.4.2 UHPLC-MS conditions for polyphenols in plasma and urine

Mobile phase composition was essential for polyphenol detection and resolution in plasma and urine. Acid modifiers suppressed 3-(4-hydroxyphenyl)propionic acid ionization in both positive and negative operating modes. The use of 0.5% formic acid was adequate to resolve most polyphenols, but 0.1% trifluoroacetic acid was required to resolve cyanidin-3-*O*-galactoside

and cyanidin-3-*O*-glucoside. The range of recovery rates of protocatechuic acid, 3,4-dihydroxyphenylacetic acid, hippuric acid, ferulic acid, 3-(4-hydroxyphenyl)propionic acid, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-arabinoside, and peonidin-3-*O*-galactoside was 70% to 110% from plasma, and 43% to 119% from urine (**Table 5.3**). The intraday relative standard deviation (RSD) of analytes was from 0.83% to 10.83%, and the interday RSD was 1.32% to 11.46% (**Table 5.7.3**).

5.4.3 Determination of anthocyanins and metabolites in plasma samples

The retention time (RT), mass to charge ratio (m/z), LOD and LOQ of aronia anthocyanins and metabolites are reported in **Table 5.2**. Protocatechuic acid, hippuric acid, 3-(4-hydroxyphenyl)propionic acid, cyanidin-3-*O*-glucoside, and peonidin-3-*O*-galactoside were recovered in plasma after aronia consumption (**Figure 5.2** & **Figure 5.4**). Concentrations of 3,4-dihydroxyphenylacetic acid, ferulic acid, cyanidin-3-*O*-galactoside, and cyanidin-3-*O*-arabinoside, were not detected. Cyanidin-3-*O*-glucoside reached a peak concentration of 0.059 ± 0.024 μg/mL at 1.6 h post-consumption, whereas polyphenol metabolites peaked at 1 h for protocatechuic acid, 2.67 h for peonidin-3-*O*-galactoside, 5.33 h for hippuric acid, and 6.33 h for 3-(4-hydroxyphenyl)propionic acid (**Table 5.5**). AUC can be used to describe the absolute bioavailability of compounds (Lappin, Rowland et al. 2006). The plasma AUC ranged from 0.054 ± 0.013 μg×h/mL for protocatechuic acid to 22.4 ± 4.4 μg×h/mL for hippuric acid. Among the plasma metabolites, hippuric acid accounted for 85.2% of the total quantitated polyphenols by their AUCs, followed by 3-(4-hydroxyphenyl)propionic acid (10.4%), peonidin-3-*O*-galactoside (2.39%), cyanidin-3-*O*-glucoside (1.76%), and protocatechuic acid (0.21%). Thus, the microbial phenolic acid catabolites represented for around 95.8% of the total of increased polyphenols after aronia consumption. In contrast, anthocyanins accounted for 4.2% of the

increase in plasma polyphenols. Creatinine concentrations varied from 15.9 to 258 mg/dL in urine samples.

5.4.4 Determination of anthocyanins and metabolites in urine samples

Protocatechuic acid, 3,4-dihydroxyphenylacetic acid, hippuric acid, ferulic acid, 3-(4-hydroxyphenyl)propionic acid, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-arabinoside, and peonidin-3-*O*-galactoside were recovered in urine samples (**Figure 5.3 & Figure 5.5**). Cyanidin-3-*O*-galactoside reached a peak concentration of 0.004 ± 0.001 mg/mg creatinine at 4.67 h post-consumption. Also, the t_{max} and C_{max} for cyanidin-3-*O*-glucoside and cyanidin-3-*O*-arabinoside were 6 h and 0.010 ± 0.006 mg/mg creatinine, and 4 h and 0.020 ± 0.006 m g/mg creatinine, respectively (**Table 5.5**). Phenolic acids peaked at 4 h for protocatechuic acid, 4.67 h for peonidin-3-*O*-galactoside, 4.83 h for 3-(4-hydroxyphenyl)propionic acid, 5.33 h for ferulic acid, and 6.33 h for both 3,4-dihydroxyphenylacetic acid and hippuric acid. The urine AUC ranged from 0.016 ± 0.005 mg×h/mg creatinine for cyanidin-3-*O*-galactoside to 2430 ± 360 µg×h/mg creatinine for hippuric acid. Among the urinary metabolites, hippuric acid accounted for 98.5% of the total increase in polyphenols after aronia supplementation, followed by ferulic acid (0.80%), 3,4-dihydroxyphenylacetic acid (0.46%), protocatechuic acid (0.15%), 3-(4-hydroxyphenyl)propionic acid (0.09%), cyanidin-3-*O*-glucoside (0.004%), cyanidin-3-*O*-arabinoside (0.004%), peonidin-3-*O*-galactoside (0.002%), and cyanidin-3-*O*-galactoside (0.001%). Thus, the polyphenol microbial catabolites represented nearly all of the increase in urinary polyphenols after aronia consumption.

5.4.5 Between-subjects differences

There was large between-subject variation in the pharmacokinetics of aronia polyphenols and metabolites (**Table 5.5**). As a result, plasma anthocyanins were below the limit of detection in 3 participants. The C_{max} interindividual CV for plasma polyphenols was generally greater than urinary polyphenols (20 - 65% vs 17 - 60%) except for hippuric acid, which had 23% and 34% CV in the urine and plasma, respectively. Also, anthocyanins had higher C_{max} variation than phenolic acids in both urine and plasma. The interindividual C_{max} range was largest for 3-(4-hydroxyphenyl)propionic acid in plasma and urine, having 14- and 29-fold differences, respectively.

5.4.6 Correlation Analysis

The utility of using overnight fasting samples of plasma or urine to estimate polyphenol bioavailability and metabolism has not been validated. If the total AUC of polyphenols is not correlated with fasting concentrations, we would expect fasting concentrations to be a poor reflection of the previous exposure. Therefore, we determined the correlation of AUC values of aronia polyphenols and metabolites with the 24 h concentrations after an overnight fast. All metabolite concentrations in plasma and urine were significantly lower at 12-24 h post consumption compared to the total AUC. The Pearson correlation coefficients between the sum of polyphenols and metabolites in fasting plasma or urine and total AUC were 0.9275 ($P < 0.0001$) and 0.9828 ($P < 0.0001$), respectively. When hippuric acid was not included in the sum of polyphenols and metabolites, the correlation between fasting plasma and total AUC was -0.1890 ($P = 0.4046$). In contrast, overnight urine and total AUC correlation of metabolites and polyphenols without hippuric acid was still significant, at 0.9573 ($P <0.0001$).

5.5 Discussion

The UHPLC-MS methods developed for polyphenol analysis in plasma and urine had acceptable reproducibility and sensitivity. Routine RSD was ≤10% without including an internal standard in the method. Including a stable isotope standard would be expected to further improve precision. The recovery rates after SPE of plasma polyphenols were 10% to 20% higher than previously reported, which was around 70% to 90% for other anthocyanin-rich foods (Zhang and Zuo 2004, Wiczkowski, Romaszko et al. 2010). However, the SPE recovery of urine polyphenols were 10% to 40% less than a previous study on aronia juice consumption (Wiczkowski, Romaszko et al. 2010). This difference may be a result of increasing the urine volume used for SPE.

The LODs of anthocyanins and their metabolites in this study were equivalent to 0.002 to 0.05 nmol/L and 0.003 to 0.02 nmol/L in the plasma and urine, respectively, and the LOQs were equivalent to 0.004 to 0.1 and 0.01 to 0.04 nmol/L in the plasma and urine, respectively. This result was consistent with other studies about the lowest detection on anthocyanins and phenolic acids measured by HPLC or HPLC-MS, which was within a range of 0.01 to 0.2 nmol/L in plasma (Kay, Mazza et al. 2004, Vitaglione, Donnarumma et al. 2007, Wiczkowski, Romaszko et al. 2010). These results indicate the sensitivity of the UHPLC-MS is in range of prior studies.

The plasma t_{max} of 1.6 h for cyanidin-3-*O*-gluoside after aronia extract consumption was in the range of 1.3 to 3.1 h, which was reported in previous studies (Wiczkowski, Romaszko et al. 2010). Plasma cyanidin-3-*O*-gluoside increased by 30 min post-consumption, which supports the hypothesis that anthocyanins can be absorbed though the stomach (Fernandes, de Freitas et al. 2012). Fernandes, et al. (2012) demonstrated that cultured stomach cells absorbed anthocyanins via a saturable transport mechanism without biotransformation. Passamonti, et al. (2002)

proposed that bilitranslocase could be a high-affinity anthocyanin transporter in epithelial cells of rat gastric mucosa. However, while rodents have stomach bilitranslocase, to the best of our knowledge, it has not been identified in human stomach (Battiston, Macagno et al. 1999).

In the present study, plasma protocatechuic acid peaked as early as 1 h, which was consistent with previous studies that reported t_{max} values of protocatechuic acid 1-2 h after anthocyanin consumption (Vitaglione, Donnarumma et al. 2007, Keane, Bell et al. 2015). The aronia supplement in the present study provided 1.2 mg of protocatechuic acid, which may contribute to its early t_{max}. Also, other aronia polyphenols were also metabolized to protocatechuic acid, e.g. chlorogenic acid, quercetin, and caffeic acid (**Figure 5.7.1**). Thus, pharmacokinetic parameters of microbial polyphenol catabolites are likely botanical- or composition-specific. Generally, the phenolic catabolites had later peaks than cyanidin-3-*O*-glucoside, which suggests these metabolites may be generated from colonic metabolism of anthocyanins. Since the major aronia anthocyanin, cyanidin-3-*O*-galactoside, was not detected in the plasma, it could be a major source of the colonic catabolites. Urinary colonic polyphenol catabolite concentrations peaked later than those in plasma, except for 3-(4-hydroxyphenyl)propionic acid. The lower concentrations of anthocyanins in both plasma and urine samples confirm their extensive metabolism and low bioavailability (Scalbert, Morand et al. 2002).

The aronia supplement provided 45.07 mg anthocyanins, which was similar to the dose provided by 250 mL juice in a prior pharmacokinetic study (Wiczkowski, Romaszko et al. 2010). The peak plasma concentration of cyanidin-3-*O*-glucoside and peonidin-3-*O*-galactoside from the aronia supplement was more than 10-fold greater than the previous study of juice, which was ~1.0 nmol/L. However, plasma peak concentration of cyanidin-3- *O*-galactoside was ~12.0

nmol/L after juice consumption, while this was undetectable after extract consumption. This suggests that cyanidin-3-*O*-galactoside in aronia extract is likely metabolized to peonidin-3-*O*-galactoside more quickly than that in juice.

Participants had significant interindividual variation of polyphenol bioavailability after aronia extract consumption. Data linking gender, age, and health status to polyphenol bioavailability is very limited. Bioavailability and colonic polyphenol catabolism can be influenced by intestinal enzyme activity, intestinal transit time, and colonic microbes (Sterchi, Mills et al. 1990). Intestinal lactase-phlorizin hydrolase (LPH) may mediate the bioavailability of cyanidin-3-*O*-glycosides and its activity varies significantly between individuals (Kay, Mazza et al. 2004, Kay 2006). Van Duynhoven, et al. (van Duynhoven, Vaughan et al. 2011) also proposed that the wide variation of polyphenol metabolism was because a result of the diversity of the colonic microbiota. Thus, variable polyphenol bioavailability may result from a number of factors. Besides differences in metabolic fate, the total bioavailability of polyphenols varies between individuals. According to Czank, et al. (Czank, Cassidy et al. 2013), the recovery of ^{13}C in blood, urine, breath, and feces was in the range of 15.1 to 99.3% in 8 patients that consumed 500 mg ^{13}C labeled cyanidin-3-glucoside. Urpi-Sarda, et al. (Urpi-Sarda, Monagas et al. 2009) have indicated that changes in human urinary levels of phenolic acids and flavanols vary from 0.001 to 59.43 nmol/mg creatinine in 21 subjects who consumed proanthocyanidin-rich cocoa powder.

In this study, we quantitated aronia anthocyanins and polyphenol catabolites. It should be noted that other metabolites are produced from aronia polyphenols beyond those measured in the present study (**Figure 5.1**) (Manach, Williamson et al. 2005). A previous study have identified 22 metabolites of cyanidin-3-*O*-glucoside using an isotopic approach (Czank, Cassidy et al.

2013). The metabolites of anthocyanins generated from intestine and liver include phase I, phase II and colonic metabolites (Wu, Cao et al. 2002, Czank, Cassidy et al. 2013, de Ferrars, Czank et al. 2014). The phase I metabolites mainly include phloroglucinaldehyde, 3,4-dihydroxybenzaldehyde, and hydroxybenzoic acid (Czank, Cassidy et al. 2013, de Ferrars, Czank et al. 2014). The phase II metabolites are glucuronidated and methylated cyanidins (Wu, Cao et al. 2002, Czank, Cassidy et al. 2013, de Ferrars, Czank et al. 2014). Colonic anthocyanin metabolites include hydroxybenzoic acid, hippuric acid, phenylpropenoic acids, and ferulic acid (Czank, Cassidy et al. 2013, de Ferrars, Czank et al. 2014). Thus, the present study did not quantitate all plausible polyphenol metabolites due to the difficulty in obtaining standards. Furthermore, the polyphenol catabolites in the present study could be products of multiple polyphenol catabolic pathways. For example, hydroxybenzoic acid and ferulic acid are also the major metabolites of proanthocyanins (Rios, Gonthier et al. 2003).

This study was a part of a larger study evaluating aronia berry polyphenol supplementation on cardiovascular disease risk. The significant correlation between fasting plasma or urine samples with total polyphenol AUC suggested that fasting polyphenol concentrations could be used as a marker of compliance and/or catabolic fate of aronia extract polyphenols. However, the correlation between plasma polyphenol AUC and fasting concentrations was low without including hippuric acid. This may result from that the wide variation in hippuric acid, and fewer types of metabolites observed in the plasma. Others have also suggested that urinary polyphenols might be a better predictor of polyphenol intake than food frequency questionnaires in analysis of association between polyphenol intake and all-cause of mortality in adults (Zamora-Ros, Rabassa et al. 2013).

5.6 Conclusions

In conclusion, we developed UHPLC-MS methods to determine bioavailability of aronia extract polyphenols and metabolites. The current study indicates that aronia extract anthocyanins are extensively metabolized. Total fasting plasma and urine polyphenols 24 h after aronia extract consumption correlated with bioavailability so they could be used as a marker of polyphenol metabolism in individuals when consuming a low-polyphenol diet. Future work should consider the extent of the significant interindividual variation in aronia polyphenol metabolism when determining intervention efficacy on health outcomes.

5.7 Chapter 5 Tables and Figures

Table 5.1 Characteristics of participant population.

Characteristic	**Male** (n = 3)	**Female** (n = 3)
Age	22.3 ± 1.5	40.3 ± 7.3
SBP (mmHg)	112.7 ± 2.9	112.0 ± 6.4
DBP (mmHg)	70.7 ± 2.0	76.3 ± 2.7
Waist circumference (cm)	81.3 ± 5.5	75.0 ± 3.0
BMI (kg/m^2)	24.9 ± 1.3	24.2 ± 1.4
Fasting TC (mg/dL)	160.7 ± 17.6	168.7 ± 24.5
Fasting HDL (mg/dL)	48.7 ± 2.8	65.3 ± 9.1
Fasting LDL (mg/dL)	92.7 ± 12.0	96.0 ± 12.5
Fasting TG (mg/dL)	96.7 ± 27.0	63.0 ± 11.5
Fasting glucose (mg/dL)	93.0 ± 4.6	99.3 ± 1.45

Data is mean ± SEM. SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; TG: Triglyceride.

Table 5.2 Optimized MS instrument parameters for UHPLC analysis of polyphenols from plasma and urine.

