Raspberries and Human Health: A Clinical Perspective on the Bioavailability and Bioactivity of Red Raspberry Antioxidants

by

Dawn Snyder

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Nutritional Sciences
University of Toronto

© Copyright by Dawn Snyder Fall 2010
Red raspberries, as an excellent source of dietary antioxidants, were investigated for their effect on oxidative stress in healthy adults. Study 1 measured effects of chronic exposure in a parallel, multi-dose intervention. Subjects consumed one-cup red raspberries (1cR) daily for two-weeks, then were randomized to consume 1cR, 2cR or 4cR for additional two-weeks (n=8, by group). There was a reduction in TBARS, indicating a decrease in lipid peroxidation, after two-weeks of intervention in the 1cR group, but effects were not significant at week 4, or for other treatment groups. Study 2 measured effects of acute exposure using a cross-over design. Subjects (n=8) consumed single treatments of 1cR, 2cR, 4cR, bread and bread plus vitamin C. Post-prandial oxidative stress responses were complex and appeared related to calorie and antioxidant load. Overall there was no clear relationship between red raspberry consumption and protection against oxidative stress.
Acknowledgments

I would like to acknowledge:

The support, guidance, knowledge and patience provided first and foremost by my supervisor, Dr. A. Venket Rao, and my advisory committee, Dr. David J. A. Jenkins and Dr. Mary Keith.

The financial contribution from Washington Red Raspberry Commission, BC Raspberry Growers Association, Investment Agriculture Foundation of British Columbia, and Canadian Institute of Health Research (CIHR), Ontario Graduate Scholarship, Master’s Award 2009-2010.

The clinic facilities provided by Dr. David J. A. Jenkins at the Clinical Nutrition and Risk Factor Modification Centre, St. Michael’s Hospital, Toronto, Ontario

The time and work contributions of the following undergraduate students: Aksa Ahmed in the laboratory for analyzing TEAC in study 1 samples; Amy Jenkins for inputting and analyzing food records; and Ye Seul Song in the clinic for assisting to organize and run study 2 clinic visits.

The laboratory assistance for HPLC analyses provided by Dr. Rong Cao and Ronghua Liu, and for MS analyses by Honghui Zhu, all at the Guelph Food Research Centre, Agriculture and Agri-Food Canada, Guelph, Ontario

The donation of IQF red raspberries, by BC Raspberry Growers Association, and of ascorbic acid powder, by Jamieson Laboratories Ltd., Windsor, Ontario

The freezer storage space, provided by Gelda Scientific and Industrial Development Corporation, Mississauga, Ontario
# Table of Contents

## Ch. 1: INTRODUCTION

1 General Introduction 2

## Ch. 2: LITERATURE REVIEW

2 Red Raspberry Composition 6
  2.1 Introduction 2.2 Nutritional Profile 2.3 Antioxidant Capacity 2.4 Polyphenol Profile 2.4.1 Classifications by chemical structure 2.4.2 Content in the Diet 2.4.3 Content in Red Raspberries 2.4.4 Ellagitannins 2.4.5 Anthocyanins 2.4.6 Maturity 2.4.7 Storage and Processing 2.4.8 Genetic and Environmental

3 Bioavailability of Major Red Raspberry Polyphenols 21
  3.1 Introduction 3.2 Ellagitannins 3.3 Anthocyanins

4 Bioactivity of Major Red Raspberry Polyphenols 26
  4.1 Introduction 4.2 Cancer 4.3 Cardiovascular Disease (CVD) 4.4 Diabetes 4.5 Obesity 4.6 Macular and Neuro Degeneration

## Ch. 3: HYPOTHESES, OBJECTIVES, STUDY DESIGN AND RATIONAL

5 Overall for Thesis 34
  5.1 Hypothesis 5.2 Objectives

6 Study 1 35
6.1 Hypotheses
6.2 Objectives

7 Study 2
7.1 Hypotheses
7.2 Objectives

8 Rational

Ch. 4: METHODOLOGY

9 Study 1 Clinical Protocol
9.1 Study Design
9.2 Ethics Approval, Recruitment and Study Location
9.3 Screening, Exclusion Criteria and Enrolment
9.4 Standard Clinic Visit for Participants
9.5 Raspberry Supplement
9.6 Questionnaires
9.7 Body Measurements
9.8 Dietary Restrictions, Food Records and Assessment
9.9 Blood Collection, Processing and Storage
9.10 Urine Collection, Processing and Storage

10 Study 2 Clinical Protocol
10.1 Study Design
10.2 Ethics Approval, Recruitment and Study Locations
10.3 Screening, Exclusion Criteria and Enrolment
10.4 Standard Clinic Visit for Participants
10.5 Raspberry and Control Treatments
10.6 Dietary Restrictions and Food Records
10.7 Blood Collection, Processing and Storage
10.8 Urine Collection, Processing and Storage

11 Laboratory Procedures for Biomarkers
11.1 Clinical Biomarkers for Chronic Disease Risk
11.2 Oxidative Stress Biomarkers
  11.2.1 Antioxidant Capacity (AOC)
  11.2.2 Protein Oxidation
  11.2.3 Lipid Peroxidation
11.3 Nutrition Biomarkers
  11.3.1 Ascorbic acid Extraction and Quantification by HPLC-DAD
  11.3.2 Polyphenol Extraction
  11.3.3 Polyphenol Quantification by U-HPLC
11.4 Red Raspberry Compositional Analyses
   11.4.1 Ascorbic acid
   11.4.2 Anthocyanins

12 Statistical Analysis
   12.1 Study 1
   12.2 Study 2

Ch. 5: RESULTS
   13 Study 1
      13.1 Baseline Participant Characteristics
      13.2 Oxidative Stress Biomarkers
      13.3 Bioavailability Biomarker
      13.4 Correlation of Bioavailability and Bioactivity Biomarkers
      13.5 Clinical Biomarkers of Chronic Disease Risk
      13.6 Participant Perceptions
   14 Study 2
      14.1 Baseline Participant Characteristics
      14.2 Oxidative Stress Biomarkers
      14.3 Bioavailability of plasma Ascorbic acid
      14.4 Bioavailability of Anthocyanins
      14.5 Correlation of Bioavailability and Bioactivity Biomarkers
      14.6 Red Raspberry Composition

Ch. 6: DISCUSSION
   15 Discussion
      15.1 Study 1
      15.2 Study 2
      15.3 Overall
   16 Future Directions

Ch. 7: CONCLUSION
   17 General Conclusion

References
List of Tables

Table 1. Nutrient composition of fresh, red raspberries. 18
Table 2. Polyphenolic composition of fresh, red raspberries. 19
Table 3. Nutrition information for the bread used as control treatments from study 2. 51
Table 4. Nutrition information for red raspberry treatments from study 2. 52
Table 5. Nutrition Information for the high-fat standard lunch from study 2, served after 4 h post-treatment. 53
Table 6. Study 1 baseline characteristics of study participants overall (n=24) and by group (n=8). 69
Table 7. Mean±SEM delta TEAC, thiols, TBARS and t12UM from week 4 compared to week 2, stratified by treatment group (n=8). 75
Table 8. Participant perceptions on taste, satiety and symptoms overall (n=24) after week 2, and by group (n=8) after week 4. 79
Table 9. Study 2 baseline characteristics of study participants (n=8). 85
Table 10. Mean±SEM net post-prandial AUC for TEAC, thiols, TBARS, ascorbic acid, and 0-8 and 8-24 h tUM. 89
Table 11. Mean±SEM delta net AUC compared to B and 1cR, for TEAC, thiols, TBARS, ascorbic acid and t24UM post-treatment (n=8). 90
Table 12. Summary of properties of compounds detected in extracts of red raspberries from study 2, following analysis by LC/MS detection. Peak numbers and retention times refer to numbers given in Figure 17. 97
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Pg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.</td>
<td>The chemical structure of major anthocyanidins</td>
<td>20</td>
</tr>
<tr>
<td>Figure 2.</td>
<td>Study 1 Design.</td>
<td>45</td>
</tr>
<tr>
<td>Figure 3.</td>
<td>Study 2 Clinic Visit Design.</td>
<td>50</td>
</tr>
<tr>
<td>Figure 4.</td>
<td>Mean±SEM serum TEAC, thiols and TBARS, at weeks 0, 2 and 8, pooled data (n=24).</td>
<td>70</td>
</tr>
<tr>
<td>Figure 5.</td>
<td>Mean±SEM serum TEAC, at weeks 0, 2, 4 and 8, stratified by treatment group (n=8).</td>
<td>71</td>
</tr>
<tr>
<td>Figure 6.</td>
<td>Mean±SEM serum TEAC for (a) pooled data (n=24), and (b) stratified by treatment group (n=8), at weeks 0, 2, 4 and 8.</td>
<td>72</td>
</tr>
<tr>
<td>Figure 7.</td>
<td>Mean±SEM serum thiols, at weeks 0, 2, 4 and 8, stratified by treatment group (n=8).</td>
<td>73</td>
</tr>
<tr>
<td>Figure 8.</td>
<td>Mean±SEM serum TBARS, at weeks 0, 2, 4 and 8, stratified by treatment group (n=8).</td>
<td>74</td>
</tr>
<tr>
<td>Figure 9.</td>
<td>t12UM stratified by treatment group (n=8), as (a) mean±SEM, at weeks 2 and 4, and (b) individual delta values at week 4 compared to week 2.</td>
<td>76</td>
</tr>
<tr>
<td>Figure 10.</td>
<td>Correlation between t12UM and serum (a) TEAC, (b) thiols and (c) TBARS, at weeks 2 and 4 (n=26, excluded 2 outliers).</td>
<td>77</td>
</tr>
<tr>
<td>Figure 11.</td>
<td>Mean±SEM fasting plasma glucose, at weeks 0, 2, 4 and 8, stratified by treatment group (n=8).</td>
<td>78</td>
</tr>
<tr>
<td>Figure 12.</td>
<td>Plasma TEAC, over 2 h post-treatment, expressed as (a) mean delta values compared to baseline and (b) individual delta net AUC values compared to bread treatment (n=8).</td>
<td>86</td>
</tr>
<tr>
<td>Figure 13.</td>
<td>Plasma thiols, over 2 h post-treatment, expressed as (a) mean delta values compared to baseline, and (b) individual delta net AUC values compared to bread treatment (n=8).</td>
<td>87</td>
</tr>
<tr>
<td>Figure 14.</td>
<td>Plasma TBARS, over 4 h post-treatment, expressed as (a) mean delta values compared to baseline and (b) individual delta net AUC values compared to bread treatment (n=8).</td>
<td>88</td>
</tr>
<tr>
<td>Figure 15.</td>
<td>Plasma ascorbic acid, over 4 h post-treatment, expressed</td>
<td>91</td>
</tr>
</tbody>
</table>
as (a) mean delta values compared to baseline, and (b) individual delta net AUC values compared to bread treatment (n=8).

Figure 16. Correlation between plasma ascorbic acid and plasma (a) TEAC, (b) thiols and (c) TBARS, across all time-points (b) and treatments (n=320).

Figure 17. t24UM post-raspberry treatments expressed as (a) mean ±SEM, and (b) individual delta values compared to 1cR (n=8).

Figure 18. Correlation between t24UM and average plasma (a) AOC, (b) thiols and (c) TBARS post-raspberry treatments (n=23).

Figure 19. U-HPLC chromatograms detected by PDA at 520 nm for anthocyanin analysis of one participant’s: (a) plasma at baseline after an overnight fast, (b) plasma pooled over 8 h following consumption of two cups red raspberries, (c) urine at baseline after an overnight fast, (d) urine pooled over 8 h following consumption of two cups red raspberries.

Figure 20. U-HPLC chromatogram of anthocyanins and anthocyanidins detected by PDA at 520 nm in an acidified methanol extract of the red raspberries from study 2.

Figure 21. Mass spectra of anthocyanins detected in red raspberries from study 2; (I) delphinidin glucoside, (II) cyanidin glucoside, (III) delphinidin arabinoside, (IV) petunidin glucoside, (V) cyanidin arabinoside, (VI) peonidin glucoside, (VII) petunidin arabinoside, (VIII) malvidin glucoside, (IX) peonidin arabinoside, (X) malvidin arabinoside.
## List of Appendices

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Appendix 1.</strong></td>
<td>Participant forms from study 1.</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>Form A – Selection, inclusion and exclusion criteria</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Form B – Consent to participate in a research study</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Form C – Study design and Participant’s Schedule</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Form D – Foods to Avoid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Form E – Instructions and food record sheet</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Form F – Instructions for raspberry consumption</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Questionnaire: Demographic, health and lifestyle</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Questionnaire: Raspberry consumption (wk 4 sample)</td>
<td></td>
</tr>
<tr>
<td><strong>Appendix 2.</strong></td>
<td>Participant forms from study 2.</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>Consent to participate in a research study</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dietary guidelines</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Study schedule</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Questionnaire: Demographic, health and lifestyle</td>
<td></td>
</tr>
<tr>
<td><strong>Appendix 3.</strong></td>
<td>Health biomarkers and chronic disease risk.</td>
<td>165</td>
</tr>
</tbody>
</table>
Abbreviations

1cR, one cup red raspberries treatment;
1cRg, one cup red raspberry treatment group;
2cR, two cups red raspberries treatment;
2cRg, two cup red raspberry treatment group;
4cR, four cups red raspberries treatment;
4cRg, four cup red raspberry treatment group;
8-iso-PGF2, 8-iso prostaglandin F2;
8-OHdG, 8-hydroxy-2′-deoxyguanosine;
ABTS(•+), 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) (radical cation);
ANOVA, analysis of variance;
AOC, antioxidant capacity;
AUC, area under the curve;
B, bread treatment;
B+vC, bread plus vitamin C treatment;
BHT, butlyated hydroxyl toluene;
BMI, body mass index
CE, cyanidin equivalents;
Cmax, maximum concentration;
COX-2, cyclooxygenase 2;
CRP, c-reactive protein;
cv., cultivar;
CV, coefficient of variation;
CVD, cardiovascular disease;
d, day;
DAD, diode array detector;
ddH20, double distilled water;
DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid);
DTPA, diethylenetriaminepentaacetic acid;
EA, ellagic acid;
EDTA, ethylenediaminetetraacetic acid;
ESD, extreme studentised deviate;
ESI, electrospray ionization;
FRAP, fluorescence recovery after photobleaching;
fw, fresh weight;
GAE, gallic acid equivalents;
GLUT4, glucose transporter 4;
GSHPx, glutathione peroxidase;
GST, glutathione S-transferase;
H2O2, hydrogen peroxide;
HbA1c, glycated haemoglobin;
HCl, hydrochloric acid;
HDL-C, HDL cholesterol;
HHDP, hexahydroxydiphenoyl;
HO-1, heme-oxygenase 1;
(U-)HPLC, (ultra) high pressure liquid chromatography;
ICAM-1, intracellular adhesion molecule 1
IL, interleukin;
IQF, individually quick frozen;
K2, dipotassium;
LC/MS, liquid chromatography/mass spectrometry;
LDL-C, LDL cholesterol;
MAPK, mitogen-activated protein kinase;
MCP-1, monocyte chemoattractant protein 1;
MDA, malondialdehyde;
MMP, matrix metalloproteinase;
NF-κB, nuclear factor-κB;
NHANES, National Health and Nutrition Examination Survey;
NOS, nitric oxide synthase;
OD, optical density;
ORAC, oxygen radical absorbance capacity;
ox-LDL, oxidized-LDL;
PDA, Photo-Diode Array;
RBP4, retinol binding protein 4;
REB, research ethics board;
RM, repeated measures;
ROS, reactive oxygen species;
RP, reverse-phase;
SEM, standard error of mean;
SOD, superoxide dismutase;
SPE, solid phase extraction;
TBA(RS), thiobarbituric acid (reactive substances);
TC, total cholesterol;
tE, time effect;
TE, treatment effect;
TEAC, trolox equivalent antioxidant capacity;
TG, triglycerides;
TNF-α, tumor necrosis factor-α;
USDA, United States Department of Agriculture;
UV, ultraviolet;
VEGF, vascular endothelial growth factor;
WHO, World Health Organisation;
yr(s), year(s);
Chapter 1

Introduction
1 General Introduction

Chronic disease morbidity and mortality are major public health problems around the globe. Cardiovascular disease (CVD) and cancer are the leading causes of death in the United States [1]. Globally, CVD accounts for 10.3% of the burden of disease and 30.9% of mortality [2]. Rising rates of obesity and type 2 diabetes mellitus are associated with CVD incidence [3]. The combination of symptoms that increase risk of developing CVD is termed metabolic syndrome [4]. They include abdominal obesity, insulin resistance, hyperglycemia, hyperlipidemia; all conditions that can be treated with diet and exercise [5]. In a world of excess, diet research has shifted away from a concern with adequacy and focused on the identification of an optimal diet for general health, longevity and reduced risk of chronic diseases [6].

Dietary pattern assessment often identifies at least two polarized diet types, which have been termed the “prudent” pattern and the “Western” pattern [7]. The first is typically characterized by higher intakes of plant-based foods, fish and poultry, resulting in high intakes of carbohydrates, especially dietary fibre, and low intakes of total and saturated fats. The second is characterized by higher intakes of meat and processed foods high in sugars and fat, and low in dietary fibre. Prospective studies have found that higher prudent pattern scores were associated with decreased risk of developing chronic diseases such as coronary heart disease [8] and type II diabetes mellitus [9], while higher Western pattern scores were associated with increased risk.

A major difference between the prudent and Western dietary patterns is intakes of fruit and vegetables. This food group’s consumption has been associated with decreased incidence of and mortality from CVD and certain types of cancer [1, 10-15]. It has been estimated that 4.4% of overall disease burden in Europe is due to low fruit and vegetable intake [16]. Increasing consumption to 400 g/day could reduce cancer incidence in the Netherlands by an estimated 19% and CVD deaths by 16% [17]. Changing the dietary habits of a population has the potential for a large impact on the population’s health, and can be achieved with the transmission of knowledge [18]. This is why most national and international health agencies have issued recommendations for the increased consumption of fruits and vegetables, often based on the World Health Organisation’s (WHO) recommendation for a minimum intake of 400 g fruits and vegetables per day (excluding starchy tubers such as potatoes) for the prevention of chronic diseases [19].
The health benefit of fruit and vegetable consumption was previously attributed to their fibre and micronutrient content. Recent scientific interest in the role of phytochemicals, which are nonnutritive bioactive plant compounds, on chronic disease pathology, has led to the postulation that phytochemicals may be required for health. One of the major phytochemical classes present in fruits are the water-soluble polyphenols, whose phenol groups enable their function as antioxidants [20]. Flavonoids, the largest and most ubiquitous subclass of polyphenols, have been associated with reduced risk of chronic diseases [21-26]. The consumption of dietary antioxidants, including polyphenols, is thought to help protect against the damage caused by reactive oxygen species (ROS) to important cellular components, including lipids, proteins and DNA [27, 28]. According to the oxidative stress hypothesis, the accumulation of such damage leads to aging and the development of chronic diseases [29].

Consumption of antioxidant-rich fruit and vegetables has been associated with lower levels of oxidative damage to LDL-cholesterol and DNA, important in CVD and cancer risk, respectively [30]. Therefore, the total antioxidant capacity (AOC) of a food may be an indication of its healthfulness [31]. Several research groups have published lists of AOC values for numerous foods using different methodologies [32-41]. Berry fruits are consistently ranked among the top sources of total phenolics and AOC, containing levels up to 4 times greater than other fruits, 10 times greater than vegetables and 40 times greater than cereals [34]. Clinical studies have demonstrated the ability of diet to alter oxidative stress and antioxidant status in the blood [42-44]. However, interventions with foodstuffs containing high in vitro AOC have not always translated into effects on in vivo AOC [45, 46], which may be due to differences in the bioavailability and antioxidant activity of phytochemicals [47].

The antioxidant capacity of phytochemicals is only one of many proposed mechanisms for the health benefit of phytochemical consumption [48]. Additional evidence from in vitro and in vivo studies has demonstrated anti-inflammatory, anti-atherosclerotic and anti-tumor capabilities [49-51]. Different plant foods contain different phytochemical compositions which may translate to different protective effects. The use of therapeutic diets as an alternative to drug therapy in the prevention and treatment of chronic disease is being investigated [52]. However, sufficient human evidence is required to prove the health benefits associated with consumption of specific foods [53].
The role of red raspberries on human health is of scientific interest because (1) they are readily available and widely consumed across Western diets; (2) have a high in vitro AOC, ranked within the top ten for commonly consumed foods [34, 36]; (3) have a unique phytochemical composition, containing high levels of anthocyanins and the less ubiquitous ellagitannin polyphenols [54-57]; and (4) are not well researched compared to other common berry fruits, including blueberries, strawberries, cranberries and black raspberries. To determine if red raspberry polyphenols are bioavailable and have antioxidant bioactivity in vivo, chronic (study 1) and acute (study 2) interventions were carried out to measure effects of raspberry consumption on oxidative stress status in a healthy adult population, as well to investigate the bioavailability and metabolism of red raspberry polyphenols.
Chapter 2

Literature Review
2  Red Raspberry Composition

2.1  Introduction

Red raspberries are a healthy food choice, low in calories, and high in fibre and nutrients. They have an appealing colour and flavor, making them not only a nutritious, but delicious snack alternative to processed foods. However, it is their high content of non-nutrient, phytochemicals which may confer additional health benefits. To investigate their potential health promoting properties, it is necessary to identify the compounds present in red raspberries. The composition of red raspberries can vary genetically by cultivar, and environmentally by growing and storage conditions, as well as by processing. To make comparisons between studies more difficult, differences in analytical methods and extraction techniques contribute variation to measurements. This section will identify the nutritional and phytochemical profiles of red raspberries, as well as review the variability within the literature and the contribution of genetic and environmental factors.

2.2  Nutritional Profile

Red raspberries are nutrient dense, containing a high ratio of nutrients to calories. Their nutrient content, as seen in Table 1, is based on a 100 g serving, and provides only 52 kcal [58]. However, their high dietary fibre content (6.5 g/100 g) may have a satiating effect. They are low in fat but a source of healthy essential fat. Raspberry seed oil is composed of 97.8% unsaturated fatty acids and has a ratio of 1.64 n-6/n-3 fatty acids [59]. Fat-soluble vitamins, including carotenoids and tocopherols, are also present in the seeds, whereas high levels of the water-soluble vitamin C are present in the flesh, at 26.2 mg/100 g fresh weight (fw). A diet low in saturated fats, simple sugars and sodium, but rich in healthy fats, dietary fibre, potassium, and other minerals, vitamins, and antioxidant phytochemicals, defines a well-balanced diet, to which red raspberries are a healthy addition.

2.3  Antioxidant Capacity

Red raspberries have a high antioxidant capacity (AOC). According to the USDA Database for the Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods, Release 2 (2010) [60], red raspberries have a hydrophilic-ORAC of 4927 μmol TE/100 g fw and a lipophilic-ORAC of 138
\( \mu \text{mol TE/100 g fw} \), reflective of a high content of water-soluble antioxidants. Overall their total-ORAC is 5065 \( \mu \text{mol TE/100 g fw} \) and their total phenolic content is 414 mg GAE/100 g fw. If you compare these values to those for other foods, such as strawberries (4302, 332), broccoli (1510, 316), apples with skin (3049, 250), tomatoes (387, 80), and even blueberries (4669, 311), red raspberries have more antioxidants and more polyphenols. This is in accordance with a review paper which summarized the results of numerous studies, and reported red raspberries to have a higher AOC than strawberries, kiwi, broccoli, leeks, apple and tomato when measured using a variety of methods, including ORAC, TEAC and FRAP [56].

To identify which compounds contribute the most to AOC, high performance liquid chromatography (HPLC), coupled to an online post-column antioxidant detection system was used to separate and analyse red raspberry extracts. It was found that ellagitannin and anthocyanin polyphenols contribute 50 and 25\% of the total AOC, respectively; whereas the antioxidant nutrient, vitamin C contributes 20\% [57].

The AOC value and its contributors vary by red raspberry cultivar. Across 17 raspberry cultivars grown in Finland, the total phenolic content, measured similarly to AOC using a radical quenching spectrophotometric method, was significantly correlated with ellagic acid content \((r = 0.98)\), the hydrolysis product of ellagitannin polyphenols, but not total anthocyanins \((r = -0.46)\) [55]. The opposite associations were found when four Spanish red raspberry cultivars were analysed for their antiradical efficiency, a measure of AOC. Correlations were significant for total anthocyanin contents \((r = 0.85)\), but not ellagic acid, nor vitamin C \((r = 0.41 \text{ and } 0.42, \text{ respectively})\) [61].

Overall, AOC is largely determined by, and therefore indicative of, the antioxidant polyphenol content. Therefore, AOC may be useful as a general indicator of one aspect of the healthfulness of a food. However, AOC values do not reveal any information on the specific antioxidants present, which requires more complex detection techniques. In conclusion, the most important antioxidants in red raspberries are the ellagitannins, anthocyanins and vitamin C.
2.4 Polyphenol Profile

2.4.1 Classifications by chemical structure

Polyphenols are a large water-soluble class of phytochemicals. They are plant products of secondary metabolism and serve many diverse biological functions, including roles in plant growth, development, and defense [20]. Several thousands of natural polyphenols have been identified in higher level plants, with several hundred found in edible plants [62].

Polyphenolic structures are characterized by the presence of one or more six-carbon aromatic rings bearing one or more hydroxyl groups. Their skeletal structure is variable and can range from simple phenolic acids, to highly polymerized tannins. They occur primarily in conjugated form, with one or more sugar residues linked to the hydroxyl groups. The associated sugars can be present as monosaccharides, disaccharides, or sometimes even oligosaccharides. Glucose is the most common sugar residue, although galactose, rhamnose, xylose, arabinose and glucuronic acid are also common. Structural diversity is further observed by the existence of stereoisomers, variations in substitution patterns, acylation by organic and phenolic acids, and by conjugation with other phenolics [63].

Polyphenols can be divided into at least 10 different classes [20]. However, the five main classes found in plant foods consist of the phenolic acids, flavonoids, stilbenes, lignans and tannins, which are grouped based on the number of phenolic rings and the structural elements that link those rings [64]. Phenolic acids are further subdivided by derivation from either hydroxybenzoic acid, or from hydroxycinnamic acid. Flavonoids all share a common structure consisting of 2 aromatic rings (A and B), that are bound together by 3 carbon atoms that form an oxygenated heterocyclic ring (C). The degree of oxidation of the oxygen heterocycle determines the subclass, which consists of flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols [65]. Stilbenes are structurally characterized by the presence of a 1, 2-diphenylethylene nucleus with hydroxyls substituted on the aromatic rings, and exist in both monomer and oligomer form. Lignans are formed of 2 phenylpropane units and metabolized by intestinal microflora into enterodiol and enterolactone, functionally known as phytoestrogens. Tannins are polymers of phenolic units, often complexed with alkaloids, polysaccharides and proteins, and with molecular weights ranging from 500 to 3,000 Da. This group consists of condensed and hydrolysable tannins. Condensed tannins are also known as proanthocyanidins,
and composed of flavan-3-ol monomers. Whereas hydrolysable tannins are further subdivided into gallotannins and ellagitannins depending on whether the compound is composed of gallic acid monomers or its dimeric condensation product, hexahydroxydiphenic acid, respectively.

2.4.2 Content in the Diet

Different plant foods have different phytochemical profiles. Content and availability are two factors which largely influence the potential relevance of a compound in the diet. However, there are major limitations in the quantification of dietary exposure to phytochemicals: 1) variability within food products, 2) variability in analytical methods, 3) lack of available databases, and 4) error in diet assessment.

Food databases have been created for some common classes of polyphenols, including the flavonoids [66]. This database was used to estimate the US population’s mean daily total flavonoid intake from NHANES dietary recall data. The American flavonoid intake was estimated at 189.7 mg/d, mainly from flavan-3-ols (83.5%), followed by flavanones (7.6%), flavonols (6.8%), anthocyanidins (1.6%), flavones (0.8%) and isoflavones (0.6%) [67]. The greatest contributing foods were tea (157 mg), citrus fruit juices (8 mg), wine (4 mg) and citrus fruits (3 mg) [67]. In contrast, flavonoid intake in Finland was lower, 80 mg/d, but anthocyanidins contributed almost 60% of the total [68]. The compositional difference in flavonoid intake is due to the high consumption of berries and berry products in Finland, where their mean adult consumption of berries is 52 g/d [68].

Berry products are the major contributor of anthocyanins in the diet, as a result of having high levels and being commonly consumed. The intake of anthocyanins can be significantly increased by small increases in berry consumption. Secondly, red raspberries are a major source of the less ubiquitous ellagitannins in the diet. Red raspberries are unique because of their high content of both anthocyanins and ellagitannins, and therefore, important because their consumption would make significant contributions to both compounds in the diet.

