EFFECTS OF ANTIOXIDANT STATUS AND ORAL DELIVERY SYSTEMS ON QUERCETIN BIOAVAILABILITY

Dissertation

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By

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ABSTRACT

Quercetin is one of the most abundant dietary flavonols that has putative cardioprotective activities. Quercetin has low bioavailability and elicits highly variable inter-individual responses following its ingestion, however, factors regulating quercetin bioavailability remain unclear. Since bioactivities of quercetin partly depend on its bioavailability, elucidating determinants for quercetin bioavailability will facilitate a better understanding of its cardioprotective activities. Therefore, this dissertation aimed to investigate the influence of endogenous factor (antioxidant status) and exogenous factor (dietary fat and nano-formulation) on quercetin bioavailability. The central hypothesis of this dissertation was that adequate antioxidant status would improve quercetin aglycone bioavailability and that its bioavailability would be enhanced by co-ingestion with dietary fat or administration as nano-emulsion. Our studies in obese adults demonstrated that dietary fat improved quercetin bioavailability by increasing its absorption. In addition, a quercetin aglycone-containing nanoemulsion was developed using self nano-emulsifying drug delivery system. Our studies in rats demonstrated that the designed quercetin nanoemulsion enhanced quercetin absorption, thereby improving its bioavailability, and increasing its intestinal and hepatic accumulation. Lastly, contrary to our hypothesis, studies in healthy adults showed that greater quercetin bioavailability was associated with inadequate plasma vitamin C status and greater plasma endotoxin. Collectively, this dissertation demonstrated that greater quercetin aglycone bioavailability could be achieved when it was ingested with dietary fat or administered orally as nano-emulsion, or when inadequate plasma vitamin C
status and greater intestinal permeability were present. The findings described herein are of significance in that they provide the foundational basis for development of effective and feasible strategies to improve quercetin bioavailability in humans. These findings will also facilitate intervention studies aiming to evaluate putative cardioprotective activities of quercetin and development of dietary recommendation for quercetin in effort to mitigate CVD risks.
DEDICATION

To my wonderful parents for all of their support, unwavering love, and encouragement.
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# TABLE OF CONTENTS

Abstract ........................................................................................................................................ ii

Dedication ...................................................................................................................................... iv

Acknowledgements ...................................................................................................................... v

Vita ................................................................................................................................................ vii

List of figures ................................................................................................................................. xiii

List of tables .................................................................................................................................... xv

Chapter 1. Introduction .................................................................................................................... 1
  1.1 Overview .................................................................................................................................. 1
  1.2 Central hypothesis and specific aims ....................................................................................... 1

Chapter 2. Literature review .......................................................................................................... 5
  2.1 Introduction ............................................................................................................................... 5
  2.2 Quercetin is a dietary flavonol ............................................................................................... 6
  2.3 Bioavailability of quercetin ...................................................................................................... 6
    2.3.1 Quercetin absorption ........................................................................................................ 6
    2.3.2 Quercetin metabolism in the small intestine .................................................................... 9
    2.3.3 Quercetin metabolism in the liver .................................................................................... 11
    2.3.4 Quercetin excretion in the colon and the kidney ............................................................... 13
    2.3.5 Quercetin bioavailability ................................................................................................. 13
    2.3.6 Quercetin bioavailability is limited by its poor absorption and biliary excretion ... 16
    2.3.7 Quercetin bioavailability varies between individuals ....................................................... 18
  2.4 Endogenous factors that may affect quercetin bioavailability .............................................. 21

viii
2.4.1 Gender ................................................................................................................. 21
2.4.2 Age ..................................................................................................................... 22
2.5 Exogenous factors ................................................................................................. 23
2.5.1 Type of sugar moieties in quercetin glycosides .................................................. 24
2.5.2 The presence of sugar moiety ............................................................................. 25
2.5.3 Dietary Fat ......................................................................................................... 26
2.5.4 Non-digestible fiber ......................................................................................... 28
2.5.5 Food matrix ...................................................................................................... 29
2.5.6 Nano-formulation of quercetin aglycone ............................................................ 30
2.6 Implications of quercetin bioavailability for studying bioactivity ...................... 32
2.7 Conclusion ............................................................................................................. 33
2.8 Figures and tables ................................................................................................. 35

Chapter 3. Dietary fat increases quercetin bioavailability in overweight adults........ 38
3.1 Abstract ................................................................................................................. 38
3.2 Introduction .......................................................................................................... 39
3.3 Materials and methods ....................................................................................... 41
3.3.1 Materials ......................................................................................................... 41
3.3.2 Subjects .......................................................................................................... 41
3.3.3 Study design .................................................................................................... 41
3.3.4 Dietary quercetin and isorhamnetin intakes ....................................................... 43
3.3.5 Sample handling ............................................................................................. 43
3.3.6 HPLC analysis of quercetin and its methylated metabolites ................................ 43
3.3.7 Clinical chemistries, antioxidants, and biomarkers of oxidative stress, and inflammation ........................................................................................................ 44
3.3.8 In vitro digestion ............................................................................................ 45
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.9 Statistical analysis</td>
<td>45</td>
</tr>
<tr>
<td>3.4 Results</td>
<td>47</td>
</tr>
<tr>
<td>3.4.1 Participants and diets</td>
<td>47</td>
</tr>
<tr>
<td>3.4.2 Antioxidants and markers of oxidative stress and inflammation</td>
<td>47</td>
</tr>
<tr>
<td>3.4.3 Pharmacokinetics of quercetin</td>
<td>47</td>
</tr>
<tr>
<td>3.4.4 Methylated metabolites of quercetin</td>
<td>48</td>
</tr>
<tr>
<td>3.4.5 Micellarization efficiency of quercetin during in vitro digestion</td>
<td>49</td>
</tr>
<tr>
<td>3.5. Discussion</td>
<td>50</td>
</tr>
<tr>
<td>3.6. Acknowledgements</td>
<td>55</td>
</tr>
<tr>
<td>3.7 Figures and tables</td>
<td>56</td>
</tr>
<tr>
<td>Chapter 4. Quercetin-containing self-nanoemulsifying drug delivery system for improving oral bioavailability</td>
<td>61</td>
</tr>
<tr>
<td>4.1 Abstract</td>
<td>61</td>
</tr>
<tr>
<td>4.2 Introduction</td>
<td>62</td>
</tr>
<tr>
<td>4.1 Materials and methods</td>
<td>64</td>
</tr>
<tr>
<td>4.3.1 Materials</td>
<td>64</td>
</tr>
<tr>
<td>4.3.2 Solubility assessment</td>
<td>64</td>
</tr>
<tr>
<td>4.3.3 Screening of surfactants for emulsifying activity</td>
<td>65</td>
</tr>
<tr>
<td>4.3.4 Construction of pseudo-ternary phase diagrams</td>
<td>65</td>
</tr>
<tr>
<td>4.3.5 Preparation and characterization of quercetin-containing SNEDDS</td>
<td>65</td>
</tr>
<tr>
<td>4.3.6 Determination of quercetin encapsulation efficiency</td>
<td>66</td>
</tr>
<tr>
<td>4.3.7 In vitro quercetin release study</td>
<td>67</td>
</tr>
<tr>
<td>4.3.8 Stability of the Q-SNEDDS</td>
<td>67</td>
</tr>
<tr>
<td>4.3.9 Transport of Q-SNEDDS in Caco-2 cells</td>
<td>68</td>
</tr>
<tr>
<td>4.3.10 Visualization of intestinal Q-SNEDDS absorption in rats</td>
<td>69</td>
</tr>
</tbody>
</table>
4.3.11 Bioavailability of quercetin in rats................................................................. 69
4.3.12 Statistical analysis............................................................................................. 71
4.4 Results and discussion ......................................................................................... 72
4.4.1 Selection of components for SNEDDS............................................................. 72
4.4.2 Construction of pseudo-ternary phase diagram and formulation optimization .... 73
4.4.3 Characterization of the optimized Q-SNEDDS............................................... 75
4.4.4 Stability of Q-SNEDDS..................................................................................... 76
4.4.5 Transport of the Q-SNEDDS across Caco-2 cell monolayer............................ 76
4.4.6 Histological evaluation .................................................................................... 78
4.4.7 Pharmacokinetics of quercetin in rats......................................................... 79
4.5 Conclusion ........................................................................................................... 82
4.6 Acknowledgement................................................................................................. 82
4.7 Figures and tables................................................................................................. 83