Method	Standard	IV (kV)	DV (kV)	IV (kV)	DLV (V)	Q array DC (V)	Q array RF (V)
1	protocatechuic acid	-3.5	3.5	-3.5	-10	-10	20
	3,4-dihydroxyphenylacetic acid	-5	3.5	-5	-70	-10	20
	hippuric acid	-5	3.5	-5	-70	-10	20
	ferulic acid	5	3	5	0	20	20
2	3-(4-hydroxyphenyl)propionic acid	-5	-3.5	-5	-70	-50	30
3	cyanidin-3-*O*-galactoside	4	3.5	4	100	0	50
	cyanidin-3-*O*-glucoside	4	3.5	4	100	0	50
	cyanidin-3-*O*-arabinoside	4	3.5	4	100	30	40
	peonidin-3-*O*-galactoside	4	3.5	4	100	30	50

DV: detector voltage; IV: interface voltage; DLV: desolvation line Voltage; Q array DC: Q array direct current; Q array RF: Q array radio frequency. Method 1: formic acid (99.5:0.5, v/v) (A) and methanol (B); Method 2: water (A) and methanol (B); Method 3: trifluoroacetic acid (99.9:0.1, v/v) (A) and methanol (B).

Table 5.3 Polyphenol retention time (RT), m/z ratio, LOD, LOQ and recovery rates of standards of optimized UHPLC-MS methods.

Standard	RT	m/z	LOD (pg/column)	LOQ (pg/column)	Recovery rate (Plasma)	Recovery rate (Urine)	Intraday RSD	Interday RSD
protocatechuic acid	3.7	(-)153	4.88	9.77	81%	73%	4.54%	7.29%
3,4-dihydroxyphenylacetic acid	4.0	(-)167	19.53	39.06	70%	92%	6.02%	5.35%
hippuric acid	5.4	(-)178	2.44	4.88	101%	104%	7.75%	11.46%
ferulic acid	17.4	(+)195	29.30	29.06	93%	114%	0.83%	6.42%
3-(4-hydroxyphenyl)propionic acid	10.5	(-)165	24.4	48.8	72%	119%	1.08&	7.29%
cyanidin-3-*O*-galactoside	8.8	(+)449	312.5	625.0	71%	43%	10.83%	9.10%
cyanidin-3-*O*-glucoside	9.3	(+)449	195.3	448.3	104%	43%	2.12%	9.98%
cyanidin-3-*O*-arabinoside	10.1	(+)419	58.6	73.2	110%	58%	6.34%	5.49%
peonidin-3-*O*-galactoside	10.3	(+)463	14.6	24.4	100%	63%	4.89%	1.32%

RT: retention time; m/z: mass to charge ratio.

Table 5.4 Polyphenol composition of aronia supplement.

Assay	**Component**	**Chokeberry Supplement** (mg/500 mg)
polyphenols by UPLC-MS	rutin	2.6 ± 0.0
	neochlorogenic acid	3.5 ± 0.0
	chlorogenic acid	31.9 ± 0.3
	protocatechuic acid	1.2 ± 0.0
	caffeic acid	0.3 ± 0.0
	quercetin-3-galactoside	3.6 ± 0.1
	quercetin-3-glucoside	2.9 ± 0.1
	quercetin	0.8 ± 0.0
	cyanidin-3-galactoside + cyanidin-3-glucoside	32.52 ± 0.7
	cyanidin-3-arabinoside	11.79 ± 0.3
	cyanidn-3-xyloside	0.76 ± 0.0
proanthocyanidins by DMAC	(+)-catechin equivalents	41.9 ± 0.4
proanthocyanidins by HPLC	(+)-catechin equivalents	2.6 ± 0.1
	% monomers	0.2 ± 0.0 (%)
	% dimers	7.1 ± 0.3 (%)
	% trimers	32.2 ± 0.7 (%)
	% 4-mers	24.2 ± 0.5 (%)

	% 5-mers	14.9 ± 1.0 (%)
	% 6-mers	6.5 ± 0.5 (%)
	% 7-mers	3.1 ±0.3 (%)
	% 8-mers	1.2 ±0.1 (%)
	% ≥ 9-mers	10.5 ±1.1 (%)
total phenol by Folin assay	gallic acid equivalents	306.0 ± 4.11

Data is mean ± SD of at least duplicate determinations.

Table 5.5 Pharmacokinetic parameters of anthocyanins and metabolites in plasma and urine samples in n = 6 individuals consuming 500 mg aronia extract.

	t_{max}	C_{max} (range)	AUC
Plasma	h	µg/mL	µg×h/mL
Protocatechuic acid	1.00 ± 0.00	0.005 ± 0.001 (0.004-0.007)*	0.054 ± 0.013
Hippuric acid	5.33 ± 0.67	2.169 ± 0.495 (0.873 to 3.488)	22.350 ± 4.441
3-(4-hydroxyphenyl)propionic acid	6.33 ± 1.45	0.211 ± 0.135 (0.033-0.475)	2.725 ± 2.391
Cyanidin-3-*O*-glucoside	1.60 ± 0.24	0.059 ± 0.024 (0.014-0.180)	0.462 ± 0.170
Peonidin-3-*O*-galacoside	2 67 ± 0.67	0.075 ± 0.049 (0.029-0.266)	0.627 ± 0.198
Urine	h	mg/mg creatinine	mg×h/mg creatinine
Protocatechuic acid	4.00 ± 0.52	0.431 ± 0.075 (0.208-0.650)	3.682 ± 0.587
3,4-dihydroxyphenylacetic acid	6.33 ± 2.25	0.700 ± 0.130 (0.552-1.238)	11/426 ± 0.881
Hippuric acid	6.33 ± 2.25	190.156 ± 42.136 (59.966-316.070)	2429.852 ± 360.197
Ferulic acid	5.33 ± 3.50	1.773 ± 0.609 (0.323-4.137)	19.770 ± 6.167

3-(4-hydroxyphenyl)propionic acid	4.83 ± 2.04	0.477 ± 0.222 (0.023-0.643)	2.299 ± 0.715
Cyanidin-3-*O*-galactoside	4.67 ± 1.03	0.004 ± 0.001 (0.002-0.010)	0.016 ± 0.005
Cyanidin-3-*O*-glucoside	6.00 ± 3.35	0.010 ± 0.006 (0.002-0.004)	0.118 ± 0.070
Cyanidin-3-*O*-arabinoside	4.00 ± 1.26	0.020 ± 0.006 (0.002-0.038)	0.088 ± 0.031
Peonidin-3-*O*-galactoside	4.67 ± 1.03	0.010 ± 0.002 (0.003-0.014)	0.053 ± 0.012

Data is mean ± SEM (n=6). The data were collected from participants whom consumed 500 mg of a polyphenol-rich aronia supplement within 24 hour after consumption.

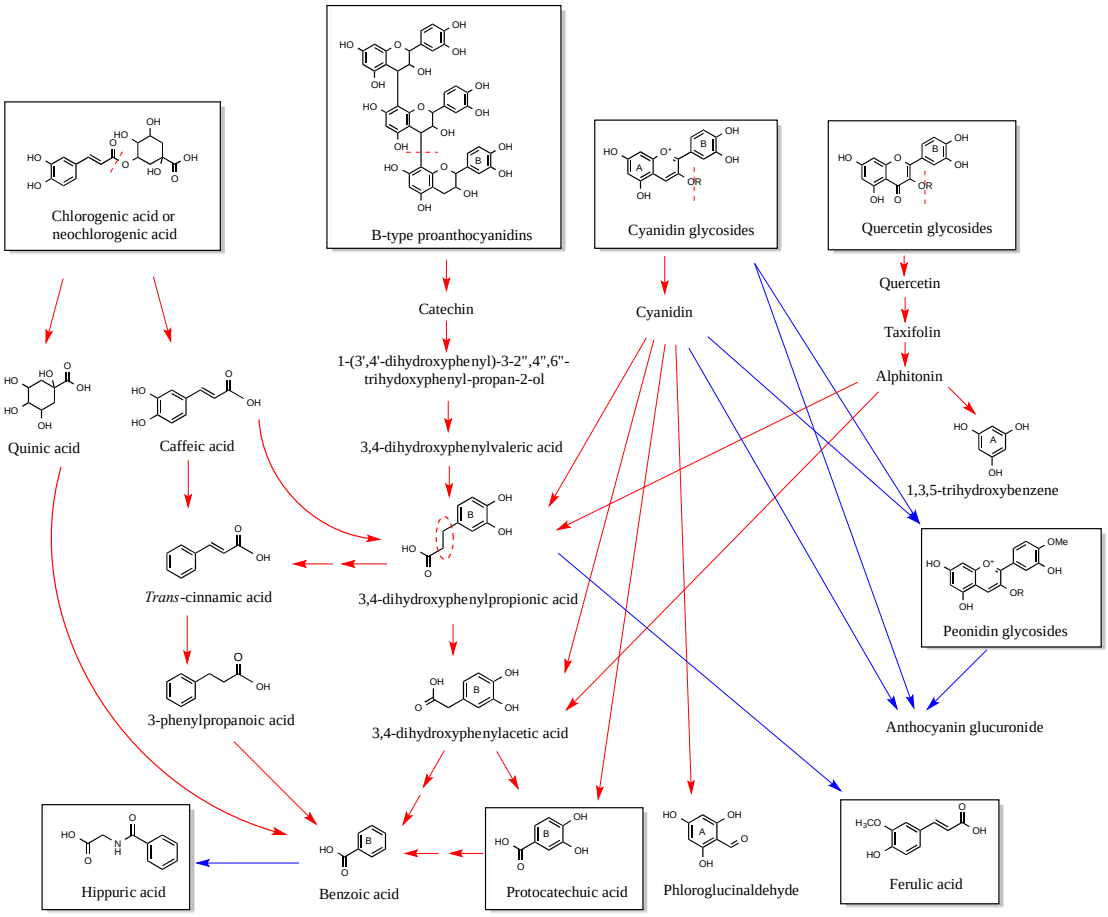

Figure 5.1 Proposed metabolic pathway of aronia polyphenols determined in the present study. Red lines represent colonic catabolic steps, while blue lines represent tissue metabolism. (Wu, Cao et al. 2002, Prior 2003, Wu, Pittman et al. 2004, Monagas, Urpi-Sarda et al. 2010, Stalmach, Edwards et al. 2013).

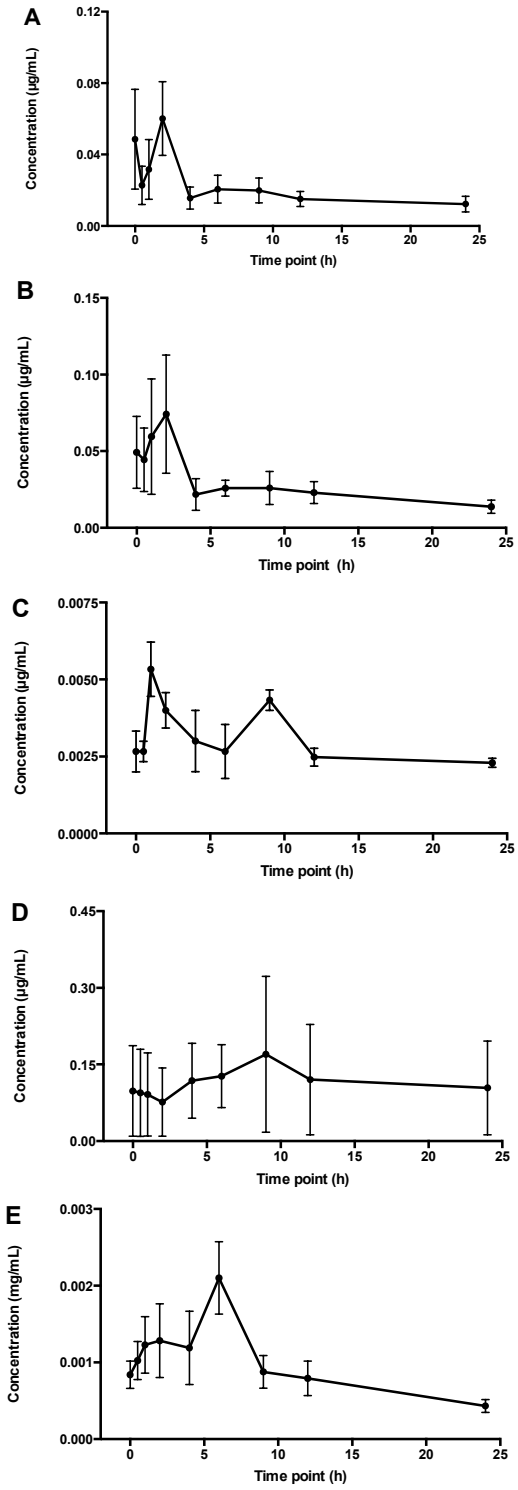

Figure 5.2 Plasma concentrations of (A) cyanidin-3-*O*-glucoside, (B) peonidin-3-*O*-galactoside, (C) protocatechuic acid, (D) 3-(4-hydroxyphenyl)propionic acid, and (E) Hippuric acid,

following consumption of 500 mg of a polyphenol-rich aronia supplement. Data is mean ± SEM of n = 6 participants.

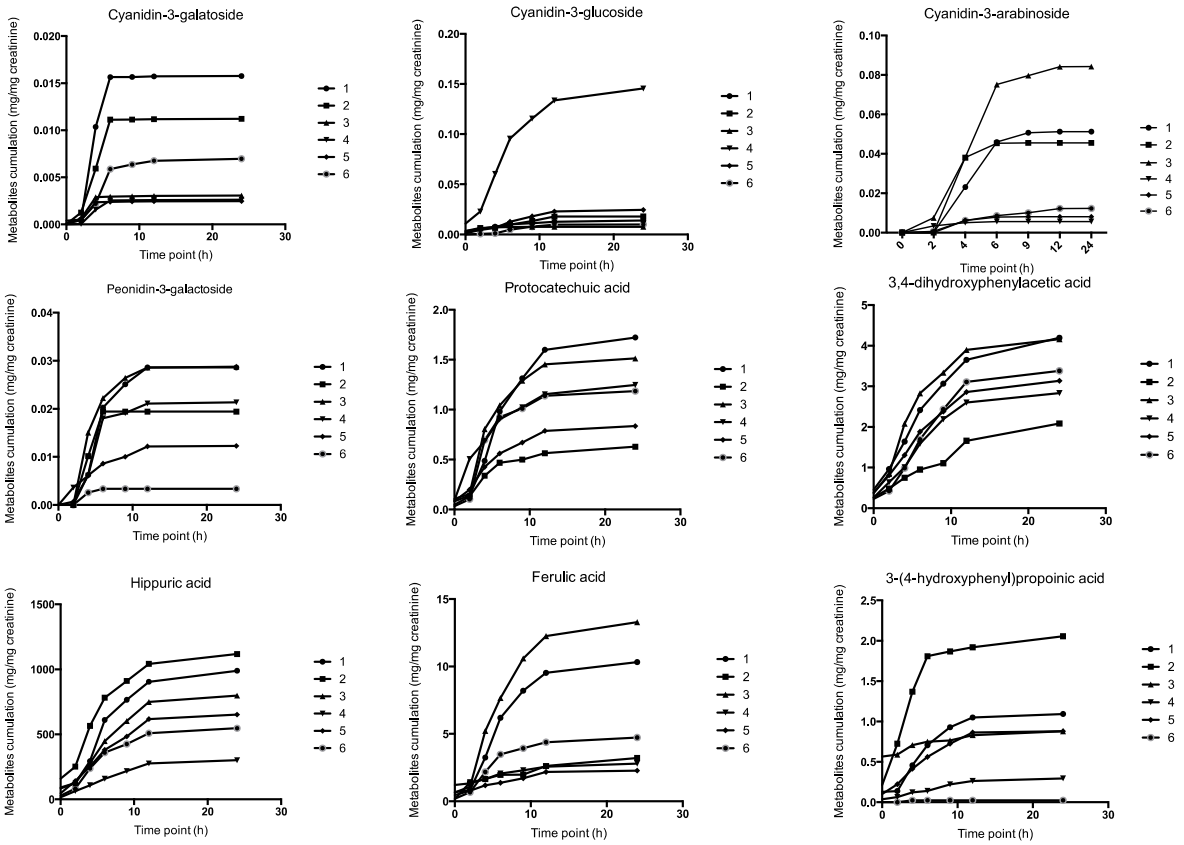

Figure 5.3 Cumulative urinary excretion of aronia polyphenols and catabolites following consumption of 500 mg of a polyphenol-rich aronia supplement.