2.4.3 Content in Red Raspberries

The polyphenolic content of red raspberries is present in Table 2. Their total flavonoid content is largely attributable to anthocyanidins. According to the USDA Database for the Flavonoid Content of Selected Foods (2003), raw red raspberries contain 47.6 mg/100 g fresh weight (fw)
anthocyanidins, primarily consisting of cyanidin, followed by pelargonidin, malvidin and delphinidin aglycones [66]. They also contain 8.26 mg/100 g fw (-)-epicatechin and 0.97 mg/100 g fw (+)-catechin, which are flavan-3-ols, and 0.83 mg/100 g fw of the flavonol quercetin. Though not reported in this database, the primary flavonol glycoside is quercetin-3-glucuronide, and kaempferol-3-glucuronide has also been detected at lower levels [69]. Red raspberries have not been reported to contain any flavones, flavonones, nor isoflavones [70].

Red raspberries contain large amounts of tannins. Though known for their ellagitannin content, they also contain proanthocyanidins. The USDA Database for the Proanthocyanidin Content of Selected Foods (2004) reports the content of raw red raspberries to be: 3.91, 8.64, 3.92, 7.70, 0.90 and 0.00 mg/100 g fw of proanthocyanidin monomers, dimers, trimers, 4-6mers, 7-10mers and polymers [71]. Higher total proanthocyanidin levels of 30.2 and 78.8 mg/100 g fw have been reported, for berries from the US and Finland [72, 73], respectively. The major forms were procyanidins, composed of (epi)catechin, and propelargonidins, composed of (epi)afzelechin units [73].

The primary phenolic acid is a precursor for the formation of ellagitannins. Gallic acid forms the dimeric ellagic acid and has been found at 27.5 mg/100 g or over three-quarters the total phenolic acid pool [74]. Other phenolic acids present in much smaller quantities include p-hydroxybenzoic acid and derivatives of the hydroxycinnamic acid class, including caffeic acid, ferulic acid, sinapic acid, p-coumaric acid, cinnamic acid, and vanillic acid[74]. Reports of polyphenol content are often expressed in gallic acid equivalents (GAE). The two ellagitannin structures identified in red raspberries are sanguin H-6 and lambertianin C, and have been reported present at 76 and 31 mg GAE/100 g fw [75].

No stilbenes have been identified in red raspberries, unlike for grapes and their resveratrol content. However, small amounts of lignans have been reported. Red raspberries were found to contain 0.02 mg/100 g of fw secoisolariciresinol [76]. Consumption of red raspberries can contribute numerous different polyphenols to the diet. Even though some may be small in quantity, all are part of a healthy diet. However, the remainder of this literature review will focus on the two major red raspberry polyphenols: ellagitannins and anthocyanins.
2.4.4 Ellagitannins

Ellagitannins are hydrolysable tannins, which are more stable than the condensed tannins. Ellagitannins have often been identified as the active principles in medicinal plants [77]. However, they are uncommon in foodstuffs. They have a wide range of structures and can be present as monomers, oligomers, and complex polymers. They are defined as hexahydroxydiphenoyl (HHDP) esters of carbohydrates and cyclitols, but also include compounds derived from additional oxidative transformations. HHDP is a product of the oxidation of galloyl groups [77]. The most common oligomers in red raspberries include lambertianin A and sanguin H-6, which are trimers and tetramers, respectively, formed by an ether link between a galloyl, hydroxyl oxygen and an HHDP group. This characteristic link between one of the hydroxyl groups of a galloyl group in one molecule and the 4,6-HHDP connected to the 4-position of the glucose core of another molecule is known as a sanguisorboyl group [77]. When ellagitannins are exposed to acids or bases, their ester bonds are hydrolysed and hexahydroxydiphenic spontaneously rearranges to yield ellagic acid. Ellagic acid is a dimer and can be further hydrolysed to gallic acid, a derivative of benzoic acid [78].

Most reports of ellagitannin content in plants are determined as hydrolysed ellagic acid; therefore all values will be reported at ellagic acid (EA). Few studies have identified and quantified the ellagitannin compounds themselves. A group from Scotland was the first to characterize the ellagitannins in red raspberries [75]. They reported the content for the cultivar (cv.) Glen Ample as 135, 55, and 0.20 mg EA/100 g fw for sanguin H-6, lambertian C, and ellagic acid, respectively.

Information on the content of ellagitannins in foodstuffs is limited. Over 500 hydrolysable tannins have been identified in various plants, with major dietary sources mainly being berries and nuts [77]. Recently, a group from Finland selected 33 commonly consumed foods and screened them for ellagitannin content [79]. They identified only 5 foods, all berries, consisting of the cloudberry with 315.1 mg EA/100 g fw, red raspberry with 297.3 mg EA/100 g fw, rose hip with 109.6 mg EA/100 g fw, strawberry with 77.1 mg EA/100 g fw and sea buckthorn with 1 mg EA/100 g fw. Ellagic acid was mostly present as ellagitannins, and the relative amount of free ellagic acid was <6% for all fruits and 1.4% in red raspberries. Another group, from the United States, analysed the content of hydrolysed ellagic acid in various fruits and nuts [80]. Red
raspberries and blackberries contained the most, with 21.4 mg EA/100 g fw, followed by strawberries with 9.0 mg EA/100 g fw, walnuts with 8.4 mg EA/100 g fw, pecans with 4.7 mg EA/100 g fw, and cranberries with 1.7 mg EA/100 g fw. Lastly, a group from Japan analysed free ellagic acid content, not considering the contribution of ellagitannins, but did detect small amounts of ellagic acid in tropical fruit, including fuenjoa, pineapple, and pomegranate [81].

The ellagitannin content of red raspberries varies genetically. Four cultivars (cv.) from Spain showed variation in hydrolysed ellagic acid content from 20.7 to 24.4 mg EA/100 g [82], whereas the variation among 17 cultivars grown in Finland varied from 38 (cv. Gatineau and cv. Nova) to 118 mg EA/100 g fw (cv. Ville) [55]. Another group from Finland reported ellagitannin values for cultivated raspberries (cv. Muskoka) as 97.7 mg EA/100 g fw, and these values were higher in yellow cultivated raspberries with 126.2 mg EA/100 g of fw, and even higher in wild raspberries with 156.0 mg EA/100 g fw [83]. Therefore, ellagitannin content is affected by environment and genetics.

### 2.4.5 Anthocyanins

Anthocyanins are glycosylated polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium. They contain two benzoyl rings (A and B) separated by a heterocyclic (C) ring. The deglycosylated or aglycone forms are known as anthocyanidins. The six most common forms of anthocyanidins are cyanidin, delphinidin, pelargonidin, malvidin, petunidin, and peonidin (Figure 1), with a distribution in nature of 50, 12, 12, 12, 7, and 7%, respectively [84]. Overall, cyanidin-3-glucoside is the most ubiquitous.

Anthocyanins are a common phytochemical and present in a large assortment of foodstuffs. Therefore, information on their quantity in the diet is more readily available than for ellagitannins. The USDA Database for the Flavonoid Content of Selected Foods [66] reported that red raspberries contain 42.17, 3.70, 1.23 and 0.50 mg/100 g fw of cyanidin, pelargonidin, malvidin, and delphinidin aglycones. Similarly, all anthocyanin values in this section will be reported as their relevant aglycone or in cyanidin equivalents (CE) if labeled as such.

A similar database was prepared containing anthocyanidin content of foods commonly consumed in Finland [79]. Red raspberries contained 38.5 mg/100 g fw cyanidin and 0.9 mg/100g fw pelargonidin. A sample from another year contained only cyanidin, but had higher levels of 53.9
mg/100 g fw. The highest anthocyanidin content was found in bilberry with a total of 563.8, followed by chokeberry with 410 and crowberry with 359.6 mg/100 g fw. Of the berries commonly consumed in North America, blueberries had 201.0, cranberries 66.7 and red raspberry and strawberry samples averaged 46.7 and 40.0 mg/100 g fw, respectively. Red raspberries grown in the US or Finland appear to have comparable anthocyanin contents.

Anthocyanidins are typically present glycosylated and/or acylated. In US sampled red raspberries, 22% of anthocyanins are mono-glycosides, 52% are di-glycosides and 26% are tri-glycosides, whereas none are acylated [85]. The sugar groups are found bound first and foremost at C3, and attachments include sophoroside, glucoside, sambubioside and rutinoside, with rhamnoside sometimes at C5 [86].

Reports of red raspberry anthocyanin content are variable. Four Spanish-grown red raspberry cultivars were analysed, and total anthocyanin content ranged from 23.59 to 74.06 mg CE/100 g fw, with early cultivars having lower values than late cultivars [87]. If a seasonal factor was linearly affecting the quantity of anthocyanins produced by the plant, it could be expected that most cultivars, ripened during the peak of the season, would have levels that fall within this range. Eleven varieties of red raspberries grown in Italy were analysed for their cyanidin content only; the mean was 47.2 mg/100 g fw, similar to the average levels previously reported for red raspberries from US and Finland; and the range was slightly lower than above, from 17.4 to 68.4 mg/100 g fw, probably due to not included the smaller contributions of other anthocyanidins [88].

As well, the anthocyanin profile of red raspberries is variable. The Spanish-grown late harvest cultivars (cv. Rubi and cv. Zeva) had greater complexity, with five and nine different anthocyanins, compared to the early harvest varieties (cv. Heritage and cv. Autumn Bliss), which had only three and four [87], respectively. Cyanidin-3-sophoroside was the predominant anthocyanin in three of the four varieties (53–58%), and cyanidin-3-rutinoside was most predominant in the other (cv. Autumn Bliss; 34%). The next predominant for all cultivars was cyanidin-3-glucoside (21–36%). The other anthocyanins identified were cyanidin-3-glucorutinoside, pelargonidin-3-sophoroside, pelargonidin-3-glucorutinoside, pelargonidin-3-glucoside, malvidin-3-glucoside, and delphinidin-3-glucoside. These anthocyanin profile characteristics are similar to those of two varieties grown in the United Kingdom (cv. Latham
and cv. Glen Moy), with anthocyanidin levels of 48.6 and 29.7 mg/100 g fw, respectively [89]. For both cultivars the relative composition was cyanidin-3-sophoroside > cyanidin-3-glucorutinoside > cyanidin-3-glucoside > cyanidin-3-rutinoside > all pelargonidin glycosides combined.

Overall, the total anthocyanin content of red raspberries is typically <100/100 g of fw, irrelative of the method used or cultivar analysed, which is similar to other red/orange berries and fruits; dark blue/red berries, on the other hand, contain levels >150 mg/100 g [79]. Cyanidin glycosides make up the majority of the total anthocyanin content of red raspberries, with pelargonidin glycosides often present, but only at levels <2% of the total. The major glycoside moieties in red raspberries include sophoroside, glucoside, rutinoside, and glucorutinoside.

2.4.6 Maturity

The phenolic composition of berries changes throughout growth and stages of ripening. While the raspberries are green, levels of tannins are high, and they decrease over the ripening period. Later, during ripening, when the red fruit fully matures, sanguin H-6 levels are not significantly affected but proanthocyanidins continue to be significantly reduced. In contrast, anthocyanins are very low in green fruit, with only cyanidin-3-glucoside present and some traces of cyanidin-3-rutinoside. While pink, small amounts of cyanidin-3-sophoroside and cyanidin-3-glucosylrutinoside are produced. By the time the fruit is red, these anthocyanins sharply increase in quantity and pelargonidin glycosides begin to form last [57]. The stage during which raspberries are picked cannot be altered, as they only detach from the cap once ripe. However, early harvest cultivars, which likely also develop quickly, have been shown to have a lower quantity and less diversity of anthocyanins [87]. While wild or late harvest cultivars have longer to develop, and therefore, the richest anthocyanin composition.

2.4.7 Storage and processing

Red raspberries traditionally have a short growing season and shelf-life. In 2008, over 60 million pounds of red raspberries were harvested in the US, and less than 3% of that was utilized fresh [90]. Therefore, it is important to understand what impact storage and processing has on the antioxidant composition of red raspberries.
Short-term storage of fresh raspberries, at temperatures >0 °C, results in significant losses of vitamin C and significant increases in total phenolics [75, 91]. After 8 d of storage at 0, 10, 20 or 30 °C, the most dramatic improvements occurred at 20 °C, with total phenolic, anthocyanin, and AOC increasing 1.5-, 2.5-, and 2.0-fold, respectively[91]. Similarly storage of fresh raspberries for 3 d at 4 °C, then for 24 h at 18 °C, resulted in significant increases in total phenols by 11%, ellagitannins by 24%, and ellagic acid by >400% [75]. The increase in total phenolics was not attributable to the same polyphenols in both studies, but both only found significant losses in vitamin C. After 8 d at 20 °C, vitamin C suffered decreases of 22%, whereas after 3 d at 4 °C and 24 h at 18 °C, it decreased by only 8%. Overall, the increase in phenolics prevented the decrease in vitamin C from having a negative impact on AOC. It seems that storing fresh berries at room temperature may improve their content of health promoting polyphenols, but decrease their shelf-life.

Berries can be frozen for long-term storage. The process of freezing has minimal effects on the phytochemical content of red raspberries. When fresh red raspberries were frozen in liquid nitrogen at -80 °C for 15 min, then thawed for 1 h at 7 °C before analysed, there were only minor compositional changes. Two varieties with low levels of anthocyanins had significant increases in anthocyanins, whereas two varieties with high levels had significant decreases [87]. One of the four varieties also had a significant decrease in ellagic acid, but overall, there were no changes in total phenolics nor AOC, measured as antiradical efficiency (AE) [82]. When fresh red raspberries berries were frozen at -30 C in a commercial plant, which is how store-bought frozen berries would have been processed, there were no effects on composition, including p-coumaric acid, kaempferol, quercetin, anthocyanins, ellagitannins, vitamin C and AOC.

The long-term storage of frozen red raspberries has greater effects on phytochemical composition. When frozen samples were stored at −20 °C for 12 months, there were significant decreases in hydrolysed ellagic acid of 14-21%, and vitamin C of 34-56%, depending on cultivar [82]. However, there were no significant decreases in total phenolics, or AOC [82, 87]. Similarly, in another study, hydrolysed ellagic acid levels decreased 30% over 9 months of storage at −20 °C [92]. Frozen storage effects on anthocyanins were cultivar dependent, with one of four cultivars having a significant decrease of 18% [87]. Of all the anthocyanins, cyanidin-3-glucoside suffered the most from degradation during freezing and storage. Therefore, the compositional differences of cultivars may be influencing their stability during storage. Overall,
the total phenolic content and AOC is maintained during freezing and storage, what is not known is whether losses in vitamin C, ellagitannins and anthocyanins are replaced by different antioxidant compounds. Frozen red raspberries are a good alternative to fresh berries, as they contribute antioxidants, ellagitannins, anthocyanins and other phenolic compounds to the diet. However, there may be implications for the type of bioactive compounds present.

2.4.8 Genetic and environmental

The post-harvest effects above are minimal when compared to the differences across cultivars, due to genetic and growing conditions. Across 17 cultivars, total phenolics varied 1.9-fold, hydrolysed ellagic acid 3.1-fold, total anthocyanins 2.7-fold, and quercetin 2.8-fold [55]. These were mostly genetic differences, as all cultivars were grown the same year, in the same location, using the same techniques. However, growing season can have a big influence, as previously mentioned when discussing compositional differences between early and late harvest varieties [82, 87]. In another study, two cultivars were crossed and the anthocyanin profiles of their progeny were assessed across two growing seasons and two growing conditions, one open field and the other covered with polytunnel. They found significant differences between growing seasons for most anthocyanins (P < 0.001), with higher anthocyanin levels present in the hotter, drier year with more sunshine hours. They also found a small significant effect of growing condition on pelargonidin glycosides (P=0.01) and an almost significant effect on some cyanidin glycosides (P<0.1), with lower anthocyanin levels in those grown under polytunnel.

The main goal of selective breeding has typically been growth, which reduces the amount of energy the plant has to put toward the production of phytochemicals. Organic produce, which is more likely to have been selected for pest and disease resistance, has higher levels of phytochemicals [93]. This is because phytochemicals are produced by the plant to protect itself from UV damage, heavy metals, bacteria, fungus, insects and so on [50]; protective effects which may be transferred to the consumer. It is logical then, that wild varieties have the highest levels of polyphenols, as they underwent natural selection for survival. As interest in the nutritional quality of food increases in popularity among consumers, the goal of breeding programs will move away from quantity and toward quality. The use of modern selective breeding practices may assist in finding a balance between these two factors. Genetic markers can identify desired traits, and allow for the selection of increased anthocyanin production, for example. Before the
phytochemical profile of red raspberries can be selectively improved for health benefits, an understanding of which compounds are most beneficial to human health is required.
Table 1. Nutrient composition of fresh, red raspberries.*

<table>
<thead>
<tr>
<th>Type</th>
<th>Nutrient</th>
<th>Per 100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximates:</td>
<td>Water (g)</td>
<td>85.75</td>
</tr>
<tr>
<td></td>
<td>Energy (kcal)</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Protein (g)</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>Total lipid (g)</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>Carbohydrate (g)</td>
<td>11.94</td>
</tr>
<tr>
<td></td>
<td>Dietary fibre (g)</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>Sugars (g)</td>
<td>4.42</td>
</tr>
<tr>
<td></td>
<td>Sucrose (g)</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Glucose (g)</td>
<td>1.86</td>
</tr>
<tr>
<td></td>
<td>Fructose (g)</td>
<td>2.35</td>
</tr>
<tr>
<td>Minerals:</td>
<td>Calcium (mg)</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Iron (mg)</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>Magnesium (mg)</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Phosphorus (mg)</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Potassium (mg)</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>Sodium (mg)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Zinc (mg)</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>Copper (mg)</td>
<td>0.090</td>
</tr>
<tr>
<td></td>
<td>Manganese (mg)</td>
<td>0.670</td>
</tr>
<tr>
<td></td>
<td>Selenium (µg)</td>
<td>0.2</td>
</tr>
<tr>
<td>Vitamins:</td>
<td>Vitamin C (mg)</td>
<td>26.2</td>
</tr>
<tr>
<td></td>
<td>Thiamine (mg)</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td>Riboflavin (mg)</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>Niacin (mg)</td>
<td>0.598</td>
</tr>
<tr>
<td></td>
<td>Pantothenic acid (mg)</td>
<td>0.329</td>
</tr>
<tr>
<td></td>
<td>Vitamin B-6 (mg)</td>
<td>0.055</td>
</tr>
<tr>
<td></td>
<td>Folate (µg)</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Choline (mg)</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>Betaine (mg)</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Vitamin B12 (µg)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Vitamin A, RAE (µg)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Lutein + zeaxanthin (µg)</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>Vitamin E, alpha-tocopherol (mg)</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>Tocopherol, beta (mg)</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Tocopherol, gamma (mg)</td>
<td>1.42</td>
</tr>
<tr>
<td></td>
<td>Tocopherol, delta (mg)</td>
<td>1.04</td>
</tr>
<tr>
<td></td>
<td>Vitamin K, phyloquinone (µg)</td>
<td>7.8</td>
</tr>
</tbody>
</table>

*Reference, [58].
**Table 2.** Polyphenolic composition of fresh, red raspberries.

<table>
<thead>
<tr>
<th>Class</th>
<th>Group</th>
<th>Compound</th>
<th>Amount (mg/100 g)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>Anthocyanins</td>
<td>Cyanidin-3-sophoroside</td>
<td>25.4</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyanidin-3-glucosylrutinoside</td>
<td>7.2</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyanidin-3-glucoside</td>
<td>3.9</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyanidin-rutinoside</td>
<td>2.3</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pelargonidin-3-sophoroside</td>
<td>0.06</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pelargonidin-3-glucosyl rutinoside</td>
<td>0.1</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pelargonidin-3-glucoside</td>
<td>0.12</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pelargonidin-3-rutinoside</td>
<td>0.005</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td>Flavan-3-ols</td>
<td>(+)-Catechin</td>
<td>2.4</td>
<td>[69]</td>
</tr>
<tr>
<td></td>
<td>Flavonoids</td>
<td>Kaempferol-3-glucuronide</td>
<td>0.6</td>
<td>[69]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quercetin-3-glucuronide</td>
<td>1.1</td>
<td>[69]</td>
</tr>
<tr>
<td>Phenolic Acids</td>
<td>Hydroxybenzoic acids</td>
<td>Gallic acid</td>
<td>21.5</td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-Hydroxybenzoic acid</td>
<td>1.82</td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td>Hydroxycinnamic acids</td>
<td>p-Coumaric acid</td>
<td>0.8</td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Caffeic acid</td>
<td>0.89</td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ferulic acid</td>
<td>0.85</td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sinapic acid</td>
<td>0.27</td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vanillic acid</td>
<td>1.04</td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cinnamic acid</td>
<td>0.27</td>
<td>[74]</td>
</tr>
<tr>
<td>Hydrolysable Tannins</td>
<td>Ellagitannins</td>
<td>Sanguin H-6</td>
<td>76</td>
<td>[79]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lambertianin C</td>
<td>31</td>
<td>[79]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ellagic acid</td>
<td>0.11</td>
<td>[79]</td>
</tr>
<tr>
<td>Condensed Tannins</td>
<td>Proanthocyanidins</td>
<td>Procyanidin, Propelargonidin</td>
<td>54.5</td>
<td>[72, 73]</td>
</tr>
<tr>
<td></td>
<td>Lignans</td>
<td>Secoisolaricirensiol</td>
<td>0.02</td>
<td>[76]</td>
</tr>
</tbody>
</table>
Figure 1. The chemical structure of major anthocyanidins*.

* ChemDraw Std. 12.0, Cambridgesoft, Cambridge, MA, USA
3 Bioavailability of Major Red Raspberry Polyphenols

3.1 Introduction

Health benefits of many phenolic phytochemicals have been identified using in vitro methods, which may not be relevant in vivo, due to poor absorption and/or metabolism. The levels of polyphenols in the blood and excreted in urine are at very low concentrations and can be in different forms from those ingested. Therefore, understanding the pharmacokinetics of potential health-promoting compounds is required in order to understand their bioactivity.

3.2 Ellagitannins

Ellagitannins are large polymers, and therefore, difficult to quantify and not directly bioavailable. In fact, most studies quantify the hydrolysed ellagic acid content of red raspberries, which turns out to be the bioactive compound of interest. Ellagitannins are partially hydrolysed in the gut to release ellagic acid [78]. This hydrolysis is not known to be catalyzed by any human enzymes, but is known to occur optimally at pH 8 or after 1 h of exposure to the microbial content of the cecum [94]. Ellagic acid can be further metabolized by colonic microbiota to yield 3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one, also called urolithin B [95]. Both metabolites, which are absorbed by humans, will be discussed further [78].

The results of three studies which have investigated the absorption and metabolism of ellagitannins in humans after raspberry consumption will be compared. The first study gave ten healthy subjects 225 g of frozen red raspberries, purchased from a supermarket, and collected urine samples at 8, 16, 32, 40 and 56 h post-intake [96]. In the second study, eleven healthy subjects were given 45 g of freeze-dried black raspberries (equivalent to 2 cups fresh), and plasma samples and urine collections were taken for 12 h post-intake [97]. The last study gave ten healthy subjects and four subjects with an ileostomy, 300 g of homogenized fresh red raspberries cv. Glen Ample, obtained from a commercial grower [98]. Blood samples were collected for the first 24 h and urine was collected for 48 h post-intake.

Ellagic acid levels peaked in plasma, with a Cmax of 3.65+-1.71 ng/mL, between 1 and 2 h, returning to baseline levels by 8 h post-intake in study two [97]. No red raspberry metabolites were detected in plasma samples from study three [98], as concentrations are often too low for
detection. In both study two and three, ellagic acid levels in the urine were greatest from 0 to 4 h post-intake, and returned to baseline levels during the 8 to 12 h and 7 to 24 h collection, respectively. Ellagic acid was also detected conjugated with glucuronide, but only in study three. Recovery of ellagic acid was low, in study two it was reported as <1%, and in study three it was reported as 0.08%, but this was an overestimation as it did not consider the contribution of ellagitannins to the ellagic acid pool. Despite a low bioavailability, ellagic acid was shown to accumulate in the body. In study two, after consuming the treatment for 7 days, baseline ellagic acid levels had increase by 40%.

Urolithin metabolites were detected in study one at 32, 40 and 56 h post-intake, with trace amounts also detected at 16 h [96]. In study three, nine of ten subjects’ urine collections from 24 to 48 h contained urolithin metabolites, whereas, five of those subjects also had detectable levels at 7 to 24 h [98]. Urolithin B was present with and without its glucoronide conjugate in both studies, but urolithin A and its glucoronide conjugate were only detected in study three. By end of study one at 56 h, the hydrolysed ellagic acid content of the red raspberry treatment had reached a mean recovery of (3.4+/−4.4)% with a range of 7.6 to 0.21% in the form of urolithin B. However, this may be an underestimation, as urinary levels were highest at 56 h and therefore, not completely cleared from the system. Mean recovery of ellagitannins/ellagic acid intake, after 24 h was 2.8% ranging from 0.6 to 8.6% in study three. This may also be an underestimation; 1) the calculation was based on the assumption that the conversion of ellagitannins to urolithins yields 4 mol of ellagic acid per 1 mol sanguin H-6; 2) as shown in the previous study, urolithins are not cleared from the system after 56 h post-intake and this study only measured 24 h post-intake.

Some subjects were fed double the quantity of red raspberries in study one. Total excretion of urolithin B was higher, but it was not proportional to the increased quantity. It is possible that bioavailability is not linear, and that is plateaus with increasing dose, but further research required to understand this relationship.

In support for the role of colonic bacteria in the production of urolithin, 1) no urolithin metabolites were detected in subjects with an ileostomy [98]; and 2) urolithin recovery was highly variable between individuals [96, 98]. The observance of high and low responders is
typical when measuring levels of colonic metabolites, due to inter-individual differences in microbiota.

Overall, systemic exposure to ellagic acid appears to be very low. Information on tissue accumulation is lacking, but it was shown to accumulate in urine with regular consumption [97]. Its colonic metabolite, urolithin B, has higher urinary recovery and therefore, may be a better candidate for in vivo bioactivity. However, this metabolite may only be produced by a fraction of the population. Overall, additional human studies are needed to determine what levels of ellagic acid and urolithin B are attainable systemically and in tissues from dietary consumption of red raspberries before their biological relevance can be understood.

3.3 Anthocyanins

Many reviews have recently been published on the absorption and metabolism of anthocyanins [99, 100]. Absorption occurs very quickly, maximum plasma concentrations are reached between 15 to 60 min post-intake. Although, levels can peak up to 4 h post-intake, when consumed with high-fat foods, which delay gastric emptying [101, 102]. Urinary recovery of anthocyanins are typically <0.1% [100], but can range from 0.03 to 4% [99]. Similar to ellagic acid, recovery is typically complete within 6–8 h. However, anthocyanins are more susceptible to phase II enzymatic modifications. Conjugation by glucuronidation [103-106], methylation [103, 105, 106], and sulfation [104] has been documented, with reports of as much as 80% [104] of total excreted anthocyanins being conjugates.

Anthocyanins are absorbed through the stomach and the small intestine. When exposed to colonic bacteria, they are deglycosylated and demethylated into their corresponding aglycones. Since aglycones are unstable at neutral pH, they rapidly degrade into their corresponding phenolic acids and aldehydes through cleavage of the C-ring [107, 108]. These low molecular weight phenolic acids can then be absorbed through the colon. A potential phenolic acid biomarker for anthocyanin microbial metabolism was identified in rats [109]. A significant increase in the plasma concentrations of protocatechuic acid following intake of cyanidin glycosides was reported, which was 8-fold higher than the plasma concentration of the parent anthocyanin. Protocatechuic acid was also found to be a major metabolic bioproduct of anthocyanins by human fecal bacteria in vitro [107]. Therefore, phenolic acids may be another pathway by which anthocyanin consumption can influence health.
Urinary recovery of a compound is not directly related to its absorption, as a portion of that absorbed may be used by the body or deposited in tissues. Anthocyanins have been found to be able to cross cell membranes in vitro. They have also been detected in the tissues of blueberry-fed pigs, including the liver, eye, cortex and cerebellum [110]. Malvidin glycosides were the most abundant in all tissues, possibly because of the stability of the structure imparted by its two methyloxylations. Different tissues had different relative abundances of each anthocyanin, with cyanidins more prevalent than delphinidins in the cortex and in the liver. This suggests that tissues selectively accumulate certain anthocyanins or that anthocyanin stability varies between tissues. However, tissue accumulation of anthocyanins in humans remains to be investigated.