Chapter 5. Quercetin bioavailability is associated with inadequate plasma vitamin C
status and greater plasma endotoxin in adults.......................................................... 96
5.1 Abstract .............................................................................................................. 96
5.2 Introduction ......................................................................................................... 98
5.3 Materials and methods ...................................................................................... 100
5.3.1 Participants .................................................................................................... 100
5.3.2 Study design .................................................................................................. 100
5.3.3 Dietary quercetin and isorhamnetin intakes .................................................. 101
5.3.4 Blood processing and clinical chemistries...................................................... 101
5.3.5 Plasma quercetin and methylated metabolites ............................................. 101
5.3.6 Biomarkers of antioxidant status, oxidative stress, and inflammation .......... 101
5.3.7 Plasma endotoxin........................................................................................... 102
5.3.8 Statistical analyses........................................................................................................... 102
5.4 Results .................................................................................................................................. 104
5.4.1 Participants and dietary intakes of quercetin and isorhamnetin............................ 104
5.4.2 Plasma pharmacokinetics of quercetin, isorhamnetin and tamarixetin ......... 104
5.4.3 Relations between quercetin pharmacokinetics and biomarkers of oxidative stress...................................................................................................................................... 105
5.4.4 Relations between quercetin pharmacokinetics, endotoxin, and vitamin C ..... 106
5.5 Discussion .......................................................................................................................... 107
5.6 Conclusion ......................................................................................................................... 111
5.7 Acknowledgements ............................................................................................................ 111
5.8 Figures and tables.............................................................................................................. 112
Chapter 6. Discussion ............................................................................................................. 118
6.1 Overview ............................................................................................................................ 118
6.2 Dietary fat improves quercetin bioavailability in humans............................................ 119
6.3 Self nano-emulsifying drug delivery system improves quercetin bioavailability ... 122
6.4 Vitamin C and intestinal permeability may mediate quercetin bioavailability ...... 127
6.5 Pharmacokinetics of isorhamnetin and tamarixetin.................................................... 129
6.6 Limitations and future direction ....................................................................................... 130
6.7 Conclusion ........................................................................................................................ 131
6.8 Figures and tables.............................................................................................................. 132
References............................................................................................................................... 135
LIST OF FIGURES

Figure 2.1 Backbone structures of benzoic acid (C1-C6), cinnamic acid (C3-C6) and flavonoids (C6-C3-C6) .......................................................... 35

Figure 2.2 Biotransformation of quercetin ......................................................... 36

Figure 3.1 Plasma concentration-time curves of quercetin, isorhamnetin, and tamarixetin for 24 h following oral ingestion of 1095 mg of quercetin aglycone with a fat-free, low-fat, or high-fat meal .......................................................... 56

Figure 3.2 In vitro micellarization efficiency of quercetin and its relation to fat content 57

Figure 4.1 Solubility of quercetin in various oils, surfactants, and co-surfactants ...... 83

Figure 4.2 Pseudo-ternary phase diagrams of formulations ................................................. 84

Figure 4.3 Particle size distribution of optimal nanoemulsions without quercetin, and optimal quercetin-loaded nanoemulsions .......................................................... 85

Figure 4.4 TEM images of optimal nanoemulsions without quercetin, and optimal Q-SNEDDS nanoemulsions at 72 h post-dilution of Q-SNEDDS .............................................. 86

Figure 4.5 Release profiles of Q-SNEDDS nanoemulsion in simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.8) containing 0.5% (w/v) Tween 80 at 100 rpm and 37 °C ........................................................................................................ 87

Figure 4.6 Stability of Q-SNEDDS nanoemulsions from two systems (castor oil/Cremophor® RH 40/Transcutol® HP and castor oil/ Tween® 80/ Cremophor® RH 40/PEG 400) stored at room temperature for 1 month ......................................................... 88
Figure 4.7 The mean cumulative transport versus time of quercetin solution and Q-SNEDDS nanoemulsions across Caco-2 cell monolayer at 37 °C for 1 h using initial quercetin concentration of 50 µg/mL ................................................................. 89

Figure 4.8 Fluorescence images of rat intestine treated with Q-SNEDDS nanoemulsions and quercetin control suspension (control) containing nile red ........... 90

Figure 4.9 Plasma concentration-time profiles of quercetin, tamarixetin and isorhamnetin in Sprague-Dawley rats followed by an oral administration of 15 mg/kg quercetin control suspension or 15 mg/kg Q-SNEDDS nanoemulsion ............... 91

Figure 5.1 Plasma concentrations of quercetin, isorhamnetin, tamarixetin, and Qtotal for 24 h following oral ingestion of quercetin aglycone (1095 mg) by healthy adults . 112

Figure 5.2 Correlations between plasma vitamin C and AUC_{0-24 h} and C_{max} of Q_{total} . 113

Figure 5.3 Correlations between plasma endotoxin and AUC_{0-24 h} and C_{max} of Q_{total}... 114

Figure 5.4 Correlations between plasma vitamin C and plasma endotoxin and plasma myeloperoxidase .................................................................................................................. 115

Figure 6.1 Relationship between dose of quercetin aglycone supplements and plasma quercetin response in chronic clinical studies shown in table 1. ........................................... 132
LIST OF TABLES

Table 2.1 Quercetin contents in Food ................................................................. 37
Table 3.1 Dietary intakes for 24 h of each trial ................................................... 58
Table 3.2 Concentrations of plasma antioxidants and biomarkers of oxidative stress and inflammation at baseline in fat-free, low-fat and high-fat trials ........................................ 59
Table 3.3 Plasma pharmacokinetic parameters of quercetin, isorhamnetin and tamarixetin in the fat-free, low-fat, and high-fat trials ......................................................... 60
Table 4.1 Formulations, droplet size and appearance of nanoemulsions composed of castor oil/Cremophor® RH 40/Transcutol® HP ......................................................... 92
Table 4.2 Formulations, droplet size and appearance of nanoemulsions composed of castor oil/Tween® 80/ Cremophor® RH 40/PEG 400 .............................................. 93
Table 4.3 Pharmacokinetic parameters of quercetin in rats after oral administration of quercetin control suspension and Q-SNEDDS (15 mg/kg) ........................................ 94
Table 4.4 Tissue concentrations of quercetin metabolites after oral administration of quercetin control suspension and Q-SNEDDS to rats at doses of 15 mg/kg .......... 95
Table 5.1 Pharmacokinetic parameters of plasma quercetin, isorhamnetin, tamarixetin, and Qtotal ............................................................................................................ 116
Table 5.2 Correlations between plasma antioxidants, biomarkers of oxidative stress and inflammation, and AUC0-24 h and Cmax of Qtotal .............................................. 117
Table 6.1 Concentrations of plasma quercetin in humans following chronic supplementation of quercetin aglycone ........................................................................ 134
Table 6.2  Plasma biomarkers of liver and kidney injury in rats prior to and 24 h after oral administration of quercetin aglycone in sodium carboxymethyl cellulose or Q-SNEDDS (30 mg/kg BW quercetin aglycone) ........................................................................................................ 135
CHAPTER 1
INTRODUCTION

1.1 Overview

Emerging evidence supports that bioactive food components derived from fruits and vegetables are a potential dietary strategy to prevent cardiovascular disease (CVD), the leading cause of morbidity and mortality in the US (1, 2). Quercetin is a bioactive food component found in fruits and vegetables in a near-ubiquitous manner and has been linked to favorable CVD-related outcomes as indicated by epidemiological observations suggesting that its higher dietary intake lowers CVD risk in healthy adults, even after for controlling for risk factors such as BMI, smoking, blood pressure, cholesterol, and diabetes (3). Although quercetin improves numerous indices of cardiovascular health, its bioavailability is poor and exhibits substantial inter-individual variability (4-6). Moreover, factors regulating its bioavailability are incompletely understood. Since cardioprotective activities of quercetin partly depend on its bioavailability, it is essential to more fully define factors influencing its bioavailability in order to better understand its putative cardioprotective activities.