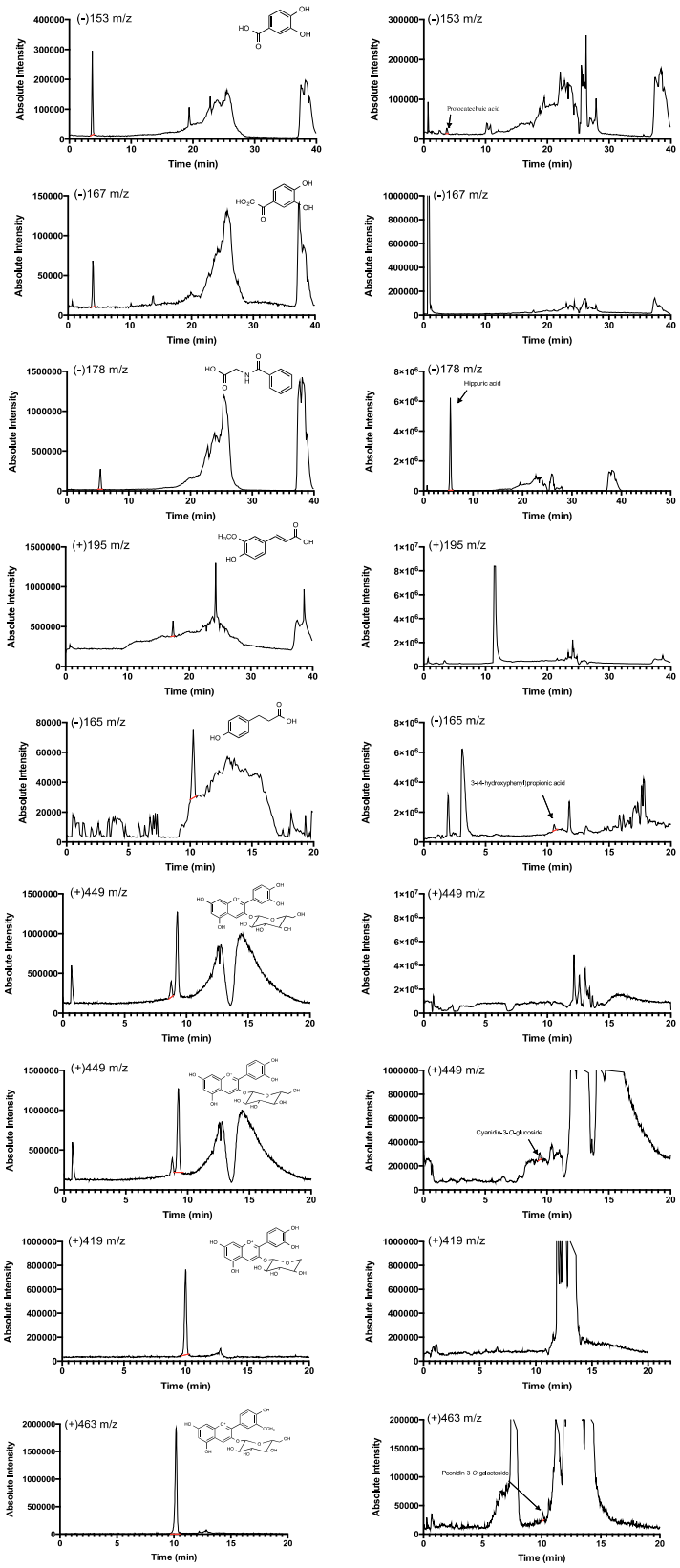

Figure 5.4 Chromatograms of (A) polyphenol standards and (B) representative plasma samples.

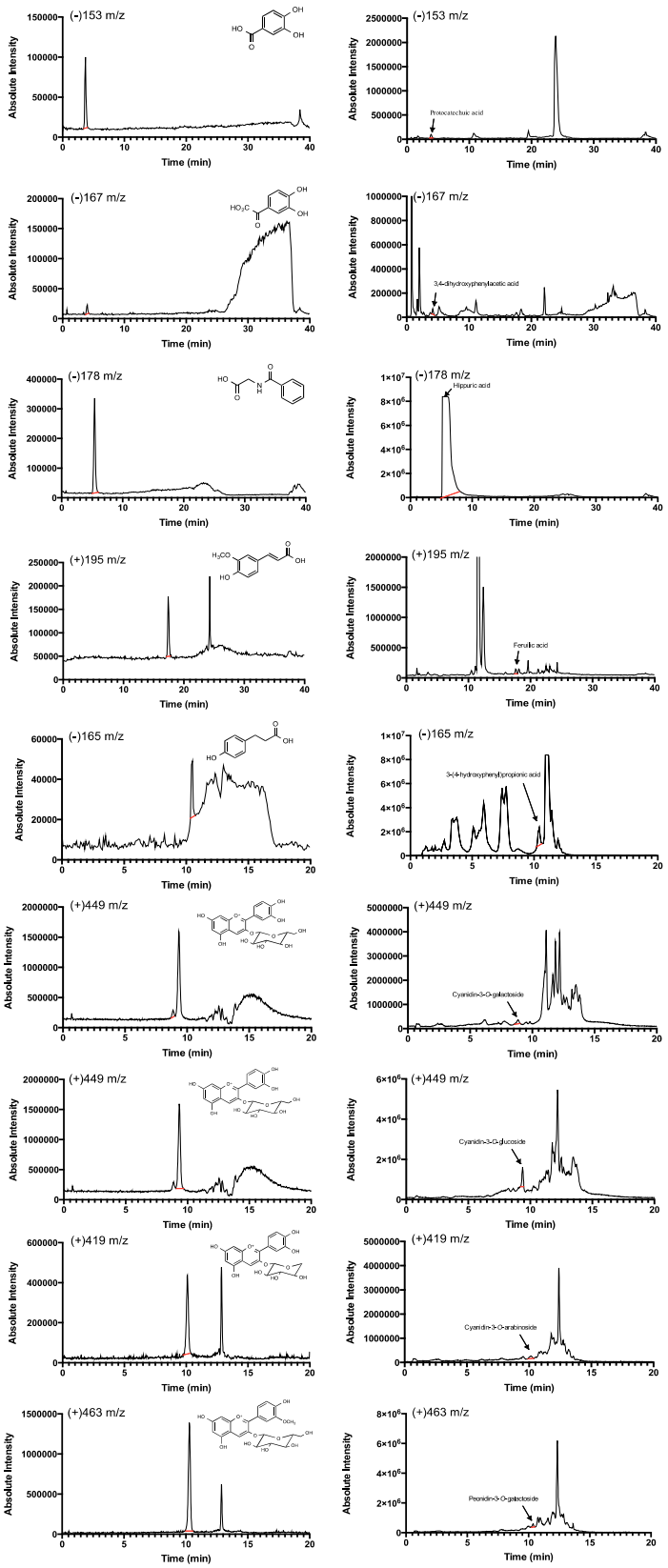

Figure 5.5 Chromatograms of (A) polyphenol standards and (B) representative urine samples.

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Chapter 6: Aronia berry polyphenol consumption reduces plasma LDL and total cholesterol in former smokers without lowering biomarkers of inflammation or oxidative stress

6.1 Abstract

Background: Former smokers are at increased risk of cardiovascular disease (CVD). Little is known about the ability of dietary polyphenols to reduce CVD risk in former smokers. Aronia berry is a rich source of anthocyanins, hydroxycinnamic acids, and proanthocyanidins. Upon consumption, these polyphenols are extensively metabolized. The significant interindividual variation of polyphenol metabolism may modulate the ability of aronia consumption to reduce CVD risk.

Objective: We hypothesized that dietary aronia polyphenols would reduce CVD risk factors and improve biomarkers of inflammation and oxidative stress in former smokers. We also tested if these effects were associated with interindividual differences in polyphenol metabolism and bioavailability.

Design: A 12-week multiple-dose, randomized, placebo-controlled trial was conducted in 49 adults (n = 24/placebo, n = 25/aronia) to evaluate if consumption of 500 mg aronia extract per day modulates plasma lipids, plasma biomarkers of inflammation, oxidative stress biomarkers in plasma and urine, and lipid transport genes of peripheral blood mononuclear cells (PBMCs).

Results: Aronia consumption reduced fasting plasma total cholesterol (TC) level by 8% during the 12-week treatment (P = 0.0140) compared with placebo group, and reduced plasma low-density lipoprotein cholesterol (LDL-C) level by 7 % and 11% at both 6 wks and 12 wks compared with placebo group, respectively (P = 0.0285). Increases in the urinary polyphenol metabolites peonidin-3-*O*-galactoside, 3-(4-hydroxyphenyl)propionic acid, and unmetabolized anthocyanin cyanidin-3-*O*-galactoside were associated with lower plasma TC and LDL-C in the aronia group. Participants in the aronia group who showed the largest decreases in TC had stronger correlations of urinary peonidin-3-*O*-galactoside and cyanidin-3-*O*-galactoside than

those who did not respond to the intervention. However, aronia and placebo consumption did not change biomarkers of inflammation and oxidative stress.

Conclusion: Aronia polyphenols reduced total and LDL-C in former smokers but did not improve biomarkers of oxidative stress and chronic inflammation. The total cholesterol-lowering effect of aronia polyphenols was most closely associated with the tissue and microbial metabolism of aronia polyphenols, particularly the formation of peonidin-3-*O*-galactoside.

6.2 Introduction

Cigarette smoking increases oxidative stress and inflammation, which increases cardiovascular disease (CVD) risk through vasomotor dysfunction, leukocyte and platelet activation, lipid peroxidation, adhesion and inflammatory molecules activation, and smooth muscle proliferation (Ambrose and Barua 2004). In the last decade, smoking cessation has increased, and in the United States, former smokers outnumber current smokers (Services 2014). Although smoking cessation reduces CVD risk, CVD risk in former smokers remains elevated for 10 to 15 years compared with never-smokers (Tilloy, Cottel et al. 2010, Godtfredsen and Prescott 2011). Former smokers also have compromised vascular and antioxidant function, suggesting the need for additional strategies to further reduce CVD risk (Helmersson, Larsson et al. , Jin, Webb-Robertson et al. 2011).

Aronia berry consumption may reduce CVD risk by improving dyslipidemia, inhibiting inflammation, and reducing oxidative stress (Naruszewicz, Łaniewska et al. 2007, Skoczyńska, Jedrychowska et al. 2007, Valcheva-Kuzmanova, Kuzmanov et al. 2007, Jurgoński, Juśkiewicz et al. 2008, Poręba, Skoczyńska et al. 2009, Broncel, Koziróg et al. 2010, Kujawska, Ignatowicz et al. 2011, Kim, Ku et al. 2013). Aronia berries has high levels of polyphenols, and its anthocyanins, hydroxycinnamic acids, and proanthocyanidins may mediate its putative health benefits (Taheri, Connolly et al. 2013). Increased aronia berry polyphenol intake reduces biomarkers of CVD risk in some populations. Daily consumption of 300 mg aronia extract for 2 months decreases blood pressure, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and total triglyceride (TG), and increased antioxidant enzyme activity in adults with metabolic syndrome (Broncel, Koziróg et al. 2010). Similarly, patients that survived a myocardial infarction and were prescribed statins whole also consuming 255 mg aronia extract

per day for 6 weeks demonstrated reduced blood pressure, oxidized low-density lipoprotein (ox-LDL), high-sensitivity C reactive protein (hsCRP) and monocyte chemoattractant protein 1 (MCP-1) when compared to patients that consumed a placebo (Naruszewicz, Łaniewska et al. 2007). Other anthocyanin-rich interventions modulate lipids and improve other biomarkers of CVD risk in individuals with dyslipidemia or metabolic syndrome (Qin, Xia et al. 2009, Basu, Du et al. 2010, Basu, Betts et al. 2011). Therefore, the consumption of aronia berries and other dietary anthocyanins appear to be a promising intervention for populations at higher risk of CVD.

Anthocyanins and polyphenols from aronia and other berries have limited bioavailability and are extensively metabolized (Manach, Scalbert et al. 2004). The interindividual variability of polyphenol pharmacokinetics is considerable (Manach, Williamson et al. 2005). Interindividual variability in polyphenol metabolism is due in part to genetic polymorphisms in xenobiotic metabolizing enzymes and transporters, as well as variation in gut microbial catabolism (Scheline 1999, Barnes 2008). The extent to which individual differences in polyphenol metabolism are associated with the efficacy of dietary polyphenol interventions for reducing CVD risk is largely unknown. Therefore, the primary objective of this study was to determine the ability of aronia polyphenols to reduce plasma lipids and biomarkers of oxidative stress and inflammation in former smokers, while the secondary objective was to determine the extent in which aronia polyphenol bioavailability is associated with changes in CVD risk biomarkers.

6.3 Materials and methods

6.3.1 Participants

Interested participants were recruited from Storrs, CT area from July 2012 to May 2015. Participants were pre-screened by phone, and then visited the University of Connecticut

Nutritional Sciences Department to provide informed consent and subsequent eligibility screening. The screening consisted of a standard medical history questionnaire and determination of height, weight, BMI, waist circumference, blood pressure, and a routine laboratory analysis of a fasting blood sample for blood sugar and TC. The inclusion criteria were: former smokers who had previously smoked ≥3 cigarettes/day for at least 1 year and ceased for at least 6 months; healthy male or female between 18-65 years of age; premenopausal status for women; serum TC <240 mg/dL and serum TG <150 mg/dL; resting blood pressure <140/90 mm Hg; stable body weight (± 5 lb) during the prior 2 months with a BMI between 18.5 kg/m^2 and 39 kg/m^2; willingness to maintain a normal exercise level (<7 h/wk) and avoid exercise 24 h prior to blood sampling; and willingness to ingest an aronia berry supplement or placebo daily for 12 wks. The exclusion criteria of this study were: previous diagnosis of CVD, diabetes, or arthritis (except for osteo-arthritis); current treatment for cancer; women with perimenopausal or menopausal symptoms or prescribed estrogen replacement therapy; adherence to a slimming or vegetarian diet; current consumption of vitamin, mineral, or botanical supplements; and consumption of alcohol exceeding the definition of moderate drinking (2 drinks/day or a total of 12/week for men or 1 drink/day or a total of 7/week for women). All participants consented to the study. The research protocol was approved by the University of Connecticut Institutional Review Board as protocol #H11-311, and listed at ClinicalTrials.gov as NCT01541826.

6.3.2 Study design

The participants were advised to consume a low polyphenol diet for a minimum of 4 weeks before the intervention and were randomly assigned to consume 500 mg of aronia berry extract or the placebo. The supplements were opaque, color-matched, and provided 250 mg of extract or placebo per capsule. The participants and study personnel were blinded to the composition of the

intervention material. The aronia extract provided 45.07 mg anthocyanins, 35.73 mg hydroxycinnamic acids, and 41.9 mg proanthocyanidins per 500 mg dose. The placebo was composed of rice powder with 0.2% beet juice concentrate. The extract and placebo passed quality control measures of microbial pathogens, heavy metals, pesticides, and residual solvents. The compositions of the aforementioned intervention materials are further described in **Table 6.1** and **Table 5.4**. The polyphenol content of placebo and supplement changed less than 10% over the course of the study.

Participants completed a three-day dietary record during the run-in period prior to the intervention. At the start of the intervention and again at week 12, participants completed an overnight fast, and baseline blood and urine samples were collected the following morning. Study personnel subsequently determined height, weight, and blood pressure. At week 6, participants were again asked to fast overnight for 12 h prior to arriving at the study center for anthropometric measurements and collection of fasting blood samples. All participants were advised to consume a low polyphenol diet, moderate alcohol consumption, and maintain their normal physical activity throughout the course of intervention.