Study two and three discussed in the ellagitannin section, also investigated the pharmacokinetics of anthocyanins, and will again be discussed here. In study two, anthocyanin levels peaked in the plasma between 1 and 2 h post-intake [97]. Cyanidin 3-rutinoside was the major anthocyanin in the berries and subsequently had the highest plasma Cmax of 22.50+/−4.86 ng/mL. However, cyanidin 3-sambubioside, followed by cyanidin 3-xylosylrutinoside had the longest mean half-lives of 6.16 and 3.39 h, respectively. Interestingly, after 6 d of treatment only the two compounds with the longest half-lives showed accumulation in the urine. Recovery in the urine remained unchanged after 7 d of intake, meaning absorption was not improved by regular consumption. Anthocyanin levels peaked in urine during 0 to 4 h post-intake, and almost returned to baseline levels by 8 to 12 h. No conjugates were reported and recovery was <1%.

In study two, no anthocyanins were detected in the plasma, and urinary levels were so low that only the three major anthocyanins were detected including, cyanidin 3-sophoroside, cyanidin 3-glucosylrutinoside, and cyanidin 3-glucoside. No conjugates were also reported and recovery was 0.04%.

Recovery of raspberry anthocyanins was quite low in the two studies discussed. It was demonstrated that sugar moieties can influence the stability or half-life of anthocyanins, but aglycone structure also plays a role. Pelargonidin, from strawberries, was found to have a recovery of 1.80%, as compared to cyanidin’s much lower recovery of 0.16% from blackberries [104, 111]. However, these two studies reported that a large proportion of the total anthocyanins recovered were conjugates. Therefore, it is possible that recovery from the raspberry studies
might have been an underestimation, if conjugated metabolites were below the detection limit and therefore, not measured.

More research is needed to understand the movement of anthocyanin and anthocyanin-derived compounds from the gastrointestinal tract and throughout the human body. It is not known whether conjugated anthocyanins or anthocyanin-derived phenolic acids significantly contribute to urinary recovery. Nor is it known whether red raspberry anthocyanins are pooled in tissues. Before their bioactivity is understood, the compounds to which one is exposed must be known.
4 Bioactivity of Major Red Raspberry Polyphenols

4.1 Introduction

The bioactivity of dietary compounds is investigated using in vitro and cell models, as well as in vivo animal models and human interventions. Berry-related polyphenols are thought to influence pathways which may prevent and/or treat certain types of cancers, cardiovascular diseases (CVD), type II diabetes mellitus, obesity, macular degeneration and neuro degeneration. Their protection from damage caused by oxidative stress and inflammation may link the etiology of these chronic conditions, but polyphenols can also have specific effects on enzyme activity, signal transduction and gene expression. To give insight into the potential bioactive roles of red raspberries on human health, the limited number of red raspberry-specific studies, as well as research on red raspberry-related polyphenols, and other berry fruits with comparable polyphenolic profiles, will be reviewed.

4.2 Cancer

Red raspberry extracts have been shown to inhibit the growth of various cancer cell lines in vitro, including stomach [112], intestine [112], colon [113, 114], breast [112], cervical [115] and prostate [112]. As well as inhibit cancer cell initiation, promotion and invasion [116]. Antioxidant activity against H2O2-induced DNA damage [116, 117] is one mechanism of action, however, anti-carcinogenic effects do not always correlate with AOC [112]. Other protective mechanisms against initiation include inhibition of mutagen absorption [118], activation and DNA-adduct formation [119]. Red raspberry extracts anti-proliferative activity has been linked to induction of cell-cycle arrest [112, 116], and inhibition of the cytokine TNF-α (tumor necrosis factor) and related COX-2 expression, as well as activation of transcription factor, NFκB [112]. Anti-metastasis effects are related to inhibition of TNF-α and related VEGF (vascular endothelial growth factor) expression [117], as well as inhibition of MMP (matrix metalloproteinases) 2 and 9 activities [120].

Ellagic acid specifically has been found to inhibit mutagen DNA binding [121-123], metabolism by phase I enzymes [124], and to induce cellular phase II enzymes [125], G1 arrest (Narayanan 99), and caspase-dependent apoptosis [126]. Effects are likely to be limited to the aerodigestive tract, as ellagic acid is not very water-soluble and accumulates in epithelial cells bound to DNA.
and proteins [127]. Recently, ellagic acid, ellagitannins and urolithin A were all found to inhibit Wnt signaling in human colon carcinoma cell lines, which is highly significant because this signaling cascade is inappropriately activated in 90% of colorectal cancers [128].

Anthocyanins are more effective than other flavonoids at suppression of tumor growth in vitro [129]. Not only do anthocyanins inhibit colon cancer cells viability and induce apoptosis [130], but their gut metabolites also inhibit colon cancer cell growth [131]. It is thought that they block phosphorylation of protein kinases, in particular MAPK [130, 132] and inhibit COX-2 expression [133-135].

Only one recent study demonstrated the anti-tumor effects of red raspberries in vivo. Dietary administration inhibited tumor progression in the rat esophagus, as well as reduced serum levels of IL-5 and IL-8 [136], which reflects COX-2 mediated anti-inflammatory effects. Ellagic acid has similarly demonstrated anti-inflammatory effects during rat colon carcinogenesis, with reductions in the expression of NFκB, COX-2, iNOS, and TNF-α, IL-6 [137]. Ellagic acid supplementation has inhibited tongue [138], liver [139], lung [140, 141], skin [142, 143] and estrogen-induced mammary [144] tumorigenesis in rodents. Cyanidin-3-glucoside supplementation has inhibited colon [145], skin [133] and lung [133] tumorigenesis in rodents, but when administered at nutritionally relevant levels was ineffective at protecting against lipid peroxidation and DNA damage in vitamin E deficient rats [146]. Numerous animal studies have been performed with black raspberries, and they have been found to positively modulate genes involved in phase I and II metabolism, oxidative damage, and oncogenes and tumor suppressor genes that regulate apoptosis, cell cycling and angiogenesis in rats after carcinogen exposure [147]. They have inhibited oral [148], esophageal [149, 150], colon [151] and cutaneous [152] tumor formation in rodents. Possibly due to low bioavailability red raspberry polyphenols may better function to reduce cancer in tissues of the aerodigestive tract, where there is more direct exposure to bioactive compounds.

Clinical trials with black raspberries are currently being performed on patients with premalignant cancer conditions of the aerodigestive tract. Dietary administration of freeze-dried black raspberries reduced oxidative stress in patients diagnosed with Barrett’s esophagus, measured by urinary 8-Iso-PGF2 and 8-OHdG [153]. Whereas, topical application of a bioadhesive black raspberry gel to oral lesions suppressed genes associated with RNA processing, growth factor
recycling and inhibition of apoptosis, as well as reduced epithelial iNOS and COX-2 protein levels. Some participants showed reduced vascular densities in the superficial connective tissues [154] and histological regression [155].

However, human studies on diet and cancer risk typically assess biomarkers of DNA damage in healthy subjects or those with risk factors for chronic disease. A 4-week intervention with a red berry juice, containing red raspberry juice, improved levels of glutathione and reduced DNA oxidative damage in healthy adult males [156]. Although, a dessert made from a similar anthocyanin-rich juice had no effect on oxidative stress status in elderly patients after 2 weeks [46]. Whereas, a beverage containing raspberry, black grape, and red currant concentrates reduced post-exercise-induced oxidative damage of proteins and DNA compared to controls [157]. Effects to reduce postprandial oxidative stress have been demonstrated for numerous studies using fruits or polyphenolic-rich foodstuffs, none of which were raspberries [102, 158-160]. It is common to find no effects on oxidative stress in healthy populations, as baseline levels are already quite low [45, 161-163]. An elevated state of oxidative stress improves the ability to measure significant antioxidant effects of dietary antioxidants, whether induced with food or exercise in a healthy population or present in an at risk population [164-166].

4.3 Cardiovascular Disease (CVD)

Red raspberries polyphenols have demonstrated bioactivities in vitro which may be protective against CVD. The antioxidant activity of numerous polyphenols have been found to inhibit copper-catalyzed human LDL-oxidation, with cyanidin being more effective than ellagic acid [167]. Bioavailable anthocyanins in the serum of cyanidin 3-glucoside fed rats have similarly demonstrated this protective effect ex vivo [168]. Anthocyanins are absorbed into cells and anthocyanin extracts from blueberries and cranberries have been shown to protect against H2O2-induced oxidative stress at both the membrane and cytosol level of endothelial cells [169]. However, their protective effects are not limited to protection against oxidative stress. They may also protect against endothelial inflammation and dysfunction, which are pathological conditions involved in the etiology of CVD. Anthocyanin extracts from blueberries and cranberries have been shown to reduce TNF-α induced up-regulation of various inflammatory mediators (IL-8, MCP-1 and ICAM-1) [169], and cyanidin-3-glucoside has been shown to dose-dependently induce NOS (nitric oxide synthase) and HO-1 (heme-oxygenase-1) in endothelial cells [170].
Whereas, ellagitannin-containing fractions of red raspberry extracts have demonstrated vasodilation properties in vitro [171].

These bioactivities of red raspberry polyphenols have improved risk factors for CVD in animals. Anthocyanin-rich blueberry extracts fed to hypertensive rats significantly improved blood pressure [172]. Whereas blueberry fed pigs showed improvements in blood lipids [173], as did insulin resistant, hyperlipidemic rats fed freeze-dried anthocyanin-rich tart cherries [174]. Ellagic acid, not present in blueberries and cherries, has also improved blood lipids in rats fed a high fat diet [175]. Beyond preventative effects, consumption of anthocyanin-rich berries may also be useful in the treatment of CVD, as a blueberry-enriched diet protected the brain [176, 177] and myocardium [178] from induced ischemic damage and the demonstrated potential to attenuate the development of post MI chronic heart failure in rats [178].

Anthocyanin containing berries have also demonstrated CVD protective effects in humans via both antioxidant and non-antioxidant mediated mechanisms. Consumption of anthocyanin-containing cranberry juice for 2 weeks significantly increased plasma AOC and reduced ox-LDL in men [179] but had no effect in women [45]. However, diabetic women with hyperlipidemia significantly responded to daily consumption of concentrated sour cherry juice with improvements in blood lipids [180]. Women with metabolic syndrome also responded to supplementation of their diets with freeze-dried strawberries for four weeks with significant improvements in blood lipids and reductions in lipid peroxidation [181]. Strawberry consumption was also effective in reducing ox-LDL in hyperlipidemic men and women above and beyond the effects of a cholesterol-lowering diet [164]. Unlike the other berries discussed, strawberries contain both anthocyanins and ellagitannins, at lower levels than typically found in red raspberries. Therefore, the combined or synergistic effects of red raspberry polyphenols may be more protective against CVD.

### 4.4 Diabetes

Red raspberries may be useful in the treatment of diabetics. Red raspberry extracts have been shown to inhibit carbohydrate digestion, with the anthocyanin containing fraction being more effective at inhibiting α-glucosidase, and the ellagitannin-containing fraction more effective at blocking α-amylase in vitro [182]. These inhibitory effects have been verified for both anthocyanins [183] and ellagitannins [184] by other groups. This bioactivity has the potential to
reduce post-prandial glucose responses, but anthocyanins may also be able to stimulate its uptake. Numerous anthocyanins have been shown to stimulate insulin secretion from rodent pancreatic cells in vitro. Cyanidin-3-glucoside, which is present in red raspberries, was the most effective anthocyanin tested [185]. Therefore, red raspberries may be able to improve glucose control by slowing glucose absorption and enhancing cellular uptake.

These bioactivities and more have been demonstrated with anthocyanins, to prevent and treat rodent models of diabetes. An anthocyanin-rich chokeberry extract inhibited sugar metabolism by decreasing maltase and sucrase activity in the mucosa of the small intestine of prediabetic, hyperlipidemic rats [186]. Whereas, anthocyanin-rich blueberries protected against high-fat diet-induced development of insulin resistance and hyperglycemia; effects which were associated with reductions in adipocyte death and the down-regulation of inflammatory and oxidative stress genes [187]. Cyanidin-3-glucoside alone reduced blood glucose and improved insulin sensitivity in type 2 diabetic mice; effects which were associated with an up-regulation of glucose transporter 4 (Glut4), and a down-regulation of retinol binding protein 4 (RBP4) and inflammatory adipocytokines (MCP-1 and TNF-α) in the white adipose tissue [188]. Similarly, anthocyanin-containing tart cherries reduced fasting hyperinsulinemia in insulin resistant, hyperlipidemic rats [174].

Despite positive results in animals, little work has been completed on the use of berries to improve blood glucose control in humans. A study in diabetic women found that consumption of concentrated sour cherry juice for 6 weeks could improve HbA1c levels, a long-term indicator of blood glucose control [180]. As well, a berry puree, containing bilberries, blackcurrants, cranberries and strawberries, when consumed with a sucrose load, significantly inhibited post-prandial glucose response in healthy subjects [189]. Therefore, more research is warranted to investigate the effects of red raspberries, and specifically their anthocyanins, on blood glucose control.

### 4.5 Obesity

The effect of phenolic compounds on weight management is a new area of research. Cyanidin and cyanidin-3-glucoside have been found to upregulate hormone sensitive lipase and lipolytic activity in vitro [190], as well as stimulate the release of adiponectin and leptin in vitro and in vivo [191] in rat adipocytes. Purified blueberry or strawberry anthocyanins, but not whole berry...
freeze-dried powder, prevented the development of high-fat diet induced obesity, measured as body weight and % body fat, in mice [192-194], whereas black red raspberry anthocyanins were less effective [195]. Tart cherry supplementation of a high fat diet reduced fat mass, abdominal fat as well as systemic and local inflammation (TNF-alpha, IL-6) in obese rats with metabolic syndrome [196]. In humans, concentrated sour cherry juice consumption for six weeks resulted in significant weight reductions in diabetic women [180], however the lack of a placebo control means that the weight reduction may not be attributable to the treatment. No human studies have investigated the effects of berry consumption on weight reduction as a primary objective, but more in vitro and animal evidence is needed to warrant such investigations, as only high pharmacological doses have demonstrated effects.

4.6 Macular and Neuro Degeneration

As oxidative stress increases with age, it contributes to the development of other age-related diseases such as macular degeneration and neuro degeneration. The antioxidant activity of anthocyanins from blueberries and bilberries have been shown to protect retinal cells against induced damage in vitro [197, 198], as well as demonstrating indirect-antioxidant effects via the upregulation of oxidative stress defense enzymes (HO-1 and GST-pi) [199]. Similarly, ellagic acid, administered via intraperitoneal injection, retarded the development of selenite-induced cataractogenesis in rats by maintaining the antioxidant defense system and reducing lipid peroxidation. [200]. Therefore, antioxidants, such as anthocyanins which have been detected in the eye tissue of animals, may serve an important role in protecting the eye from damage caused by oxygen exposure.

Anthocyanins have also been detected in brain tissue and are being investigated for their role in cognitive performance. Blueberry extracts, administered orally and intraperitonealy, have repeatedly been shown to improve performance on memory-related tasks in rats [201-206]. Effects have been shown to correlate with anthocyanin levels detected in the task-related brain region [202] and with improved antioxidant properties [205, 207]. Improvements in cognitive performance have also been demonstrated following consumption of strawberries [208-210], blackberries [211] and anthocyanin-rich mulberry extracts [212]. Some positive effects of anthocyanin supplementation on cognitive function have even been seen in humans [213].
Therefore, consumption of antioxidant- and anthocyanin-rich berries, like red raspberries, may protect against some of negative effects of aging.
Chapter 3
Hypotheses, Objectives, Study Design and Rational
5 Overall

5.1 Hypotheses

1. Chronic and acute exposure to antioxidants, from red raspberry consumption, will reduce oxidative stress in healthy adults by:

   (i) increasing TEAC, a biomarker for total antioxidant capacity;

   (ii) increasing thiols, a biomarker for protection against protein oxidation;

   (iii) decreasing TBARS, a biomarker for lipid peroxidation.

2. Effects on the measured oxidative stress biomarkers will be dose-dependent with greater reductions in oxidative stress following consumption of larger doses of red raspberries.

3. Red raspberry antioxidants, including vitamin C and anthocyanins, will be dose-dependently bioavailable.

5.2 Objectives

1. Determine the chronic and acute effects of red raspberry consumption on oxidative stress in healthy adults.

2. Investigate the effect of red raspberry dose on oxidative stress (bioactivity) in healthy adults.

3. Investigate the bioavailability of red raspberry antioxidants in healthy adults.

4. Assess the relationship between red raspberry bioactivity and the bioavailability of red raspberry antioxidants.

   *Use results to provide guidelines for future investigations on red raspberries.*
6  Study 1

6.1  Hypotheses

1. Daily consumption of red raspberries by healthy subjects for two and four weeks will reduce fasting oxidative stress (↑ TEAC, ↑ thiols and ↓ TBARS).

2. Effects on the measured oxidative stress biomarkers will be dose-dependent with greater reductions in oxidative stress following consumption of larger doses of red raspberries for two weeks (4cR>2cR>1cR).

3. Red raspberry anthocyanins will have dose-dependent bioavailability and therefore, they will be detected in 12 h overnight urine collections following raspberry consumption, with greater recovery following larger doses (4cR>2cR>1cR).

4. Red raspberry’s bioactivity will relate to the bioavailability of red raspberry anthocyanins, such that higher concentrations of anthocyanins will correlate with reduced oxidative stress (↑ TEAC, ↑ thiols and ↓ TBARS).

6.2  Objectives

1. Determine the effect of chronic exposure to red raspberries and their antioxidants on fasting oxidative stress in healthy adults by supplementing 24 subjects’ diets with one cup red raspberries (1cR) for two weeks, followed by 1cR, 2cR or 4cR for additional two weeks and comparing oxidative stress biomarkers to levels before and after intervention.

2. Investigate the effect of red raspberry dose on fasting oxidative stress (bioactivity).

3. Investigate bioavailability and effect of dose on recovery of anthocyanins in 12 h overnight urine collections following consumption of red raspberries.

4. Assess the relationship between red raspberry bioactivity and anthocyanin bioavailability.

+ Investigate the effect of the raspberry intervention on clinical biomarkers, used to assess chronic disease risk, including body weight, blood pressure, fasting plasma glucose, lipid profile and C-reactive protein (CRP) levels.
7 Study 2

7.1 Hypotheses

1. Consumption of a single dose of red raspberries by healthy adults will reduce post-prandial oxidative stress (↑ TEAC, ↑ thiols and ↓ TBARS), over 8 h post-treatment.

2. Effects on the measured oxidative stress biomarkers will be dose-dependent with greater reductions in post-prandial oxidative stress following consumption of larger doses of red raspberries and antioxidants (4cR>2cR>1cR>B+vC>B).

3. Red raspberry antioxidants will have dose-dependent bioavailability and therefore, ascorbic acid will be detected in plasma, and anthocyanins in plasma and urine, with greater concentrations following larger doses (4cR=B+vC>2cR>1cR>B for ascorbic acid, and 4cR>2cR>1cR>B+vC=B for anthocyanins).

4. Red raspberry’s bioactivity will relate to the bioavailability of red raspberry antioxidants, such that greater concentrations of ascorbic acid and anthocyanins will correlate with reduced oxidative stress (↑ TEAC, ↑ thiols and ↓ TBARS).

7.2 Objectives

1. Determine the effect of acute exposure to red raspberries and their antioxidants on post-prandial oxidative stress in healthy adults by giving 10 subjects a meal of 1cR, 2cR, 4cR, bread (B) or bread plus vitamin C (B+vC), and comparing oxidative stress biomarkers over 8 h post-treatment to fasting levels.

2. Investigate the effect of treatment on post-prandial oxidative stress (bioactivity).

3. Investigate bioavailability and effect of dose on concentrations of ascorbic acid in plasma over 8 h post-treatment, and anthocyanins in plasma and urine, from 24 h post-treatment.

4. Assess the relationship between red raspberry bioactivity and antioxidant bioavailability.

+ Determine the anthocyanin composition of the red raspberry treatment and identify the structure of anthocyanins and their metabolites in plasma and urine samples.
8 Rational

The investigation of red raspberry’s antioxidant activity in vivo is warranted because 1) berry fruits, including red raspberries have a high in vitro AOC [214], 2) among the berries, red raspberries are unique for their high content of the dietary antioxidants, anthocyanins and the less ubiquitous ellagitannins [214], and 3) dietary antioxidants have been found to protect against oxidative stress, a condition hypothesized to increase chronic disease risk [215]. However, there have been a limited number of human intervention studies with berries and none of these have investigated the bioactivity of red raspberries.

Frozen, whole red raspberries were used for treatments because, 1) freezing lengthens shelf-life, enabling consistency of treatments, 2) whole raspberries have an appealing taste, eliminating the need to improve palatability with added sugars, and 3) whole foods are nutritious and an important part of a healthy diet, validating their investigation over that of berry extracts. However, the use of whole foods is not conducive to the use of placebo controls. Therefore, in study 1, treatment effects were compared to the non-treatment effects of the background diet. In study 2, bread was selected as a negative control treatment because 1) it is devoid of antioxidants and phytochemicals, 2) its macronutrient composition is primarily composed of carbohydrates, and 3) it has previously been used as a standard food for comparison in the nutrition literature. Bread plus vitamin C was used as a positive control to verify the effectiveness of antioxidants on oxidative stress in the context of the study 2 design.

Effects of red raspberry consumption were measured on a healthy adult population because 1) they are the target population for prevention of chronic disease, 2) it is a large population, easy to recruit, and 3) results are generalisable and can be used to direct future research in at risk populations. Effects on TEAC, thiols and TBARS were measured because 1) they are commonly used indicators of oxidative stress [216], 2) they are quick and cheap assays, and 3) they each measure a different extracellular target of oxidative stress in the blood, total AOC, protein oxidation, and lipid peroxidation, respectively. Protective effects of dietary antioxidants have been more successful in interventions on populations with elevated oxidative stress. Since energy metabolism is a major source of free radical generation, one state of elevated oxidative stress is following food consumption. Therefore, in study 1, treatments were consumed with meals and chronic effects on fasting oxidative stress were measured. Whereas, in study 2,
treatments were consumed alone and acute effects on post-prandial oxidative stress were determined. Therefore, both studies were designed with the intent to give a different perspective on the effect of raspberry consumption on oxidative stress in healthy adults.

Chronic (short-term interventions) and acute (single-dose interventions) studies on berry consumption have been reported in the literature and were considered in the design of study 1 and 2, respectively. Chronic studies with a primary objective to investigate effects on oxidative stress were 2-4 weeks in duration [45, 46, 156, 163, 164, 181], and had samples sizes from 12-28 subjects [45, 46, 156, 164, 181, 217], and studies with whole berries used doses that ranged from approximately 1-4 cups [103, 158, 164, 218]. This justifies the intervention duration of 2-4 weeks and sample size of 24. Since, no studies had previously investigated the effect of dose from chronic exposure to berries, doses of 1, 2 and 4 cups were investigation for dose effects because they had been previously reported as a treatment dose in the literature. During the first phase of the investigation all participants consumed one cup of red raspberries daily for two weeks because effects 1) had full statistical power (n=24), and 2) could be used as a baseline effect for dose effect comparisons. Stratification of subjects into treatment dose groups for the second half of the intervention resulted in 1) a loss of power (n=8), but 2) randomization controlled for time effects between doses.

Similar acute studies, looking at post-prandial oxidative stress following berry consumption used sample sizes from 5-10 subjects and measured in vivo AOC between 0.5-6 h post-intake of berries consumed alone or with a meal [102, 158, 161]. This justifies the use of 8 subjects, and the measure of oxidative stress biomarkers over 8 h post-intake. Dose effects of 1, 2 and 4 cups red raspberries were investigated similar to study 1; therefore, berries were consumed alone due to the satiating effect of the 4cR dose.

The tertiary objective to investigate the bioavailability of red raspberry antioxidants also influenced the study design. In study 1, overnight 12 h urine collections following raspberry consumption were provided 1) to quantify recovery of anthocyanins and their metabolites, while 2) minimizing the participant burden of a 24 h collection. In study 2, 24 h urine collections were provided to quantify recovery of anthocyanins and their metabolites, but were separated into 0-8 h and 8-24 h fractions because 1) recovery of anthocyanins is almost complete by 8 h post-intake [97], and 2) recovery of colonic metabolites typically does not begin until after 8 h post-intake
Anthocyanins appear in the blood within 15 min, peak at 1 h, and plateau around 8 h [97]. Therefore, measures from these time-points are important to investigating anthocyanin bioavailability, explaining the collection of blood samples at 15 and 30 min, as well as at 1, 2, 4, 6 and 8 h post-treatment.

The primary objective was to investigate the effect of red raspberry consumption on oxidative stress. However, human interventions with berry fruits have found other bioactivities on health biomarkers associated with CVD risk, including CRP [219], cholesterol levels [165, 166, 181], and blood pressure [165, 220]. Additionally, the protective effect of anthocyanins against diabetes and obesity has been demonstrated in animals but not humans [194, 221]. Therefore, measures of CRP, lipid profile, blood pressure, fasting glucose and body weight were also tested for an effect from chronic exposure as supplementary objectives in study 1.
Chapter 4

Methodology
9 Study 1 Clinical Protocol

9.1 Study Design

This was a 10-week, parallel intervention. Twenty-four healthy adults (12 male and 12 female) meeting the selection criteria of the study, consumed IQF red raspberries for four weeks. Week -1 was the pre-study phase to assess baseline characteristics. Week 0 was the washout phase, when participants began avoiding the consumption of anthocyanin and ellagitannin-containing food sources, which was continued for the duration of the study. Weeks 1-4 were the treatment phase. During the first two weeks all participants consumed one-cup red raspberries (1cR) daily, and during the second two weeks all participants were randomized to consume 1cR, 2cR or 4cR daily. Weeks 5-8 were the post-treatment washout or non-treatment phase. Study visits occurred at the end of each phase. Food records were completed for each of the seven days during week -1, and one-day food records were completed the day prior to each study visit. Twelve-hour urine collections were collected before each visit; and during each visit a raspberry consumption questionnaire was answered, and body measurements of weight and blood pressure, as well fasting blood samples were taken. The study protocol is shown in Figure 2 and all participant forms from study 1 are available in Appendix 1.

9.2 Ethics Approval, Recruitment and Study Location

Ethics approval was obtained from the University of Toronto, Health Sciences Research Ethics Board (REB) in April of 2008 (entitled: Raspberries and Human Health; REB # 22069). This study also obtained approval from the St. Michael’s Hospital REB in January of 2009 (REB # 08-306). Recruitment took place at these two locations using poster advertisements, as well as via an online posting on the http://my.utoronto.ca bulletin. The goal was to recruit 24 healthy adult subjects (12 male and 12 female). All responders were contacted by telephone and given a brief description of the study protocol, if interested they attended an initial consultation in order to determine their eligibility. No participants were excluded due to ineligibility. Meetings with participants were held at the Clinical Nutrition and Risk Factor Modification Centre, a part of St. Michael’s Hospital in Toronto, Ontario. All participant forms can be found in Appendix 1.
9.3 Screening, Exclusion Criteria and Enrolment

To determine eligibility, answers to a health questionnaire, as well as measurements of blood pressure, body weight and height, were assessed at an initial consultation. Potential participants would have been excluded if they were ≥45 yrs old, obese (BMI ≥32 kg/m²), had high blood pressure (>140/90 mm Hg), a history of chronic disease, or were currently taking medication(s). They would also have been excluded if they were pregnant, lactating or had a suspected allergy to raspberries. Signed consent was obtained from participants during the initial consultation after having read over the REB consent information form, having had the study protocol explained verbally, and having been given the opportunity to ask questions. There were two study dropouts, one due to distaste for the raspberry treatment and the other due to relocation for work. Replacements were recruited and a total of 24 participants completed the study.

9.4 Standard Clinic Visit for Participants

Study visits to the clinic lasted approximately 20 min. Participants came to the clinic after an overnight fast with their urine collection and food record. They sat and spoke with the study coordinator, who inquired about time of urine collection, missed voids, enjoyment of raspberries, symptoms, illness and completion of the diet record. The study coordinator took the participant’s blood pressure, weight and height measurements, and a registered nurse took a fasting blood sample. Participants were provided with a clean urine collection container, and reminded of their participation requirements for the next phase of the study, as well as the time of their next study visit.

9.5 Raspberry Supplement

The red raspberries used in study 1 had been individually quick frozen (IQF) by a commercial processing plant and were donated by the British Columbia Berry Council. They were stored at -20 °C until distributed to participants in sealed plastic bags. Participants were directed to store the berries in their home freezer, removing only a day’s serving to thaw at a time. Servings were measured as a heaping standard cup. Berries could not be heated or microwaved, but could be thawed, and were to be consumed in a single serving with dinner. Participants were asked to record any missed days of treatment, and any symptoms experienced. Compliance was high, as only one participant reported having missed a day.
9.6 Questionnaires

Questionnaires were used to obtain and track information throughout the study. A demographic, health and lifestyle questionnaire was given to participants to fill-out during their initial consultation. Information on age, sex, medical history, medication use, smoking status, alcohol consumption, and exercise habits was collected. The study coordinator filled out a raspberry consumption questionnaire with participants during every study visit, except the week 1 washout visit, regarding their personal enjoyment of red raspberries, how and when they consumed the raspberries, the number of missed treatment days, and the experience of any symptoms or illness. A urine collection sheet was also used by the study coordinator to record volume, start and end time, and number of missed voids, as reported by participants.