1.2 Central hypothesis and specific aims

To better define the potential benefits of quercetin on cardiovascular health, an understanding of endogenous and exogenous factors affecting its absorption and metabolism are needed. This is of considerable importance because our work demonstrates that dietary supplementation of quercetin aglycone (1095 mg) decreases blood pressure in overweight hypertensive adults 10 h after its ingestion despite its
limited bioavailability. Blood pressure in overweight hypertensive adults 10 h after its ingestion despite its limited bioavailability (7). Thus, the research of this dissertation aims to define the influence of dietary fat, endogenous antioxidant status, and quercetin-containing nanoemulsions on quercetin bioavailability and will be accomplished by conducting a series of pharmacokinetics studies in humans and rodents. Findings from this research are expected to contribute to development of strategies for improving quercetin bioavailability and a greater understanding of the relationship between antioxidant status and quercetin bioavailability. This work is also of significance because it is anticipated to provide the foundational basis to inform future studies aiming to better define the cardioprotective effects of quercetin and to formulate targeted dietary recommendations for quercetin in effort to mitigate CVD risk and other disorders implicated by oxidative stress and inflammation. The central hypothesis of this dissertation is that adequate antioxidant status improves bioavailability of quercetin aglycone and that its bioavailability can be enhanced by co-ingestion with dietary fat and by delivered as nanoemulsions. To test this hypothesis, the following specific aims will be evaluated:

**Aim 1. Define the extent to which dietary fat co-ingestion improves bioavailability of quercetin aglycone in overweight adults.**

The working hypothesis is that co-ingestion of quercetin aglycone with dietary fat will dose-dependently improve its bioavailability in overweight adults. This hypothesis was tested by conducting a randomized, crossover pharmacokinetics study in overweight men and overweight postmenopausal women who ingested 1095 mg of quercetin aglycone with standardized meals that provided varying levels of fat. An in vitro digestion study was also performed to evaluate the extent to which dietary fat enhanced micellarization of quercetin aglycone. The findings of this study demonstrated that
dietary fat improves bioavailability of quercetin aglycone by increasing its absorption, likely mediated through enhancing its micellarization at the small intestine.

**Aim 2. Define the influence of antioxidants, oxidative stress and pro-inflammatory responses on bioavailability of quercetin aglycone in healthy adults.**

The *working hypothesis* is that greater bioavailability of quercetin aglycone will occur in the presence of adequate antioxidant status, whereas lower quercetin aglycone bioavailability will be associated with greater oxidative stress and pro-inflammatory responses. This hypothesis was tested by conducting a pharmacokinetics study in healthy adults who ingested 1095 mg quercetin aglycone with a standardized meal to assess whether quercetin aglycone bioavailability is affected by individual differences in circulating antioxidants, oxidative stress and inflammation, and intestinal permeability. The findings of this study indicated that quercetin pharmacokinetics were highly variable between participants and contrary to our hypothesis, greater quercetin bioavailability was associated with poor vitamin C status. It was also observed that quercetin bioavailability was associated with greater plasma endotoxin, supporting that vitamin C status and intestinal permeability are potential modulators of quercetin bioavailability.

**Aim 3. Define the influence of quercetin-containing nanoemulsions on bioavailability of quercetin aglycone in rats.**

The *working hypothesis* is that a quercetin-containing nanoemulsion developed using self-nanoemulsifying drug delivery system (SNEDDS) will improve quercetin aglycone bioavailability. This hypothesis was tested by conducting a pharmacokinetics study in rats following oral administration of quercetin aglycone containing-SNEDDS (Q-SNEDDS) or quercetin aglycone suspended in a solution of sodium carboxymethyl cellulose by oral gavage. Findings from this study demonstrated that Q-SNEDDS increases quercetin aglycone absorption, thereby improving its bioavailability and
accumulation in small intestine and liver. These findings support Q-SNEDDS as a promising approach to improve quercetin aglycone bioavailability.
CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Quercetin is a flavonoid that is present ubiquitously in fruits and vegetables (8). Although controversy exists regarding its role in improving human health, studies suggest that it exerts cardioprotective, hepatoprotective, anti-diabetic, and anti-cancer activities (9-12). Health-promoting activities of quercetin are dependent upon its bioavailability as exemplified by studies showing that inhibition of platelet aggregation occurs with increases in plasma quercetin (13). However, quercetin bioavailability is poor, similar to other polyphenols (14), and has an estimated bioavailability of 17% in pigs (4, 15). Its bioavailability also varies widely between individuals, and substantial differences exist in its plasma concentrations when participants ingest quercetin supplements regardless of standardization of their administration (16). Thus, the putative health benefits of quercetin are likely to be marginalized among those who absorb quercetin to a limited extent. Further complicating is the limited understanding of exogenous and endogenous factors regulating the variable responses in quercetin bioavailability. Undoubtedly, quercetin bioavailability is an important determinant of its bioactivities, thereby indicating a need to better define factors affecting its bioavailability in order to more fully understand and realize its benefits on human health. Therefore, this review aims to summarize the knowledge related to the bioavailability of dietary quercetin and to describe its biotransformation that is mediated through xenobiotic metabolism while also emphasizing endogenous factors that may be responsible for the high variability.
observed in its bioavailability. This discussion will also identify potential strategies for improving quercetin bioavailability, particularly in the context of its potential role for reducing cardiovascular disease (CVD) risk.

2.2 Quercetin is a dietary flavonol

Polyphenols are phytochemicals derived from plants. They have a phenolic structure, and are classified into phenolic acids and flavonoids (17). Phenolic acids are divided into benzoic acid and cinnamic acid derivatives based on their C1-C6 and C3-C6 backbones whereas flavonoids are characterized by a C6-C3-C6 backbone (Figure 2.1) (17).

Flavonols are a subgroup of flavonoids with quercetin being among the most abundant dietary flavonol (8, 17). In nature, quercetin exists primarily as quercetin glycosides (18), and consists of quercetin aglycone conjugated to sugar moieties including glucose and rutinose (Figure 2.2) (19). Quercetin is distributed widely in fruits, vegetables, nuts, tea, and wine (8, 20). Quercetin-rich foods include lettuce, chili pepper, cranberry, onion, black chokeberry, tomato, broccoli, and apple (Table 2.1) (21). Cohort studies suggest that dietary quercetin intakes range from 6-18 mg/d in the United States, China, and the Netherlands (22-24).

2.3 Bioavailability of quercetin

2.3.1 Quercetin absorption

Quercetin bioavailability depends upon its absorption, metabolism, and elimination. Quercetin absorption occurs in the stomach and/or the small intestine (25-27). Stomach uptake of quercetin aglycone has been demonstrated in an in situ gastric perfusion model, in which the pylorus of unconscious rats was ligated prior to perfusing quercetin aglycone prepared in a gastric buffer (pH 4.5) into the gastric lumen (26). After quercetin aglycone (15 μM) was incubated in the stomach for 30 min, 62% of the perfused dose was recovered from the gastric contents, accompanied by the appearance of quercetin
and its methylated metabolite in the bile. This finding suggested that 38% of the perfused dose was absorbed in the stomach. In the same experimental model, concentrations of quercetin glucoside or quercetin rutinoside remained unchanged after these quercetin glycosides were incubated in the stomach, indicating that glycosylated forms of quercetin are not absorbed at the stomach (26).

Studies in an *in situ* intestinal perfusion rat model demonstrate that quercetin aglycone absorption also occurs in the small intestine (27). In this study, quercetin aglycone (15 μM) was perfused through a cannula inserted into the jejunum of unconscious rats, and the effluent was collected from a cannula placed in the distal ileum. Following perfusion, 14% of the perfused quercetin aglycone disappeared in the effluent, which was concomitant with increases in plasma quercetin. This demonstrated that quercetin aglycone was absorbed in the small intestine.