6.3.3 Preparation of biological samples

Blood samples (80 mL) were drawn from a brachial vein into EDTA vacutainer tubes (BD, Franklin Lakes, NJ, USA) to recover plasma and peripheral blood mononuclear cells (PBMCs). Another 10 mL blood sample was drawn from a brachial vein into evacuated glass tubes (BD, Franklin Lakes, NJ, USA) to recover serum. The blood samples were immediately placed on ice, and centrifuged at 1,500 ×g for 15 min at 4 °C to recover plasma. For serum collection, the blood samples were allowed to clot at room temperature for 25 min and then centrifuged at 1000 ×g for 15 min at 4 °C. Plasma and urine aliquots for polyphenol analysis were acidified using 0.5% HCl

and centrifuged at 2,500 ×g for 15 min at 4 °C. The supernatants were collected and stored at -80 °C until analysis. PBMCs were isolated from whole blood samples using Ficoll-Paque premium (GE Healthcare, Pittsburgh, PA, USA) by density gradient centrifugation. Whole blood samples were diluted 1:1 with sterile PBS, and the diluted blood samples were layered over 2:1 with Ficoll-Paque premium, and centrifuged at 700 ×g for 20 min to separate the PBMCs. The buffy coat-containing PBMCs were collected, washed twice with PBS, and the cells were resuspended in ice-cold FBS. The cell suspensions were diluted 1:1 with cryopreservation media (fetal bovine serum, 20% dimethyl sulfoxide) to a final concentration of 10^7 cells/mL and frozen in CoolCell containers (BioCision, LLC, Larkspur, CA, USA) at −80 °C for 24 h. The plasma were then transferred to liquid nitrogen for storage.

6.3.4 Anthropometric measurements

In a quiet location, participants were seated for at least 10 min in a chair with their back supported, feet on the floor, legs uncrossed, having an empty bladder, and the upper arm supported at heart level (Pickering, Hall et al. 2005). Before all visits, participants were asked to refrain from exercise or ingesting caffeine the day of the measurement. Blood pressure (BP) was measured in the left arm using an Omron HEM-780 automatic BP monitor (Omron Healthcare, Inc., Bannockburn, IL, 60015) three times with one minute intervals between measurements. If the readings were within 5 mmHg, the readings were averaged and recorded as resting systolic and diastolic blood pressure. If a difference of > 5 mmHg between readings occurred, the measurements were repeated until three readings were within 5 mmHg. Body weight and height were measured on a digital physician scale (Detecto 758C, Cardinal Scale Manufacturing Company). Waist circumference was determined by placing a measuring tape evenly around a bare abdomen at hipbone level.

6.3.5 Plasma lipid analysis

As previously described, plasma lipid concentrations were determined enzymatically using Cholesterol Reagent (Pointe Scientific, Canton, MI) and a L-Type TG M kit (Wako Chemical, Richmond, VA) for TC and TG, respectively (Kim, Ku et al. 2013). High-density lipoprotein cholesterol (HDL-C) was measured after precipitation of apolipoprotein B-containing lipoprotein in plasma, and non-HDL-C was calculated by subtraction of HDL-C from TC. Precinorm L (Roche, Indianapolis, IN) was used as a control. The Friedeman LDL formula was used to determine LDL-C concentration (Equation 1).

Equation 1: $LDL\text{-}C = TC - HDL - TG/5$

6.3.6 Analysis of inflammatory biomarkers

Inflammatory biomarkers were measured in plasma samples by ELISA. Plasma adiponectin (eBioscience, San Diego, CA), interleukin-6 (IL-6) (eBioscience, San Diego, CA), interleukin-1β (IL-1β) (eBioscience, San Diego, CA), MCP-1 (eBioscience, San Diego, CA), tumor necrosis factors-α (TNF-α) (eBioscience, San Diego, CA), and C-reactive protein (CRP) (eBioscience, San Diego, CA) were determined as described by the respective manufacturers' protocols.

6.3.7 Analysis of biomarkers related to oxidative status

Biomarkers of oxidative stress and total antioxidant activity were measured in both plasma and urine to investigate the antioxidant effects of aronia polyphenol supplementation. Assay kits were used following manufacturers' instructions to measure plasma oxidized LDL by immunoassay (MyBiosource, San Diego, CA), catalase activity (Cayman Chemical, Ann Arbor, MI), glutathione peroxidase (GSH-PX) activity (Cayman Chemical, Ann Arbor, MI), and superoxide dismutase (SOD) activity (Cayman Chemical, Ann Arbor, MI). Plasma total

antioxidant capacity was measured using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assay and expressed as mg vitamin C equivalent (VCE)/mL as previously described (Wang, Yang et al.). Urinary 8-isoprostanes were also measured by immunoassay (Cayman Chemical, Ann Arbor, MI) and the amount was normalized with urinary creatinine.

6.3.8 Analysis of plasma biomarkers of vascular reactivity

Concentrations of cell adhesion molecules including inter-cellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), and P-selectin in serum were measured using assay kits for Bio-Plex Magpix multiplex reader with xPotent software (Luminex, Austin, TX).

6.3.9 Gene expression analysis by quantitative realtime PCR (qRT-PCR)

Cryopreserved PBMCs were thawed and slowly added to 15 mL of phosphate-buffered saline (PBS), and then 0.25 mL of 1 mg/mL DNAse were added to prevent PBMC clumping. Pelleted PBMCs were obtained by centrifugation at 300 ×g for 6 min. Total RNA was isolated from pelleted PBMCs using TRIzol reagent (Invitrogen, Grand Island, NY, USA) and qRT-PCR analysis of gene expression was conducted as previously described using the SYBR Green procedure and CFX96 realtime PCR detection system (Bio-Rad, Hercules, CA) (Park, Rasmussen et al. 2008, Rasmussen, Blobaum et al. 2008). Primer sequences were designed according to GeneBank database using the Beacon Designer software (Premier Biosoft, Palo Alto, CA) (Rasmussen, Blobaum et al. 2008). GAPDH was used as an internal control.

6.3.10 Western blot analysis

Protein was extracted from pelleted PBMCs as previously described (Rasmussen, Blobaum et al. 2008). Western blot analysis was performed using antibodies against 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGR) and β-actin (Sigma, St. Louis, MO) as previously described (Rasmussen, Blobaum et al. 2008). The blots were developed using a horseradish peroxidase system (Pierce), and densitometry analysis was performed using a Chemidoc XRS+ (Bio-Rad) and Image Lab software (Bio-Rad). β-Actin was used as a loading control.

6.3.11 Urinary creatinine analysis

The urine creatinine was measured by a urinary creatinine colorimetric assay (Cayman Chemical, Ann Arbor, MI). Creatinine concentrations varied from 15.26 to 573.92 mg/dL in urine samples.

6.3.12 Plasma and urinary polyphenol analysis

Polyphenols were isolated from plasma and urine by solid phase extraction (SPE) and quantitated by UHPLC-MS as we previously described in Chapter 5. Briefly, plasma and urine samples were applied to HyperSep C18 SPE cartridges (Thermo Scientific, Waltham, MS) attached to a vacuum manifold. The eluate was collected with 0.5% formic acid in methanol (v:v) and dried under nitrogen gas. Dried residue was reconstituted with methanol. UHPLC–MS analysis was conducted using a Shimadzu Nexera UHPLC equipped with LCMS-2020 mass spectrometer. Separate chromatographic methods were used to detect 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, hippuric acid, and ferulic acid; 3-(4-hydroxyphenyl)propionic acid; and cyanidin-3-*O*-glucoside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, and

peonidin-3-*O*-galactoside. Limits of detection (LOD), limits of quantification (LOQ), and intra- and inter-day variance have been listed in Chapter 5.

6.3.13 Statistics

Changes in plasma lipids and anthropometric markers after supplementation were log-transformed and analyzed by a repeated measures MANOVA model using JMP® Pro 11 (SAS Institute, Inc., Cary, NC). All other data were analyzed using SAS (9.4, SAS Institute, Inc., Cary, NC). Baseline differences between the placebo and treatment groups were analyzed by two sample t-test and chi square test. Group differences in biomarkers over time were analyzed by repeated measures ANOVA and assuming 100% compliance. For these data, a univariate approach was applied when the sphericity assumption was violated. A multivariate approach was applied when the sphericity assumption was not violated (Austin). A log transformation was applied when the normal assumption was violated. Principle component analysis and Pearson correlation were used to measure the association of changes in urinary polyphenol and plasma lipids. Data was expressed as mean ± standard error of mean.

6.4 Results

6.4.1 Participant characteristics

After informed consent, 56 participants were enrolled and randomized (26 men and 30 women) and 49 participants completed the study (**Figure 6.1**). The reasons for drop-out included not responding to communication from study personnel (n = 2 placebo, and n = 3 supplement), weight gain (n = 1 placebo), and headache (n = 1 placebo). Participant characteristics are listed in **Table 6.2**. There were no significant differences between the two groups, other than the

average years of smoking in the aronia group that was five years less than that of the placebo group.

6.4.2 Anthropometric measurements

Participant waist circumferences were 85.57 ± 2.87 cm for the aronia group and 86.16 ± 4.86 cm for the placebo group at baseline (P = 0.9163) (**Table 6.2)**. Over the course of the study participant waist circumferences were reduced with the aronia and placebo groups having 1 and 5% lower waist circumference, respectively, at week 12 (P = 0.0075) (**Table 6.4**). At baseline, systolic blood pressures were not different between groups, at 117 ± 3 and 121 ± 3 mmHg in the aronia and placebo groups, respectively (P = 0.3643). However, the placebo group had lower diastolic pressure than the aronia group at week 6 (74.8 ± 1.8 vs 80.2 ± 2.2 mmHg) (P = 0.0331) which had persisted through week 12. The BMI of both groups were not different at baseline, but the placebo group mean BMI decreased from 27.0 ± 0.9 to 26.8 ± 0.9 over the course of the study (P = 0.0354).

6.4.3 Plasma lipids

Participants had borderline high TC at baseline with 220.11 ± 12.59 mg/dL for the aronia group and 203.41 ± 7.25 mg/dL for the placebo group (P = 0.2037). Aronia polyphenol consumption reduced TC by 8 % at 12 wks, compared to no significant change in the placebo group (P = 0.0140) (**Table 6.3**). The mean LDL-C for each group was above optimal, with 175.42 ± 10.80 and 158.26 ± 6.07 mg/dL for the aronia and placebo group, respectively (P = 0.1289). The aronia group had 7 and 11% less LDL-C at both 6 and 12 wks from baseline, with no significant changes to LDL-C in the placebo group (P = 0.0285). Participants had low TG with 89.58 ± 9.08 mg/dL for the aronia group and 82.08 ± 7.62 mg/dL for the placebo group (P =

0.4858), and low HDL with 26.77 ± 1.52 mg/dL for the aronia group and 28.75 ± 1.25 mg/dL for the placebo group (P = 0.2715). Neither intervention significantly changed HDL-C or TG from baseline values.

6.4.4 Expression of hepatic genes involved in lipid metabolism

LDL receptor (LDLR) and HMGR mRNA expression of PBMCs were not different between the placebo and aronia groups at baseline or 12 wk (**Table 6.5**). However, aronia supplement consumption reduced LDLR protein in PBMCs 56 % from baseline at 12 wk (P = 0.0036). In contrast, LDLR protein of PBMCs in the placebo group was unchanged from baseline at 12 wk.

6.4.5 Other biomarkers relevant to CVD risk

Plasma cytokines (IL-6, IL-1β, and TNF-α), CRP, adiponectin, vascular adhesion molecules (sICAM and sVCAM), chemokines (MCP-1), antioxidant enzyme activity (CAT, GSH-Px, and SOD), and markers of lipid oxidation (urinary 8-isoprostanes and oxLDL) were determined at baseline, 6, and 12 wk in both groups (**Table 6.5)**. None of these biomarkers significantly changed in either group at 6 and 12 wk.

6.4.6 Urinary and plasma polyphenols

Analysis of dietary records did not reveal differences of polyphenol intake between the groups at any time point (**Table A.6.1**). Aronia polyphenols and their key intermediates were determined in plasma and urine to evaluate the relative enrichment and metabolism of the supplemental polyphenols in participants. The baseline level of peonidin-3-*O*-galactoside and sum of anthocyanins in aronia group were 6.2 μg/mg creatine and 0.332 mg/mg creatine, 7-8 times higher than that in placebo group (P = 0.0461 and P = 0.0441) (**Table 6.6**). At 12 wk,

protocatechuic acid, 3,4-dihydroxyphenylacetic acid, hippuric and ferulic acid were detected in all individuals in both groups without differences at the baseline. However, at least 25% of participants did not accumulate urinary 3-(4-hydroxyphenyl)propionic acid, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside or peonidin-3-*O*-galactoside in significant quantities, as all were below the LOD. At 12 wk, the change from baseline of total urinary polyphenol excretion was not significantly different for either intervention. However, the polyphenol profiles of the aronia and placebo group could be distinguished by canonical discriminate analysis with 80% confidence (**Figure A.6.1**). At baseline and 12 wk, fasting plasma polyphenols were lower than the LOD for most of the participants in both placebo and aronia groups, except for hippuric acid (**Table 6.7**). The range of plasma polyphenol and metabolite concentrations for the aronia group was similar to those of the placebo group.

6.4.7 Association of urinary polyphenols and lipids changes in aronia group

A multivariate approach was used to determine the association of urinary polyphenols to changes in plasma lipids at 12 wk from baseline. In the aronia group, changes of urinary polyphenol and its metabolites were associated with the changes of lipid in the plasma (**Figure 6.2**). Cohen's standard was used to determine the strength of the correlation between the difference of plasma lipids and urinary polyphenols at 12 wk from baseline (Δ), where correlation coefficients between 0.10 and 0.29, 0.30 and 0.49, and greater than 0.50 represented small, medium and large associations, respectively. Peonidin-3-*O*-galactoside Δ had a medium inverse association with plasma TC Δ and LDL-C Δ (P-value = 0.0645 and P-value = 0.1104, respectively). Cyanidin-3-*O*-galactoside Δ and 3-(4-hydroxyphenyl)propionic acid Δ both had small inverse associations with plasma TC Δ (P-value = 0.1912 and P-value =0.4993, respectively) and LDL-C Δ (P-value = 0.2586 and P-value = 0.4176, respectively). A strong

positive association was observed between 3,4-dihydroxyphenylacetic acid Δ and plasma HDL-C Δ. A medium positive association was observed between protocatechuic acid Δ and plasma HDL-C Δ. Hippuric acid Δ and ferulic acid Δ also had small positive associations with plasma HDL-C Δ. Cyanidin-3-*O*-glucoside Δ was strongly inversely associated with plasma TG Δ. Small inverse associations were also observed between plasma TG Δ and urinary polyphenol metabolites including ferulic acid Δ, cyanidin-3-*O*-galactoside Δ, cyanidin-3-*O*-arabinoside Δ, and peonidin-3-*O*-galactoside Δ. In contrast, the placebo group did not have medium or large associations between urinary polyphenols Δ and plasma lipids Δ.

6.4.8 Analysis by respondent to total cholesterol

To further examine participant response to aronia consumption, we also classified participants as responders or non-responders to the intervention. An aronia responder was defined as a participant who lost more than 10 mg TC/dL from baseline at 12 wk, and an aronia non-responder was a participant who lost less than 10 mg TC/dL. Aronia responders (n = 14) had higher baseline TC at baseline (248.09 ± 17.06 vs 188.96 ± 13.12 mg/dL, P = 0.0117) as well as a slightly higher urinary peonidin-3-*O*-galactoside Δ (0.01577 ± 0.00890 vs -0.00400 ± 0.00222, P = 0.0998). Aronia responders had a medium association of peonidin-3-*O*-galactoside Δ and plasma TC Δ, whereas these were not associated in non-responders (**Figure 6.3**). In addition, responders consuming aronia extract had distinct polyphenol metabolite profiles from non-responder. Particularly, responders had more closely correlated peonidin-3-*O*-galactoside Δ and cyandin-3-*O*-galactoside Δ than non-responders (**Figure 6.4**).