9.7 Body Measurements

Measurements of blood pressure, and weight and height for the calculation of Body Mass Index (BMI), were taken at the initial consultation and all study visits. Blood pressure was measured with a digital blood pressure monitor (OMRON IntelliSense®, HEM-907XL), which uses the oscillometric method. The occluding cuff was wrapped around the participant’s non-dominant arm, and systolic and diastolic measurements were taken three times, with one minute intervals, and the average values were recorded. Subjects were weighed fasting, without shoes, in indoor clothing on a clinical mechanical balance beam scale and recorded to the nearest hectogram. Height was measured to the nearest millimeter.

9.8 Dietary Restrictions, Food Records and Assessment

To minimize participants’ exposure to anthocyanins and ellagitannins during the study, participants were asked to avoid the consumption of most red, blue or purple coloured fruits, vegetables and their related products, as well as avoid nuts, seeds, herbal teas and nutritional supplements. A list of the foods to avoid can be found in Appendix 2.

Food records were recorded at baseline to analyse participants’ typical diet. They were also recorded the day before each study visit to monitor compliance to dietary restrictions. Instructions for completing a food record, as well as blank food record sheets, were provided. Participants were asked to record the time, quantity and give a detailed description of all food consumed during the pre-specified day.
All food records were inputted into the Diet Analysis Plus 8.1 software. Records were used to assess dietary trends and changes, such as average daily number of cups of fruits and vegetables consumed.

9.9 Blood Collection, Processing and Storage

Fasting blood samples were taken at each study visit for the measurement of health biomarkers. Registered nurses collected ~40 mL by venipuncture into three 10 mL red-top no additive BD Vacutainers® and two 6 mL pink-top BD Vacutainers® containing 10.8 mg K$_2$EDTA. Pink-top tubes were stored away from light in the fridge at 4 °C, and red-top tubes were wrapped in aluminum foil and left on the counter for an hour to coagulate. Blood was separated by centrifugation at 2000 rpm for 10 min at 4 °C. Serum from the red-top tubes and plasma from the pink-top tubes were aliquoted into 1.5 mL microcentrifuge tubes. Samples were stored at –20 °C for three to five days, and transferred on dry ice to -80 °C until analysed.

9.10 Urine Collection, Processing and Storage

Twelve-hour urine collections were obtained from participants prior to each study visit for the analysis of red raspberry polyphenolic metabolites. Special containers were provided at the previous clinic visit. Collections were to begin following dinner; and during the raspberry intervention period, the entire raspberry dose was to be consumed with dinner. The study coordinator measured and recorded the total volume of the urine to the nearest centiliter. Urine samples were aliquoted into three 1.5 mL microcentrifuge tubes. Samples were stored at -20 °C for three to five days, and then transferred on dry ice to -80 °C until analysed, with a temporary period of storage at -20 °C during this time.
Figure 2. Study 1 Design.

AM = Anthropometric Measures (blood pressure, body weight, height)
fBS = fasting Blood Sample
UC = 12hr Urine Collection
FR = Food Record (7d or 1d)
Q = Questionnaire (health or raspberry)
10 Study 2 Clinical Protocol

10.1 Study Design

This was an acute intervention. Treatments of one, two and four cups IQF red raspberries, or two slices of white bread with and without a vitamin C supplement, were tested in eight healthy adult participants (5 male and 3 female). All treatments were randomly given in a cross-over design with one-week intervals, following a two-day washout period, whereby participants consumed a low polyphenol diet. Urine samples were provided before each visit, and all urinary voids were collected during the 8 h study visit, as well as after the visit, until 24 h post-treatment. Blood samples were taken at each visit, prior to treatment and at 0.25, 0.5, 1, 2, 4, 6 and 8 h post-treatment. A standard high-fat lunch was provided after the 4 h blood sample and water was provided ad libitum. The study visit protocol is shown in Figure 3. Nutrition information for the bread treatments can be found in Table 3, the raspberry treatments in Table 4 and the standard lunch in Tables 5. All participant forms from study 2 are available in Appendix 2.

10.2 Ethics Approval, Recruitment and Study Locations

Ethics approval was obtained from the St. Michael’s Hospital REB (entitled: Pharmacokinetics of Red Raspberries; REB # 09-297) and the University of Toronto Health Sciences REB (REB # 24774) in February of 2010. Recruitment took place at these two locations using poster advertisements, as well as via an online posting on the http://my.utoronto.ca bulletin. The goal was to recruit 10 healthy adult subjects (5 males and 5 females). All responders were contacted by telephone and given a brief description of the study protocol, if interested they attended an initial consultation in order to determine their eligibility. One individual was determined ineligible at consultation due to age. Meetings with participants were held at the Clinical Nutrition and Risk Factor Modification Centre, a part of St. Michael’s Hospital in Toronto, Ontario.

10.3 Screening, Exclusion Criteria and Enrolment

To determine eligibility, answers to a health questionnaire, as well as measurements of blood pressure, body weight and height, were assessed at an initial consultation. Note that the same demographic, health and lifestyle questionnaire, as well as the same body measurement...
equipment and techniques were used as previously described for study 1 (see sections 9.5 and 9.6). Potential participants were excluded if they were >30 yrs old, overweight (BMI ≥25 kg/m²), had high blood pressure (>120/80 mm Hg), a history of chronic disease, or were taking medication(s). They would also have been excluded if they were pregnant or lactating, or had a suspected allergy to raspberries. Signed consent was obtained from participants during the initial consultation after having read over the REB consent information form, having had the study protocol explained verbally, and having been given the opportunity to ask questions. There was one study dropout due to personal reasons. A replacement was recruited, who completed four of five study visits. A total of eight participants (5 males and 3 females), including the replacement, completed the study and were used in the analysis.

10.4 Standard Clinic Visit for Participants

Study visits to the clinic lasted approximately 8 h and 30 min. Participants arrived after an overnight fast and provide a urine sample of their second morning void. A registered nurse inserted a catheter into their brachial vein to be used for blood collection throughout the day. After a baseline fasting blood sample was taken, they received their treatment breakfast to be consumed within 15 min, end of consumption marked zero time. Additional blood samples were taken at 0.25, 0.5, 1, 2, 4, 6 and 8 h. All urinary voids during the study visit were collected in the provided container. Water was provided ad libitum and a standardized high fat lunch was provided after the 4 h blood sample. A pictorial representation of the clinic visit protocol is presented in Figure 2. At the end of the study visit participants were provided with a second container to be used at home for their 8-24 h urine collection, and returned to the clinic the following morning.

10.5 Raspberry and Control Treatments

The red raspberries used in study 2 were of the same cultivar and harvest, but different from those used in study 1. They too had been individually quick frozen (IQF) by a commercial processing plant and were donated by the British Columbia Berry Council. They were stored at -20 °C until the night before their use. Treatment doses of 125, 250 and 500 g red raspberries were measured frozen with an electronic kitchen scale, and left in the fridge overnight to thaw. Participants were instructed to consume all the thawed berries and their juices.
The white bread used for the control treatments was made with enriched white flour and purchased from a local grocery store. Participants were given two plain slices of the white bread and a glass of water to consume. For the bread plus vitamin C treatment, 200 mg of an ascorbic acid fine powder, donated by Jamieson Laboratories Limited (Windsor, Ontario) was dissolved in the water provided. This quantity is comparable to that found in the four cup red raspberry treatment.

10.6 Dietary Restrictions and Food Records
For the two days before each study visit and 24 h post-treatment, participants were asked to consume an animal-based, low polyphenol diet, while keeping a food record to monitor compliance. Allowable foods primarily consisted of: white rice, white bread, meat, poultry, fish, cheese, and milk, a complete table of the dietary guidelines can be found in Appendix 2. Compliance was high, with only one incident; one participant consumed seaweed once while on the low polyphenol diet.

10.7 Blood Collection, Processing and Storage
For blood collections, participants were cannulated with an intravenous catheter in the brachial vein by a registered nurse. At each blood draw approximately 6 mL of blood was collected into a 10 mL green-top BD Vacutainer® spray-coated with sodium heparin. Tubes were covered in foil and left at room temperature for <1 h before processing. To process, they were centrifuged at 1500 rpm for 10 min at 4 °C. Plasma was aliquoted into black microcentrifuge tubes. Aliquots for vitamin C analysis were acidified with 6% metaphosphoric acid containing 1 mmol diethylenetriaminepentaacetic acid (DTPA) in a 1:1 ratio [222]. Aliquots for polyphenolic analyses were acidified with 15 μL of 50% formic acid and 50 μL of 10 mmol/L ascorbic acid, per mL of plasma, method modified from Mullen et al. [223]. Additional aliquots were for analysis of oxidative stress biomarkers. All samples were flash frozen in liquid nitrogen and stored at -20 °C for 3 to 5 days then transferred on dry ice to -80 °C until analysed.

10.8 Urine Collection, Processing and Storage
A baseline sample of participants’ second morning void was collected at the beginning of each study visit. Following consumption of the treatment breakfast, all voids during the following 8 h
study visit were collected in a container as a pooled sample. At home from 8 to 24 h post-treatment, all urinary voids were collected in another container as a second pooled sample. The study coordinator measured and recorded the total volume of the urine samples and collections to the nearest 10 mL. Urine was aliquoted into 15 mL conical centrifuge tubes and acidified with 50% formic acid in a 100:1 ratio, method modified from Bitsch et al. [224]. Samples were flash frozen in liquid nitrogen and stored at -20 °C for 3 to 5 days then transferred on dry ice to -80 °C until analysed.
Figure 3. Study 2 Clinic Visit Design.
Table 3. Nutrition information for the bread used as control treatments from study 2.

<table>
<thead>
<tr>
<th>Nutrition Facts*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serving Size 2 slices (84.0 g)</td>
</tr>
<tr>
<td><strong>Amount Per Serving</strong></td>
</tr>
<tr>
<td>Calories 200</td>
</tr>
<tr>
<td>Total Fat 2.0 g</td>
</tr>
<tr>
<td>Saturated Fat 0.4 g</td>
</tr>
<tr>
<td>Sodium 390 mg</td>
</tr>
<tr>
<td>Total Carbohydrates 37.0 g</td>
</tr>
<tr>
<td>Dietary Fibre 3.0 g</td>
</tr>
<tr>
<td>Sugars 3.0 g</td>
</tr>
<tr>
<td>Protein 8.0 g</td>
</tr>
<tr>
<td>% Daily Value (based on a 2000 calorie diet)</td>
</tr>
<tr>
<td>Vitamin A 0%</td>
</tr>
<tr>
<td>Vitamin C 0%</td>
</tr>
<tr>
<td>Calcium 6%</td>
</tr>
<tr>
<td>Iron 15%</td>
</tr>
</tbody>
</table>

* referenced from Dempster’s Soft Slice® packaging.
**Table 4.** Nutrition information for red raspberry treatments from study 2.

<table>
<thead>
<tr>
<th>Serving Size:</th>
<th>Nutrition Facts*</th>
</tr>
</thead>
<tbody>
<tr>
<td>125 g</td>
<td>250 g</td>
</tr>
<tr>
<td>Calories:</td>
<td>65</td>
</tr>
<tr>
<td>Total Fat:</td>
<td>0.8 g</td>
</tr>
<tr>
<td>Saturated Fat:</td>
<td>0 g</td>
</tr>
<tr>
<td>Sodium:</td>
<td>1 mg</td>
</tr>
<tr>
<td>Total Carbohydrates:</td>
<td>15 g</td>
</tr>
<tr>
<td>Fibre:</td>
<td>8 g</td>
</tr>
<tr>
<td>Sugars:</td>
<td>6 g</td>
</tr>
<tr>
<td>Protein:</td>
<td>1.5 g</td>
</tr>
</tbody>
</table>

% Daily Value (based on a 2000 calorie diet)

| Vitamin A:    | 0.25%            | 0.5%             | 1%               |
| Vitamin C**:  | 78% (47 mg)      | 156% (94 mg)     | 312% (188 mg)    |
| Calcium:      | 3%               | 6%               | 12%              |
| Iron:         | 6%               | 12%              | 24%              |

* referenced from USDA Nutrient Database [58].

** referenced from laboratory analysis of study 2 red raspberries.
Table 5. Nutrition Information for the high-fat standard lunch from study 2, served after 4 h post-treatment.

<table>
<thead>
<tr>
<th>Nutrition Facts*</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Food Item:</strong></td>
<td><strong>Big Mac® sandwich</strong></td>
<td><strong>French Fries, Medium</strong></td>
<td><strong>1% Partly Skimmed Milk</strong></td>
</tr>
<tr>
<td><strong>Serving Size:</strong></td>
<td>209 g</td>
<td>113 g</td>
<td>200 mL</td>
</tr>
<tr>
<td><strong>Calories:</strong></td>
<td>430</td>
<td>360</td>
<td>90</td>
</tr>
<tr>
<td><strong>Total Fat:</strong></td>
<td>19 g</td>
<td>17 g</td>
<td>2 g</td>
</tr>
<tr>
<td><strong>Saturated Fat:</strong></td>
<td>8 g</td>
<td>2 g</td>
<td>1 g</td>
</tr>
<tr>
<td><strong>Cholesterol:</strong></td>
<td>60 mg</td>
<td>0 mg</td>
<td>10 mg</td>
</tr>
<tr>
<td><strong>Sodium:</strong></td>
<td>38 mg</td>
<td>11 mg</td>
<td>4 mg</td>
</tr>
<tr>
<td><strong>Total Carbohydrates:</strong></td>
<td>38 g</td>
<td>11 g</td>
<td>4 g</td>
</tr>
<tr>
<td><strong>Fibre:</strong></td>
<td>2 g</td>
<td>4 g</td>
<td>0 g</td>
</tr>
<tr>
<td><strong>Sugars:</strong></td>
<td>6 g</td>
<td>0 g</td>
<td>10 g</td>
</tr>
<tr>
<td><strong>Protein:</strong></td>
<td>24 g</td>
<td>4 g</td>
<td>7 g</td>
</tr>
<tr>
<td><strong>% Daily Value</strong> (based on a 2000 calorie diet)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin A:</td>
<td>8%</td>
<td>0%</td>
<td>8%</td>
</tr>
<tr>
<td>Vitamin C:</td>
<td>2%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Calcium:</td>
<td>20%</td>
<td>2%</td>
<td>25%</td>
</tr>
<tr>
<td>Iron:</td>
<td>30%</td>
<td>6%</td>
<td>0%</td>
</tr>
</tbody>
</table>

* referenced from McDonald’s® website (www.mcdonalds.ca/en/food/calculator.aspx)
11 Laboratory Procedures for Biomarkers

11.1 Clinical Biomarkers for Chronic Disease Risk

Analysis of fasting plasma samples for clinical biomarkers were completed by St. Michael’s Hospital’s Diagnostics Laboratories’ biochemistry service (Toronto, ON, CAN) using the Beckman SYNCHRON LX System. In brief, glucose was determined by an oxygen rate method; total cholesterol and triglycerides by a timed-endpoint method; HDL-C by a homogenous assay; LDL-C was calculated; and CRP was complexed with an antibody and measured by turbidimetric method.

11.2 Oxidative Stress Biomarkers

11.2.1 Antioxidant Capacity (AOC)

The TEAC-ABTS\(^{++}\) (Trolox Equivalent Antioxidant Capacity) assay is based on the ability of antioxidant molecules in the sample to quench the long-lived ABTS\(^{++}\) (2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid radical cation), a blue-green chromophore with characteristic absorption at 734 nm. Decolourisation occurs after the addition of antioxidants to the preformed radical cation, which becomes reduced to the colourless ABTS compound. Results are expressed in Trolox Equivalents (TE) using a standard curve.

A modified version of ABTS\(^{++}\) colourimetric assay was used [225]. A stable stock solution of ABTS\(^{++}\) was produced by reacting 7.4 mmol/L aqueous solution of ABTS with 2.45 mmol/L potassium persulfate (final concentration). The mixture was stored in the dark at 4 °C for 12-60 h before use to allow for colour development. An ABTS\(^{++}\) working solution was produced by dilution of the stock solution with double distilled water (ddH\(_2\)O) to an absorbance of 0.75±0.05 at 734 nm (Ultrrospec 2000, Pharmacia Biotech). The absorbance or optical density (OD) of the working solution was recorded, and then the serum or plasma sample was added in a ratio of 1:150. The change in OD after 1 min was used to calculate the TEAC, expressed in mmol of Trolox per L of sample. The coefficient of variation (CV) for serum samples from study 1, analysed in triplicate, was 1.35%, and for plasma samples from study 2 was 3.23%.
11.2.2 Protein Oxidation

The loss of a protein’s sulfhydryl groups or thiols (-SH) is indicative of oxidation. A colourimetric method developed by Hu [226] uses DTNB (5,5’-Dithio-bis(2-nitrobenzoic acid)) for the determination of protein thiols in plasma as a biomarker for oxidative stress. The DTNB is cleaved by the thiol and a mixed disulfide of one NTB moiety with the thiol is formed, which has an intense absorption band at 412 nm.

A modified version of the DTNB colourimetric assay for determination of thiols [226] was used. Serum from study 1 or plasma from study 2, were diluted with 0.25 M Tris Buffer containing 20 mM of the disodium salt of ethylene diamine tetraacetic acid (EDTA) and incubated with 10mM DTNB in absolute methanol for 15 min at room temperature. After centrifugation at 3,000 rpm for 10 min at 4 °C, the supernatant’s OD was read at 412 nm (Ultrospec 2000, Pharmacia Biotech). Protein thiols were measured for each sample in triplicate, against the OD of a DTNB blank sample. Results were calculated with a molar absorption coefficient of 13 600 M⁻¹ cm⁻¹ per thiol and expressed as total thiols in μmol per L of sample. The CV for serum samples from study 1 was 3.05%, and for plasma samples from study 2 was 3.31%.

11.2.3 Lipid Peroxidation

RONS degrade polyunsaturated lipids, forming malondialdehyde (MDA), which is used as a biomarker for oxidative stress. In the TBA-MDA assay, two equivalents of thiobarbituric acid (TBA) condense with MDA and other “thiobarbituric acid reactive substances” (TBARS) to give a fluorescent red derivative which can be measured spectrophotometrically [227].

Serum or plasma samples were incubated for 45 min at 90 °C with 10mM butylated hydroxyl toluene (BHT) in ethanol, 1% orthophosphoric acid and 0.6% TBA in 0.1 N HCl. After being cooled to room temperature, n-butanol was added and the sample was vortexed then centrifuged at 3,000 rpm for 10 min at 4 °C. The supernatant’s OD was read at 535 nm (Ultrospec 2000, Pharmacia Biotech). The concentration of TBARS was calculated with a molar absorption coefficient of 156 000 M⁻¹ cm⁻¹ and expressed as TBARS in μmol per L of sample. The CV for serum samples from study 1 was 2.37%, and for plasma samples from study 2 was 4.56%.
11.3 Nutrition Biomarkers

11.3.1 Ascorbic acid Extraction and Quantification by HPLC-DAD

Plasma samples from study 2, acidified at time of storage in a 1:1 ratio with 6% MPA containing 1 mmol DTPA, were used for the analysis of vitamin C concentration. Samples were thawed and centrifuged at 15 700 xg for 1 min. The supernatant was filtered through 0.45μ PVDF syringe filters and aliquoted into brown vials. Reversed-Phase High Pressure Liquid Chromatography-Diode Array Detector (RP-HPLC-DAD) method modified from a LifeLabs protocol [228] was used for analysis. The HPLC system (Model 110, Agilent Technologies, Palo Alto, CA, USA) was equipped with a quaternary pump (G1311A), an autosampler (G1313A), and a DAD (G1315B). A reversed-phase C-18 column (Phenosphere ODS (2), 5 μm particle size, 150 X 4.6 mm; Phenomenex, Torrance, CA, USA) was used for separation. The mobile phase was 0.1% metaphosphoric acid operated isocratically at a flow rate of 1.5 mL/min. Samples and standards were injected at a volume of 10 μL and detected at 265 nm. Peak areas were converted to μmol/L using a standard curve.

11.3.2 Polyphenol Extraction

Plasma and urine samples from study 2, acidified at time of storage with formic acid, as well as urine samples from study 1, were used for the analysis of red raspberry polyphenols. A modified extraction method reported by Bitsch et al. [229] was used. Plasma samples were thawed and centrifuge at 15 700 xg for 1 min, and then the supernatant was filtered through 0.45μ PVDF syringe filters. Urine samples were thawed and centrifuged at 3220 xg for 3 minutes. A Polymeric RP-Solid Phase Extraction (SPE) 96-well plate (Strata-X, 33 μm particle size, 30 mg/well; Phenomenex) was used to extract the polyphenols in the samples. Under vacuum, the columns were conditioned with 5% formic acid in methanol, and then equilibrated with an aqueous solution of 5% formic acid. The sample supernatants (1.5 mL plasma from study 2, 6 mL urine from study 2 or 3 mL urine from study 1) were loaded onto the plate and slowly vacuumed through the columns, followed by a rinse with 5% formic acid in water, and a slow elution with 5% formic acid in methanol. The elute was partially evaporated under fumehood for 24-72 h, then evaporated to dryness with a centrifugal vacuum concentrator (Heto Maxi Dry-Lyo), and stored at -80 °C. The residual was redissolved in 200 μL of 5% formic acid in
methanol by vortex. Extracts were centrifuged at 15 700 xg for 1 min, filtered through 0.2μ PVDF syringe filters, aliquoted into brown HPLC vials, and stored at -80 °C until analysed.

11.3.3 Polyphenol Quantification by U-HPLC

An Ultra-High Performance Liquid Chromatography (U-HPLC) system (Thermo Scientific Accela™, San Jose, CA) with a quaternary pump, a thermostatic autosampler, and a photodiode array (PDA) detector was used for analysis of polyphenolic metabolites in blood and urine samples. A reversed-phase C-18 column (Phenomenex Kinetex®, 2.6 μm particle size, 100 Å pore size, 100 X 4.6 mm; Phenomenex, Torrance, CA, USA) was used for separation. Method was modified from McCallum et al. [230]. The binary mobile phase consisted of 5% formic acid (A) and 95% methanol + 5% formic acid (B). The column was equilibrated with 10% B, and a gradient program from 10 to 100% B in 25 min was used. 100% B was maintained for 1 min, and then back to 10% B in 2.5 min, which was maintained for 5 min. The flow rate was 1 mL/min for a total run time of 33.5 min. The injection volume was 10 μL for all samples and standards. Identification of compounds was attempted by comparing retention times with standards. Quantification of total metabolites was carried out by integration of the peak areas at 520 nm. Values were converted to cyanidin equivalents (CE) using a standard curve and total urinary metabolites were expressed in nmol.

11.4 Red Raspberry Compositional Analysis

11.4.1 Ascorbic acid

For extraction, 60 g of frozen red raspberry sample (from study 2, stored at -80 °C) was blended with ~150 mL 0.8% metaphosphoric acid using an electric blender (Waring), a method modified from Sanchez Moreno et al. [231]. The slurry was vortexed, mixed on a shaker for 10 min, and then centrifuged at 3220 xg for 5 min. The supernatant was transferred to a volumetric flask and the pellet was similarly extracted two more times. The combined supernatants were made to a final volume of 500 mL, and then a portion was filtered through a 0.45 μm syringe filter, aliquoted into brown HPLC vials and stored at -80 °C until analysed. Extraction was performed in duplicate. Samples were shielded from light. The HPLC conditions were the same as for analysis of the plasma samples (see section 11.3.1). Peak area was converted to mg/100g fresh weight using a standard curve.
11.4.2 Anthocyanins

For extraction, 10 g of red raspberry sample (from study 2, stored at -80 °C) was blended with 100 mL acidified methanol (1% HCl) using an electric blender (Waring), a method modified by de Ancos et al. [232]. The slurry was filtered through no. 2 filter paper, which was rinsed with another 100 mL of acidified methanol (1% HCl). The extract was evaporated by rotary evaporator at 40 °C to a final volume of 25 mL and stored at -20 °C until analysed. Extraction was performed in duplicate.

LC-MS experiments were carried out using a Finnigan LCQ DECA ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. The samples were analysed in the same UPLC chromatographic condition as for the plasma samples (see section 11.3.3). Positive mode was selected for data collection. Before sample analysis, the instrument was tuned by using cyanidin standard to reach its optimum performance. As a result, the shear gas and auxiliary flow rates were set at 96 and 3 (arbitrary unit), respectively. The capillary voltage was set at 32.5 kV and its temperature was controlled at 350°C. The entrance lens voltage was fixed at –58.0 V and the multipole RF amplitude was set at 770 V. The ESI needle voltage was controlled at 5 kV. The tube lens offset was 55.0 V, the multipole lens 1 offset was -4.40 V and the multipole lens 2 offset was –8.00 V. The electron multiplier voltage was set at -1030 V for ion detection.
12 **Statistical Analysis**

All analyses, unless otherwise noted, were performed using Graphpad Prism version 4.02 for Windows (Graphpad Software, San Diego, California), with P<0.05 considered statistically significant.

12.1 Study 1

Baseline participant characteristics were reported as mean (SEM) for continuous variables, with the exception of age and CRP, which were reported as median (range). All categorical variables were reported as number of participants (%). The non-binomial categories’ cutoffs were arbitrarily selected to show the distribution within the study population. To test for differences in baseline characteristics between treatment groups, continuous variables were analysed by either analysis of variance (ANOVA), or the nonparametric Kruskal-Wallis test. The chi-squared test was used for all categorical variables.

All effect analyses compare biomarker levels during the intervention (week 2 and 4) with each other and to levels before (week 0) and after treatment (week 8). The baseline values were not used. Values from the same participant were matched. Due to small sample size (n=24; n=8 by treatment group) and limitations in the statistical methods used, missing values or excluded outliers were replaced, as defined by the situation. Data on all health biomarkers measured in the plasma were missing for one participant, from one study visit. These missing values were replaced with the average of the value before and after the missing time-point.

All data was checked for outliers with Grubb’s Test (also known as the extreme studentised deviate, or ESD method), using GraphPad Software’s free online QuickCalcs program (http://www.graphpad.com/quickcalcs/). A CRP, HDL-C and HDL-C/LDL-C ratio outlier from the same person and same visit were identified and explained by illness. Two additional CRP outliers from sick participants were identified. These values were excluded and replaced with the average of the value before and after the time-point. For total 12 h urinary metabolites (t12UM), one significant outlier was identified and excluded from relevant analyses. The high levels could be explained by the participant having consumed a large fruit salad with their raspberry dose before week 2’s study visit.
Deviation from a Gaussian distribution was tested for with the D’Agostino-Pearson omnibus K2 normality test. Data which did not pass the normality test were visually inspected to determine whether failure was attributable to chance. The distributions of both CRP and TG values had pronounced lower limits, the first being attributable to a methodological limit of detection, and the second a physiological plateau. Attempts to normalize the data by logarithmic and reciprocal transformations were unsuccessful. Therefore, these datasets were analysed with nonparametric tests.

To answer the primary objective of whether raspberry consumption has an effect on health biomarkers, time effects (tE), by treatment group or pooled, were analysed by one-way repeated measures analysis of variance (RM ANOVA). Time-points (week 2, 4 and 8) were compared to week 0 by Dunnett’s post-test for multiple comparisons. Delta time effects during the intervention only, were compared by two-tailed one sample t-test. If a linear relationship was visible post hoc, the trend was tested for by linear regression. The Friedman two-way analysis of variance by ranks test, performed in combination with the Dunn’s posttest, was used as the nonparametric alternative.

To answer the secondary objective of whether effects on health biomarkers varied by raspberry dose, treatment effects (TE) were analysed by two-way RM ANOVA. Treatment groups (2cRg and 4cRg) were compared to 1cRg by Bonferroni post-test for multiple comparisons. Delta treatment effects during the intervention only, were compared by one-way ANOVA. No nonparametric test alternatives were available through the selected software.

To answer the tertiary objective of whether red raspberry polyphenols are (a) bioavailable and (b) bioactive, the total 12 h urinary metabolites (t12UM), expressed in nanograms of cyanidin equivalents (CE), were analysed for treatment effect (TE) and their correlation with oxidative stress biomarkers. Treatment effects were determined between treatment groups (comparing 1cRg, 2cRg and 4cRg) by one-way ANOVA, and within treatment groups (comparing week 2 and 4) by paired, two-tailed t-test. Pearson’s correlation compared t12UM with fasting serum AOC, thiols and TBARS levels from weeks 2 and 4.
12.2 Study 2

Baseline participant characteristics were reported as mean (SEM) for continuous variables, with the exception of age, which was reported as median (range). All categorical variables were reported as number of participants (%). The non-binomial categories’ cut-points were arbitrarily selected to show the distribution within the study population.