The manner by which quercetin aglycone is absorbed by the stomach remains unclear. Studies in Caco-2 cells suggest that quercetin aglycone is primarily absorbed by passive diffusion and secondarily by organic anion transporting polypeptide (OATP) in the small intestine (28). In this study, media containing quercetin aglycone was added to apical side of Caco-2 cell monolayer. Subsequently, quercetin aglycone concentrations were measured in the media collected from the basolateral side to determine the permeability coefficient following its transfer from the apical to the basolateral side. The permeability coefficient of quercetin aglycone was 4-times higher compared with a paracellular passive transport marker (mannitol), but 7-times lower compared with a transcellular passive transport marker (propranol). This suggests that transport of quercetin aglycone through the intestinal epithelium is mediated through a transcellular-passive diffusion pathway. The permeability coefficient of quercetin aglycone was decreased by 39% in the presence of estrone-3 sulfate, an OATP inhibitor, suggesting
that intestinal uptake of quercetin is mediated by OATP. Although OATP-B was proposed to be responsible for transporting quercetin aglycone in this study, other OATP isoforms (e.g. OATP1A2) are also expressed in the human intestine and may participate in quercetin aglycone absorption in vivo (28, 29). Also, quercetin aglycone is weakly acidic (pK_a = 6.3) and its ionized form exists in intestinal fluid (pH = 5-7), which may lend itself to being a substrate of OATP (28, 30, 31).

Studies using an in situ small intestinal perfusion model demonstrate that the small intestine is the site of uptake of certain quercetin glycosides (i.e. quercetin glucosides, quercetin galactoside, quercetin arabinoside) (25). Perfusion of these quercetin glycosides in rats resulted in decreased concentrations of intact quercetin glycosides in the intestinal perfusate, with parallel increases in plasma quercetin and isorhamnetin, a methylated metabolite of quercetin (Figure 2.2) (25). Intestinal uptake of these quercetin glycosides involves luminal deglycosylation by lactase phlorizin hydrolase (LPH), a β-glucosidase residing at the brush border membrane of small intestine, and passive absorption of the hydrolyzed quercetin aglycone (32, 33). This absorption pathway of quercetin glycosides has been demonstrated in studies using quercetin-4-glucoside in an everted-jejunal sac model in rats (32). After quercetin-4-glucoside was incubated in jejunal sacs in the presence of a LPH inhibitor, intestinal mucosa concentrations of quercetin aglycone were 83% lower compared with incubation of quercetin-4-glucoside in the jejunal sacs in the absence of the LPH inhibitor. In addition, following incubation of quercetin-4-glucoside in the jejunal sacs (no LPH inhibitor present), quercetin glucoside was not detected in the jejunum, whereas 89% of quercetin metabolites were detected in the mucosa, with the remaining portion found in the tissue and the serosa. These findings indicate that deglycosylation is a prerequisite for absorption and metabolism of quercetin glycosides in the small intestine.
Quercetin rutinoside absorption occurs in the colon (34). Indeed, ileostomy patients who ingested tomato juice fortified with quercetin rutinoside had no detectable quercetin metabolites in plasma or urine, however, ~86% of the ingested quercetin rutinoside was recovered from ileal fluid (34). In contrast, when healthy participants with an intact colon ingested the same tomato juice, quercetin metabolites, including quercetin-3-glucuronide and isorhamnetin-3-glucuronide (Figure 2), were detected in plasma and urine. Since β-glucosidase has been found in gut microbiota (35), quercetin rutinoside may be converted into quercetin aglycone that is then absorbed by the colon. In addition, studies using an in vitro model of anaerobic human fecal fermentation showed that 60% of quercetin rutinoside was degraded to phenylacetic acid within 2 h by colonic microbiota (36). This suggests that the majority of quercetin rutinoside is probably first deglycosylated to quercetin aglycone, which is then degraded into phenylacetic acid rather than being absorbed in the colon.

2.3.2 Quercetin metabolism in the small intestine

Xenobiotic metabolism consists of three phases that occur independently and/or additively to eliminate xenobiotics: phase I modification, phase II conjugation, and phase III elimination (37). Phase I modification, which includes oxidation, reduction and hydrolysis, is primarily catalyzed by cytochrome P450 (CYP) enzymes. During phase II metabolism, xenobiotics are converted into their conjugated metabolites by various transferases. Phase III elimination of xenobiotic compounds is mediated though active membrane transporters, including breast cancer resistance protein (BCRP) and multidrug resistance associated-protein (MRP).

Quercetin is a dietary xenobiotic, but is not subjected to phase I metabolism, because its flavonoid structure makes it an unfavorable substrate for CYPs (38). Although quercetin is not metabolized by CYPs, quercetin differentially affects CYPs
activities (39). For example, quercetin supplementation (500 mg/d, 12 d) decreased CYP1A2 activity but increased CYP2A6 activity in adults (39). CYP1A2 and CYP2A6 activities were assessed after providing participants a single oral dose of caffeine 2 wk prior to and immediately after quercetin supplementation and measuring the changes in plasma and urinary caffeine metabolites, which included 1,7-dimethylnxanthine and 1,7-dimethylurate (39). Lower urinary levels of 1,7-dimethylnxanthine indicated that quercetin supplementation inhibited CYP1A2 activity. CYP2A6 activity was increased by quercetin supplementation as suggested by greater urinary levels of 1,7-dimethylurate. These findings suggest that quercetin-mediated alterations in CYPs activities may affect bioavailability of compounds metabolized by CYPs such as caffeine.

Phase II conjugation of quercetin in the small intestine involves glucuronidation, sulfation, and methylation. This is evidenced by the detection of glucuronidated, sulfated, and methylated metabolites of quercetin in the small intestine, after rats were fed a diet containing 0.45% (w/w) quercetin aglycone for 6 wk (40). Phase II quercetin-conjugating enzymes include catechol-O-methyltransferase (COMT), sulfotransferase (SULT), and uridine 5’-diphospho-glucuronosyltransferase (UGT) (27, 41). The resultant conjugated metabolites include quercetin monoglucuronide, quercetin diglucuronide, methylated quercetin monoglucuronide sulfate, and quercetin sulfate (40). Among a variety of quercetin metabolites, isorhamnetin and tamarixetin are methylated metabolites, and are also positional isomers (Figure 2.2) (27, 41).

Phase III elimination of quercetin metabolites occurs in the small intestine, as evidenced by studies in an in situ intestinal perfusion rat model showing that quercetin glucuronides and sulfates, isorhamnetin, and tamarixetin were detected in the effluent collected from the intestinal lumen (27). Intestinal efflux of quercetin metabolites is mediated through BCRP1 and MRP2, which are phase III transporters located at the
apical side of enterocytes (42, 43). The excretion of quercetin glucuronides into luminal perfusate was decreased after rat small intestine was perfused in situ with quercetin-3'-glucoside and fumitremorgin C, a BCRP1 inhibitor (42). Decreases in secretion of quercetin glucuronide into media were also observed when Caco-2 cells were incubated with MK-571, a MRP2 inhibitor (43).

In addition to their intestinal efflux, quercetin metabolites can also be secreted into both the portal vein and the lymph (25, 44). Concentrations of quercetin metabolites (i.e., sum of glucuronidated and sulfated metabolites of quercetin) in the portal vein increased to 5 μM after small intestine was perfused with 50 μM quercetin-4'-glucoside for 30 min in the small intestine- and portal vein-cannulated rats (25). After quercetin aglycone suspended in a saturated glucose solution (10 mg/kg body weight) was administered intraduodenally for 8 h in rats, cumulative lymph concentration of quercetin metabolites (i.e., sum of glucuronidated and sulfated metabolites of quercetin) was 8.3 nM (44). Quercetin aglycone concentrations in portal vein or lymph samples were below detection limits (25, 44), suggesting that most of the absorbed quercetin is metabolized and becomes conjugated metabolites in the small intestine.