6.5 Discussion

In the present study, consumption of 500 mg of an anthocyanin-rich aronia berry extract daily for 12 weeks did not change plasma biomarkers of chronic inflammation in former smokers (**Table 6.5**). This contrasts prior studies that have characterized the mechanism by which anthocyanins may inhibit chronic inflammation. Increased anthocyanin consumption or direct application of anthocyanins to cells reduces NF-κB activation, thereby inhibiting the expression of pro-inflammatory chemokines and cytokines (Blanco-Colio, Valderrama et al. 2000, Kim, Tsoy et al. 2006, Karlsen, Retterstøl et al. 2007, Xia, Ling et al. 2007, Martin, Taheri et al. 2014). Anthocyanins can also inhibit the pathogenesis of atherosclerosis and plaque formation by reducing IL-6, IL-8, MCP-1 and ICAM-1 induced by TNF-α and CD40 in human endothelial cells (Atalay, Gordillo et al. 2003, García-Alonso, Rimbach et al. 2004, Xia, Ling et al. 2007). Anthocyanin pretreatment of cultured human umbilical vein endothelial cells inhibited VCAM-1, nuclear *GATA-4*, nuclear *GATA-6* and nuclear interferon regulatory transcription factor-1 (IRF-1) expression in human, which suggests a role of the Jak/STAT pathway in preventing monocyte adhesion and subsequent inflammation (Nizamutdinova, Kim et al. 2009). Large doses of anthocyanin-rich foods or extracts reduce biomarkers of inflammation in healthy individuals. Daily consumption of 280 g cherries reduced plasma CRP after 4 weeks of intervention (Kelley, Rasooly et al. 2006). Additionally, a single 12 g dose of an anthocyanin-rich extract from red wine grape decreased plasma level of MCP-1 24 h after intake (Garcia-Alonso, Minihane et al. 2009).

Aronia berry consumption has an inconsistent anti-inflammatory effect among participants with increased CVD risk. Participants with metabolic syndrome that consumed a 300 mg dose of aronia berry extract per day did not have significant changes in CRP from a baseline of 2.62 ±

2.5 mg/dL after 8 weeks (Broncel, Koziróg et al. 2010). However, participants who had suffered myocardial infraction and consumed 255 mg of aronia berry extract and statins daily for 6 weeks, had 23% lower CRP from a baseline of 4.48 ± 2.56 mg/L (Naruszewicz, Łaniewska et al. 2007). In the same study, aronia consumption reduced MCP-1 by 23% from a baseline of 305.9 ± 87.5 pg/mL (Naruszewicz, Łaniewska et al. 2007). The former smokers in the present study had baseline levels of CRP and MCP-1 that were at least 70% lower than the population of participants that suffered a myocardial infarction (7). Thus, aronia consumption may only have an anti-inflammatory effect in populations with relatively higher levels of chronic inflammation.

Aronia berry extract consumption did not modulate markers of antioxidant function and oxidative stress in former smokers. However, over 8 wk, aronia berry extract consumption modulates the serum antioxidant capacity of participants with metabolic syndrome by reducing erythrocyte CAT activity and increasing erythrocyte SOD and GSH-Px activities (Broncel, Koziróg et al. 2010). Blueberry anthocyanins (1.2 g) consumed with high-fat meals for 7 days improves serum antioxidant capacity (oxygen radical absorbance capacity) of healthy male individuals compared with placebo consumption, with the change positively correlated to serum anthocyanin content (Mazza, Kay et al. 2002). Anthocyanins may directly protect membrane fatty acids of endothelial cells from oxidation and DNA cleavage induced by increased inflammation (Youdim, McDonald et al. 2002, Acquaviva, Russo et al. 2003). The 500 mg dose of the anthocyanin-rich aronia extract used in the present study did not appear to sufficiently enrich plasma to exert antioxidant activity, as peak concentrations were 0.004 to 3.488 µg/mL. In contrast, a lower dose of aronia berry extract reduces plasma ox-LDL and urinary 8-isoprostanes in participants that suffered from myocardial infarction (Naruszewicz, Łaniewska et al. 2007). The ox-LDL and 8-isoprostanes reported by Naruszewicz, et al (Naruszewicz, Łaniewska et al.

2007) are not comparable to the present study due to significant differences in analytical methods. However, when compared to a report using similar methods, urinary isoprostanes of former smokers in the present study was 3-fold lower than that of participants with coronary artery disease (Kim, Hyun et al. 2008, Noriega, Pennanen et al. 2009). Former smokers in the present study had less oxidative stress and more favorable plasma lipid profiles (lower plasma LDL-C, TG, and TC and higher HDL-C) than participants in other trials where aronia intake improved antioxidant function (Naruszewicz, Łaniewska et al. 2007, Broncel, Koziróg et al. 2010).

Consumption of anthocyanin-rich foods also lowered TC, LDL-C, and TG in previous studies (Qin, Xia et al. 2009, Basu, Du et al. 2010, Broncel, Koziróg et al. 2010, Basu, Betts et al. 2011). Low CVD risk is defined as TC less than 200 mg/dL, LDL-C between 100 to 120 mg/dL, and HDL-C more than 40 mg/dL (Services). The former smokers in the present study had increased risk of CVD due to greater than optimal levels of TC and LDL-C. Aronia extract consumption lowered plasma LDL-C level at 6 and 12 wks, and TC at 12 wk. Responders to the aronia intervention had higher baseline TC than non-responders, suggesting that individuals with hypercholesterolemia may benefit the most from increased polyphenol consumption. Our prior pre-clinical work suggests that aronia berry polyphenols reduce TC through mechanisms independent of the modulation of hepatic mRNA expression of lipid-metabolizing genes, and possibly by intestinal mechanism of cholesterol efflux (Kim, Ku et al. 2013, Martin, Taheri et al. 2014). In the present study, LDLR protein of PBMCs was reduced by aronia extract consumption without a corresponding change of LDLR mRNA. Post-translational regulation of LDLR protein by proprotein convertase subtilisin/kexin type 9 (PCSK9) might explain this observation (Park,

Moon et al. 2004), but further studies are needed to identify how aronia polyphenols modulate LDLR expression.

Aronia berry consumption reduced blood pressure, waist circumference, and BMI of participant with metabolic syndrome or abdominal obesity (Kardum, Petrovi et al. 2014, Sikora, Broncel et al. 2014). These parameters were not affected by aronia consumption in the present study. The mean blood pressure of the aronia group met the criteria of prehypertension (120-139/80-89 mm Hg) (Association). However, in trials where aronia reduced blood pressure, participants had higher blood pressure (127.6 ± 16.9/83.5 ± 9.9 to 136.8 ± 10.9/86.8 ± 16.0 mmHg), larger waist circumference (95.0 ± 9.4 cm to 104.8 ± 10.1 cm) and greater BMI (30.9 ± 3.7 kg/m^2 to 36.1 ± 4.4 kg/m^2) (Kardum, Petrovi et al. 2014, Sikora, Broncel et al. 2014). It was suggested that aronia juice and polyphenols could reduce the blood pressure of spontaneously hypertensive rat model (Hellström, Shikov et al. 2010). The observed effect might result from of the angiotensin converting enzyme (ACE)-inhibitory activity of cyanidin-3-*O*-glucoside, proanthocyanidin oligomers and phenolic metabolites of aronia polyphenols (Ottaviani, Actis-Goretta et al. 2006, Hellström, Shikov et al. 2010, Hidalgo, Martin-Santamaria et al. 2012).

Under well-controlled dietary conditions, the dose of aronia polyphenols consumed in the present study could increase transient accumulation and excretion of polyphenols. However, the same dose did not significantly enrich aronia polyphenols and microbial metabolites in these biofluids after 12 wk consumption. Given the low abundance in plasma, urinary excretion of polyphenols and metabolites are a better indicator of polyphenol bioavailability than plasma concentrations (Tresserra-Rimbau, Rimm et al. 2014).

Although polyphenol-rich aronia berries may have atheroprotective effects, compounds or metabolites may be associated with these effects are unknown. Within the aronia group, greater

reductions of plasma TC and LDL-C were associated with increases in urinary cyanidin-3-*O*-galactoside, peonidin-3-*O*-galactoside, and 3-(4-hydroxyphenyl)propionic acid. Thus, aronia polyphenol metabolites peonidin-3-*O*-galactoside and 3-(4-hydroxyphenyl)propionic acid and parent compounds (cyanidin-3-*O*-galactoside) were associated with the cholesterol-lowering mechanism of aronia berry. A precursor of 3,4-dihydroxyphenylacetic acid and ferulic acid, 3-(4-hydroxyphenyl)propionic acid can also be derived from microbial metabolism of aronia anthocyanins, chlorogenic acid, or proanthocyanidins. 3-(4-Hydroxyphenyl)propionic acid catabolites have the potential to reduce plasma TC and LDL-C levels in rodents (Lee, Park et al. 2007). Thus, 3-(4-hydroxyphenyl)propionic acid might be a functional precursor to cholesterol-modulating compounds. A large inverse association was found between cyanidin-3-*O*-glucoside and plasma TG levels in the aronia group, which was also observed in another study that suggested cyanidin-3-*O*-glucoside could improve TG metabolism by the activation of lipoprotein lipase (LPL) activity in plasma and skeletal muscle but suppression of LPL activity in visceral adipose (Wei, Wang et al. 2011). Comparative data on anthocyanin structure-function activity and mechanisms of lipid metabolism are lacking. However, even minor structural differences can alter anthocyanin anti-inflammatory activity in vitro (Martin, Taheri et al. 2014).

There were several limitations of this study. First, participants did not synchronize daily consumption of the intervention. The interindividual variation of fasting urinary and plasma polyphenol concentrations could result from the time of consumption and do not solely reflect bioavailability. Furthermore, participants did not strictly adhere to low-polyphenol diets, as anthocyanins were detected at the baseline. Therefore, consumption of other polyphenol-containing foods might significantly affect polyphenol excretion.

6.6 Conclusions

Consumption of 500 mg aronia berry extract for 12 wks improved biomarkers of CVD risk in former smokers by reducing LDL-C by 10% and TC by 8%. However, this intervention did not change CRP, 8-isoprostane, and other biomarkers of chronic inflammation and oxidative stress. Regular consumption of aronia extract in high dose may be impractical, and given the apparently lower baseline markers of inflammatory and oxidative stress, have limited benefits. Increased excretion of peonidin-3-*O*-galactoside, and to a lesser extent, cyanidin-3-*O*-galactoside and 3-(4-hydroxyphenyl)propionic acid were most associated with reductions in TC and LDL-C, which suggested host and microbial metabolism have a role in the cholesterol-lowering mechanism of aronia berry extract consumption. These specific associations could arise from structure-function differences between polyphenols and metabolites and may begin to explain why polyphenol-interventions are more effective in some individuals with metabolic pathways favoring production of these compounds.

6.7 Chapter 6 Tables and Figures

Table 6.1 Non-polyphenol contents analysis in aronia berry supplement

Assay	Aronia berry Supplement (mg/500 mg)
Total sugar	7.5
Total Sugar Alcohol	4.1
Vitamin C	< 0.005
Minerals	< 0.4
Vitamin B	< 0.02
Organic Acids	< 4.9
Sterol	< 0.005

Table 6.2 Baseline characteristics of study population

Measurement	**Placebo** (n=24)	**Aronia** (n=25)	**P Value**
Age	37.37 ± 3.02	32.6 ± 2.57	0.2337
Gender (%Male)	46%	52%	0.6660
Average years of Smoking	14.85 ± 2.43	8.96 ± 1.34	0.0404
Average Num. of Cigarettes/d			
3-5 Cigarettes/d	2	4	0.5447
6-10 Cigarettes/d	4	7	
11-15 Cigarettes/d	4	4	
16-20 Cigarettes/d	11	6	
>20 Cigarettes/d	3	4	
Height (cm)	172.97 ± 1.88	171.44 ± 1.66	0.5447
Weight (kg)	81.64 ± 3.47	77.18 ± 4.15	0.4151
BMI (kg/m^2)	27.04 ± 0.93	25.64 ± 1.15	0.3429
Waist Circumference (cm)	85.57 ± 2.87	86.16 ± 4.86	0.9163
Blood Pressure (mmHg)			
Systole	121.00 ± 2.95	116.96 ± 3.29	0.3643
Diastole	76.00 ± 2.12	75.28 ± 2.41	0.8224
Total Cholesterol (mg/dL)	203.41 ± 7.25	220.11 ± 12.59	0.2037
HDL-C (mg/dL)	28.75 ± 1.25	26.77 ± 1.52	0.2715
Triglycerides (mg/dL)	82.08 ± 7.62	89.58 ± 9.08	0.4858
LDL-C (mg/dL)	158.26 ± 6.07	175.42 ± 10.80	0.1289

TC/HDL Ratio	7.27 ± 0.31	8.54 ± 0.53	0.0284*

Data was expressed as mean ± standard error of mean and analyzed by t-test.
*P < 0.05.

Table 6.3 Changes in plasma lipids in former smokers after daily consumption of 500 mg aronia extract or a placebo

Biomarker	**Week**	**Δ baseline (mg/dL)**		**P-value RM-MANOVA**		
		Aronia (n = 25)	**Placebo (n = 24)**	**Group**	**Time**	**Time*Group**
Total Cholesterol	6	1.4 ± 2.5	-0.1 ± 4.6	0.2300	0.0016*	0.0140*
	12	-17.9 ± 7.6	-1.3 ± 6.4			
LDL-C	6	-12.6 ± 5.4	0.9 ± 4.2	0.0285*	0.1046	0.4547
	12	-18.5 ± 8.0	-0.4 ± 5.9			
HDL-C	6	0.2 ± 0.8	-0.9 ±1.0	0.4181	0.8881	0.9153
	12	0.2 ± 0.8	-0.7 ± 1.4			
TG	6	1.0 ± 5.9	-0.6 ± 2.7	0.7534	0.2326	0.8699
	12	2.0 ± 8.5	-1.0 ± 5.9			

Data was expressed as mean ± standard error of mean, transformed and analyzed by repeated measures MANOVA.

*P < 0.05.

TG, triglycerides

Table 6.4 Changes in anthropometric measures following of consumption of placebo or 500 mg aronia extract/d for 12 wk

Marker	**Week**	**Δ baseline**		**P-value RM-MANOVA**		
		Aronia (n = 25)	**Placebo (n = 24)**	**Group**	**Time**	**Time*Group**
Weight	6	0.5 ± 0.3	0.7 ± 0.6	0.7364	0.0775	0.1842
(kg)	12	0.5 ± 0.5	-0.1 ± 0.6			
BMI	6	0.3 ± 0.4	0.2 ± 0.2	0.3800	0.0354*	0.0464*
(kg/m^2)	12	0.5 ± 0.4	-0.2 ± 0.3			
Waist	6	0.0 ± 1.2	1.5 ± 1.1	0.8832		0.0619
circumference					0.0075*	
(cm)	12	-0.8 ± 1.0	-1.4 ± 0.7			
Blood pressure						
Systole	6	2.8 ± 1.7	-0.8 ± 1.5	0.3606	0.5179	0.5002
(mmHg)	12	1.2 ± 2.4	0.2 ± 2.9			
Diastole	6	5.0 ± 1.3	-1.2 ± 2.4	0.0331*	0.6855	0.1661
(mmHg)	12	2.0 ± 1.5	-1.1 ± 1.7			

Data were expressed as mean ± standard error of mean, transformed and analyzed by repeated measures MANOVA. *P-value < 0.05.