All effect analyses compare biomarker levels across time-points during each treatment, or between treatments. Levels are expressed as both mean absolute values and net area under the curve (AUC). Net AUC was calculated for each participant by study visit, as the sum of the area of peaks above and peaks below the mean baseline value. Net AUC was calculated over 2 h post-intake for plasma AOC and thiols, and over 4 h post-intake for plasma TBARS and ascorbic acid. Cutoffs were selected as the time-point after which levels stabilized across treatments. Values from the same participant were matched.

Due to small sample size (n=8), and limitations in the statistical methods used, missing values or excluded outliers were replaced, as defined by the situation. There was no data on one participant’s response to the vitamin C plus bread treatment because they were a replacement and only completed four of the five study visits. These missing values were replaced with averages for each time-point from the participant’s other four visits. Other missing data includes a missed blood collection for one participant and a missing TEAC and thiol result for another participant from two different time-points. All data was also checked for outliers with Grubb’s Test (GraphPad Software’s free online QuickCalcs program, http://www.graphpad.com/quickcalc/). A TBARS, thiol and ascorbic acid outlier were identified from the same participant, same visit and same time-point, explained by hemolysis. These additional missing values and three outliers were replaced with an average of the value before and after the time-point. All data sets were assumed to come from a Gaussian distribution.

To answer the primary objectives of whether raspberry consumption has a post-prandial effect on oxidative stress biomarkers, time effects (tE) were analysed by one-way RM ANOVA. Time-points were compared to baseline using Dunnett’s post-test for multiple comparisons.

To answer the secondary objective of whether effects on oxidative stress biomarkers varied by antioxidant dose, treatment effects (TE) were analysed by two-way RM ANOVA for mean
absolute values and one-way RM ANOVA for net AUC values. Treatments were compared to bread (B) using post-tests for multiple comparisons. Delta net AUC values were also compared to B and 1cR treatments by two-tailed one sample t-tests.

To answer the tertiary objective of whether red raspberry antioxidants are (a) bioavailable and (b) bioactive, plasma ascorbic acid and total 24 h urinary metabolites (t24UM) were analysed for treatment effect (TE) and their correlation with oxidative stress biomarkers. Plasma ascorbic acid was also analysed for time effect (tE). Plasma ascorbic acid tE and TE were analysed as described above. Pearson’s correlation compared plasma ascorbic acid with plasma AOC, thiols and TBARS. Total urinary metabolites was analysed using 24 h values and not the 8 and 16 h fractions separately, because their separation contributed no new information. Raspberry treatments were compared for t24UM by one-way RM ANOVA. Pearson’s correlation compared t24UM with mean plasma AOC, thiols and TBARS post-raspberry consumption.
Chapter 5

Results
13 Study 1

13.1 Baseline Participant Characteristics

Baseline characteristics of the study participants are presented in Table 6. The study participants (n=24) were healthy adults with a median age of 27 yrs, ranging from 19 to 44 yrs. Their mean BMI was 24.9±0.8 kg/m², which is within healthy range (BMI, 18.5-24.9 kg/m²; see Appendix 3 for clinical biomarker’s associated health risk). Seven participants were overweight (BMI, 25.0-29.9 kg/m²), and four were borderline class I obese (BMI, ≥30.0 kg/m²). Three of the four obese individuals had elevated CRP (1.0-3.0 mg/L); one was at high risk (>3.0 mg/L), and had a high TC/HDL-C (≥5.0). Average blood pressure was 114/66 mm Hg, which is in the healthy range (<120/80 mm Hg); one participant had elevated blood pressure in the normal to high range (130/85-139/89 mm Hg). None of the participants had a high fasting plasma glucose, but six were hypoglycemic (<4.0 mM) on at least one visit. Participants’ lipid profile levels were mostly within healthy range. Total cholesterol (TC) and triglycerides (TG) had healthy mean values of 4.02±0.18 and 0.70±0.06 mM, respectively (data not presented in Table 3). Two participants had slightly elevated levels of LDL-C (>3.5 mM), but were otherwise healthy with an overall mean of 2.44±0.14 mM. Half the men and half the women had low HDL-C levels (<1.0 and 1.3 mM for men and women, respectively), with means of 1.19±0.13 and 1.26±0.10 mM HDL-C for the men and women, respectively (data not presented in Table 3). Although mean TC/HDL-C ratios were healthy with a mean of 3.32±0.16 mM, with the exception of one elevated level already mentioned. All participants reported being in good health and free of chronic disease. Therefore, as expected, most participants’ had low risk levels of the measured clinical biomarkers (see Appendix 3).

The measured oxidative stress biomarkers are not used clinically and do not have recommended levels. Participants had a mean TEAC of 3.08±0.06 mM TE, and a mean of 399±13 µM protein thiols and 5.10±0.20 µM TBARS, all measured in fasting serum, and within the ranges previously detected in healthy subjects by our laboratory.

Qualitative information on lifestyle habits were collected and reported for insight only. Overall participants were nonsmokers and nondrinkers, with one participant being both a casual smoker and frequent consumer of alcoholic beverages. The majority of participants did not meet
recommended levels of weekly physical activity nor daily fruit and vegetable intake, according to the USDA MyPyramid guidelines [233]. MyPyramid recommends 30 min of physical activity most days of the week. For a 2000 calorie diet, 2 ½ cups of vegetables and 2 cups of fruit is recommended daily. Ten participants (42%) met the minimum recommendations for physical activity, with at least 2 h of physical activity weekly. Three participants (13%) consumed ≥4.5 cups of fruits and vegetables daily.

The randomization of participants into three treatment dose groups (n=8) did not result in significant heterogeneity between groups for any of the baseline characteristics measured.

13.2 Oxidative Stress Biomarkers

The effects of short-term, daily red raspberry consumption on fasting oxidative stress, as measured by serum TEAC, thiols and TBARS, are presented. Time effects (tE) are the change in oxidative stress levels between study visits, and address the primary objective to determine an effect of treatment. Treatment effects (TE) are the differences in oxidative stress levels between treatment groups, and address the secondary objective to investigate the effect of dose. Data are presented as mean values in bar graphs by study visit, stratified by treatment group to assess tEs and TE (n=8; see Figures 5, 7 and 8) and pooled to assess tE (n=24; see Figure 4). Delta values for week 4 compared to week 2, are also reported to assess tEs and TE, but during the intervention phase only (see Table 7).

For serum TEAC, there was an overall significant tE (P=0.0005, two-way RM ANOVA; see Figure 5), but no TE (P=0.20). The 1cRg had a significant tE (P<0.0001, one-way RM ANOVA) with week 2 and 8 levels significantly higher than week 0 (P<0.05 and 0.01, respectively, Dunnett’s post-test). The 4cRg had increases in serum TEAC at week 4 compared to week 2 (P=0.05, two-tailed one sample t-test; see Table 7), though the tE was not significant. Pooled data from week 0, 2 and 8 had a significant tE (P<0.001, one-way RM ANOVA; see Figure 8) with wk 8 levels significantly higher than week 0 (P<0.01, Dunnett’s post-test). Linear regression of pooled individual TEAC values was not significant (P=0.08; data not shown), but when analysed as mean values was significant for pooled data (P=0.005; see Figure 6a), 1cRg and 4cRg (P=0.01 and 0.02, respectively; see Figure 6b). Overall, there was a linear increase in TEAC across time, representing an increase in serum AOC and reduction in oxidative
stress. Therefore, the tE of raspberry consumption on TEAC cannot be separated from the confounding effect of time.

For serum thiols, overall there was no tE (P=0.10, two-way RM ANOVA; see Figure 7) and no TE (P=0.19). The significant tE in the 1cRg (P=0.0211, one-way RM ANOVA; see Figure 7) had significant reductions in thiols at week 2 and week 8 compared to week 0 (P<0.05, Dunnett’s post-test), representing an increase in protein oxidation and oxidative stress. Thiol levels increased in the 1cRg at week 4 compared to week 2 (P=0.076, two-tailed one sample t-test; see Table 7), although the tE was not significant and levels non-significantly lower than at week 0. Pooled data from week 0, 2 and 8 did not have a tE (P=0.10, one-way RM ANOVA; see Figure 4), therefore overall, 1cR did not result in reduced thiols after 2 weeks of intervention. The 4cRg had the greatest increases in thiols at week 4 compared to week 2 (P=0.171, two-tailed one sample t-test; see Table 7), though not significant. Therefore, there were no significant tEs on thiols toward reduced oxidative stress.

For TBARS, overall there was no tE (P=0.14, two-way RM ANOVA; see Figure 8) and no TE (P=0.92). The 1cRg had a significant tE (P=0.056, one-way RM ANOVA) with week 2 levels significantly lower than week 0 (p<0.05, Dunnett’s post-test), but this tE was not seen in pooled data (P=0.12, one-way RM ANOVA; see Figure 4), and did not remain significant at week 4 in the 1cRg. The 2cRg and 4cRg had reductions in TBARS at week 4 compared to week 2 (P=0.633 and 0.506, respectively, two-tailed one sample t-test; see Table 8), though not significant. Therefore, a significant tE of raspberry consumption on reducing serum TBARS, representing a decrease in lipid peroxidation and oxidative stress, was found but was not replicated.

13.3 Bioavailability Biomarker

The tertiary objective to investigate the bioavailability of red raspberry anthocyanins could not be fully addressed because mass spectrometry was unable to identify anthocyanin metabolites in the urine, due to low detection limits. However, dose effects on total 12 h urinary metabolites (t12UM) were analysed by comparing week 2 and week 4 levels, as well as week 4 levels between treatment groups. There were no significant dose effects in the 1cRg, 2cRg or 4cRg when comparing week 2 and week 4 levels (P=0.78, 0.80 and 0.059, respectively, two-tailed paired t-test; see Figure 9a). There were no significant dose effects at week 4 when comparing
absolute (P=0.21, one-way ANOVA) or delta levels (P=0.12, one-way ANOVA; see Table 7) between treatment groups. However, the 4cRg had the largest delta increase in t12UM at week 4 compared to week 2 (+58.5±25.1 ng CE, Table 7). Therefore, no significant dose effects on t12UM were found during the intervention phase.

13.4 Correlation of Bioavailability and Bioactivity Biomarkers

The quaternary objective to assess the relationship between red raspberry bioactivity and bioavailability of red raspberry anthocyanins was addressed by correlating t12UM with corresponding fasting levels of the oxidative stress biomarkers. There was a significant correlation with increased thiols (P=0.016, $r^2=0.13$, Pearson’s correlation; see Figure 8), representing reduced protein oxidation and oxidative stress. However, t12UM was not correlated with the other oxidative stress biomarkers, TEAC and TBARS (P=0.56 and 0.76, respectively). Therefore, higher tUM relates to reduced oxidative damage to proteins in the blood.

13.5 Clinical Biomarkers of Chronic Disease Risk

To address the study 1 supplementary objectives, the effects of short-term, daily red raspberry consumption on clinical biomarkers of chronic disease risk, including weight, blood pressure, fasting plasma glucose, lipid profile and CRP were analysed for tEs and TEs. Overall, there were no tEs and no TEs on weight, blood pressure, lipid profile or CRP levels (P>0.05, one-way and two-way RM ANOVA; data not shown). However, fasting plasma glucose, overall had a significant tE (P=0.026, two-way RM ANOVA; see Figure 11) and no TE (P=0.73). There was a tE in the 2cRg (P= 0.022, one-way RM ANOVA) with levels at week 2 and 8 significantly higher than week 0 (P<0.05, Dunnett’s post-test). The mean 2cRg values were tested for linear trend over time by regression, but the relationship was not significant (P=0.08, $r^2=0.69$; data not shown). Fasting plasma glucose levels were stable across time-points in the other treatment groups. The majority of hypoglycemic subjects at baseline were randomized to the 2cRg by chance. At week 8, only one, of the six previously hypoglycemic subjects, was hypoglycemic. Therefore, significant increases in fasting plasma glucose were related to the stabilization of fasting plasma glucose in hypoglycemic subjects, and effects were strongest at week 8, after the non-treatment phase.
13.6 Participant Perceptions

Self-reported, qualitative data on participants’ perceived like/dislike for the berries, satiety following consumption of the berries and symptoms experienced during the intervention phase are presented (see Table 8). Pooled data from week 2, following consumption of 1cR daily for two weeks, found 88% of participants reporting the taste of the red raspberries as *enjoyable*, and 100% reported the serving size as *not filling*. At week 4, TE on perceptions of taste non-significantly decreased with increasing dose (P=0.12, chi-squared test), and TE on satiety significantly increased with increasing dose (P=0.004). There was one report of diarrhea during the four cup intervention only, which was considered an undesirable symptom. There were ten reports of increased ease and/or frequency of bowel movements, and two reports of an increased feeling of well-being, which were considered desirable symptoms. Overall, the red raspberries were well tolerated during chronic exposure at serving sizes of 1 and 2 cups. At 4 cups, one negative symptom was reported, and three participants reported the serving size to be *very filling*. These findings contribute to the thesis’ supplementary objective to provide guidelines for future investigations.
Table 6. Study 1 baseline characteristics of study participants overall (n=24) and by group (n=8).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Descriptor</th>
<th>Overall</th>
<th>1cRg</th>
<th>2cRg</th>
<th>4cRg</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr), median (range)</td>
<td></td>
<td>27.0 (19 - 44)</td>
<td>26.0 (19 - 39)</td>
<td>28.0 (19 - 44)</td>
<td>25.5 (20 - 36)</td>
<td>0.71</td>
</tr>
<tr>
<td>Sex (male), N (%)</td>
<td></td>
<td>12 (50)</td>
<td>6 (75)</td>
<td>3 (38)</td>
<td>3 (38)</td>
<td>0.22</td>
</tr>
<tr>
<td>BMI (kg/m²), mean (SEM)</td>
<td></td>
<td>24.9 (0.8)</td>
<td>24.6 (1.1)</td>
<td>25.6 (1.4)</td>
<td>24.3 (1.8)</td>
<td>0.80</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg), mean (SEM)</td>
<td></td>
<td>114 (2)</td>
<td>119 (4)</td>
<td>110 (3)</td>
<td>112 (4)</td>
<td>0.20</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg), mean (SEM)</td>
<td></td>
<td>66 (2)</td>
<td>68 (3)</td>
<td>60 (2)</td>
<td>68 (3)</td>
<td>0.13</td>
</tr>
<tr>
<td>Fasting plasma glucose (mM), mean (SEM)</td>
<td></td>
<td>4.5 (0.1)</td>
<td>4.5 (0.3)</td>
<td>4.3 (0.3)</td>
<td>4.7 (0.2)</td>
<td>0.49</td>
</tr>
<tr>
<td>Fasting TC/HDL-C, mean (SEM)</td>
<td></td>
<td>3.32 (0.16)</td>
<td>3.17 (0.29)</td>
<td>3.40 (0.21)</td>
<td>3.39 (0.34)</td>
<td>0.82</td>
</tr>
<tr>
<td>Calc. LDL-C (mM), mean (SEM)</td>
<td></td>
<td>2.44 (0.14)</td>
<td>2.57 (0.38)</td>
<td>2.36 (0.17)</td>
<td>2.40 (0.16)</td>
<td>0.83</td>
</tr>
<tr>
<td>CRP (mg/L), median (range)</td>
<td></td>
<td>0.3 (0.2 - 7.2)</td>
<td>0.3 (0.2 - 1.1)</td>
<td>0.3 (0.2 - 2.7)</td>
<td>1.0 (0.2 - 7.2)</td>
<td>0.20</td>
</tr>
<tr>
<td>Fasting serum TEAC (mM TE), mean (SEM)</td>
<td></td>
<td>3.08 (0.06)</td>
<td>3.04 (0.12)</td>
<td>3.25 (0.05)</td>
<td>2.95 (0.10)</td>
<td>0.08</td>
</tr>
<tr>
<td>Fasting serum thiols (μM), mean (SEM)</td>
<td></td>
<td>399 (13)</td>
<td>430 (14)</td>
<td>366 (23)</td>
<td>401 (25)</td>
<td>0.13</td>
</tr>
<tr>
<td>Fasting serum TBARS (μM), mean (SEM)</td>
<td></td>
<td>5.10 (0.20)</td>
<td>5.38 (0.33)</td>
<td>5.05 (0.44)</td>
<td>4.89 (0.30)</td>
<td>0.62</td>
</tr>
<tr>
<td>Non-smokers, N (%)</td>
<td></td>
<td>23 (96)</td>
<td>8 (100)</td>
<td>8 (100)</td>
<td>7 (88)</td>
<td>0.35</td>
</tr>
<tr>
<td>Alcohol consumption (serv./wk), N (%)</td>
<td>never</td>
<td>11 (46)</td>
<td>3 (38)</td>
<td>3 (38)</td>
<td>5 (63)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 - 5</td>
<td>10 (42)</td>
<td>3 (38)</td>
<td>5 (63)</td>
<td>2 (25)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 - 10</td>
<td>2 (8)</td>
<td>2 (25)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt; 10</td>
<td>1 (4)</td>
<td>0</td>
<td>0</td>
<td>1 (13)</td>
<td>0.23</td>
</tr>
<tr>
<td>Exercise frequency (h/wk), N (%)</td>
<td>never</td>
<td>7 (29)</td>
<td>1 (13)</td>
<td>2 (25)</td>
<td>4 (50)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>7 (29)</td>
<td>4 (50)</td>
<td>3 (38)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-3</td>
<td>5 (21)</td>
<td>2 (25)</td>
<td>1 (13)</td>
<td>2 (25)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt; 3</td>
<td>5 (21)</td>
<td>1 (13)</td>
<td>2 (25)</td>
<td>2 (25)</td>
<td>0.37</td>
</tr>
<tr>
<td>Fruit and vegetable consumption (cups/d), N (%)</td>
<td>&lt; 2.5</td>
<td>13 (54)</td>
<td>6 (75)</td>
<td>4 (50)</td>
<td>3 (38)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5 - 5</td>
<td>8 (33)</td>
<td>1 (13)</td>
<td>4 (50)</td>
<td>3 (38)</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>&gt; 5</td>
<td>3 (13)</td>
<td>1 (13)</td>
<td>0</td>
<td>2 (25)</td>
<td></td>
</tr>
</tbody>
</table>

* One-way ANOVA for quantitative characteristics, except Kruskal-Wallis test for CRP; Chi-squared test for qualitative characteristics.
** p<0.01, compared to week 0, Dunnett's post-test.

**Figure 4.** Mean±SEM serum TEAC, thiols and TBARS, at weeks 0, 2 and 8, pooled data (n=24).
one-way RM ANOVA:
P=0.001  P=0.44  P=0.21

two-way RM ANOVA
Treatment:
P=0.20
Time:
P=0.0005
Interaction:
P=0.10

* p<0.05 and ** p<0.01, compared to week 0, Dunnett's post-test.

**Figure 5.** Mean±SEM serum TEAC, at weeks 0, 2, 4 and 8, stratified by treatment group (n=8).
Figure 6. Mean±SEM serum TEAC for (a) pooled data (n=24), and (b) stratified by treatment group (n=8), at weeks -1, 0, 2, 4 and 8.
two-way RM ANOVA
Treatment:
P=0.28
Time:
P=0.10
Interaction:
P=0.19

* $P<0.05$, compared to week 0, Dunnett's post-test.

**Figure 7.** Mean±SEM serum thiols, at weeks 0, 2, 4 and 8, stratified by treatment group (n=8).
one-way RM ANOVA:
P=0.056  P=0.42  P=0.77

RM two-way ANOVA
Treatment:
P=0.92
Time:
P=0.14
Interaction:
P=0.26

* p<0.05, compared to week 0, Dunnett's post-test.

**Figure 8.** Mean±SEM serum TBARS, at weeks 0, 2, 4 and 8, stratified by treatment group (n=8).
Table 7. Mean±SEM delta TEAC, thiols, TBARS and t12UM from week 4 compared to week 2, stratified by treatment group (n=8).

<table>
<thead>
<tr>
<th></th>
<th>wk 4 – wk 2</th>
<th>1cRg</th>
<th>2cRg</th>
<th>4cRg</th>
<th>P-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ TEAC (μM)</td>
<td>Mean</td>
<td>-10.7</td>
<td>-30.5</td>
<td>50.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>42.1</td>
<td>38.2</td>
<td>21.3</td>
<td></td>
</tr>
<tr>
<td>P-value*</td>
<td></td>
<td>0.80</td>
<td>0.45</td>
<td>0.050</td>
<td>0.26</td>
</tr>
<tr>
<td>Δ Thiols (μM)</td>
<td>Mean</td>
<td>12.9</td>
<td>-5.5</td>
<td>23.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>6.2</td>
<td>6.1</td>
<td>15.7</td>
<td></td>
</tr>
<tr>
<td>P-value*</td>
<td></td>
<td>0.08</td>
<td>0.40</td>
<td>0.17</td>
<td>0.15</td>
</tr>
<tr>
<td>Δ TBARS (μM)</td>
<td>Mean</td>
<td>0.19</td>
<td>-0.07</td>
<td>-0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.28</td>
<td>0.13</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>P-value*</td>
<td></td>
<td>0.51</td>
<td>0.63</td>
<td>0.51</td>
<td>0.52</td>
</tr>
<tr>
<td>Δ t12UM (ng CE)</td>
<td>Mean</td>
<td>-6.3</td>
<td>-6.4</td>
<td>58.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>21.7</td>
<td>24.6</td>
<td>25.1</td>
<td></td>
</tr>
<tr>
<td>P-value*</td>
<td></td>
<td>0.78</td>
<td>0.80</td>
<td>0.059</td>
<td>0.12</td>
</tr>
</tbody>
</table>

* two-tailed one sample t-test; ** one-way ANOVA.
Figure 9. t12UM stratified by treatment group (n=8), as (a) mean±SEM, at weeks 2 and 4, and (b) individual delta values at week 4 compared to week 2.
Figure 10. Correlation between t12UM and serum (a) TEAC, (b) thiols and (c) TBARS, at weeks 2 and 4 (n=26, excluded 2 outliers).
one-way RM ANOVA:

- Treatment: $P=0.73$
- Time: $P=0.029$
- Interaction: $P=0.14$

two-way RM ANOVA
- Treatment: $P=0.73$
- Time: $P=0.029$
- Interaction: $P=0.14$

* $P<0.05$, compared to week 0, Dunnett's post-test.

**Figure 11.** Mean±SEM fasting plasma glucose, at weeks 0, 2, 4 and 8, stratified by treatment group (n=8).
Table 8. Participant perceptions on taste, satiety and symptoms overall (n=24) after week 2, and by group (n=8) after week 4.

<table>
<thead>
<tr>
<th>Perception</th>
<th>Scale</th>
<th>Overall</th>
<th>1cRg</th>
<th>2cRg</th>
<th>4cRg</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taste (6 = highly enjoyable), N (%)</td>
<td>5 - 6</td>
<td>21 (88)</td>
<td>7 (88)</td>
<td>7 (88)</td>
<td>3 (38)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 - 4</td>
<td>3 (13)</td>
<td>1 (13)</td>
<td>0</td>
<td>3 (38)</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>1 - 2</td>
<td>0</td>
<td>0</td>
<td>1 (13)</td>
<td>2 (25)</td>
<td></td>
</tr>
<tr>
<td>Satiety (1 = very filling), N (%)</td>
<td>5 - 6</td>
<td>24 (100)</td>
<td>8 (100)</td>
<td>6 (75)</td>
<td>1 (13)</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>3 - 4</td>
<td>0</td>
<td>0</td>
<td>2 (25)</td>
<td>4 (50)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 - 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3 (38)</td>
<td></td>
</tr>
<tr>
<td>Change in bowel movements (yes), N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 (38)</td>
<td>3 (38)</td>
<td>5 (63)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Chi-squared test; P<0.05 is significant.
14 Study 2

14.1 Baseline Participant Characteristics

Baseline characteristics of the study participants (n=8) are presented (see Table 9). The study participants were mostly undergraduate students, with a median age of 22 yrs, ranging from 20-30 yrs. Their mean BMI was 21.5 kg/m² and within healthy range for all participants. Their mean blood pressure was 106/60 mm Hg and within healthy range for all participants. The baseline oxidative stress biomarkers, as measured in fasting plasma, had a mean of 2.80±0.07 mM TE, 549±12 µM thiols and 5.54±12 µM TBARS. Participants were nonsmokers, and rarely drank alcohol, but only three (38%) engaged in at least 30 min of moderate physical activity daily. Overall, this was a healthy, young adult study population.

14.2 Oxidative Stress Biomarkers

The effects of acute red raspberry consumption on post-prandial oxidative stress are presented. Time effects (tEs) are comparisons of post-prandial oxidative stress levels across study visit time-points, and address the primary objective to determine the effect of treatment. Treatment effects (TEs) are comparisons of post-prandial oxidative stress levels between different treatments, and address the secondary objective to investigate dose effects. Effects on TEAC, thiols and TBARS are presented in line graphs of mean delta values compared to baseline (see Figures 12a, 13a and 14a, respectively). For simplicity, values over the entire 8 h study visit are not presented because after 2 h for TEAC and thiols, and 4 h for TBARS oxidative stress responses stabilized. TEs are also analysed as net AUC values, and reported as means (see Table 10), and as delta values compared to both B and 1cR treatments (see Table 11). Variability in individual subjects’ responses to treatments are also presented in scatter plots, as delta net AUC values compared to bread (see Figures 12b, 13b and 14b).

For plasma TEAC, overall there was a significant tE (P=0.0001, two-way RM ANOVA; see Figure 12a) and no TE (P=0.46). There was a significant tE post-B and -4cR treatments (P=0.03 and 0.02, respectively, one-way RM ANOVA) both with significant decreases peaking at 30 min post-treatment (P<0.05, Dunnett’s post-test). TEs were also not significant when analyzing net AUC values by comparing all treatments (P=0.43, one-way RM ANOVA; see Tables 10 and
or raspberry treatments only (P=0.15). The treatments’ order of effect from smallest to largest was as follows: 1cR<2cR<B<B+vC<4cR, with the largest effect representing the largest oxidative stress response. However, the 4cR treatments’ oxidative stress response was not significantly different from the smallest oxidative stress response, induced by the 1cR treatment (P=0.06, two-tailed one sample t-test; see Table 11). Overall, there was a significant post-prandial tE toward decreased plasma TEAC, representing an increase in oxidative stress, but no significant TEs between treatments.

For plasma thiols, overall there were no tEs or TEs (P=0.13 and 0.97, respectively, two-way RM ANOVA; see Figure 13a). However, there was a non-significant decrease in post-prandial thiols, representing an increase in oxidative stress, similar to the tE on TEAC. TEs were also not significant when analyzing net AUC values by comparing all treatments (P=0.95, one-way RM ANOVA; see Tables 10 and 11), or raspberry treatments only (P=0.82). The treatments’ order of effect from smallest to largest was as follows: 2cR<B+vC<4cR<B<1cR, with the largest effect representing the largest oxidative stress response. Overall, tEs and TEs on plasma thiols were small and did not come close to reaching significance. Therefore, there were no significant effects on plasma thiols post-treatment.

For plasma TBARS, overall there was a significant tE (P=0.0005; two-way RM ANOVA; see Figure 14a) and no TE (P=0.61). There was a significant tE post-B and -1cR treatments (P=0.017 and 0.034, respectively; one-way RM ANOVA) with significant decreases at 15, 30 and 60 minutes post-1cR treatment only (P<0.05, Dunnett’s post-test). The 1cR treatment response was the only one where TBARS levels remained below baseline, and remained significantly reduced compared to baseline at 8 h post-treatment (P<0.05, Dunnett’s post-test; data not shown), representing reduced oxidative stress damage to lipid. TEs were not significant when analyzing net AUC values by comparing all treatments (P=0.39, one-way RM ANOVA; see Tables 10 and 11), or raspberry treatments only (P=0.27). The treatments’ order of effect from smallest to largest was as follows: 4cR<B+vC<2cR<B<1cR, with the largest effect representing the largest reduction in oxidative stress. However, the 1cR treatments’ reduction in oxidative stress was not significantly different from the smallest oxidative stress reduction, induced by the 4cR treatment (P=0.056, two-tailed one sample t-test; see Table 11). Overall,
there was a significant post-prandial tE toward decreased plasma TBARS, and no significant TEs between treatments.