2.3.3 Quercetin metabolism in the liver

Quercetin metabolites reach the liver through the portal vein (25). Uptake of quercetin metabolites by hepatocytes likely involves passive diffusion and organic anion transporters (OATs) and/or OATPs-mediated transport. These uptake pathways have been studied in HepG2 cells treated with quercetin-3'-sulfate or quercetin glucuronides (45). Uptake of quercetin-3'-glucuronide, quercetin-7'-glucuronide and quercetin-3'-glucuronide were 7-times lower than that of quercetin-3'-sulfate after HepG2 cells were separately treated (10 min, 37°C) with these quercetin metabolites. In addition, uptake of quercetin glucuronides was not affected by temperature, indicating that these
metabolites passively diffuse into HepG2 cells. In contrast to quercetin glucuronides, uptake of quercetin-3'-sulfate by HepG2 cells was 8-times lower when HepG2 cells were incubated at 4°C compared with 37°C, which suggests that quercetin-3'-sulfate uptake involves carrier-mediated transport. Intracellular accumulation of quercetin-3'-sulfate was decreased following incubation of HepG2 cells with quercetin-3'-sulfate and inhibitors of OATs and OATPs, such as estrone-3-sulfate, digoxin and sulfobromophthalein. Further, uptake of quercetin-3'-sulfate was 2.3- or 1.4-times higher in human embryonic kidney cells transfected with OAT4 and OATP4C1 than control cells. Collectively, these findings indicate that quercetin glucuronides passively diffuse into hepatocytes, whereas OATs and OATPs mediate hepatic uptake of quercetin-3'-sulfate.

Following their hepatic uptake, quercetin metabolites are further metabolized by phase II conjugating enzymes in the liver. For example, after quercetin-7-glucuronide was incubated in HepG2 cells for 48 h, 44% of total quercetin detected in the culture media were 3'-methylquercetin-7-glucuronide and 4'-methylquercetin-7-glucuronide, and 7% of total quercetin was quercetin-3'-sulfate (46). These findings suggest that methylation of quercetin glucuronides occurs in the liver. The formation of quercetin-3'-sulfate also suggests that quercetin glucuronides are de-glucuronidated followed by sulfation in the liver, because β-glucuronidase is expressed in human liver (47), and no evidence supports the presence of quercetin metabolites-specific sulfatase in humans.

Hepatic quercetin metabolites are either secreted into the circulation or excreted into bile (25). In bile duct cannulated rats, two additional cannulas were inserted into the duodenum and the cecum (25). After quercetin-3-glucoside was perfused through the small intestine for 30 min, the appearance of quercetin metabolites, including glucuronidated and sulfated quercetin, isorhamnetin and tamarixetin, were detected in both plasma and bile. In addition, biliary excretion of quercetin metabolites is possibly
mediated through MRP2, a phase III transporter on the apical side of hepatocyte. This is evidenced by a study showing that methylated quercetin glucuronides secreted in the media was decreased by 73% when HepG2 cells were co-incubated with quercetin glucuronides and MK-571, a MRP2 inhibitor (46).

2.3.4 Quercetin excretion in the colon and the kidney

Unabsorbed quercetin and quercetin metabolites that are effluxed by small intestine are eliminated rapidly via feces and urine (48). After rats received a single dose of radiolabeled quercetin glucoside by oral gavage, 61% of radioactivity was recovered in feces and urine at 12 h, and 87% of radioactivity was eliminated at 72 h (48). A majority of radiolabeled compounds were identified as 3-hydroxyphenylacetic acid, benzoic acid, and hippuric acid (48). These metabolites suggest that quercetin aglycone, which is derived from quercetin glucoside, is subjected to ring fission in the colon and the kidney to produce 3,4-dihydroxyphenylacetic acid. Subsequently, 3,4-dihydroxyphenylacetic acid may undergo dehydroxylation to yield 3-hydroxyphenylacetic acid, which then may be oxidized and dehydroxylated to produce hippuric acid and benzoic acid. In addition, although little is known whether deconjugation enzymes exist for methylated and sulfated metabolites of quercetin in vivo, β-glucuronidase exists in the colon microflora and the kidney (36, 49). This suggest that quercetin glucuronides are hydrolyzed to quercetin aglycone in the colon and the kidney, and then quercetin aglycone is catabolized to phenolic acids for excretion.

2.3.5 Quercetin bioavailability

Bioavailability of dietary constituents is defined as the fraction of an orally administered food-derived substance that is utilized for normal physiological functions or storage (50). This indicates that a major determinant of bioavailability is the proportion of a substance that is absorbed and reaches systemic circulation (50). Pharmacokinetics
studies are an approach to assess bioavailability in humans and animal models. In pharmacokinetics studies, the change in plasma concentrations of the food-derived substance are evaluated at regular intervals following oral and/or intravenous (i.v.) administration of a single dose of this substance.

Bioavailability is classified as absolute bioavailability and relative bioavailability. Absolute bioavailability is calculated as the area under the plasma concentration-time curve (AUC$_{oral}$) of this substance after its oral administration relative to AUC$_{i.v.}$ following its i.v. administration. For example, AUC$_{oral}$ is 34.1 mg/L x h and AUC$_{i.v.}$ is 46.6 mg/L x h after 500 mg vitamin C solubilized in water is administered orally or intravenously in healthy participants (51). Vitamin C bioavailability is calculated as the ratio of AUC$_{oral}$ to AUC$_{i.v.}$ or 73% (51). Relative bioavailability is calculated as AUC$_{oral}$ after single oral intake of food-derived substance without comparison to a response following i.v. administration. In addition to AUC, other pharmacokinetics parameters derived from plasma concentration-time curves include plasma maximum concentration (C$_{max}$) and time to C$_{max}$ (T$_{max}$), and elimination half-life (t$_{1/2}$). However, measuring plasma concentration-time curves does not yield information regarding the extent of food-derived substances that accumulate at tissues or exert physiological activity.

Absolute bioavailability of quercetin in humans has been examined in two pharmacokinetics studies (52, 53). In one study, participants ingested quercetin aglycone supplements with water or were administered quercetin solubilized in an ethanol-water mixture by i.v. administration (52). Quercetin bioavailability could not be determined because plasma quercetin was not detected in the samples from participants who ingested quercetin aglycone supplements. In this study, plasma quercetin was assessed using a fluorometric method following plasma extraction using water-saturated methyl-ethylketone, derivitization with tetraphenyldiboroxide, and subsequent extraction
with di-isopropyl ether. This fluorometric method aimed to measure quercetin aglycone, however, most plasma quercetin metabolites were glucuronidated and sulfated conjugates, and only a trace amount of quercetin aglycone was found (41, 54). It is possible that this fluorometric method is not suitable for quantitation of plasma quercetin metabolites. In another study, radiolabeled quercetin aglycone solubilized in ethanol was administered orally or intravenously to participants, and plasma quercetin concentrations were determined by measuring total radioactivity (53). Absolute bioavailability of quercetin was calculated to be as high as 44.8%. This study is limited by the fact that degradation of quercetin aglycone occurs during its absorption (55), and absorbed radiolabelled compounds could be quercetin aglycone-derived phenolic acids. In addition, co-administration of quercetin with ethanol enhances its absorption (56). Therefore, absolute bioavailability of quercetin may be overestimated in this study (53).

In pharmacokinetics studies, quercetin bioavailability is determined as relative bioavailability following an oral intake of quercetin provided as supplements or quercetin-rich foods in humans or animals (56, 57). Assessing relative oral bioavailability is easier and less invasive compared to absolute bioavailability, but is clearly less precise. However, in order to produce an adequate plasma response, >50 mg quercetin aglycone or quercetin aglycone equivalents are usually provided, which is higher than that obtained from typical dietary sources (i.e. 6-18 mg/d) (22-24).

Lastly, it is worth noting that the most common quantification method for plasma quercetin in pharmacokinetics studies is to measure total quercetin, total isorhamnetin and total tamarixetin (i.e. the sum of free and glucuronidated/sulfated conjugates of each compound) by HPLC-UV or -FL or -Coularray detection following enzymatic hydrolysis of plasma with β-glucuronidase and sulfatase. Compared to LC-MS methods that measure each metabolite of quercetin, these conventional HPLC approaches are more widely
used because standards for many quercetin metabolites are not yet available commercially.

2.3.6 Quercetin bioavailability is limited by its poor absorption and biliary excretion

Quercetin has low bioavailability when it is orally administered as suspension or ingested with meals (58, 59). Absolute bioavailability of quercetin was 16.2% in rats following oral administration of quercetin aglycone suspended in sodium carboxymethyl cellulose or intravenous administration of quercetin aglycone dissolved in dimethyl sulfoxide and polyethylene glycol (PEG) 200 (59). Relative bioavailability of quercetin is also low, as evidenced by studies where distribution of radioactivity was measured after rats were fed a single meal containing radiolabelled quercetin glucoside (58). In this study, ~6% of ingested radioactivity was present in plasma and internal organs such as liver, kidney and spleen, and >95% of these radiolabeled compounds were conjugated quercetin metabolites. Meanwhile, >85% of ingested radioactivity remained in gastrointestinal tract.