Table 6.5 Changes in peripheral blood mononuclear cell gene and protein expression, and biomarkers of inflammation and antioxidant function following of consumption of placebo or 500 mg aronia extract per day for 12 wk

	Placebo (n=24)			**Aronia (n=25)**			**P**
	Baseline	**6 wk**	**12 wk**	**Baseline**	**6 wk**	**12 wk**	**value**
HMGR mRNA (fold change relate to baseline)	0.90 ± 0.06	ND	1.16 ± 0.28	0.91 ± 0.06	ND	0.79 ± 0.12	0.3781
LDLR mRNA (fold change relate to baseline)	0.90 ± 0.07	ND	0.93 ± 0.22	0.84 ± 0.07	ND	0.88 ± 0.26	0.9666
LDLR protein (fold change relate to baseline)	1.71 ± 0.33	ND	2.47 ± 0.80	1.45 ± 0.58	ND	0.64 ± 0.07	0.0036 *

Adiponectin (μg/mL)	13.21 ± 1.92	14.15 ± 2.01	12.45 ± 1.64	12.75 ± 1.54	13.82 ± 1.68	13.24 ± 1.47	0.9994
IL-1β (pg/mL)	9.33 ± 2.43	9.50 ± 2.73	10.08 ± 3.04	16.20 ± 8.55	17.03 ± 9.67	14.74 ± 7.38	0.4438
IL-6 (pg/mL)	1.94 ± 0.34	1.87 ± 0.38	1.76 ± 0.29	4.68 ± 1.34	5.16 ± 1.54	5.34 ± 1.52	0.0782
MCP-1 (pg/mL)	97.01 ± 5.78	96.41 ± 5.24	90.93 ± 5.32	132.76 ± 31.15	166.85 ± 42.74	140.99 ± 26.78	0.0679
TNF-α (pg/mL)	4.36 ± 1.83	4.70 ± 1.89	4.86 ± 1.85	5.90 ± 1.63	6.44 ± 1.83	6.10 ± 1.72	0.5977
CRP (pg/mL)	2.54 ± 0.60	2.18 ± 0.42	2.24 ± 0.38	2.01 ± 0.34	2.32 ± 0.39	2.29 ± 0.49	0.7222
sICAM (ng/mL)	97.06 ± 7.19	97.63 ± 7.16	100.12 ± 7.70	96.59 ± 5.40	99.42 ± 5.68	97.52 ± 5.82	0.4613
sVCAM (ng/mL)	585.43 ± 22.55	603.93 ± 22.86	604.11 ± 25.53	601.09 ± 31.04	621.97 ± 30.91	599.35 ± 27.24	0.5902
P-selectin (ng/mL)	129.95 ± 13.39	110.66 ± 10.92	130.82 ± 11.41	123.76 ± 11.77	126.60 ± 11.75	144.61 ± 16.75	0.1321
OxLDL	209.55 ± 3.70	214.26 ± 3.44	209.16 ± 3.97	207.66 ± 3.24	211.43 ± 3.42	206.57 ± 4.41	0.5701

(ng/mL)							
TAC (µg VCE/mL)	210.46 ± 4.51	215.70 ± 4.00	214.49 3.28	221.20 ± 4.51	225.87 ± 4.66	224.31 ± 4.90	0.0775
CAT (nmol/min/mL)	41.71 ± 1.36	36.53 ± 1.17	41.65 ± 2.28	41.07 ± 2.13	38.68 ± 1.52	41.20 ± 1.84	0.2552
GSH-Px (nmol/min/mL)	2554. 35 ± 139.90	2561.51 ± 133.91	2468.65 ± 110.19	2554.37 ± 143.63	2497.00 ± 163.72	2654.65 ± 186.52	0.8323
SOD (U/mL)	1186.69 ± 54.27	1192.81 ± 51.87	1158.84 ± 58.25	1181.85 ± 40.92	1160.11 ± 43.85	1160.19 ± 39.76	0.8479
Isoprostane (ng/mmol creatinine)	71.01 ± 8.09	ND	81.59 ± 7.81	58.60 ± 7.00	ND	68.42 ± 8.98	0.1715

Data was expressed as mean ± standard error of mean, transformed and analyzed by repeated measures ANOVA.

*P-value < 0.05.

ND: Not determined.

Abbreviations: HMGR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; IL1β, Interleukin 1 beta; IL6, Interleukin 6; MCP1, monocyte chemoattractant protein 1; LDLR: Low-Density Lipoprotein (LDL) Receptor; OxLDL, oxidized low density lipoprotein; TAC, total antioxidant capacity; TNF-α, tumor necrosis factor alpha; CAT, catalase; CRP, C-reactive protein; GSH-Px, glutathione peroxidase; sICAM, soluble intercellular adhesion molecule 1; sVCAM, soluble vascular cell adhesion molecule1; SOD, superoxide dismutase.

Table 6.6 Overnight urinary polyphenols and metabolites at baseline and 12 weeks following daily consumption of 500 mg aronia berry extract or placebo.

	Placebo (mg/mg creatinine)				Supplement (mg/mg creatinine)			
	Baseline	12 wk			Baseline	12 wk		
Polyphenol/Metabolite	mean	Q1	Q2	Q3	mean	Q1	Q2	Q3
protocatechuic acid	1.89 ± 0.41	0.94	1.38	2.08	1.41 ± 0.20	0.64	1.20	2.03
3,4-dihydroxyphenylacetic acid	4.85 ± 0.92	2.36	5.04	7.45	4.93 ± 0.81	2.18	4.56	9.09
hippuric acid	161 ± 22	104	133	234	208 ± 40	62	138	292
ferulic acid	5.81 ± 4.57	0.242	0.684	1.20	1.71 ± 0.48	0.233	0.709	3.571
3-(4-hydroxyphenyl)propionic acid	5.56 ± 1.28	ND**	3.91	9.95	4.93 ± 0.14	ND	3.36	9.68
cyanidin-3-*O*-glucoside	0.036 ± 0.017	ND	ND	0.055	0.170 ± 0.094	ND	0.00907	0.1993
cyanidin-3-*O*-galactoside	0.004 ± 0.003	ND	ND	0.014	0.0175 ± 0.0081	ND	0.00466	0.0341
cyanidin-3-*O*-arabinoside	0.0090 ± 0.0057	ND	ND	0.011	0.138 ± 0.073	ND	ND	0.053
peonidin-3-*O*-galactoside	0.000796 ± 0.000547	ND	ND	ND	0.006159 ± 0.002571*	ND	0.00075 6	0.0106

Sum anthocyanins	0.051 ± 0.022	ND	0.014	0.091	0.332 ± 0.136*	0.028	0.134	0.442

Data are mean ± standard error of mean of n = 24 placebo, n = 25 aronia extract with non-missing value.
*P < 0.05 by 2-tailed paired t-test vs 4 wk control group.
**ND: less than LOD and not detectable, assumed to be 0 for calculation of mean

Table 6.7 Fasting plasma polyphenols and metabolites at baseline, 6 weeks, and 12 weeks following daily consumption of 500 mg aronia berry extract or placebo.

	Placebo (mg/mL plasma)			Supplement (mg/mL plasma)		
	Baseline	6 wk	12 wk	Baseline	6 wk	12 wk
Polyphenol/Metabolite	range (number of detections lower than the LOD)					
protocatechuic acid	0.0017-0.0042 (22)	0.0010-0.0016 (22)	0.0015-0.0053 (18)	0.0024 (24)	0.0016-0.0038 (21)	0.0028-0.0091 (22)
3,4-dihydroxyphenylacetic acid	0.0571 (23)	0.0540 (23)	0.0407 (23)	0.0191 (23)	0.0156-0.0335 (23)	- (25)
hippuric acid	0.0968-2.0232 (0)	0.0643-2.2765 (0)	0.0319-2.3277 (0)	0.0362-1.3358 (0)	0.0694-3.0580 (0)	0.0636-1.2587 (0)
ferulic acid	0.0028-0.0190 (8)	0.0025-0.0477 (7)	0.0024-0.0300 (8)	0.0039-0.1368 (13)	0.0035-0.0372 (14)	0.0030-0.0224 (15)
3-(4-hydroxyphenyl)propionic acid	0.1237-0.1806 (20)	0.2107-0.2536 (22)	0.1782-0.2059 (21)	0.1468-0.1581 (23)	0.1347-0.2470 (22)	0.2311 (24)
cyanidin-3-*O*-glucoside	-	-	-	-	-	-

	(24)	(24)	(24)	(25)	(25)	(24)
	-	-	0.0095-0.0153	0.0025-0.0316	0.0002-0.0065	-
cyanidin-3-*O*-galactoside	(24)	(24)	(22)	(22)	(23)	(24)
	-	-	-	-	0.0069	-
cyanidin-3-*O*-arabinoside	(24)	(24)	(24)	(25)	(24)	(24)
	-	0.0001-0.0009	-	0.0005	0.0003	-
peonidin-3-*O*-galactoside	(24)	(21)	(24)	(24)	(24)	(24)
	-	0.0001-0.0009	0.0095-0.0153	0.0005-0.0316	0.0002-0.0009	-
Sum anthocyanins	(24)	(21)	(22)	(21)	(22)	(24)

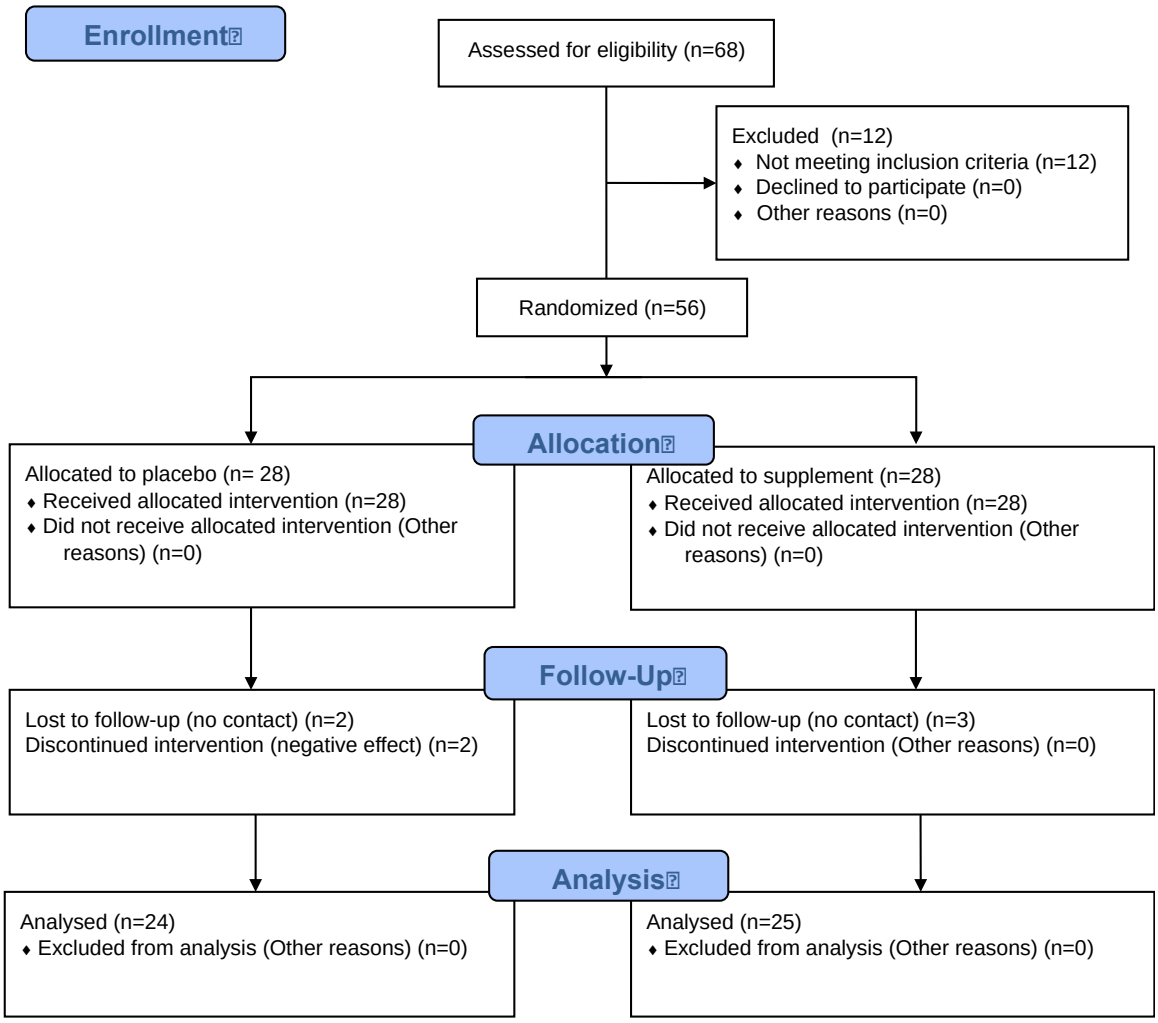
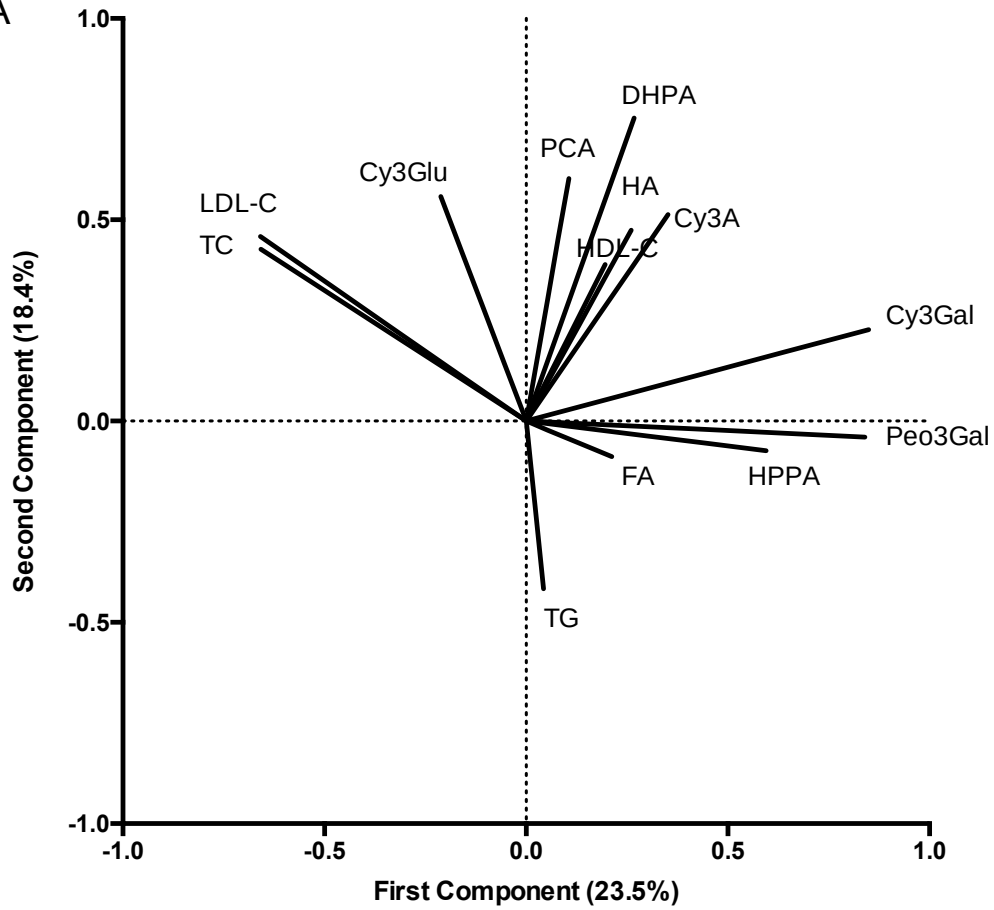


Figure 6.1 Flowchart of enrollment.