14.3 Bioavailability of plasma Ascorbic acid

The tertiary objective to investigate the bioavailability of red raspberry antioxidants was addressed by analyzing tEs and TEs, over 4 h post-prandial, as done for oxidative stress biomarkers. Overall, plasma ascorbic acid levels had a significant tE, TE and interaction effect (P<0.0001, P=0.037 and P=0.0001, respectively, two-way RM ANOVA; see Figure 15a) There was a significant tE post-B+vC and -4cR treatments (P=0.0001 and 0.0008, respectively, one-way RM ANOVA) with significant increases at 30 min post-4cR, and at 1, 2 and 4 h post-intake of both treatments (p<0.05, Dunnett’s post-test). Analyses on net AUC values found significant TEs when comparing all treatments (P=0.012, one-way RM ANOVA; see Tables 10 and 11), but not when comparing raspberry treatments only (P=0.19). The treatments’ order of effect from smallest to largest was as follows: B<1cR<2cR<4cR<B+vC, with the largest effect representing the highest absorption of ascorbic acid. However, only the B+vC and 4cR treatments resulted in significant increases in plasma ascorbic acid compared to the ascorbic acid devoid bread treatment (P=0.008 and 0.001, respectively, two-tailed one sample t-test; Table 11). Bioavailability was proportional to ascorbic acid dose with the exception of the 1cR treatment, which had ~36% higher relative bioavailability than the higher doses (data not shown). Overall, red-raspberry ascorbic acid was bioavailable, and dose-dependent, with higher relative bioavailability at the lowest dose of 1cR.

14.4 Bioavailability of Anthocyanins

The tertiary objective was also to investigate the bioavailability of red raspberry anthocyanins. Plasma anthocyanin metabolites were undetectable in extracts of most participants’ plasma. The U-HPLC chromatogram of a 7x concentrated extract of plasma samples from one participant at all time-points post- 2cR is presented in Figure 19b. Five metabolites, eluted between 12.2 and 13.8 min, were detected by PDA at 520 nm. Levels were still too low for identification by LC/MS, but it is interesting to note that cyanidin and petunidin aglycones were detected in red raspberry extracts at 12.43 and 13.10 min using the same conditions (see Table 12). Therefore, these peaks were likely anthocyanins absorbed from the red raspberry treatment.
Numerous metabolites were detected in the urine using U-HPLC-PDA. Urine samples following raspberry consumption (and the high fat meal) tended to have a broader range of peaks eluted between 4-24 min, whereas control samples had peaks between 6-20 min (see Figure 19d and e, respectively). However, mass spectrometry was unable to identify any of the metabolites.

Since red raspberry-specific metabolites could not be identified, dose effects on total 12 h urinary metabolites (t12UM) were analysed by comparing levels following red raspberry treatments. Overall, there were no significant TEs on t24UM (P=0.45, one-way RM ANOVA; see Figure 17a and Table 11), t8hUM or t8-24UM (P=0.61 and 0.10, respectively, one-way RM ANOVA; see Table 10). Therefore, red raspberry anthocyanins may be bioavailable, but tUM are not dependent on raspberry dose.

14.5 Correlation of Bioavailability and Bioactivity Biomarkers

The quaternary objective to assess the relationship between red raspberry bioactivity and the bioavailability of red raspberry antioxidants was addressed by correlating plasma ascorbic acid with corresponding oxidative stress biomarkers, and t24UM with mean oxidative stress biomarkers post-raspberry treatments. Plasma ascorbic acid had a significant correlation with increased plasma TEAC (P<0.0001, r²=0.05, Pearson’s correlation; see Figure 16a), and decreased plasma TBARS (P<0.0001, r²=0.16; see Figure 16c) and thiols (P=0.007, r²=0.02; see Figure 16b). Therefore, ascorbic acid is related to increased plasma AOC, and protection against lipid peroxidation, but also increased protein oxidation.

Alternatively, t24UM had a significant correlation with decreased TEAC (P=0.024; r²=0.23, Pearson’s correlation; see Figure 18a), a non-significant correlation with increased TBARS (P=0.057, r²=0.17, see Figure 18c), and no relationship with thiols (P=0.52, r²=0.17; see Figure 18b). Therefore, t24UM is related to increased oxidative stress, decreasing plasma AOC and possibly increasing lipid peroxidation.

14.6 Red Raspberry Composition

Red raspberry samples from study 2 were analysed for their ascorbic acid and anthocyanin content, as a supplementary objective. The ascorbic acid content was 37.42 ± 3.55 mg/100g fw. The anthocyanidin composition was diverse, containing: cyanidin, peonidin, delphinidin,
petunidin and malvidin. Glycosylated forms of each aglycone were also present with glucoside and arabinoside sugar groups. However, the location of the sugar attachment could not be determined. The UPLC-PDA chromatogram is shown in Figure 20, with the peaks, identified by LC/MS, numbered and reported in Table 12. The positive ion mass spectra for some of the identified anthocyanidins’ are displayed in Figure 21.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Descriptor</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr), median (range)</td>
<td>22</td>
<td>(20-30)</td>
</tr>
<tr>
<td>Sex (male), N (%)</td>
<td>5</td>
<td>(62.5)</td>
</tr>
<tr>
<td>BMI (kg/m^2), mean (SEM)</td>
<td>21.5</td>
<td>(1.8)</td>
</tr>
<tr>
<td>Systolic blood pressure, mean (SEM)</td>
<td>106</td>
<td>(11)</td>
</tr>
<tr>
<td>Diastolic blood pressure, mean (SEM)</td>
<td>60</td>
<td>(6)</td>
</tr>
<tr>
<td>Fasting plasma AOC (mM TE), mean (SEM)</td>
<td>2.80</td>
<td>(0.07)</td>
</tr>
<tr>
<td>Fasting plasma protein thiols (µM), mean (SEM)</td>
<td>549</td>
<td>(12)</td>
</tr>
<tr>
<td>Fasting plasma TBARS (µM), mean (SEM)</td>
<td>5.54</td>
<td>(0.12)</td>
</tr>
<tr>
<td>Non-smokers, N (%)</td>
<td>8</td>
<td>(100)</td>
</tr>
<tr>
<td>Alcohol consumption (serv./wk), N (%)</td>
<td>0</td>
<td>3 (37.5)</td>
</tr>
<tr>
<td></td>
<td>&lt;3</td>
<td>5 (62.5)</td>
</tr>
<tr>
<td>Exercise (min/day) N (%)</td>
<td>&lt;30</td>
<td>5 (62.5)</td>
</tr>
<tr>
<td></td>
<td>30-60</td>
<td>3 (37.5)</td>
</tr>
</tbody>
</table>
*p < 0.05, compared to baseline, Dunnett's post-test.

**Figure 12.** Plasma TEAC, over 2 h post-treatment, expressed as (a) mean delta values compared to baseline and (b) individual delta net AUC values compared to bread treatment (n=8).
Figure 13. Plasma thiols, over 2 h post-treatment, expressed as (a) mean delta values compared to baseline, and (b) individual delta net AUC values compared to bread treatment (n=8).
**Figure 14.** Plasma TBARS, over 4 h post-treatment, expressed as (a) mean delta values compared to baseline and (b) individual delta net AUC values compared to bread treatment (n=8).
Table 10. Mean±SEM net post-prandial AUC for TEAC, thiols, TBARS, ascorbic acid, and 0-8 and 8-24 h tUM.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Net AUC</th>
<th>Treatment</th>
<th>1cR</th>
<th>2cR</th>
<th>4cR</th>
<th>B</th>
<th>B+vC</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 h plasma TEAC (μM*h)</td>
<td>Mean</td>
<td>-1.5</td>
<td>-11.1</td>
<td>-41.4</td>
<td>-25.9</td>
<td>-26.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>15.1</td>
<td>9.9</td>
<td>25.2</td>
<td>15.5</td>
<td>14.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P*</td>
<td>0.15</td>
<td>0.43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 h plasma thiols (nM*h)</td>
<td>Mean</td>
<td>-35.6</td>
<td>-6.4</td>
<td>-11.4</td>
<td>-22.6</td>
<td>-9.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>27.2</td>
<td>40.9</td>
<td>31.7</td>
<td>36.5</td>
<td>14.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P*</td>
<td>0.82</td>
<td>0.95</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h plasma TBARS (nM*h)</td>
<td>Mean</td>
<td>-36.1</td>
<td>-4.9</td>
<td>27.3</td>
<td>-17.7</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>17.5</td>
<td>34.0</td>
<td>25.7</td>
<td>21.9</td>
<td>16.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P*</td>
<td>0.27</td>
<td>0.39</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 h plasma ascorbic acid (μM*h)</td>
<td>Mean</td>
<td>10.6</td>
<td>18.0</td>
<td>49.9</td>
<td>-16.8</td>
<td>59.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>20.5</td>
<td>20.8</td>
<td>8.0</td>
<td>7.7</td>
<td>16.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P*</td>
<td>0.19</td>
<td>0.012</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-8 h urinary metabolites (ng CE)</td>
<td>Mean</td>
<td>89.7</td>
<td>68.6</td>
<td>90.0</td>
<td>20.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>24.5</td>
<td>11.8</td>
<td>20.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P*</td>
<td>0.61</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-24 h urinary metabolites (ng CE)</td>
<td>Mean</td>
<td>74.8</td>
<td>54.6</td>
<td>44.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>22.2</td>
<td>11.6</td>
<td>10.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P*</td>
<td>0.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* one-way RM ANOVA; first value is a comparison of raspberry treatments and second value is a comparison of all five treatments.
Table 11. Mean±SEM delta net AUC compared to B and 1cR, for TEAC, thiols, TBARS, ascorbic acid and t24UM post-treatment (n=8).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Δ net AUC</th>
<th>Treatment – Bread</th>
<th>Treatment – 1 cup</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>B+vC</td>
</tr>
<tr>
<td>2 h plasma TEAC (mM*h)</td>
<td>Mean</td>
<td>0</td>
<td>-0.001</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.022</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>P*</td>
<td>0.96</td>
<td>0.12</td>
</tr>
<tr>
<td>2h plasma Thiols (μM*h)</td>
<td>Mean</td>
<td>0</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.039</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>P*</td>
<td>0.74</td>
<td>0.78</td>
</tr>
<tr>
<td>4 h plasma TBARS (μM*h)</td>
<td>Mean</td>
<td>0</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.028</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>P*</td>
<td>0.54</td>
<td>0.47</td>
</tr>
<tr>
<td>4 h plasma Ascorbic acid (μM*h)</td>
<td>Mean</td>
<td>0</td>
<td>76.2</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>20.7</td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td>P*</td>
<td>0.008</td>
<td>0.22</td>
</tr>
<tr>
<td>24 h urinary metabolites (ng CE)</td>
<td>Mean</td>
<td>0</td>
<td>-41.3</td>
</tr>
</tbody>
</table>

* two-tailed one sample t-test; ** one-way RM ANOVA.
**Figure 15.** Plasma ascorbic acid, over 4 h post-treatment, expressed as (a) mean delta values compared to baseline, and (b) individual delta net AUC values compared to bread treatment (n=8).
Figure 16. Correlation between plasma ascorbic acid and plasma (a) TEAC, (b) thiols and (c) TBARS, across all time-points and treatments (n=320).
Figure 17. t24UM post-raspberry treatments expressed as (a) mean±SEM, and (b) individual delta values compared to 1cR (n=8).
Figure 18. Correlation between t24UM and average plasma (a) AOC, (b) thiols and (c) TBARS post-raspberry treatments (n=23).
Figure 19. U-HPLC chromatograms detected by PDA at 520 nm for anthocyanin analysis of one participant’s: (a) plasma at baseline after an overnight fast, (b) plasma pooled over 8 h following consumption of two cups red raspberries, (c) urine at baseline after an overnight fast, (d) urine pooled over 8 h following consumption of two cups red raspberries.
Figure 20. U-HPLC chromatogram of anthocyanins and anthocyanidins detected by PDA at 520 nm in an acidified methanol extract of the red raspberries from study 2.
Table 12. Summary of properties of compounds detected in extracts of red raspberries from study 2, following analysis by LC/MS detection. Peak numbers and retention times refer to numbers given in Figure 17.

<table>
<thead>
<tr>
<th>Peak</th>
<th>t&lt;sub&gt;R&lt;/sub&gt; (min)</th>
<th>m/z</th>
<th>Anthocyanidin</th>
<th>Sugar group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.35</td>
<td>303, 465</td>
<td>Delphinidin</td>
<td>glucoside</td>
</tr>
<tr>
<td>2</td>
<td>7.75</td>
<td>303, 465</td>
<td>Delphinidin</td>
<td>glucoside</td>
</tr>
<tr>
<td>3</td>
<td>8.05</td>
<td>287, 449</td>
<td>Cyanidin</td>
<td>glucoside</td>
</tr>
<tr>
<td>4</td>
<td>8.52</td>
<td>303, 435</td>
<td>Delphinidin</td>
<td>arabinoside</td>
</tr>
<tr>
<td>5</td>
<td>8.90</td>
<td>317, 479</td>
<td>Petunidin</td>
<td>glucoside</td>
</tr>
<tr>
<td>6</td>
<td>9.27</td>
<td>287, 419</td>
<td>Cyanidin</td>
<td>arabinoside</td>
</tr>
<tr>
<td>7</td>
<td>9.58</td>
<td>301, 463</td>
<td>Petunidin</td>
<td>glucoside</td>
</tr>
<tr>
<td>8</td>
<td>9.95</td>
<td>317, 449</td>
<td>Petunidin</td>
<td>arabinoside</td>
</tr>
<tr>
<td>9</td>
<td>10.23</td>
<td>301, 463</td>
<td>Peonidin</td>
<td>glucoside</td>
</tr>
<tr>
<td>10</td>
<td>10.50</td>
<td>301, 433</td>
<td>Peonidin</td>
<td>arabinoside</td>
</tr>
<tr>
<td>11</td>
<td>11.12</td>
<td>331, 493</td>
<td>Malvidin</td>
<td>glucoside</td>
</tr>
<tr>
<td>12</td>
<td>12.43</td>
<td>331, 463</td>
<td>Delphinidin</td>
<td>arabinoside</td>
</tr>
<tr>
<td>13</td>
<td>13.10</td>
<td>287</td>
<td>Cyanidin</td>
<td>arabinoside</td>
</tr>
<tr>
<td>14</td>
<td>14.20</td>
<td>317</td>
<td>Petunidin</td>
<td>arabinoside</td>
</tr>
<tr>
<td>15</td>
<td>14.63</td>
<td>331</td>
<td>Peonidin</td>
<td>arabinoside</td>
</tr>
</tbody>
</table>

<sup>t<sub>R</sub></sup>, retention time; m/z, mass to charge ratio.
Figure 21. Mass spectra of anthocyanins detected in red raspberries from study 2; (I) delphinidin glucoside, (II) cyanidin glucoside, (III) delphinidin arabinoside, (IV) petunidin glucoside, (V) cyanidin arabinoside, (VI) peonidin glucoside, (VII) petunidin arabinoside, (VIII) malvidin glucoside, (IX) peonidin arabinoside, (X) malvidin arabinoside.
Chapter 6
Discussion
Discussion

Results from studies do not support a clear relationship between the chronic consumption of red raspberries and the reduction of fasting oxidative stress. However, some significant observations ($P<0.05$) were made and will be discussed.

15.1 Study 1

The protective effect on increasing serum TEAC and decreasing TBARS following daily consumption of 1cR for 2 weeks was significant in the 1cRg only. However, the overall tE was toward an increasing TEAC across all time-points, with significant increases at week 8, implying a non-treatment related effect of time confounding the tEs of the intervention. In contrast, there was no overall tE on TBARS. Therefore, tEs on TBARS in the 1cRg are attributable to the raspberry intervention with more certainty than the tEs on TEAC, which were confounded by a significant effect of time.

These tEs at week 2, were not observed when data was pooled to increase power ($n=24$). Therefore, the 1cRg may have been more responsive to the raspberry intervention. Even though there were no significant differences between treatment groups for baseline characteristics measured, power to detect a difference was low due to a small sample size. It is interesting to note that the 1cRg had the lowest consumption of fruits and vegetables, as well as the highest LDL-C and lowest TC/HDL-C levels. Therefore, low dietary antioxidants and slight dyslipidemia may have heightened the raspberry treatment’s effects on increasing serum AOC and decreasing lipid peroxidation, respectively. Alternatively, the responsiveness of the 1cRg could have been due a combination of other unknown factors, or occurred by chance.

In contrast to the protective effects observed on TEAC and TBARS, there was a concurrent decrease in thiols at week 2 in the 1cRg, implying an increase in protein oxidation. Similar to the observed effects on TEAC and TBARS, this effect on thiols was not observed in pooled data or other treatment groups, and may have occurred by chance. However, the inconsistency may also imply that protective effects on TEAC and TBARS are not protective on thiols. Therefore, dietary antioxidants may have differing targets.
Interestingly, t12UM was correlated with increased thiols, implying the presence of antioxidants which target the protection of thiols. Even though there was no significant protective effects of the raspberry intervention on thiols, the largest increase in thiols (P=0.17) and t12UM (P=0.056) was concurrently observed in the 4cRg at week 4 compared to week 2. Therefore, the antioxidants contributing to t12UM may be different than those contributing protective effects on TEAC and TBARS. However, the source of observed protective effects cannot be attributable to red raspberry-derived antioxidants with confidence, because their bioavailability was indeterminable.

There were no significant dose responses (TEs) of raspberry consumption on bioavailability or bioactivity. However, t12UM was highest following consumption of 4cR for 2 weeks. Assuming that t12UM does capture the recovery of red-raspberry derived anthocyanins, this observation may reflect dose-dependent bioavailability. The lack of difference in t12UM at lower does may be explained by high background variability from non-raspberry derived metabolites masking the contribution from raspberry metabolites. As previously noted, this increase in t12UM occurs concurrently with the highest increases in thiols (P=0.17), as well as the highest increases in TEAC (P=0.05) and decreases in TBARS (P=0.51) at week 4 compared to week 2. Therefore, bioactivity may also be dose-dependent.

The tE toward an increase in fasting plasma glucose was greatest at week 8 in the 2cRg and attributable to a reduction in the presence of fasting hypoglycemia. This effect may be attributable to a confounding effect of time, but linear regression was not significant (P=0.08), and an increase was also observed at week 2, during the intervention. Therefore, red raspberry consumption may have had a beneficial effect on stabilizing blood glucose levels.

No effects were found on the other clinical biomarkers for chronic disease risk, including body weight, blood pressure, lipid profile and CRP. These were measured in case a trend was identified for future investigation, but were not expected to be significantly changed. Other studies which have found significant effects of nutrition interventions on some of these biomarkers had sample sizes of 138 for weight reduction and blood pressure [234], 72 for blood pressure and cholesterol [165], and 233 for CRP [219]. Additionally, since the study population was healthy, improvements in these biomarkers were further unlikely, as levels were already within the healthy recommended range for most participants.
Although red raspberries do not have high calorie content, there was a significant TE toward increased satiety with increased dose, which may be explained by the satiating effect of red raspberry’s high dietary fibre content. The consumption of 4cR was too large a serving for some participants to easily consume with a meal, therefore, if in future studies raspberries are to be consumed with a meal, smaller serving sizes should be given. Alternatively, rather than consuming the entire dose in one sitting, smaller doses could be given with each meal.

Results from study 1 have identified some other key factors to be considered when planning future investigations. For example, there is a strong need to control for the effect of time, as TEAC was significantly confounded by this factor. The randomization during weeks 3 and 4, to investigate dose effects, was not confounded by time, and had the hypothesized dose effect been present it would have verified an effect of raspberry consumption, independent of time. However, stratification reduced power and no significant TEs were observed. Therefore, a randomized, cross-over design should be used for future short-term investigations, to maintain power and control for time.

Overall effects on oxidative stress markers were unclear, as they were inconsistent across intervention periods and doses, often not significant and confounded by time. In addition, the bioavailability of red raspberry antioxidants was not verified to support the possibility of an in vivo antioxidant effect from raspberry consumption. Therefore, further research is needed to determine red raspberry’s bioactivity, bioavailability and dose response in vivo following consumption by healthy adults.

15.2 Study 2

All treatments induced a post-prandial increase in oxidative stress, as indicated by the significant tE toward decreased plasma TEAC. Meal-induced decreases on in vivo AOC have been previously reported [102], but typically consumption of antioxidant-rich foods has resulted in an increase in in vivo AOC [47]. However, depending on the antioxidant-rich food, protective effects can occur in the hydrophilic or lipophillic fraction of the blood, or both [47], therefore measures of total AOC may mask differing effects on different blood fractions. The absence of significant TEs does not support the bioactivity of red raspberry antioxidants in vivo. In fact, high doses of red raspberries induced greater oxidative stress responses, with decreases in TEAC greater post-4cR than -1cR (P=0.061), though not significantly. It is hypothesized that the 4cR
treatment’s higher calorie content contributed to the larger oxidative stress response. Therefore, calorie-load may be confounding the potential bioactivity of dietary antioxidants.

In contrast, all treatments caused a post-prandial decrease in oxidative damage to lipids, as indicated by the significant decrease in plasma TBARS. This implies that lipid fractions are being protected against oxidative stress, possibly by antioxidants which contribute to TEAC, explaining the observed reduction in plasma TEAC. However, the 4cR treatment was less protective against TBARS than 1cR (P=0.056), though not significant, supporting the presence of higher oxidative stress at higher calorie-load, as TEAC responses showed.

For a decrease in lipid peroxidation to occur concurrently with an increase in oxidative stress, there must also have been an increase in antioxidant activity. Post-prandial decreases in TBARS have previously been related to levels of lipid-bound phenolics, following consumption of a berry treatment [235]. Therefore, 1cR’s greatest protective effect on TBARS may not only have been attributable to it having the lowest calorie-load and therefore, post-prandial induced oxidative stress response, but also attributable to its antioxidant content. In fact, the 1cR treatment was found to have the highest relative bioavailability of ascorbic acid, resulting in the highest ratio of bioavailable antioxidant-load to calorie-load, and explaining its significant effects.

There was a lack of significant tE and TE on plasma thiols is worth. However, there was an observed post-prandial decrease across treatments (P=0.13), supporting the evidence for a post-prandial oxidative stress response. Though the overall decrease in thiols was unexpected, this observation is in accordance with the findings of a previous study, which found post-prandial increases in protein oxidation following consumption of a berry treatment [235]. Overall, thiols were unresponsive to acute consumption of red raspberries.

The negative B control was antioxidant devoid, and had a calorie load in between the 4cR and 2cR treatment. The positive B+Vc control had ascorbic acid content comparable to 4cR, and a calorie-load equivalent to B. B’s effect on TEAC was comparable to the decrease induced by 4cR, but the negative peak was narrower, meaning the effect had a shorter duration, which could possibly be attributable to faster digestion. B+Vc did not induce a drop in TEAC as dramatic as that of B, but overall had a net AUC response comparable to B. Therefore, supplemental ascorbic acid may reduce the size of oxidative stress responses. This is also supported by the
quicker rebound in thiol levels at 1 h post-B+vC, as compared to 2 h post-B. In contrast, protective effects on TBARS were greater following B than B+vC, possibly explained by the post-B spike in oxidative stress which may have induced endogenous antioxidant defenses. It appears that ascorbic acid supplementation may reduce post-prandial oxidative stress responses, possibly protecting against oxidative damage. Alternatively, that oxidative stress response may induce endogenous antioxidant systems, also protecting against oxidative damage. Overall, there were no significant differences between control treatments, implying that the study was likely not powered to detect TEs on oxidative stress biomarkers between any of the treatments.

The plasma ascorbic acid levels were dose-dependent, as indicated by the significant tE, TE and interaction effect, in addition to the increases in peak and net AUC levels with increasing ascorbic acid dose. Therefore, the red raspberry antioxidant, vitamin C, was bioavailable to contribute bioactivity in vivo. Since plasma ascorbic acid significantly correlated with increased TEAC and decreased thiols, the TEs on these biomarkers may be attributable to ascorbic acid contributing to AOC and protecting against lipid peroxidation. However, plasma ascorbic acid also had a significant negative correlation with thiols, indicating a relationship with increased oxidative stress. This correlation is further supported by the observed post-prandial increases in ascorbic acid and decreases in thiols. To explain this unexpected finding, it is proposed that thiols’ antioxidant activity had a sparing effect on ascorbic acid, which is biologically plausible given that thiols levels were 6x higher in the plasma than ascorbic acid.

The contribution of red raspberry polyphenols to the observed bioactivity of red raspberry consumption could not be determined because exposure could not be quantified. The detection of unidentified anthocyanins in pooled plasma reveals they were more than likely bioavailable, but at low concentrations. Baseline levels had too many peaks to determine which were the results of the raspberry intervention. This may explain the lack of dose response in tUM, as background non-raspberry derived metabolites were high, and may have masked any contribution from red raspberry metabolites. This may further explain the correlation of t24UM with decreasing TEAC and increasing TBARS. If metabolites were not derived from the raspberries they would have been derived from food consumption before the study visit. Therefore, they represent recent food exposure and resulting exposure to post-prandial oxidative stress, explaining the correlations with decreased plasma AOC and increased lipid peroxidation. The contribution from bioavailable anthocyanins and other antioxidant polyphenols to red
raspberry bioactivity cannot be ruled out, however due to low bioavailability it is likely that the acute effects of raspberry consumption are primarily attributable to ascorbic acid.

Overall, these observations identify some important considerations for the planning of future studies. The confounding effect of calorie-load on oxidative stress response highlights the need to control for this factor, for example by using a control food to make treatment doses iso-caloric. Additionally the difficulty in detecting and identifying red raspberry-derived polyphenolic metabolites has revealed 1) that large volumes of plasma are needed to detect the low concentrations of anthocyanins, and 2) a 2 day low-polyphenol diet is not a sufficient amount of time to clear polyphenolic metabolites out of the body. Further research is needed to elucidate the contrasting effect of calorie and antioxidant load on oxidative stress, requiring the identification and quantification of in vivo exposure to dietary antioxidants and their metabolites.

15.3 Overall

Direct comparisons between the two studies cannot be made because study 1 investigated the chronic effects of red raspberry consumption and study 2 investigated the acute effects. However, in combination, the two studies give a more complete perspective on the role of dietary antioxidants on oxidative stress status in vivo.

In both studies, significant reductions in TBARS, representing a decrease in oxidative damage to lipids, was observed. In the acute study, post-prandial reductions were immediate, and post-1cR levels were still significantly reduced at 8 h. In the chronic study, fasting reductions in TBARS were seen in the 1cRg after 2 weeks of intervention. Therefore, dietary antioxidants from red raspberries protect against TBARS.

Protective effects on TBARS occurred concurrently, in both studies, with increases in TEAC. In the acute study, plasma AOC was reduced the least post-1cR. In the chronic study, TEAC levels were significantly increased in the 1cRg after 2 weeks of intervention. In addition, both biomarkers significantly correlated with plasma ascorbic acid in study 2, such that there was an association with decreased oxidative stress. Whereas, their correlation with tUM was such that there was either, no association or an association with increased oxidative stress. Therefore, ascorbic acid may be the red raspberry antioxidant which contributes the majority of antioxidant bioactivity in vivo.
Thiols were not very responsive to treatment in either study. However, they did correlate to t12UM in the chronic study, such that there was a decrease in oxidative stress. This implies that there may be bioactive compounds in the urine which target the protection of thiols. It is proposed that accumulation of colonic metabolites following chronic red raspberry exposure contributed 1) to the increase in t12UM seen at week 4 in the 4cRg, and 2) to the positive correlation with thiols. This makes biological sense, since anthocyanins are known to be metabolized by microbiota into phenolic acids [107], and ellagitannins into urolithins [95], both compounds with in vitro AOC [236]. Additionally, biokinetic studies have found urolithins to have 1) a much higher bioavailability than their parent compounds, 2) delayed exposure beginning after 8 h post-intake and 3) prolonged exposure, lasting beyond 52 h post-intake [96, 98]. This means that the acute study may not have allowed sufficient time for the accumulation of colonic metabolites in t12UM, explaining the lack 1) dose response and 2) correlation with increased thiols. Therefore, chronic red raspberry consumption may result in the accumulation of colonic metabolites that increase thiols and protect against oxidative damage to proteins.

The absence of a clear relationship between red raspberry consumption and protection against oxidative stress may be attributable to 1) there being a weak relationship, as anthocyanins have low bioavailability, and despite berries high in vitro AOC, some reports have found other fruits with lower in vitro AOC to be more bioactive in vivo [47]; 2) a lack of power, as sample size was selected based on previous studies, but stratification in study 1 and not meeting the recruitment goal of 10 participants, in study 2, both decreased sample size; and 3) confounders masking effects, such as time in study 1 and calorie-load in study 2. Therefore, future studies need to take these factors into consideration.
16 Future Directions

The results of study 2 revealed that there may be complex interactions between calorie load, dietary antioxidant exposure and endogenous antioxidant defense systems. Post-prandial induced oxidative stress has not been well investigated in the literature. It is known that energy metabolism produces free radicals, and that spikes in blood glucose and triglycerides are associated with increased oxidative stress, inflammation and reduced endothelial function [237]. However, the effect of calorie load and macronutrient composition on post-prandial induced oxidative stress has not been investigated. Therefore, more acute studies are needed to understand the basic physiology of post-prandial induced oxidative stress and the responses of endogenous antioxidant systems.