Quercetin bioavailability is dependent upon its absorption. In clinical studies, the extent to which quercetin is absorbed can be estimated by multiplying quercetin $C_{\text{max}}$ by plasma volume (estimated as 3 L for adults; (60, 61)) and subsequently dividing by the administered dose. Pharmacokinetics studies routinely demonstrate low absorption of quercetin in humans. For example, after healthy participants ingested grape juice containing 10 mg quercetin aglycone, quercetin $C_{\text{max}}$ was 0.16 μM and ~1.4% of ingested quercetin was absorbed (62). The extent of quercetin aglycone absorbed was similarly low as that of catechins, since absorption of 25 mg catechin in healthy participants was ~1.7% based on $C_{\text{max}}$ value (0.49 μM) (62). Quercetin glucoside is also poorly absorbed as evidenced by an estimated absorption of ~6.9% in healthy
participants who ingested onion-derived quercetin glucoside at a dose corresponding to 100 mg quercetin aglycone (57).

Although the precise mechanisms explaining quercetin’s poor absorption are not fully understood, studies in rats investigating absolute bioavailability of quercetin aglycone provides evidence that its absorption is related to its solubility in the vehicle used for its administration (59). In this study, absolute bioavailability of quercetin aglycone was 16.2% and quercetin $C_{\text{max}}$ was 2.01 μM following administration of quercetin suspended in aqueous solution. Administration of quercetin aglycone dissolved in an ethanol and PEG200 solution increased absolute bioavailability of quercetin aglycone to 27.5% and its $C_{\text{max}}$ reached 3.44 μM. These findings suggest that quercetin aglycone bioavailability is limited by its low solubility in water or aqueous solution. Indeed, quercetin aglycone is a relatively lipophilic compound with low water solubility. The octanol-water partition coefficient of quercetin aglycone (1.82 ± 0.32) is nearly double that of quercetin-3-glucoside (0.76 ± 0.01), but is lower than that of kaempferol (3.11 ± 0.54) (63). The water solubility of quercetin ranges 1.53-12.5 mg/L at pH values similar to those found in gastrointestinal tract (pH 2-7) (31). Limited water solubility of quercetin aglycone likely precludes its ability to pass through the unstirred water layer and diffuse into enterocytes, thereby limiting its intestinal uptake and absorption. Thus, poor water solubility of quercetin aglycone is one explanation for its low absorption.

Poor absorption of quercetin aglycone and quercetin glycosides may also be attributed to the propensity of it or its metabolites to be excreted back into the intestinal lumen following enterocyte uptake (27, 64). After quercetin aglycone was perfused from the jejunum to the ileum of unconscious rats for 30 min, conjugated quercetin metabolites detected in the collected effluent accounted for 52% of total perfused quercetin aglycone (27). This indicates that a majority of absorbed quercetin aglycone is
rapidly metabolized and excreted back into intestinal lumen. Intestinal efflux of quercetin glycosides has also been demonstrated in an in situ rat intestinal perfusion studies showing that 39% of perfused quercetin-3-glucoside was detected as conjugated metabolites in the luminal effluent (64). In addition to quercetin metabolites, quercetin aglycone or quercetin glycosides are also effluxed across the apical membrane of enterocytes into intestinal lumen. This is supported by studies in Caco-2 cells showing that the permeability of quercetin aglycone or quercetin glucosides from basolateral to apical sides was >2-times higher compared with its apical to basolateral permeability (28, 65). Together, these findings support that quercetin absorption is limited by intestinal efflux of quercetin and its metabolites.

Quercetin bioavailability may also be limited by its biliary excretion from the liver. This notion is supported by studies in bile duct-cannulated rats showing that 30% of ingested quercetin appeared in the bile as glucuronidated, sulfated, and methylated metabolites over a 9-h period following duodenal administration of an aqueous solution containing quercetin glucosides (66). The excretion of quercetin metabolites through the bile into the intestinal lumen suggests that only a limited portion of quercetin metabolites enters circulation.

2.3.7 Quercetin bioavailability varies between individuals

Marked inter-individual variability exists in quercetin bioavailability as evidenced by studies demonstrating substantial variations in quercetin pharmacokinetics among participants ingesting the same dose and form of quercetin. For example, plasma quercetin AUC in healthy participants ranged from 8.9-89.1 μM ● h following an oral intake of onion-derived quercetin glucosides at a dose equivalent to 100 mg quercetin aglycone (57). Quercetin C\text{max} ranged from 0.29-2.26 μM when healthy participants ingested a beverage providing 500 mg quercetin aglycone (16). The coefficient of
variation for $T_{\text{max}}$ and $t_{1/2}$ of quercetin in healthy participants were 69% and 122% following ingestion of 100 mg apple-derived quercetin glycosides (67). In addition to acute pharmacokinetics studies, increases in plasma quercetin were highly variable between individuals (64-370%) following chronic supplementation of quercetin aglycone in healthy adults (2 wk, 50 mg/d) (5).

Wide inter-individual variability in quercetin bioavailability is not a rare phenomenon, but clear explanations for variations in absorption, metabolism and elimination of quercetin are not fully understood. Since β-glucosidase-mediated hydrolysis of quercetin glycosides is an important determinant for absorption of quercetin glycosides in the small intestine (32, 33), individual differences in β-glucosidase activity may account for inter-individual variations in intestinal uptake of orally administered quercetin glycosides. This notion is suggested by studies where activities of human intestinal β-glucosidase were assessed by measuring quercetin glucoside-hydrolyzing capacity (33). There were up to 87-times difference between the lowest and the highest activities (33). However, it remains unknown whether individual differences in β-glucosidase activity are resulted from genetic variation.

Individual differences in intestinal and hepatic expression of phase II quercetin-metabolizing enzymes (UGT, specifically UGT1A family; SULT, specifically SULT1A family; COMT) are speculated to contribute to inter-individual variation in quercetin metabolism. In support, 8 UGT1A isoforms and 5 SULT1A isoforms have been identified in human small intestine and liver, and their mRNA and protein expression vary between individuals (68, 69). For example, UGT1A1 mRNA expression was observed in 60% of collected human duodenum samples, and its expression was absent in 40% of duodenum samples (70). UGT1A4 mRNA expression in collected human liver samples differed by 28-times between individuals (71). Likewise, differences between the lowest
and highest protein levels of SULT1A1 were 7-times in human small intestine samples (68). Further, catalytic activities toward quercetin are different between these isoforms such that higher glucuronidation capacity of UGT1A1 was 29-times greater than that of UGT1A4 in vitro (72). These findings suggest that different expression patterns of UGT1A and SULT1A isoforms may contribute to inter-individual variation in plasma levels of quercetin glucuronides and sulfates. Additionally, individual difference exists in COMT activity as exemplified by a 5-times variation in human hepatic COMT activity when COMT activity was measured using protocatechuic acid as the substrate in vitro (73). However, it remains unclear whether specific COMT isoforms are related to individual variation in hepatic COMT activity.

Activity or expression of quercetin phase III transporters may also vary between individuals. For example, MRP2 is encoded by the ATP-binding cassette sub-family C member 2 or ABCC2 genes (28, 46), and its mRNA expression in human liver samples exhibited a 12-times difference between the lowest and the highest individuals (74). Although it is unknown whether individual differences in MRP2 expression affects quercetin efflux, individuals having a SLCO2B1*1/*1/ABCC2-24CC genotype had significant lower plasma AUC of fexofenadine, a drug for seasonal allergies and chronic hives, compared to individuals having other polymorphisms for fexofenadine metabolism (75). This supports that a MRP2 polymorphisms affects drug bioavailability, and the possibility that similar effects may occur for quercetin.