A

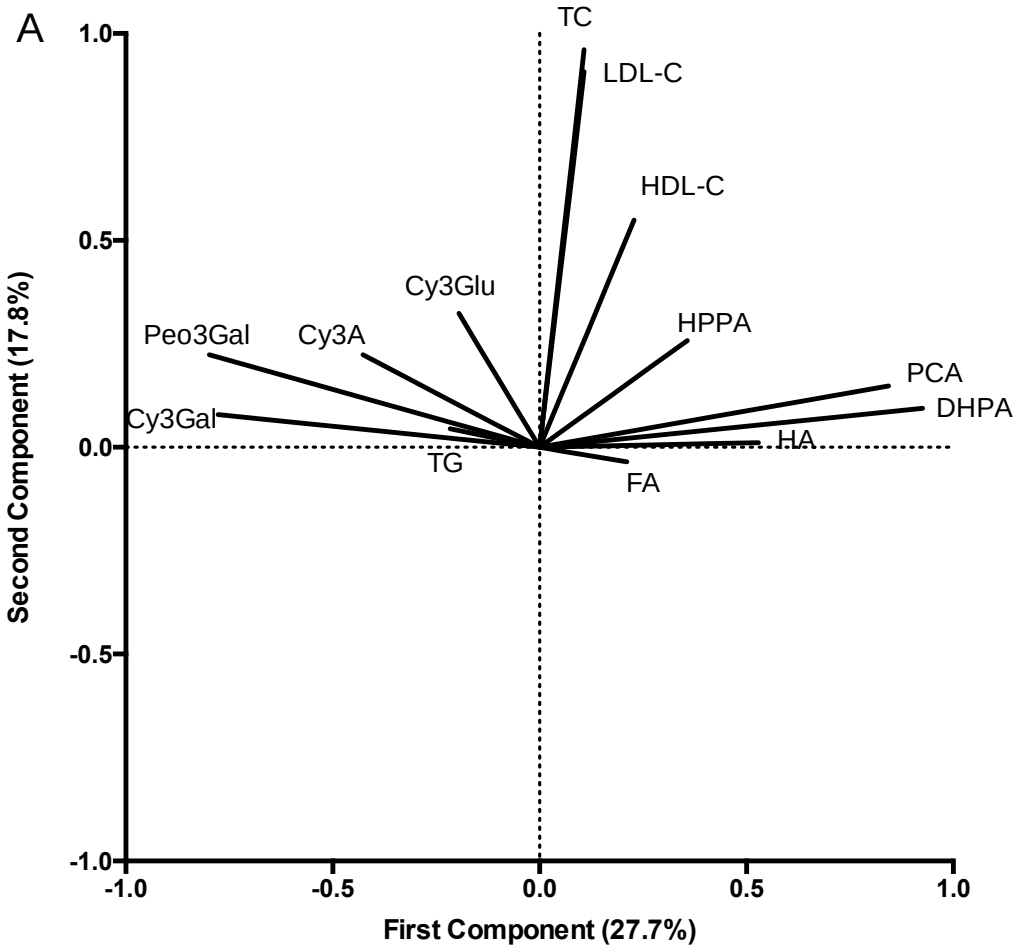


B

	TC	LDL-C	HDL-C	TG
Peo3Gal	-0.3833	-0.3342	-0.0889	-0.1144
Cy3Gal	-0.2763	-0.2400	0.0321	-0.1318
HPPA	-0.1449	-0.1734	0.0025	0.1613
FA	-0.0880	-0.0776	0.1781	-0.1145
DHPA	-0.0366	-0.0753	0.5398	-0.0620
Cy3A	0.0307	0.0711	0.0480	-0.2185
PCA	0.0358	0.0103	0.4043	-0.0753
HA	0.0705	0.0471	0.1371	0.0324
Cy3Glu	0.2750	0.3750	0.0671	-0.5549

Figure 6.2 Principle component analysis correlation of urinary polyphenol and lipid changes A) loading plot and B) correlation coefficients of participants in the aronia extract supplemented group.

A



B

	TC	LDL-C	HDL-C	TG
Cy3Glu	-0.0720	-0.07220	0.24130	-0.3078
FA	-0.0437	-0.03260	0.13000	-0.2231
HA	0.0095	0.09890	-0.06890	-0.3609
Peo3Gal	0.0690	0.09020	-0.10710	0.0477
HPPA	0.0750	0.09650	-0.11380	0.0562
Cy3Gal	0.1416	0.16180	-0.11960	0.0975
Cy3A	0.1652	0.12180	0.15010	0.1131
PCA	0.1668	0.12850	0.34560	-0.1370
DHPA	0.1744	0.16530	0.22280	0.1369

Figure 6.3 Principle component analysis correlation of urinary polyphenol and lipid changes A) loading plot and B) correlation coefficients of participants in the placebo group.

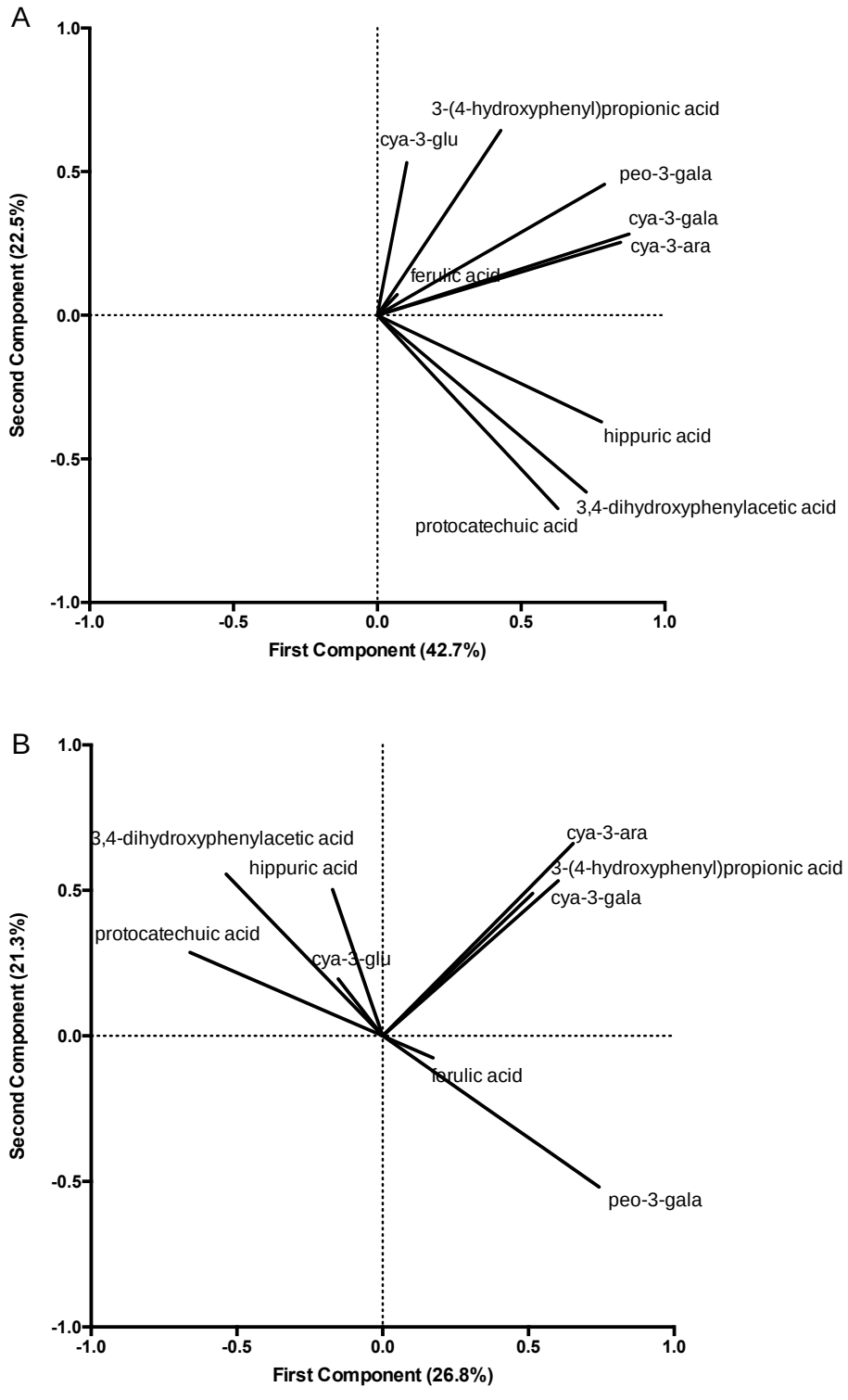


Figure 6.4 Principle component analysis of urinary polyphenol change in aronia supplement group A) responder and B) non-responder.

6.8 Reference

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Chapter 7: Conclusion

The bioefficacy of polyphenols is often assessed by *in vitro* cell models. In these studies, cells are treated with either polyphenol aglycones or polyphenol-rich extracts derived from plants and foods. However, after consumption, human plasma and tissues are not exposed to these forms of polyphenols. **Table 7.1** indicates the potential metabolic fates of polyphenols in humans. Based on human studies, only phenolic acids and flavonoids are present as parent compounds in human fluids in significant quantities after consumption. Polyphenols mainly exist as metabolites in the human body after consumption (Shahrzad and Bitsch 1998, Shahrzad, Aoyagi et al. 2001, Holt, Lazarus et al. 2002, Rechner, Kuhnle et al. 2002, Scalbert, Morand et al. 2002, Setchell, Brown et al. 2002, Olthof, Hollman et al. 2003, Rios, Gonthier et al. 2003, Sano, Yamakoshi et al. 2003, Talavéra, Felgines et al. 2003, Walle, Hsieh et al. 2004, Seeram, Henning et al. 2006, Fernandes, de Freitas et al. 2012, Czank, Cassidy et al. 2013). Thus, the overall hypothesis for this study was that polyphenol metabolites contribute more to bioactivity than their dietary polyphenol parent compounds. The rationale for this hypothesis is that 1) few dietary polyphenols are absorbed intact; 2) for those that are absorbed intact, the rate of first-pass and microbial metabolism leads to a much larger pool of metabolites than parent polyphenols; 3) chronic polyphenol consumption will reduce biomarkers of chronic disease and polyphenol metabolites would be most closely associated with efficacy.

Table 7.1 Metabolic fates of polyphenols (Shahrzad and Bitsch 1998, Shahrzad, Aoyagi et al. 2001, Holt, Lazarus et al. 2002, Setchell, Brown et al. 2002, Bowey, Adlercreutz et al. 2003, Lu,

Meng et al. 2003, Olthof, Hollman et al. 2003, Rios, Gonthier et al. 2003, Sano, Yamakoshi et al. 2003, Walle, Hsieh et al. 2004, Seeram, Henning et al. 2006, Czank, Cassidy et al. 2013).

Classes	Location	Metabolites
Phenolic acids	Small intestine	NE[1]
	Colon	parental compounds, phenolic acids
	Liver	parental compounds, phenolic acids
Flavonoids	Small intestine	parental compounds, flavonoid glucuronide conjugates, flavonoid sulfate conjugates, flavonoid methyl conjugates
	Colon	flavonoid aglycones, phenolic acids
	Liver	parental compounds, flavonoid glucuronide conjugates, flavonoid sulfate conjugates, flavonoid methyl conjugates
Stilbenes	Small intestine	stilbene glucuronide conjugates, stilbene sulfate conjugates
	Colon	stilbene reductive products
	Liver	stilbene glucuronide conjugates, stilbene sulfate conjugates
Lignans	Small intestine	lignan glucuronide conjugates
	Colon	lignan reductive products
	Liver	lignan glucuronide conjugates
Hydrolysable tannins	Small intestine	NE
	Colon	tannic acid, hydrolysable tannin reductive products
	Liver	tannic acid, tannic acid methyl conjugates, tannic acid glucuronide conjugates
Condensed tannins	Small intestine	PAC dimers
	Colon	PAC dimers, phenolic acids
	Liver	NE

[1]NE: No evidence

In order to develop a more accurate assessment of the potential pool of dietary metabolized polyphenols, we characterized tannins and stilbenes in almond (*Prunus dulcis*). Almonds contribute to dietary polyphenol intake, and are ranked as one of the top 40 richest food sources of polyphenols (Perez-Jimenez, Neveu, Vos, & Scalbert, 2010). A prior study identified more almond flavonoids and phenolic acids than reported in prior studies (Bolling, Chen et al. 2011). This study demonstrated the need to more thoroughly characterize specific polyphenol classes in almonds and other foods. Accurate compositional data are needed to understand polyphenol bioactivity and metabolism. Almond phenolic acids and flavonoids have been well-characterized (**Table 7.2**) (Milbury, Chen et al. 2006, Bolling, Dolnikowski et al. 2010). Eighteen flavonoids and two phenolic acids, a total of 25.04 mg/100 mg, were reported, which were catechin, epicatechin, isorhamnetin, isorhamnetin-3-*O*-rutinoside, isorhamnetin-3-*O*-glucoside, isorhamnetin-3-*O*-galactoside, quercetin, quercetin-3-*O*-galactoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, kaempferol, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-galactoside, kaempferol-3-*O*-glucoside, dihydroxykaempferol, eriodictyol, naringenin, naringenin-7-O-glucoside, procatechuic acid, and vanillic acid. These two polyphenol classes would be expected to contribute a minor portion of parent compounds and a major portion of phase II metabolites and microbial catabolites in humans after consumption (**Table 7.1**). Almond also has lignan polyphenols (**Table 7.2**) (Smeds, Eklund et al. 2007). However, lignans are extensively metabolized, and parent compounds are not observed after consumption (**Table 7.1**). Although, proanthocyanidins (PACs) in almond has also been determined (Gu, Kelm et al. 2004), almond PAC content may be underestimated because solvent extraction does not completely liberate polyphenols covalently bound to cell walls (Arranz, Saura-Calixto et al. 2009). Furthermore,

almond stilbenes and hydrolysable tannins have not been determined previously, and may significantly contribute to almond polyphenol content.

The studies undertaken in Chapters 3 and 4 expanded the knowledge of the polyphenol content of almonds. In Chapter 3, bound PACs were recovered from almonds that contributed 3-21% of the total PAC content among three almond varieties. Also, a significant quantity of hydrolysable tannins, ellagitannins (27.4 mg/100 mg almond, ellagic acid equivalents) and gallotannins (54.7 mg/100 mg almond, gallic acid equivalents), were characterized in almond. In Chapter 4, we report the presence of stilbenes (8.33-9.55 µg/100 g almond) in almond. Polydatin (resveratrol-3-glucoside) was the most abundant stilbene in almond, and piceatannol or oxyresveratrol were also tentatively identified in almond. To our knowledge, these were the first reports of both hydrolysable tannins and stilbenes are in almond. Thus, this research has redefined almond polyphenol abundance, with proanthocyanidins being the most abundant class followed by hydrolysable tannins, flavonoids, lignans, phenolic acids, and stilbenes.

Hydrolysable tannins and bound PACs have limited bioavailability and only their microbial catabolites are absorbed. Stilbenes circulate mainly as phase II metabolites. (**Table 7.1**). Only dimers of extractable PACs can be absorbed as their intact form in the small intestine and colon (Holt, Lazarus et al. 2002, Sano, Yamakoshi et al. 2003). Extractable PACs have degree of polymerization more than 2 are metabolized in colon and absorbed as phenolic acids (Rios, Gonthier et al. 2003). The metabolic fate of bound PACs is still unknown, but if liberated in digestion, these would also be subjected to extensive microbial catabolism. Hydrolysable tannins are metabolized by colon to corresponding phenolic acids and colonic metabolites, such as urolithins (Seeram, Henning et al. 2006). Similar, only daughter compounds of stilbenes were found in human plasma (Walle, Hsieh et al. 2004). Thus, PACs, stilbenes and hydrolysable

tannins constitute of the pool of parent polyphenols compounds with limited or no bioavailability.

Chapter 3 and 4 largely support the hypothesis that few almond polyphenols remain unmetabolized after absorption. Although the polyphenol databanks such as USDA databank and Phenol-explore provide comprehensive database on polyphenol contents in food (Neveu, Perez-Jiménez et al. 2010, Bhagwat, Haytowitz et al. 2013), these studies developed a more comprehensive understanding of the almond polyphenol profile. Many nuts, berries, and other polyphenol-rich foods have not been well characterized. Utilizing a similar analytical approach can likely improve the compositional data for other foods.