Overall the antioxidant bioactivity of red raspberries warrants further investigation. Other acute studies have found fruits and berries to increase post-prandial AOC over 4 h when consumed alone or with a meal [47]. However, the effectiveness of fruits has been variable, with berry fruits having lower than expected effects due to low anthocyanin bioavailability. In study 2, the effect of red raspberry consumption on AOC and TBARS unexpectedly resulted in increased oxidative stress with increasing raspberry dose, attributed to calorie-load. Therefore, further research on red raspberries and other antioxidant-rich foods is required in order to determine the amounts of dietary antioxidants required to inhibit different levels of induced oxidative stress.

Ascorbic acid was found to be dose-dependently bioavailable and possibly bioactive, as a result of having had a significant correlation with reduced oxidative stress levels in study 2. However, treatment with only ascorbic acid did not result in significant protective effects against oxidative stress. Therefore, other red raspberry-derived antioxidants, not identified, may contribute bioactivity in vivo. For example, the increases in tUM, observed in study 1, were postulated to be the accumulation of colonic metabolites from chronic red raspberry exposure. The significant positive correlation between total urinary metabolites and serum thiols may be identifying the antioxidant activity of microbial-derived phenolic acids, from raspberry-derived parent compounds. Few studies have investigated the bioavailability of colonic metabolites from berry fruits in humans [96, 98], and no studies have investigated their bioactivity. Further research is
needed to link the bioavailability of red raspberry polyphenols to antioxidant activity in vivo. Ascorbic acid has a much higher bioavailability than anthocyanins and therefore, may be the only red raspberry-derived antioxidant with relevant antioxidant activity in vivo. However, investigation into colonic metabolites may alter this perspective, finding polyphenolic compounds to be more bioavailable than currently known.

Overall, the three main focuses for further research in this area should involve investigation on:

1. basic physiology of post-prandial induced oxidative stress and endogenous antioxidant responses
2. use of antioxidant-rich foods to inhibit meal-induced oxidative stress
3. bioavailability of polyphenols and their metabolites, and how this relates to bioactivity.
4. long-term benefits of dietary antioxidants on chronic disease outcomes
Chapter 7
Conclusion
17 General Conclusion

Red raspberries are an important source of dietary antioxidants in the Western diet. Their consumption significantly contributes ellagitannins and anthocyanins to the diet, which are polyphenols with the potential to function as antioxidant, anti-inflammatory, anti-carcinogenic and anti-atherosclerotic agents in vivo. Although, human intervention studies with other berries such as blueberries and strawberries have been reported in the literature, red raspberries have not yet been studied.

Two intervention studies were undertaken to investigate the bioactivity and bioavailability of red raspberry antioxidants in humans. Study 1 investigated the chronic effect of daily red raspberry consumption for four weeks on fasting oxidative stress status. Study 2 investigated the acute effect of a single dose of red raspberries on post-prandial oxidative stress over 8 h post-intake. To assess oxidative stress, TEAC, thiols and TBARS were measured as biomarkers of AOC, protection from protein oxidation and levels of lipid peroxidation. Both studies investigated the effect of dose, using treatments of one, two and four cups red raspberries. Also, the recoveries of red raspberry polyphenols in the urine were measured in both studies. However, red raspberry anthocyanins could not be identified due to methodological limitations, so total urinary metabolites were reported in cyanidin equivalents (CE) and assumed to represent recovery. In addition, plasma ascorbic acid was measured in study 2.

The primary objective was to determine the effect of raspberry consumption on oxidative stress. There were protective effects against lipid peroxidation observed in both studies. However, in study 1 reduction in TBARS was only significant in the 1cRg after 2 weeks of intervention, and in study 2, reductions were only significant post-1cR treatment. This inconsistency in the observed effect at higher doses and across other time-points brings the validity of the effect into question, especially because study 1 was confounded by time and study 2 by calorie load.

The secondary objective was to determine the dose effect of raspberry consumption, however no significant differences were found. Although in study 2, higher doses of raspberries appeared to induce a greater oxidative stress response, which was attributed to the increased calorie load.
The tertiary objective was to determine the bioavailability of red raspberry antioxidants. In study 1, total urinary metabolites were higher following 2 week consumption of 4cR, though not significantly. It could not be verified whether increases in metabolites were derived from the red raspberry intervention, but it was proposed that increases were attributable to the accumulation of colonic metabolites. In study 2, urinary metabolite levels were not influenced by raspberry dose. However, unidentifiable peaks in the plasma were detected, which were thought to be attributable to red raspberry anthocyanins. Vitamin C bioavailability was confirmed in study 2, with increased levels in plasma dependent on dose.

The quaternary objective was to determine whether bioactivity of red raspberry consumption was related to bioavailability of red raspberry antioxidants. In study 1, t12UM was significantly correlated with increased thiols. In study 2, plasma ascorbic acid was significantly correlated with decreased TBARS, and TEAC. Therefore, unidentified urinary metabolites, thought to be colonic metabolites, may protect against protein oxidation, and ascorbic acid may protect against lipid peroxidation.

Overall, the results revealed no clear relationship between the consumption of red raspberries by healthy human subjects and protection against oxidative stress. Further research is needed to verify the bioactivity of red raspberry antioxidants, and to attribute effects to bioavailable red raspberry-derived metabolites. In general, investigation on the use of dietary antioxidants to protect against acute oxidative stress is needed, as the mechanisms are not well understood. Alternatively, investigation on chronic effects is warranted because they may extend beyond protection against oxidative stress. For example, in vitro studies have found red raspberry polyphenols to have antioxidant-independent effects on cellular functioning via modulation of enzyme activity, signaling pathways and gene expression. Therefore, long-term investigations into the effects of dietary antioxidant exposure from red raspberries, and other antioxidant-rich food sources are needed to verify the bioactivity of polyphenols in vivo and link their protective effects to more biologically relevant outcomes, such as disease incidence.
References


137. Umesalma, S. and G. Sudhandiran, Differential inhibitory effects of the polyphenol ellagic acid on inflammatory mediators NF-ÎºB, iNOS, COX-2, TNF-Î«, and IL-6 in 1,2-


225. Fellegrini, N., et al., Screening of dietary carotenoids and carotenoid-rich fruit extracts for antioxidant activities applying 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic


228. Boss, S., Ascorbic acid (vitamin C) analysis on varian prostar HPLC systems. LifeLabs, 2007. Doc. # 6276(Ver. 4.0).


Appendix 1

Participant forms from Study 1

Form A – Selection, inclusion and exclusion criteria 132
Form B – Consent to participate in a research study 133
Form C – Study design and participant’s schedule 142
Form D – Foods to avoid 143
Form E – Instructions and food record sheet 144
Form F – Instruction for raspberry consumption 146
Questionnaire: Demographic, health and lifestyle 147
Questionnaire: Raspberry consumption questionnaire (wk 5 sample) 150
FORM A

Selection, Inclusion and Exclusion Criteria

TITLE OF STUDY: *Raspberries and Human Health*

Selection and Inclusion Criteria:

- Healthy men and women aged 18-40

  Participants who are willing to participate should agree to provide fasting blood samples and maintain their dietary records as needed. Those using any form of nutritional supplement will be asked to refrain from use for the duration of the study.

Exclusion Criteria:

- Those who have a body mass index $\geq 25\text{kg/m}^2$ (ratio of weight and height)
- Those who have blood pressure $\geq 140/90\text{mmHg}$
- Those who are currently taking any medications
- Those who have any chronic health conditions, such as cancer, CVD, diabetes
- Those who are allergic to raspberries
- Those who are pregnant or lactating/plan to become pregnant
FORM B
Consent to Participate in a Research Study

INFORMATION

TITLE OF STUDY: Raspberries and Human Health

INTRODUCTION:

Before agreeing to take part in this research study, it is important that you read the information in this research consent form. It includes details we think you need to know in order to decide if you wish to take part in the study. If you have any questions, ask a study doctor or study staff. You should not sign this form until you are sure you understand the information. All research is voluntary. You may also wish to discuss the study with your family doctor, a family member or close friend. If you decide to take part in the study, it is important that you are completely truthful about your health history and any medications you are taking. This will help prevent unnecessary harm to you.

INVESTIGATORS:

Dr. A.V. Rao, Principal Investigator, M.Sc., Ph.D.
Professor Emeritus, Department of Nutritional Sciences
University of Toronto
Tel #: (416) 978-3621, Tues – Thurs, 9:00 AM – 12:00 PM
Email: v.rao@utoronto.ca

Dr. David J. A. Jenkins, M.D., Ph.D., D.Sc.
Staff Physician, St. Michael’s Hospital
Professor, Department of Nutritional Sciences
University of Toronto
Tel #: (416) 867-7475, Mon – Fri, 10:00 AM – 5:00 pm
Email: cyril.kendall@utoronto.ca

Ms. Dawn Snyder, study coordinator
M.Sc. student, Department of Nutritional Sciences
University of Toronto
Tel #: (416) 978-3621, Mon – Fri, 9:00 AM – 5:00 PM
Email: dawn.snyder@utoronto.ca

MASTER’S OF SCIENCE RESEARCH PROJECT:

This study is to be conducted as part of the study coordinator, Ms. Dawn Snyder’s Master’s of Science degree requirements. Dr. A. V. Rao is Ms Dawn Snyder’s advisor and the Principal Investigator on this study. This means that he will be supervising the conduct of the study. Dr. David J. A. Jenkins is a staff member at St. Michael’s Hospital and is providing facilities at the Risk Factor Modification Center, where the study will be conducted. He is a collaborator in this study and will also be supervising the conduct of the study.
CONFLICTS OF INTEREST:

Dr. A.V. Rao, declares that he has no conflict of interest in conducting this study. Ms. Dawn Snyder declares no conflicts of interest.

STUDY SPONSORS:

1. Washington Red Raspberry Commission
2. Raspberry Industry Development Council

PURPOSE OF THE RESEARCH:

You are invited to participate in a nutrition research study on the effects of red raspberry consumption on biological markers of health. You have been selected as a potential participant because you are a healthy adult, who has met all criteria in Form A, which is included in the package with this consent form.

Dietary guidelines recommend that people increase their consumption of fruits and vegetables in order to improve their health and reduce their risk of developing chronic diseases. Fruits and vegetables are not only good sources of essential nutrients and fibre, but they contain beneficial ‘phytochemicals’. Phytochemicals are compounds found in plants that are not considered essential in the diet, but are believed to play a role in improving human health. We are specifically interested in red raspberries because of their unique phytochemical composition and their high antioxidant content.

Some of the phytochemicals function as antioxidants. An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation reactions can produce free radicals (molecules), which start chain reactions that damage cells. Your body naturally produces free radicals, for example, when it breaks down food. However, you are also exposed to free radicals in the environment from cigarette smoke, ultra-violet radiation, and other pollutants. Oxidative stress is the harmful condition that occurs when there is an excess of free radicals as a result of aging, poor nutrition and/or lifestyle. Long-term damage caused by oxidative stress is thought to contribute to the development of chronic diseases, such as cancer and cardiovascular disease. It is possible to reduce oxidative stress by consuming foods that are rich in antioxidants, like raspberries.

The evidence supporting the benefits of raspberry consumption comes mostly from experiments on cells and animals. Very few studies have tested on humans. It is the purpose of this study to determine whether red raspberry consumption can: 1) increase the concentration of raspberry phytochemicals; 2) increase the antioxidant capacity; 3) reduce oxidative stress levels; 4) reduce inflammation; and 5) improve the status of chronic disease risk factors, in the blood of healthy adult participants. Additionally, this study will determine if these effects can be increased by increasing the quantity of the red raspberries consumed daily. The results of this study will contribute knowledge on the role of red raspberries in the diet for improving human health and the prevention of chronic disease.

DESCRIPTION OF THE RESEARCH:
This is a 10-week study and will require six visits to the Risk Factor Modification Center, St. Michael’s Hospital, 61 Queen St. E. There will be a total of 24 eligible participants (12 male and 12 female) recruited for this study, and this recruitment is only taking place at this one location.

The following schedule is to be followed by willing, eligible participants:

Pre-study: On your first visit, the study will be explained to you and your eligibility will be determined. You will be given Forms A through F and asked to read them over. In order to determine your eligibility as outlined in Form A, your weight, height and blood pressure will be measured by study staff, and you will fill out a ‘demographic, health & lifestyle questionnaire’. Provided that you meet all requirements as outlined in Form A, and give consent to participate in this research study by signing this consent form, your future study visits will be scheduled.

Questions regarding your medical history must be answered to ensure that you meet study eligibility requirements. Additional questions regarding the frequency of your smoking, drinking and exercise habits must also be answered. These lifestyle factors can influence oxidative stress levels and must be taken into consideration when interpreting your study results.

Baseline (week1): During this week you will maintain your regular dietary habits and record the type and quantity of all foods consumed on Form E-2, as explained in Form E-1.

Your second visit is the morning following the first week of the study. The night before you will be asked to collect your total urine excretion in the provided container beginning after dinner and continuing the next morning until 12 hours has passed since your last meal. During all study visits you will have your height, weight and blood pressure measured by study staff. You will also give an overnight fasting blood sample (no food or drink, with the exception of water, for 12-14 hours previous). A study nurse will collect 40ml (approximately 2.5 tablespoons) of blood from your vein. You will hand in your completed 7-day Food Record and your 12hr urine collection.

Washout Period (week 2): During this week you will refrain from consuming any foods mentioned in Form D, while still maintaining your regular dietary and lifestyle habits.

Your third visit is the morning following the second week of the study. The day before you will record your food intake on Form E-2 and you will collect your total 12hr urine excretion following dinner. During the visit you will have your height, weight and blood pressure measured, and then provide a fasting blood sample. You will also fill-out a brief opinion-based questionnaire on your pre-study raspberry consumption and hand in your Food Record and 12hr urine collection.

Treatment Period (weeks 3-6): Throughout this period of the study you will continue to refrain from consuming any foods mentioned in Form D. You will consume one cup of frozen raspberries daily for the first two weeks, as described in Form F. On your fourth visit you will be randomized into one of three groups, which will determine whether or not you will consume one, two or four cups of red raspberries daily for the remaining two weeks of the treatment period.
You will have a one in three chance of receiving one of the three treatments. Please try to maintain your lifestyle and dietary habits as normal as possible.

Your fourth visit is the morning following the fourth week of the study. The day before you will record your food intake on Form E-2 and you will collect your total 12hr urine excretion following dinner. During the visit you will have your height, weight and blood pressure measured, and then provide a fasting blood sample. You will also fill out a brief opinion-based questionnaire on your raspberry consumption during the study and hand in your Food Record and 12hr urine collection.

Your fifth visit is the morning following the sixth week of the study. The day before you will record your food intake on Form E-2 and you will collect your total 12hr urine excretion following dinner. During the visit you will have your height, weight and blood pressure measured, and then provide a fasting blood sample. You will also fill out a brief opinion-based questionnaire on your raspberry consumption during the study and hand in your Food Record and 12hr urine collection.

Post-Treatment Period (weeks 7-10): Throughout this period of the study you will continue to refrain from consuming any foods mentioned in Form D, while still maintaining your regular dietary and lifestyle habits.

Your sixth visit is the morning following the tenth and final week of the study. The day before you will record your food intake on Form E-2 and you will collect your total 12hr urine excretion following dinner. During the visit you will have your height, weight and blood pressure measured, and then provide a fasting blood sample. You will also fill out a brief opinion-based questionnaire on your study and post-study raspberry consumption and hand in your Food Record and 12hr urine collection.

Total time commitment: Participation in this study requires one pre-study visit and 5 study visits to the Risk Factor Modification Center, part of St. Michael’s Hospital, at 61 Queen St. E. Appointments will be scheduled to your convenience, and all study visits must be between Monday-Friday from 7:30 AM to 9 AM. The pre-study visit should take approximately half an hour and each study visit should take approximately 15 minutes. Additionally, participants will be required to record their diet for 7 consecutive days prior to their second visit, and for an additional day prior to their remaining 4 visits. They will also be required to collect their total urine excretion in the provided container for 12 hours following dinner the night before each of the 5 study visits. Participants will be contacted by either telephone or email to confirm these appointments and to monitor progress throughout the study. If any new information relevant to the study is revealed during the study, participants will be contacted immediately by telephone.

Supplement: The red raspberries to be consumed during the treatment period will be provided to participants. Depending on your treatment group you will receive 3.5, 5.25, or 10.75 kg of frozen raspberries in total, which will be provided in sealed bags each weighing up to 1.2 kg. These bags must be kept frozen and can be picked up from the Risk Factor Modification Center after participant’s first visit. If it is not possible for participants to transport their raspberries home in a timely fashion, in order to keep them frozen, then alternative arrangements can be made with the study coordinator to have them delivered.
One cup of the provided raspberries is to be consumed daily for two weeks, during weeks 3 and 4 of the study. Then one, two or three cups are to be consumed for two weeks, during weeks 5 and 6, depending on the group you are randomly assigned. Raspberries are to be kept frozen at all times, with the exception of removing one serving a day for consumption. Be sure to measure the raspberries while frozen; one cup is equivalent to 250ml or 123g. They must be consumed with a meal, and in a single serving. Do not add new foods to your diet in order make the supplement more palatable. The raspberries cannot be heated or microwaved, but they can be thawed at room temperature or in the refrigerator. The entire quantity, including juices from thawing, must be consumed daily. Any missed days or discomforts experienced should be recorded. Further directions and meal suggestions for consuming the raspberries can be found in Form F.

**Blood and Urine Samples:** Blood samples will be taken at the Risk Factor Modification Center, part of St. Michael’s Hospital, at 61 Queen St. E., by experienced study nurses. The study coordinator will be present at all times. Blood samples are overnight fasting samples; we ask that for 12 hours prior to the appointment you consume no food or beverages; however you may drink as much water as you like. Approximately 40mL (approximately 2.5 tablespoons) of blood will be taken.

Urine samples will be collected by the participant. Participants will be provided with containers in order to store their 12hr urine collections which will be collected prior to each study visit, beginning after dinner. Urine collections will be handed in at each study visit and a new container will be provided to participants.

Blood and urine samples will be labeled with subjects’ I.D. number and used only for the purpose of this research study. The samples will be processed at the Risk Factor Modification Center and stored at the University of Toronto, in a -80°C freezer. Phytochemical content, total antioxidant capacity, antioxidant enzyme activity, oxidative stress parameters, blood lipid profile, blood glucose and inflammatory markers will be analyzed at the University of Toronto, FitzGerald Building, 150 College St. Extra samples will be stored for 5 years, and used only to verify results of the study. This may be necessary if methodological advancements allow for improved detection of parameters of interest. All samples will be destroyed after this period.

**POTENTIAL HARMS (INJURIES, DISCOMFORT, INCONVENIENCES):**

Some individuals may be allergic to consuming raspberries and be at risk of experiencing an allergic reaction in response to treatment. To minimize such risk, you will be asked at the time of screening if you are allergic to consuming raspberries. If your response is positive, then you will not be selected to participate in the study and the reason explained. If after starting your participation in the study you experience an allergic response to consuming raspberries, you will be asked not to consume raspberries further, and asked to withdraw from the study. The reason of the withdrawal request will be explained to you.

There may be a small amount of bleeding when blood is taken from the vein, and there may be slight discomfort and bruising or redness. This will usually disappear in a few days. If a more
severe reaction occurs, such as infection, please consult a physician and inform the study coordinator.

We ask participants to record their diet - everything they eat and drink as well as quantities for 7 consecutive days prior to their second visit, and for a total of 4 days prior to their remaining study visits - and submit the completed records to the study coordinator at each appointment. We will provide you with blank diet records (Form E-2, E-3a, E-3b) on which to record this information. Some participants find this process to be an inconvenience as it may be time consuming.

We ask participants to collect their total urine excretion for 12 hours following dinner before each of their 5 study visits. Urine collection containers must be carried to and from the centre. The transportation and actual collection of samples is viewed as an inconvenience to many participants.

POTENTIAL BENEFITS:

You may receive no direct benefits from being in this study. However, results from this study may further medical or scientific knowledge in the area of diet, health and disease.

ALTERNATIVES TO PARTICIPATION:

The alternative to participation in the study is early withdrawal or completion of the study as originally consented. There are no likely consequences to you if you decide not to participate in the study. Recruitment will be continued until the required number of participants is attained.

PROTECTING YOUR HEALTH INFORMATION:

Confidentiality will be respected and no information that discloses your identity will be released if results of this study are published or presented. Your identity will not be disclosed without your permission unless required by law.

Any medical records, documentation, study samples or information related to you will be coded by I.D. numbers to ensure that persons outside of the study will not be able to identify you. Your contact information on page 1 of the Pre-Study Questionnaire will be the only documentation linking your linking your I.D. number to you name. This will be stored separately from study data and will be destroyed following the end of the study by the study coordinator. All information will be stored in a secure place, either a locked filing cabinet or a password protected electronic file.

By signing this form, you are authorizing access to your study records by the study personnel and the St. Michael’s Hospital Research Ethics Board. Such access will be used only for purposes of verifying the authenticity of the information collected for the study, without violating your confidentiality, to the extent permitted by applicable laws and regulations.

National and Provincial Data Protection regulations, including the Personal Information Protection and Electronic Documents Act (of Canada) or PIPEDA and the Personal Health
Information Protection Act (PHIPA) of Ontario, protect your personal information. They also give you the right to control the use of your personal information, including personal health information, and require your written permission for your personal information (including personal health information) to be collected, used or disclosed for the purposes of this study, as described in this consent form. You have the right to review and copy your personal information. However, if you decide to be in this study or choose to withdraw from it, your right to look at or copy your personal information related to this study will be delayed until after the research is completed.

It is important to understand that despite these protections being in place, experience in similar studies indicates that there is the risk of unintentional release of information. The study personnel will protect your records and keep all the information in your study file confidential to the greatest extent possible. The chance that this information will accidentally be given to someone else is small.

All study records will be kept confidential for 5 years. De-identified samples will be frozen in a –80°C freezer at University of Toronto for analyses as described above, all samples will be disposed of after 5 years.

STUDY RESULTS:

Participants may contact the Study Coordinator or Principal Investigator by telephone for the results of the study, both individual and overall, once the study has been completed. Additionally, if requested, the participants will be given the information on any published manuscript related to the study.

The results of this study will be published in peer-reviewed scientific journals and/or presented at conferences, seminars or other public forums without breaking the confidentiality and privacy as stated above. Your identity will not be disclosed in any presentations or publications of the results of the study.

POTENTIAL COSTS OF PARTICIPATION AND REIMBURSEMENT TO PARTICIPANT:

Those who participate and successfully complete the study will be provided $250.00 compensation for time and travel costs. The participant will be provided with $50 per visit to cover these costs (5 visits in total for the duration of the study), which will be given at one time, either at premature withdrawal or upon completion of the study. The participant will be asked to sign a receipt indicating the sum to be reimbursed and the university will issue a check within 2 months of study completion.

COMPENSATION FOR INJURY:

If you suffer a physical injury from consuming the provided raspberries and/or as a direct result of the administration of this study, medical care will be provided to you in the same manner as you would ordinarily obtain any other medical treatment. In no way does signing this form waive your legal rights nor release the study doctors, the study sponsor or involved institutions from their legal and professional responsibilities.
PARTICIPATION AND WITHDRAWAL:

Participation in research is voluntary. If you choose not to participate, you and your family will continue to have access to customary care at St Michael’s Hospital. If you choose to participate in this study you can withdraw from the study at any time without any effect on the care you or your family will receive at St Michael’s Hospital.

Premature withdrawal from the study will be followed by destruction of all your personal data. Compensation will only be provided for the duration of the study completed. Participants may not complete the study by their own choice, or upon request by the study doctors because it is in their best interest to stop participation or because they do not follow study directions.

NEW FINDINGS OR INFORMATION:

We may learn new things during the study that you may need to know. We can also learn about things that might make you want to stop participating in the study. If so, you will be notified about any new information in a timely manner. You may also be asked to sign a new consent form discussing these new findings if you decide to continue in the research study.

RESEARCH ETHICS BOARD CONTACT:

The study protocol and consent form have been reviewed by a committee called the Research Ethics Board at St. Michael’s Hospital. The Research Ethics Board is a group of scientists, medical staff, and individuals from other backgrounds (including law and ethics) as well as members from the community. The committee is established by the hospital to review studies for their scientific and ethical merit. The Board pays special attention to the potential harms and benefits involved in participation to the research participant, as well as the potential benefit to society. This committee is also required to do periodic review of ongoing research studies. As part of this review, someone may contact you from the Research Ethics Board to discuss your experience in the research study.

If you have any questions regarding your rights as a research participant, you may contact Dr. Julie Spence, Chair, Research Ethics Board at 416-864-6060 ext. 2557 during business hours.

STUDY CONTACTS:

In case of questions or emergency please contact Dr. Venket Rao or Ms Dawn Snyder at (416) 978-3621, or Dr. David Jenkins at (416) 867-7475.
TITLE OF STUDY: Raspberries and Human Health

CONSENT:

The research study has been explained to me, and my questions have been answered to my satisfaction. I have been informed of the alternatives to participation in this study. I have the right not to participate and the right to withdraw without affecting the quality of medical care at St. Michael’s Hospital for me and for other members of my family. As well, the potential harms and benefits of participating in this research study have been explained to me.

I have been told that I have not waived my legal rights nor released the investigators, sponsors, or involved institutions from their legal and professional responsibilities. I know that I may ask now, or in the future, any questions I have about the study. I have been told that records relating to me and my care will be kept confidential and that no information will be disclosed without my permission unless required by law. I have been given sufficient time to read this consent information in Form B.

I consent to participate. I have been told I will be given a signed copy of this consent form.

Name of Participant (Print): Signature: Date:

_____________________________          ___________________    ________________

Name of Person Obtaining Consent: Signature: Date:

_____________________________          ___________________    ________________
FORM C

Study Design and Participant’s Schedule

TITLE OF STUDY: Raspberries and Human Health

WEEK 1: Baseline - _________________________________________________________

consume regular diet, including nutritional supplements if taken
- complete 7-day food record

VISIT #2: ________________________ - come fasting

WEEK 2: Washout Period - ____________________________________________________

- consume regular diet, excluding:
  - berries, red grapes and pomegranates (fresh/frozen/dried/baked/juiced)
  - nutritional supplements (including fortified beverages) and herbal tea

VISIT #3: ________________________ - come fasting

WEEK 3 - 6: Treatment Period - _______________________________________________

- consume regular diet, excluding:
  - other berries, red grapes and pomegranates (fresh/frozen/dried/baked/juiced)
  - nutritional supplements (including fortified beverages) and herbal tea
- consume 1 cup of frozen raspberries with a meal, daily
  - do not heat
  - if thawed, also consume juices
- complete 3-day food record, during week 6

VISIT #4: ________________________ - come fasting

VISIT #5: ________________________ - come fasting

WEEK 7 - 10: Post-Treatment Period - _____________________________________________

- consume regular diet, excluding:
  - berries, red grapes and pomegranates (fresh/frozen/dried/baked/juiced)
  - nutritional supplements (including fortified beverages) and herbal tea
- complete 3-day food record, during week 10

VISIT #6: ________________________ - come fasting

All visits take place at the Risk Factor Modification Center which is located on the 6th floor of 61 Queen St. E., across from St Michael’s hospital and east of the Queen Subway station.
FORM D

Foods to Avoid by Participants

TITLE OF STUDY: Raspberries and Human Health

Please avoid consuming the following during the washout, treatment and post-treatment periods of your participation in this study (weeks 2-10):

- Berry fruits of any variety, fresh or frozen – identifiable by a ‘-berry’ suffix in their name
- Berry fruit products, in such forms as juices, jams, baked goods and so on
- Red/purple grapes and its juice
- Pomegranates and its juice
- Red wine
- Green tea and other herbal teas
- Purple/red coloured fruits and vegetables, excluding tomatoes
- Nuts, excluding peanuts
- Nutritional supplements, including multi-vitamins, antioxidants, minerals, herbals, botanicals and fortified beverages
FORM E-1  
Instructions for Completing Food Records

TITLE OF STUDY: Raspberries and Human Health

Please record all food consumed for the period of 7 consecutive days or additional select days as outlined in your study schedule (Form C). It is important to maintain your usual eating habits during these days and throughout your participation in the study. If you have any questions, please contact the Principal Investigator, Dr. A.V. Rao, or the Study Coordinator, Dawn Snyder, at (416) 978-3621; if there is no answer, leave a message and your call will be returned promptly.