Collectively, the activity and/or expression level of proteins involved in quercetin metabolism vary between individuals. However, whether there is a causal relation between inter-individual variability in quercetin bioavailability and these xenobiotic metabolizing proteins remains unknown. Additional studies are needed to examine this relationship to better define individual differences in quercetin bioavailability.
2.4 Endogenous factors that may affect quercetin bioavailability

2.4.1 Gender

No clear evidence exists demonstrating that gender affects pharmacokinetics of total quercetin (i.e. the sum of quercetin aglycone, glucuronidated and sulfated quercetin). A study conducted in healthy young adults (8 men and 7 women) reported no significant differences between gender in parameters of absorption kinetics ($C_{\text{max}}$, $T_{\text{max}}$, and rate of absorption), or quercetin AUC, after participants ingested quercetin aglycone supplements (5). This study was unable to determine elimination parameters because of insufficient sampling time points following supplementation, and thus gender differences in elimination kinetics parameters were not examined. Findings from another study suggested a potential gender difference in quercetin elimination in individuals (8 men and 8 women) following an ingestion of quercetin glycosides provided from onions or apples at an equivalent dose of 100 mg quercetin aglycone (67). Regardless of dietary source of quercetin, $t_{1/2}$ in women (15.2 h for onions and 93.8 h for apples) was higher than men (13.4 h for onions and 29.9 h for apples), though these differences did not reach statistical significance. It is worth noting that these two studies reported substantial inter-individual variations in quercetin pharmacokinetics (5, 67). The high degree of variation makes it difficult to detect gender differences, especially when the study population is small. Thus, studies in larger cohorts are needed to determine the extent to which pharmacokinetics parameters of quercetin or its metabolites differ between genders.

Gender may affect quercetin biotransformation (76). After participants (8 men and 8 women) ingested onions-derived quercetin glycosides, AUCs of quercetin sulfate and quercetin glucuronide sulfate were 1- or 4-times greater, respectively, in women compared with men (76). AUCs of quercetin glucuronide, quercetin diglucuronide, and
methylated quercetin glucuronide were similar between men and women. Since this study did not examine gender differences in pharmacokinetics parameters other than AUC, it remains unknown whether higher AUCs of quercetin sulfate and quercetin glucuronide sulfate in women are resulted from greater formation of these metabolites or their slower elimination compared with men.

Gender may also affect bioavailability of quercetin rutinoside but not quercetin aglycone (77). Quercetin AUC was greater in women than men after participants (5 women and 7 men) ingested quercetin rutinoside supplements, whereas quercetin AUCs were similar between genders following ingestion of quercetin aglycone supplements (77). Quercetin rutinoside is absorbed in the large intestine after its hydrolysis by microbiota, and thus it was speculated that sex hormones might affect intestinal microbiota composition and increase intestinal microbiota-producing enzymes capable of hydrolyzing quercetin rutinoside (34, 77, 78). Future studies that collect fecal samples and are conducted in a larger population are needed to confirm this finding.

2.4.2 Age

It remains unclear whether aging affects quercetin bioavailability. A 12-wk quercetin aglycone supplementation study of individuals aged 18–85 y demonstrated that age was not associated with individual differences in plasma concentrations of total quercetin (79). However, aging is associated with numerous physiological and biochemical changes that may affect bioavailability of food components, such as increased gastrointestinal transit time, decreased hepatic blood flow, and decreased glomerular filtration (80). Therefore, randomized clinical trials that investigate the influence of aging on quercetin pharmacokinetics are needed to confirm this observational result (79).

Although the influence of aging on quercetin pharmacokinetics has not been explained, aging may alter the activity of UGT, the enzyme responsible for formation of
quercetin glucuronides (81). UGT was isolated from liver microsomes in 4, 18 and 28 months old rats (81). Hepatic UGT activity was assessed by using quercetin aglycone (30 μM) as the substrate, and then measuring the formation of quercetin-7-glucuronide over 30 min. Quercetin-7-glucuronide production was 12% greater in 28 mo old rats compared with 4 mo old rats, indicating age-associated changes in UGT activity. It remains unclear whether plasma and tissue levels of quercetin glucuronides differ in humans in an age-related manner, which presents an area for further investigation.

2.5 Exogenous factors

2.5.1 Type of sugar moieties in quercetin glycosides

Quercetin is primarily present as quercetin glycosides in plant foods (18). Studies have been performed comparing the bioavailability of quercetin glycosides from onions or apples in healthy adults (67). The AUC and C\text{max} of quercetin following consumption of onions were 7.8 μM • h and 0.9 μM, respectively, which were 2- and 3-times higher than the AUC and C\text{max} of quercetin after consuming apples. Quercetin T\text{max} was similar between onions (2 h) and apples (3 h), but quercetin t\text{1/2} was more than 4-times faster after consuming onions (15 h) than apples (65 h). These findings indicate that the bioavailability of onion-derived quercetin is greater than apple-derived quercetin, resulting from higher absorption of onion-derived quercetin.

The differences in absorption of quercetin glycosides between onions and apples are related to the type of sugar moieties conjugated to quercetin aglycone. The predominant forms of quercetin in onions are quercetin glucosides, whereas quercetin rhamnoside and quercetin galactosides are more abundant in apples (18, 67). Studies using an \textit{in situ} intestinal perfusion model in rats show that uptake of quercetin glucosides is faster and occurs to a greater extent compared with quercetin galactoside and quercetin rhamnoside, while no differences exist between the uptake of quercetin-3-
glucoside and quercetin-4'-glucoside (25). These findings indicate that the type of sugar moiety, not their position of conjugation, determines absorption of quercetin glycosides. This notion is supported by a pharmacokinetics study in healthy participants who ingested equal amounts of quercetin-3-glucoside or quercetin-4'-glucoside supplements (82). Absorption kinetics parameters of quercetin-3-glucoside \( (C_{\text{max}}: 5 \mu\text{M}; T_{\text{max}}: 37 \text{ min}) \) were similar to those of quercetin-4-glucoside \( (C_{\text{max}}: 4.5 \mu\text{M}; T_{\text{max}}: 27 \text{ min}) \). This indicates that absorption of quercetin glycosides is unaffected by the position of conjugation of the sugar moiety. In addition, no differences were detected between \( t_{1/2} \) of quercetin-3-glucoside \( (t_{1/2}: 19 \text{ h}) \) and quercetin-4-glucoside \( (t_{1/2}: 18 \text{ h}) \) (82). The bioavailability of quercetin-3-glucoside (quercetin AUC: 19.1 μM ● h) was also similar to quercetin-4'-glucoside (quercetin AUC: 17.5 μM ● h). Therefore, the bioavailability of quercetin glycosides differs by the type, rather than the position, of the glycoside moiety.

The specificity of LPH may explain why the type of sugar moiety determines absorption of quercetin glycosides (32, 33). LPH is the \( \beta \)-glucosidase catalyzing deglycosylation of quercetin glycosides during their absorption at the small intestine (33). LPH prefers to hydrolyze quercetin glucosides, as demonstrated by a study comparing LPH-mediated deglycosylation rates of quercetin glycosides by measuring quercetin aglycone release following incubation of quercetin glycosides with a highly purified LPH collected from sheep small intestine (33). The tested quercetin glycosides include quercetin glucosides, quercetin galactoside, and quercetin rutinoside. The highest deglycosylation rate \( (3.08-3.16 \mu\text{mol/min/mg protein}) \) was obtained when quercetin glucosides were incubated, whereas deglycosylation rate was the lowest for quercetin galactoside \( (0.13 \mu\text{mol/min/mg protein}) \). The preferential deglycosylation of quercetin glucoside may explain its greater intestinal uptake compared with quercetin galactosides (25). In addition, the release of quercetin aglycone was not detectable following
incubation of quercetin rutinoside, suggesting that quercetin rutinoside is not a substrate for LPH. This may also explain why quercetin rutinoside absorption occurs in the colon but not the small intestine (34).