Next, we hypothesized thatplasma and urinary polyphenol metabolite concentrations would be greater than bioavailable unmetabolized polyphenols in adults that consumed a polyphenol-rich aronia berry extract,. This hypothesis was strongly supported by data presented in Chapter 5. The polyphenol profile in aronia berry supplements is listed in **Table 5.4**, which contained 36.9 mg/500 mg phenolic acid (neochlorogenic acid, chlorogenic acid, protocatechuic acid, and caffeic acid), 9.9 mg/500 mg flavonoids (quercetin, quercetin-3-glucoside, quercetin-3-galactoside, and quercetin-3-rutinoside), 45.07 mg/500 mg anthocyanins (cyanindin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-arabinoside, and cyanidin-3-xyloside), and 41.9 mg/500 mg PACs. Previous studies suggested quercetin aglycone and glycoside consumption mainly generated daughter compounds such as quercetin-3-glucuronide, methyl-quercetin-3-glucuronide and quercetin-3-sulfate in plasma (Day, Mellon et al. 2001, Sesink, O'Leary et al. 2001). Chlorogenic acid ingested by humans is hydrolyzed into caffeic acid and quinic acid (Plumb, Garcia-Conesa et al. 1999). Only 1.7 % of ingested chlorogenic acid was recovered in urine unmetabolized (Olthof, Hollman et al. 2003). Caffeic acid is an intermediate metabolite of many

polyphenols, including chlorogenic acid (**Table 5.1**) (Stalmach, Mullen et al. 2009). Protocatechuic acid is an abundant metabolite of anthocyanins (Vitaglione, Donnarumma et al. 2007). Anthocyanins are extensively metabolized to phenolic acids, phase I metabolites and conjugated anthocyanins, and more than 99% of the bioavailable portion of anthocyanins are metabolites (Czank, Cassidy et al. 2013). However, it is unclear the polyphenol metabolism profile in human consumed polyphenol rich food. Thus, in this study, polyphenol metabolism and pharmacokinetics were determined in adults that consumed an anthocyanin and PAC -rich aronia berry extract.

In Chapter 5, after former smokers consumed a 500 mg dose of aronia berry extract, polyphenol metabolites constituted at least 99% of the total polyphenol pool measured in plasma and urine. This is likely because the aronia extract had abundant condensed tannins and anthocyanins, which are highly metabolized (Sano, Yamakoshi et al. 2003, Czank, Cassidy et al. 2013). This result suggested plasma and urinary polyphenol metabolite concentrations would be greater than bioavailable unmetabolized polyphenols in adults consumed aronia berries extract. The C_{max} of hippuric acid was the highest among the studied compounds, followed by 3-(4-hydroxyphenyl)propionic acid peonidin-3-glucoside, cyanidin-3-glucoside, and protocatechuic acid in the plasma, subjected the concentration of polyphenol metabolites is higher than that of the parent compounds. Studies utilizing isotopically-labelled polyphenols are needed to more specifically track the metabolic fate of polyphenols. Also, this method can be applied to other types of polyphenol rich food.

Our hypothesis for Objective 3 was that chronic consumption of polyphenols would reduce biomarkers of CVD risk and that increases in polyphenol metabolites would be more associated with modulating these biomarkers than unmetabolized polyphenols. This hypothesis was

partially supported by the results of the study described in Chapter 6. The aronia supplement significantly reduced fasting plasma total cholesterol in former smokers after the 12-week treatment, and reduced plasma LDL-C level by 6 weeks of consumption. Peonidin-3-*O*-galactoside, a phase II metabolite of cyanidin-3-*O*-galactoside, was associated with lower plasma total cholesterol and LDL-C in the aronia supplemented group. It is presently unclear why peonidin-3-*O*-galactoside has more bioactivity than cyanidin-3-*O*-galactoside, and further studies are needed to resolve its potential cholesterol-lowering mechanism. Despite changes in polyphenol excretion after supplementation, the increase of total urinary polyphenols from baseline was not significantly different between the placebo and aronia-supplemented groups. This implies that the bioavailability of aronia polyphenols was insufficient to lead to significant polyphenol accumulation beyond the background diet. Also, aronia berry supplement consumption did not change biomarkers of inflammation and oxidative stress, which might be a function of the participant population having lower oxidative stress and more favorable plasma lipid profiles (lower plasma LDL-C, TG, and total cholesterol and higher HDL-C) than participants in other trials where aronia intake improved antioxidant function (Naruszewicz, Łaniewska et al. 2007, Broncel, Koziróg et al. 2010). Future studies should consider examining the specific function of polyphenol metabolites.

Thus, this dissertation supports the central hypothesis that the sum of polyphenol metabolites is more responsible than polyphenol parental compounds for *in vivo* bioactivity. Also, despite efforts to databank polyphenol values, there is still a strong need to utilize analytical chemistry to define the polyphenol composition of foods. The polyphenol contents of foods may be more abundant than previously reported. Large interindividual differences were observed for polyphenol metabolism in Chapter 5, which is informs the new field of personalized

nutrition, as individuals have apparently unique polyphenol profiles after consuming aronia berry. Colonic polyphenol metabolism and peonidin-3-galactoside were related to cholesterol-lowering activity of aronia berry polyphenols in former smokers. Although we cannot suggest a causal relationship for these compounds, studies of these compounds should be pursued to identify their intrinsic cholesterol-lowering activities and mechanisms.

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Appendix

A.1 Appendix Materials for Chapter 3

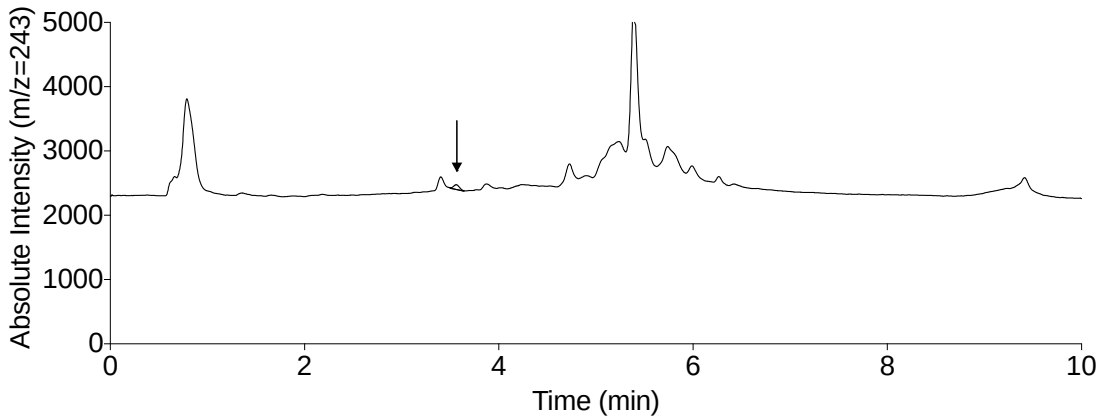

Figure A.4.1 Chromatogram of blanch water

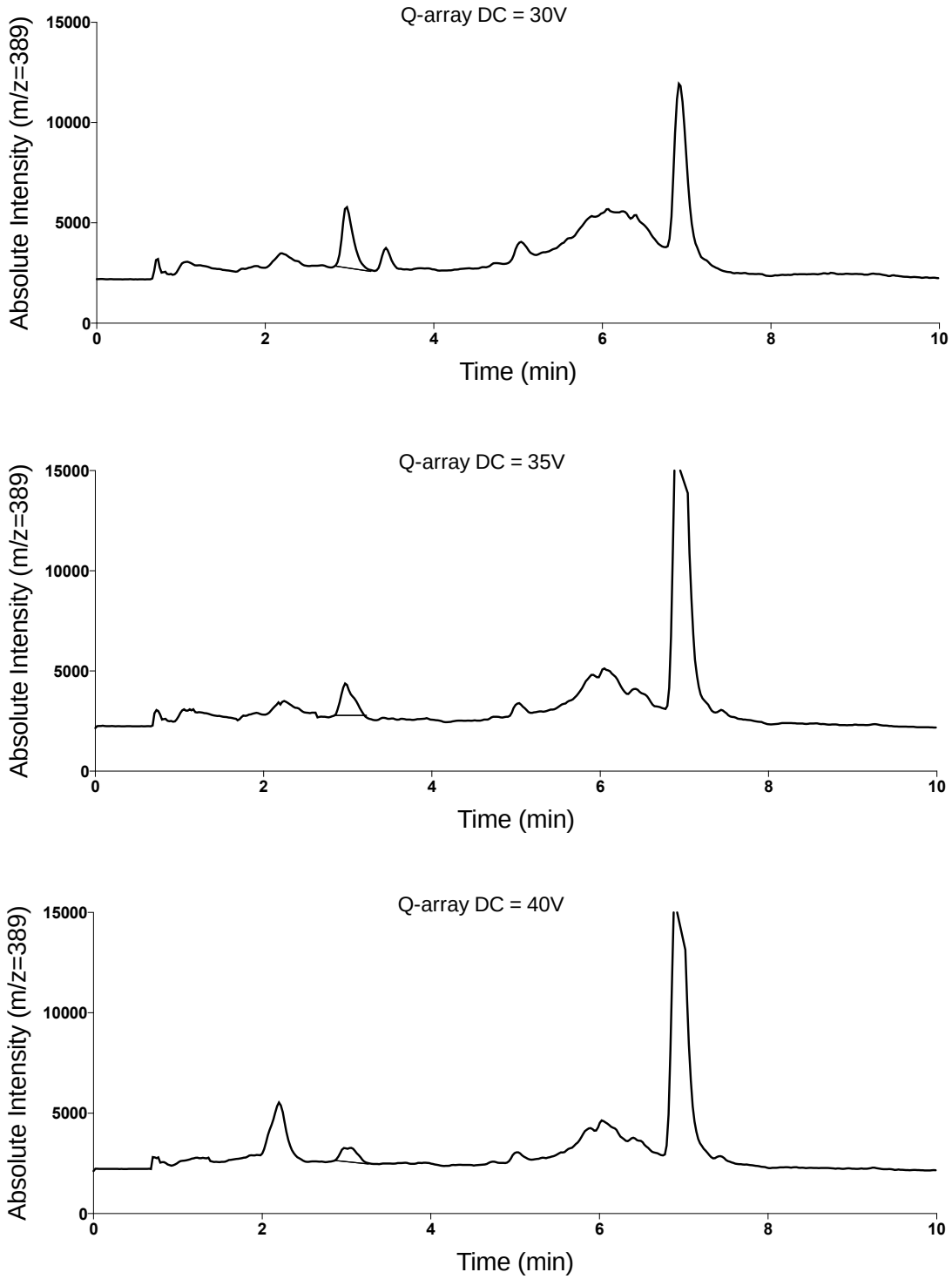

Figure A.4.3 Chromatogram of almond sample under different condition

A.2 Appendix Materials for Chapter 6

Table A.6.1 Baseline characteristics of nutrients intake

Nutrient	Placebo	Aronia	P-Value
Macronutrients			
Energy (kcal/d)	2225.6 ± 734.8	1952.7 ± 525.8	0.146
Carbohydrate (g/d)	237.9 ± 9.1	241.1 ± 8.5	0.791
Protein (g/d)	89.2 ± 3.3	90.3 ± 3.1	0.812
Fat (g/d)	87.8 ± 4.0	77.6 ± 29.5	0.553
Fiber (g/d)	23.5 ± 1.6	21.0 ± 1.5	0.282
Micronutrients			
Sodium (mg/d)	3490.3 ± 156.1	3479.7 ± 146.2	0.961
Calcium (mg/d)	946.5 ± 58.2	1066.8 ± 53.2	0.139
Iron (mg/d)	16.9 ± 1.3	16.9 ± 1.2	0.974
Vitamin D (mg/d)	5.0 ± 0.9	7.1 ± 0.8	0.109
Vitamin E (α-tocopherol mg/d)	10.6 ± 1.1	10.8 ± 1.1	0.904
Vitamin C	80.9 ± 12.9	90.9 ± 12.1	0.579
Vitamin A (retinol equiv mcg/d)	3278.0 ± 738.0	2688.5 ± 691.3	0.566
Polyhenols			
Isoflavones (mg/d)	3.86 ± 2.7	8.5 ± 2.6	0.233
Flavanols (mg/d)	73.9 ± 20.0	81.7 ± 18.7	0.779
Flavones (mg/d)	54.1 ± 29.1	50.3 ± 27.2	0.924
Flavanones (mg/d)	28.8 ± 11.4	22.4 ± 10.7	0.686

Flavan-3ols (mg/d)	20.6 ± 7.4	26.0 ± 6.9	0.601
Anthocyanins (mg/d)	18.2 ± 5.2	26.6 ± 4.8	0.247
Proanthocyanins (mg/d)	221.8 ± 162.4	417.9 ± 152.1	0.388

Data are mean ± standard error of mean of n = 24 placebo, n = 25 aronia extract with non-missing value.

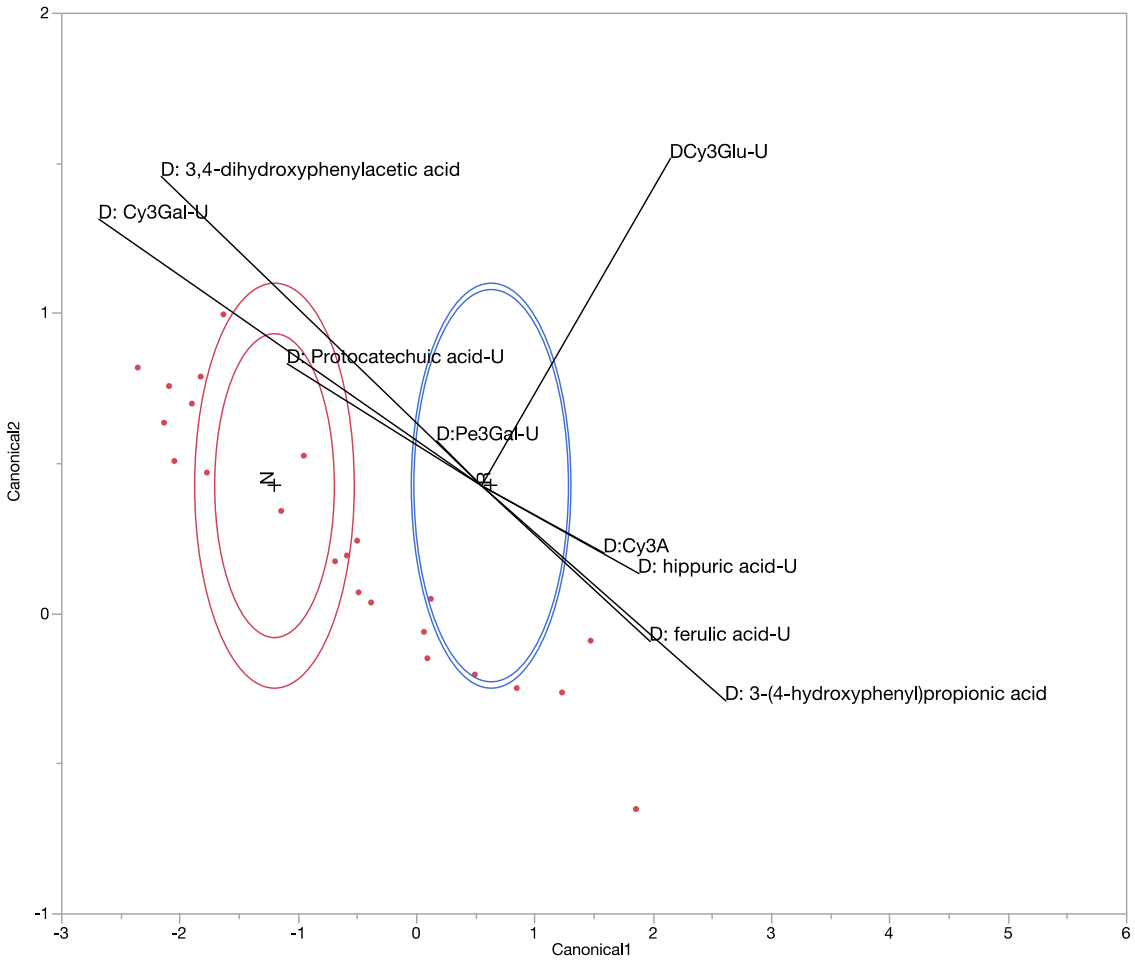

Figure A.6.1 canonical discriminate analysis based on respondent