1. Record foods and beverages as soon as possible after consumption.

2. Provide a complete description (cooking method, brand name) of the type of food or beverage.
   e.g., baked/boiled/pan-fried/deep-fried
   McDonald’s/Kellogg’s/Lean Cuisine

3. Record the amount of the food or beverage consumed in units as accurate as possible.
   e.g., 1 slice of white bread               1 cup of Kellogg’s corn flakes
        2 heaping tsp of brown sugar        8 oz 2% milk

   Meat portions are often difficult to estimate; 1 deck of playing cards is typically the same size as 3 oz of cooked meat. If you are uncertain, try measuring the item’s dimensions (e.g., roast beef, 5x1/2 “x 41/2” x 1/4”).

4. For combination dishes, please provide a detailed description by listing the individual ingredients or provide recipes.

<table>
<thead>
<tr>
<th>Detailed description</th>
<th>Vague description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 medium plain bagel</td>
<td>1 cheese sandwich</td>
</tr>
<tr>
<td>1 tsp margarine</td>
<td></td>
</tr>
<tr>
<td>2 oz cheddar cheese</td>
<td></td>
</tr>
<tr>
<td>1 1/2 cups cooked white rice</td>
<td>Chicken stir fry</td>
</tr>
<tr>
<td>1/2cup broccoli, boiled</td>
<td></td>
</tr>
<tr>
<td>2 oz chicken breast</td>
<td></td>
</tr>
<tr>
<td>1 tsp soy sauce</td>
<td></td>
</tr>
</tbody>
</table>

5. For vitamins and other nutritional supplements, please record the type of supplement, amount, frequency taken, and the brand name.

Additional pages detailing your diet (food/supplement labels, recipes) should be attached to your food record as you see fit.
FORM E-2
Food Record

TITLE OF STUDY: Raspberries and Human Health

<table>
<thead>
<tr>
<th>Time Eaten</th>
<th>Food/Beverage and Description (include nutritional supplements if applicable)</th>
<th>Quantity (cups, ml, g, tsp, etc)</th>
<th>For Official Use Only</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Is this a usual day? Yes _____  No ______

If “No” please explain:
__________________________________________________________________________
__________________________________________________________________________
FORM F

Instructions for Consuming Raspberry Supplement

TITLE OF STUDY: Raspberries and Human Health

Please consume one cup of the provided frozen raspberries daily during the treatment period of the study (weeks 3-6).

When measuring the raspberries:
- Measure while frozen
- Keep remaining raspberries stored in the freezer
- Use any of the following units to measure your daily portion:
  - 1 cup, standard imperial system
  - 250 ml
  - 123 g

When preparing the raspberries for consumption:
- Do not heat, bake or cook
- Do not microwave
- If thawing is desired, leave at room temperature or in the fridge overnight

When consuming the raspberries:
- Eat them with dinner
- Eat the entire one cup as a single portion
- If thawed, consume all the juices
- Do not add new foods to your regular diet to make the raspberries more palatable *(i.e. eating ice cream and raspberries every night during the treatment period)*
- Change up how you eat them so that you do not get bored with the same food combination

Suggestions for incorporating frozen raspberries into your diet:
- Spread on toast
- Blend with a drink while frozen
- Sprinkle lightly with sugar for desert
PRE-STUDY QUESTIONNAIRE
Demographic, Health and Lifestyle

STUDY TITLE: Raspberries and Human Health

I.D. Code:_______________  Date:_______________

Part A: Contact Information

First name:________________________  Last name:________________________

Residential address:

_____________________________________________________________________________________________________

ADDRESS   APT. #

_____________________________________________________________________________________________________

CITY   POSTAL CODE

Preferred number to be reached at:

   Home:________________________

   Work:________________________

   Mobile:_______________________

Preferred time of day to be contacted:________________________

Email:_________________________________________________

Preferred method of communication:  Phone / Email
I.D. Code:_________________________ Date:____________________

**Part B: Demographic Information**

Gender: Male / Female

Date of Birth:___________________________________________

**Part C: Health Information**

Current medications:________________________________________________

Current health conditions:________________________________________________

History of medication use:________________________________________________

History of health conditions:________________________________________________

Current nutritional supplements:___________________________________________

Are you pregnant, lactating, or planning on becoming pregnant?:  Y / N

Are you allergic to raspberries?:  Y / N
I.D. Code:_________________________ Date:_________________________

**Part C: Lifestyle Information**

Are you a habitual smoker, casual smoker, or non-smoker?:______________________________

If you smoke, how many cigarettes do you smoke in an average day/week?:_________________

Do you drink alcohol?: Y / N

If yes, how many alcoholic beverages do you consume in an average week?:______________

Do you regularly engage in continuous intense physical activity for ≥ 2 hours?: Y / N

If yes, how many times per week/month?:______________________________________________

**Please sign below to indicate completion of Parts A-D of this questionnaire:**

PARTICIPANT_________________________________________ COORDINATOR_________________________________________

DATE_________________________________________ DATE_________________________________________
WEEK 5 QUESTIONNAIRE
Raspberry Consumption

STUDY TITLE: Raspberries and Human Health Study

I.D. Code:_______________                      Date:_______________

Part A: Please circle your answer to the following questions. 1 means you definitely DO NOT agree with the statement; 5 means you definitely DO agree with the statement.

I find one cup of frozen raspberries a comfortable quantity to consume in a single serving.

1 2 3 4 5 6

I find consuming frozen raspberries enjoyable.

1 2 3 4 5 6

Part B: Please answer the following questions in as much detail as required.

How are you consuming the frozen raspberries in this study?

____________________________________________________________________

____________________________________________________________________

____________________________________________________________________

Have you been experiencing any unusual symptoms while consuming the raspberries (ie. itching, bloating, loose bowel movements)?:

____________________________________________________________________

____________________________________________________________________

What are you finding to be the most difficult part about participating in this study?:

____________________________________________________________________

____________________________________________________________________

Please sign below to indicate completion of this questionnaire:

PARTICIPANT_________________________ COORDINATOR_________________________

DATE_________________________ DATE_________________________
Appendix 2

Participant forms from Study 2

Consent to participate in a research study ........................................... 152
Dietary guidelines ............................................................................. 161
Study schedule .................................................................................. 162
Questionnaire: Demographic, health and lifestyle .............................. 163
Consent to Participate in a Research Study

INFORMATION

TITLE OF STUDY: Pharmacokinetics of Red Raspberries

INTRODUCTION:

Before agreeing to take part in this research study, it is important that you read the information in this research consent form. It includes details we think you need to know in order to decide if you wish to take part in the study. If you have any questions, ask a study doctor or study staff. You should not sign this form until you are sure you understand the information. All research is voluntary. You may also wish to discuss the study with your family doctor, a family member or close friend. If you decide to take part in the study, it is important that you are completely truthful about your health history and any medications you are taking. This will help prevent unnecessary harm to you.

INVESTIGATORS:

Dr. A.V. Rao, Principal Investigator, M.Sc., Ph.D.
Professor Emeritus, Department of Nutritional Sciences
University of Toronto
Tel #: (416) 978-3621, Tues – Thurs, 9:00 AM – 12:00 PM
Email: v.rao@utoronto.ca

Dr. David J. A. Jenkins, M.D., Ph.D., D.Sc.
Staff Physician, St. Michael’s Hospital
Professor, Department of Nutritional Sciences
University of Toronto
Tel #: (416) 867-7475, Mon – Fri, 10:00 AM – 5:00 pm
Email: GalbraithK@smh.toronto.on.ca

Ms. Ye Seul Song, Student Co-investigator
Undergraduate student, Department of Nutritional Sciences
University of Toronto
Tel #: (647) 680-6597, Anytime
Email: yeseul.song@utoronto.ca

Ms. Dawn Snyder, Student Co-investigator
M.Sc. candidate, Department of Nutritional Sciences
University of Toronto
Tel #: (289) 892-2270, Mon – Fri, 9:00 AM – 6:00 PM
Email: dawn.snyder@utoronto.ca
STUDENT RESEARCH PROJECT:

The study is being conducted as the fourth year undergraduate research project of student co-investigator, Ms. Ye Seul Song. As well, the study results will contribute to the Master’s of Science (M.Sc.) research thesis of student co-investigator, Ms. Dawn Snyder. Dr. A. V. Rao is Ms. Ye Seul Song and Ms. Dawn Snyder’s advisor, and the Principal Investigator on the study. This means that he will be supervising the conduct of the study. Dr. David J. A. Jenkins is a staff member at St. Michael’s Hospital and is providing facilities at the Clinical Nutrition and Risk Factor Modification Centre for the conduction of the study. He is also collaborator in the study and will be supervising its conduct.

CONFLICTS OF INTEREST:

None of the study personnel involved in the study declare any conflicts of interest.

STUDY SPONSORS:

1. Washington Red Raspberry Commission
2. Raspberry Industry Development Council

PURPOSE OF THE RESEARCH:

You are invited to participate in a nutrition research study on the absorption, metabolism and excretion of red raspberry phytochemicals. You have been selected as a potential participant because you are a healthy adult, who has no history of chronic disease, is not currently taking medications, has not taken antibiotics in the past 3 months, if female, is not pregnant or lactating, and has no allergy to berry fruits.

Dietary guidelines recommend that people increase their consumption of fruits and vegetables in order to improve their health and reduce their risk of developing chronic diseases. Fruits and vegetables are not only good sources of essential nutrients and fibre, but they contain beneficial ‘phytochemicals’. Phytochemicals are compounds found in plants that are not considered essential in the diet, but are believed to play a role in improving human health. We are specifically interested in red raspberries because of their unique phytochemical composition and their high antioxidant content.

Some phytochemicals function as antioxidants. An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation reactions can produce free radicals (molecules), which start chain reactions that damage cells. Your body naturally produces free radicals, for example, when it breaks down food. However, you are also exposed to free radicals in the environment from cigarette smoke, ultra-violet radiation, and other pollutants. Oxidative stress is the harmful condition that occurs when there is an excess of free radicals as a result of aging, poor nutrition and/or lifestyle. Long-term damage caused by oxidative stress is thought to
contribute to the development of chronic diseases, such as cancer and cardiovascular disease. It is possible to reduce oxidative stress by consuming foods that are rich in antioxidants, like raspberries.

The evidence supporting the benefits of raspberry consumption comes mostly from experiments on cells and animals. Very few studies have tested on humans. It is the purpose of this study to determine whether red raspberry consumption leads to: 1) the absorption and excretion of red raspberry phytochemicals detectable in the blood and urine; and 2) an increase in the antioxidant capacity of the blood. Additionally, this study will determine if these effects can be increased by increasing the quantity of red raspberries consumed. The results of this study will contribute knowledge on the role of red raspberries in the diet for improving human health and the prevention of chronic disease.

DESCRIPTION OF THE RESEARCH:

This is a 5-week study and will require a short pre-study visit and five full-day study visits to the Clinical Nutrition and Risk Factor Modification Center, St. Michael’s Hospital, 61 Queen St. E. There will be a total of 10 eligible participants (5 male and 5 female) recruited for this study, and recruitment is only taking place at this one location.

Participation in this study involves the following:

Pre-Study Visit: During this short visit you will be given a paper copy of the Consent form, which you would have received an electronic copy of to review prior; and you will have the opportunity to ask study personnel any questions before signing. To participate you must be willing and able to attend five of the pre-specified study visit dates, of which the study personnel will inform you of. In order to verify your eligibility and obtain participant characteristic data, you will complete a Health Questionnaire and have your body measures taken. The questionnaire asks questions regarding your age, gender, health history, medication and nutritional supplement use, as well as smoking, drinking and exercise habits. Measurements of your height, weight and blood pressure will be taken by study personnel.

Low Polyphenol Diet: The two days prior to each study visit you will be asked to avoid the consumption of most plant-based foods. A list of ‘allowable foods’ and ‘foods to avoid’, as well as a few meal suggestions, is provided in the Dietary Guidelines (see attached form). Since this diet is primarily meat-based, it may not be appropriate for some people, and therefore, willingness and ability to follow the low polyphenol diet is required for participation in the study. You will also be asked to record all food and beverages consumed during these days on the provided Food Record Sheets (see attached forms), which should be handed in to study personnel during the following study visit.

Preparation for Study Visits: The day before each study visit, you will be asked to consume at least 2 L of water to ensure adequate hydration for blood sampling. The day of the study visit, you will not consume breakfast or any beverages, with the exception of water, prior to coming into the centre for your appointment. You will also provide a sample of your second morning urinary void in the container given to you during the previous visit. This sample may be
collected before or after your arrival at the centre, but it must be provided before you receive your raspberry treatment.

**Study Visits:** Upon arrival to the centre, you will have a catheter inserted into a vein on your arm by a registered nurse. A baseline fasting blood sample will be taken. You will then be given a breakfast, consisting of a red raspberry beverage or white bread, to consume within 15 min, and zero time will be marked by its completion. Additional blood samples will be taken at 0.25, 0.5, 1, 2, 4, 6 and 8 hours. All your urinary voids during the study visit must be collected in a large plastic container. You will be provided with an 8 oz bottle of water after 2, 4 and 6 hours, as well as a standard meal after 4 hours. The standard meal will consist of a McDonald’s BigMac with cheese, mayonnaise-style sauce, and no other toppings, plus a medium order of french fries. No other food or beverage will be allowed during the study visit. All participants must stay at the centre during the study visit. You can bring books, games, a laptop, or work to occupy yourself during this time. Movies, magazines, board games, and a limited number of computers with internet access will be available to you at the centre.

**Post-Study Visit Urine Collections:** At the end of each study visit you will be provided with another large urine collection container to take home. All your urinary voids during the 16 hours following the study visit will be collected. You will continue to follow the low polyphenol diet during this 16 hour period. The container will then be returned to study personnel at the centre the following morning.

**Red Raspberry Treatment:** Throughout the study you will receive three different red raspberry doses (1, 2, or 4 cups) and two control meals consisting of two slices of white bread and a glass of water, containing vitamin C or nothing. The breakfasts will be given to you in a random order, one at the beginning of each study visit. Your red raspberry treatment will be made up of frozen red raspberries, thawed and blended with water to a total volume of 750 ml. Sweetener will be added to the beverage to improve its palatability. Following your first blood sample, you will be given 15 minutes to consume the entire breakfast.

**Total Time Commitment:** Participation in the study requires one pre-study visit of approximately 30 minutes in length and five 8 hour and 20 minute study visits to the Clinical Nutrition and Risk Factor Modification Center, St. Michael’s Hospital, 61 Queen St. E. Plus five 5 minute visits the morning following each study visit. Additional time may be required in order to follow the low polyphenol diet and record your food intake during the two days prior to each study visit.

**Collection of Blood Samples:** A catheter will be inserted into a vein on your arm and left in place for the duration of the study for ease of repeat blood sampling. All blood samples will be taken by experienced study nurses. Study personnel will be present at all times. A total of eight blood samples, 6 ml in quantity, will be taken throughout each study visit, for a total of 48 ml of blood drawn.

**Collection of Urine Samples:** Urine samples will be collected by you, in the provided containers. You are responsible to collect a sample of your second morning void, the day of each study visit, and hand this in to study personnel. During each study visit, beginning post raspberry consumption, you will collect all urinary voids into a large container. This container will be
labeled with your name and kept in the washroom during the study visit for easy access. At the end of each study visit another urine collection container will be provided to you to take home. All your urinary voids during the 16 hours following the study visit will be collected. The container will then be returned to study personnel at the centre the following morning.

Analysis of Blood and Urine Samples: All blood and urine samples will be labeled with participants’ study I.D. Code and used only for the purpose of this research study. The samples will be processed at the Risk Factor Modification Center and stored at the University of Toronto, in a -70°C freezer. Phytochemical content of the samples, as well as total antioxidant capacity of the blood, will be analyzed at the University of Toronto, FitzGerald Building, 150 College St. Extra samples will be stored for 5 years, and used only to verify results of the study. This may be necessary if methodological advancements allow for improved detection of parameters of interest. All samples will be destroyed after this period.

POTENTIAL HARMS (INJURIES, DISCOMFORT, INCONVENIENCES):

Some individuals may be allergic to consuming raspberries and be at risk of experiencing an allergic reaction in response to treatment. To minimize such risk, you are asked at the time of screening and in the health questionnaire whether you have or suspect an allergy to any type of berry fruits. If your response is positive, then you will not be selected to participate in the study and the reason explained. If after starting your participation in the study you experience an allergic response to consuming raspberries, you will be asked to withdraw from the study and the reason explained.

There may be a small amount of bleeding when blood is taken from the vein, and there may be slight discomfort and bruising or redness. This will usually disappear in a few days. If a more severe reaction occurs, such as infection, please consult a physician and inform study personnel.

The urine sample to be provided at the beginning of each study visit, and the urine collection to be completed during each study visit, may be viewed as an inconvenience to some.

The low polyphenol diet, involving avoidance of most plant-based foods, is limited, and can therefore be an inconvenience as well as potentially less enjoyable than your typical diet. Additionally, recording your food intake can also be an inconvenience as it may be time consuming.

POTENTIAL BENEFITS:

You may receive no direct benefits from being in the study. However, results from the study may further medical or scientific knowledge in the area of diet, health and disease.

ALTERNATIVES TO PARTICIPATION:
The alternative to participation in the study is to not participate. There are no likely consequences to you if you decide not to participate in the study. Recruitment will be continued until the required number of participants is attained.

PROTECTING YOUR HEALTH INFORMATION:

Confidentiality will be respected and no information that discloses your identity will be released if results of the study are published or presented. Your identity will not be disclosed without your permission unless required by law.

Any medical records, documentation, study samples or information related to you will be de-identified and labelled with a study I.D. Code to ensure that persons outside of the study will not be able to identify you. All information will be stored in a secure place, either a locked filing cabinet or a password protected electronic file. A password protected electronic file will be the only documentation linking your study I.D. Code to your identity. This file will be deleted following completion of the study and all analyses.

By signing this form, you are authorizing access to your study records by the study personnel and the St. Michael’s Hospital Research Ethics Board. Such access will be used only for purposes of verifying the authenticity of the information collected for the study, without violating your confidentiality, to the extent permitted by applicable laws and regulations.

National and Provincial Data Protection regulations, including the Personal Information Protection and Electronic Documents Act (of Canada) or PIPEDA and the Personal Health Information Protection Act (PHIPA) of Ontario, protect your personal information. They also give you the right to control the use of your personal information, including personal health information, and require your written permission for your personal information (including personal health information) to be collected, used or disclosed for the purposes of this study, as described in this consent form. You have the right to review and copy your personal information. However, if you decide to be in this study or choose to withdraw from it, your right to look at or copy your personal information related to this study will be delayed until after the research is completed.

It is important to understand that despite these protections being in place, experience in similar studies indicates that there is the risk of unintentional release of information. The study personnel will protect your records and keep all the information in your study file confidential to the greatest extent possible. The chance that this information will accidentally be given to someone else is small.

All identifiable records will be destroyed following study completion. All de-identified study records will be kept for 5 years, and then destroyed. De-identified blood and urine samples will be frozen in a –70°C freezer at University of Toronto and used for the analyses identified above; remaining samples will be stored and then disposed of after 5 years.

STUDY RESULTS:
Participants may contact study personnel by telephone or email for both individual and/or overall results, once the study has been completed. Additionally, you can request information on any published manuscript related to the study.

The results of the study will be published in peer-reviewed scientific journals and/or presented at conferences, seminars or other public forums without breaking the confidentiality and privacy as stated above. Your identity will not be disclosed in any presentations or publications of the results of the study.

POTENTIAL COSTS OF PARTICIPATION AND REIMBURSEMENT TO PARTICIPANT:

Those who participate and successfully complete the study will be provided compensation for time and travel costs. You will be given $60 per visit, plus $10 per overnight urine collection, for a total of up to $350, which will be received in a single payment following study completion or early withdrawal. You will be asked to sign a receipt indicating the sum to be reimbursed and the University of Toronto will issue you a check within 2 months.

COMPENSATION FOR INJURY:

If you suffer a physical injury from consuming the provided food and/or as a direct result of the administration of this study, medical care will be provided to you in the same manner as you would ordinarily obtain any other medical treatment. In no way does signing this form waive your legal rights nor release the study doctors, the study sponsor or involved institutions from their legal and professional responsibilities.

PARTICIPATION AND WITHDRAWAL:

Participation in research is voluntary. If you choose not to participate, you and your family will continue to have access to customary care at St Michael’s Hospital. If you choose to participate in this study you can withdraw from the study at any time without any effect on the care you or your family will receive at St Michael’s Hospital.

Premature withdrawal from the study will be followed by destruction of all your personal data. Compensation will only be provided for the duration of the study completed. Participants may not complete the study by their own choice, or upon request by the study doctors because it is in their best interest to stop participation or because they do not follow study directions.

NEW FINDINGS OR INFORMATION:

We may learn new things during the study that you may need to know. We can also learn about things that might make you want to stop participating in the study. If so, you will be notified
about any new information in a timely manner. You may also be asked to sign a new consent form discussing these new findings if you decide to continue in the research study.

RESEARCH ETHICS BOARD CONTACT:

The study protocol and consent form have been reviewed by a committee called the Research Ethics Board at St. Michael’s Hospital. The Research Ethics Board is a group of scientists, medical staff, and individuals from other backgrounds (including law and ethics) as well as members from the community. The committee is established by the hospital to review studies for their scientific and ethical merit. The Board pays special attention to the potential harms and benefits involved in participation to the research participant, as well as the potential benefit to society. This committee is also required to do periodic review of ongoing research studies. As part of this review, someone may contact you from the Research Ethics Board to discuss your experience in the research study.

If you have any questions regarding your rights as a research participant, you may contact Dr. Julie Spence, Chair, Research Ethics Board at 416-864-6060 ext. 2557 during business hours.

STUDY CONTACTS:

In case of questions or emergency, please contact Dr. A. Venket Rao at (416) 978-3621, or Dr. David J. A. Jenkins at (416) 867-7475.
Consent to Participate in a Research Study
SIGNATURE PAGE

TITLE OF STUDY: Pharmacokinetics of Red Raspberries

CONSENT:

The research study has been explained to me, and my questions have been answered to my satisfaction. I have been informed of the alternatives to participation in this study. I have the right not to participate and the right to withdraw without affecting the quality of medical care at St. Michael’s Hospital for me and for other members of my family. As well, the potential harms and benefits of participating in this research study have been explained to me.

I have been told that I have not waived my legal rights nor released the investigators, sponsors, or involved institutions from their legal and professional responsibilities. I know that I may ask now, or in the future, any questions I have about the study. I have been told that records relating to me and my care will be kept confidential and that no information will be disclosed without my permission unless required by law. I have been given sufficient time to read the consent information.

I consent to participate. I have been told I will be given a signed copy of this consent form.

Name of Participant (Print): ___________________________ Signature: ___________________________ Date: ___________________________

Name of Person Obtaining Consent: ___________________________ Signature: ___________________________ Date: ___________________________

Principal Investigator:

Dr. A. Venket Rao

Phone #: 416-978-3261

Email: v.rao@utoronto.ca
DIETARY GUIDELINES

Low Polyphenol Diet

TITLE OF STUDY: Pharmacokinetics of Red Raspberries

For the two days prior to each of the three study visits, only consume the allowable foods indicated in the table below and avoid consumption of all other plant-based foods. All foods consumed on these days must be recorded on the Food Record Sheets provided.

<table>
<thead>
<tr>
<th>FOOD TYPE</th>
<th>ALLOWABLE FOODS</th>
<th>FOODS TO AVOID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beverages</td>
<td>milk, water, club soda</td>
<td>fruit juices, vegetable juice, coffee, tea, soda pop, alcohol</td>
</tr>
<tr>
<td>Grains</td>
<td>white bread, white rice, white pasta</td>
<td>all other breads, baked goods, breakfast cereals, noodles, crackers, etc.</td>
</tr>
<tr>
<td>Vegetables</td>
<td>none</td>
<td>all</td>
</tr>
<tr>
<td>Fruit</td>
<td>none</td>
<td>all</td>
</tr>
<tr>
<td>Protein</td>
<td>fish, poultry, meat without fillers</td>
<td>sausage, hot dogs, bologna, etc.</td>
</tr>
<tr>
<td></td>
<td>eggs</td>
<td>no meat alternatives, legumes, nuts (including peanut butter), seeds, tofu</td>
</tr>
<tr>
<td>Dairy</td>
<td>cheese, plain or vanilla yogurt</td>
<td>no fruit, nut, or chocolate flavoured dairy products</td>
</tr>
<tr>
<td>Other</td>
<td>butter, cooking oil</td>
<td>no olive oils or margarine</td>
</tr>
<tr>
<td></td>
<td>sugar, honey, jell-o, custard, vanilla ice cream</td>
<td>all other deserts and sweets</td>
</tr>
<tr>
<td></td>
<td>Mayonnaise, salt, pepper</td>
<td>all other condiments, sauces and spices</td>
</tr>
</tbody>
</table>

Meal Suggestions:

**Breakfast**
- White toast with butter and honey
- Eggs on white toast
- Grilled cheese sandwich on white bread (no ketchup)

**Lunch/Dinner**
- Canned tuna with mayonnaise on white bread
- Grilled chicken, beef or pork on rice (no sauce)
- Beef, chicken or seafood noodle bowl in meat stock (no vegetables or sauce)
- All beef hamburger with cheese (no toppings)
- Plain white pasta with butter

**Snack/Desert**
- Plain yogurt
- Jell-O
- Vanilla ice cream
STUDY SCHEDULE (EXAMPLE)

STUDY TITLE: Pharmacokinetics of Red Raspberries

On each of the coloured days, please follow the appropriate guidelines as outlined below.

<table>
<thead>
<tr>
<th>Monday</th>
<th>Tuesday</th>
<th>Wednesday</th>
<th>Thursday</th>
<th>Friday</th>
<th>Saturday</th>
<th>Sunday</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Study visit at 7:10am</td>
<td>Study end at 7:30am</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Study visit at 7:10am</td>
<td>Study end at 7:30am</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>15</td>
<td>16</td>
<td>17</td>
<td>18</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Study visit at 7:10am</td>
<td>Study end at 7:30am</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>22</td>
<td>23</td>
<td>24</td>
<td>25</td>
<td>26</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Study visit at 7:10am</td>
<td>Study end at 7:30am</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Study visit at 7:10am</td>
<td>Study end at 7:30am</td>
<td></td>
</tr>
</tbody>
</table>

Guidelines:

- Follow low polyphenol diet
- Follow low polyphenol diet; drink 2L of water
- Collect 2nd morning urinary void; do not eat or drink before study visit; follow low polyphenol diet; after study visit collect all urinary excretions
- Follow low polyphenol diet until time of study end; collect all morning urinary excretions; bring urine collection container to centre
HEALTH QUESTIONNAIRE

STUDY TITLE: Pharmacokinetics of Red Raspberries

I.D. Code:____________________  Date:____________________

Please answer the following questions:

1. **Date of Birth:**

2. **Gender:** Male / Female
   
   *If female:*
   
   i. Are you pregnant, lactating, or planning on becoming pregnant?: Yes / No

3. **Do you have or suspect an allergy to any type of berry fruit?:** Yes / No

4. **Do you regularly take any medications?:** Yes / No
   
   *If yes:*
   
   i. Please List:

5. **Do you regularly take any nutritional supplements?:** Yes / No
   
   *If yes:*
   
   i. Please List:
6. Have you taken antibiotics in the past 3 months?: Yes / No ; year?: Yes / No

7. Have you ever been diagnosed with a chronic health condition?: Yes / No

   If yes:
   i. Please List:______________________________________________________________
      _________________________________________________________________

8. Do you smoke cigarettes or any other substances?: Yes / No

   If yes:
   i. Are you a casual or habitual smoker?:__________________________

9. Do you drink alcohol?: Yes / No

   If yes:
   i. How many drinks do you consume in a standard week?:_______________

10. How many minutes of physical activity do you engage in daily?: <30 / 30-60 / >60

Please sign below to testify your accurate completion of the above questionnaire:

COORDINATOR_____________________________________________________

DATE_____________________________________________________________
Appendix 3

Health biomarkers and chronic disease risk.

<table>
<thead>
<tr>
<th>Health Marker*</th>
<th>Risk of Developing Health Problems</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Least:</td>
<td>Increased:</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>18.5 - 24.9</td>
<td>25.0 - 29.9</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>&lt;120</td>
<td>130 - 139</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>&lt;80</td>
<td>85 - 89</td>
</tr>
<tr>
<td>Glucose fasting (mmol/L)</td>
<td>&lt;6.1</td>
<td>6.1 - 6.9</td>
</tr>
<tr>
<td>TC/HDL-C fasting</td>
<td>&lt;5.0</td>
<td>≥5.0</td>
</tr>
<tr>
<td>LDL-C Calc. fasting (mmol/L)</td>
<td>&lt;3.5</td>
<td>≥3.5</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>&lt;1.0</td>
<td>1.0 - 3.0</td>
</tr>
</tbody>
</table>

* To convert glucose, cholesterol and triglycerides to milligrams per deciliter, multiple by 18.02, 38.67 and 88.57, respectively.