2.5.2 The presence of sugar moiety

Quercetin bioavailability depends on the presence of sugar moiety. This is demonstrated by studies comparing the bioavailability of quercetin aglycone and quercetin glucoside in pigs (83). In this study, pigs were fed diets containing quercetin aglycone or quercetin-3-glucoside (50 mg/kg body weight) on a single occasion. Quercetin AUC was higher following ingestion of quercetin-3-glucoside (605.7 μM ● min) than ingestion of quercetin aglycone (392.9 μM ● min). Although elimination kinetics were not evaluated, a greater amount of quercetin-3-glucoside was absorbed, as evidenced by the significantly higher quercetin C_max (1.78 μM) compared with the quercetin C_max (1.19 μM) following quercetin aglycone ingestion. This indicates that quercetin glucoside is more bioavailable, which may, at least in part, be resulted from its greater absorption.

Several hypotheses may explain the preferential absorption of quercetin glucoside compared with quercetin aglycone (83). First, quercetin glucoside is more water-soluble than quercetin aglycone, as reflected by its lower octanol-water partition coefficient (0.76 ± 0.01) than quercetin aglycone (1.82 ± 0.32) (63). Greater water solubility facilitates quercetin glucoside diffusion across the unstirred water layer to reach the brush border membrane where quercetin glucoside is preferentially hydrolyzed by LPH. Second, another absorption pathway exists for quercetin glucoside, which is mediated through sodium-dependent glucose transporter 1 (SGLT1). This absorption pathway is evidenced in studies where concentrations of quercetin-3-glucoside and quercetin aglycone were measured in mucosal solution following incubation of rats jejunum.
segments with quercetin-3-glocoside (84). The amount of quercetin-3-gluco-side disappearing from mucosal solutions paralleled the appearance of quercetin aglycone. Further, disappearance of quercetin-3-glucoside decreased in the presence of a SGLT1 inhibitor, which was accompanied by decreases in the amounts of quercetin aglycone appearing in the mucosal solution. Therefore, the SGLT1-mediated pathway may also contribute to greater intestinal uptake of quercetin glucoside, but additional studies are needed to more fully define this possibility.

### 2.5.3 Dietary Fat

Quercetin aglycone is a relatively lipophilic compound, as evidenced by its substantially lower solubility in water than in soybean oil (0.55 vs. 100 μM) (56). Its hydrophobicity suggests that co-ingestion of quercetin aglycone with dietary fat may improve its propensity to be micellarized in the intestinal lumen, thereby increasing its absorption and bioavailability. This hypothesis is supported by a greater quercetin AUC in pigs following co-ingestion of quercetin aglycone with the meal containing 32% fat (%weight) (176.0 μM • min) compared with the meal containing 3% fat meal (%weight) (117.3 μM • min) (85). Quercetin $C_{\text{max}}$ was greater after pigs ingested quercetin aglycone with the meal containing 32% fat (0.56 μM) compared with the meal containing 3% fat meal (0.52 μM), though this difference did not reach statistical significance. Dietary fat also increased the rate of quercetin aglycone absorption, as evidenced by an earlier appearance of quercetin $T_{\text{max}}$ following co-ingestion of quercetin aglycone with the meal containing 32% fat (51 min) compared with the meal containing 3% fat meal (103 min). The speculative explanation is that transport of quercetin aglycone across unstirred water layer is faster when it is incorporated into micelles, but additional studies are needed to examine this hypothesis. In addition, plasma quercetin at 12 h was also significantly higher after pigs ingested quercetin aglycone with the meal containing 32%
fat (0.07 μM) compared with the meal containing 3% fat (0.02 μM). This indicated that dietary fat delayed quercetin aglycone elimination, but the explanation remains unknown. Collectively, dietary fat improves quercetin aglycone bioavailability in pigs. Despite pigs being a model commonly used to recapitulate gastrointestinal physiology of humans, additional studies such as those proposed in this dissertation are being conducted to confirm this relation in humans.

Increases in dietary fat content also enhance quercetin glucoside bioavailability, as evidenced by a greater quercetin AUC following ingestion of quercetin-3-glucoside with the meal containing 32% fat (%weight) (249.7 μM ● min) compared with the meal containing 3% fat (205.5 μM ● min) (85). Quercetin elimination was also delayed after ingestion of quercetin-3-glucoside with the meal containing 32% fat, as demonstrated by a significantly higher concentration of quercetin at 12 h (0.09 μM) compared with the meal containing 3% fat (0.03 μM). In contrast to the absorption-enhancing effect of dietary fat on quercetin aglycone, absorption of quercetin-3-glucoside was decreased in response to the meal containing 32% fat (quercetin C_max: 0.64 μM) compared with the meal containing 3% fat (quercetin C_max: 0.91 μM). These results suggest that increased quercetin-3-glucoside bioavailability related to higher dietary fat intake is likely due to delayed elimination. Further studies are needed to investigate mechanisms explaining the influence of dietary fat on quercetin glucoside absorption.

In addition to dietary fat content, dietary fat composition affects quercetin bioavailability (44). Quercetin aglycone was orally administered with a glucose solution (i.e., control group), oils containing medium chain fatty acids, or oils containing long-chain fatty acids to thoracic lymph duct-cannulated rats (44). Quercetin absorption and bioavailability were significantly increased by co-administration with oil containing long-chain fatty acids, as evidenced by a greater C_max (1.94 μM) and AUC (512.8 μM ● min)
compared with the control group (C\text{max} : 1.28 \mu M and AUC: 328.8 \mu M \cdot \text{min}). In contrast, quercetin C\text{max} (1.43 \mu M) and AUC (367.0 \mu M \cdot \text{min}) were unaffected following co-administration with oil containing medium chain-fatty acids. In addition, greater amounts of quercetin metabolites (i.e. the sum of aglycone, glucuronidated and sulfated form of quercetin and isorhamnetin) were detected in the lymph fluids over 8 h after co-administration quercetin aglycone with oils containing long-chain fatty acids (14.1 nmol), compared with medium-chain fatty acids (8.5 nmol) or glucose (8.3 nmol) (44). This indicated that co-ingestion of quercetin aglycone with long-chain fatty acids enhanced lymphatic transportation of quercetin aglycone and its metabolites. Increased lymphatic transportation of quercetin aglycone and its metabolites may result in decreases in their intestinal efflux and biliary excretion, thereby improving absorption and bioavailability of quercetin aglycone. Furthermore, no changes in total quercetin concentrations were detected when lipoproteins were removed from the lymph collected from rats that were co-administered quercetin aglycone with oils containing long-chain fatty acids (44). This suggested that quercetin aglycone and its metabolites were not incorporated into chylomicrons in the small intestine. Additional studies are needed to define the manner to which quercetin metabolites are transported in the lymph.

2.5.4 Non-digestible fiber

Short-chain fructooligosaccharides (FOS), a non-digestible fiber, has been suggested to improve quercetin bioavailability (86). In a 2 wk feeding study, rats were fed a 0.68% (weight%) quercetin-3-glucoside diet with 3% (weight%) FOS, and rats in the control group were fed with a 0.68% (weight%) quercetin-3-glucoside diet devoid of FOS (86). Total flavonols in the plasma (i.e., the sum of aglycone, glucuronidated and sulfated form of quercetin, isorhamnetin and tamarixetin) were 2-times greater in FOS-fed rats compared with the control group. Greater plasma levels of total flavonols may be
related to FOS-mediated alterations in colonic degradation of quercetin-3-glucoside and quercetin aglycone, because the colon is the site where FOS is fermented, and where microbial enzyme-catalyze quercetin-3-glucoside deglycosylation and quercetin aglycone catabolism occur (34, 86). In support, after cecal contents collected from FOS-fed rats were incubated with quercetin-3-glucoside for 120 min in vitro, quercetin-3-glucoside was hydrolyzed to quercetin aglycone without degradation. Conversely, 30% of quercetin-3-glucoside was degraded following its incubation with cecal contents collected from control rats. The degradation of quercetin aglycone was 70% following its incubation with cecal contents collected from the control rats for 120 min in vitro, whereas its degradation was decreased to 30% after incubation of cecal contents collected from FOS-fed rats. These findings indicate that FOS feeding inhibits colonic degradation of quercetin-3-glucoside and quercetin aglycone, which in turn may improve their absorption and bioavailability. Additional studies are needed to investigate whether inhibitory action of FOS on colonic degradation of quercetin is mediated through altered gut microbiota community.

2.5.5 Food matrix

Improvements in quercetin aglycone bioavailability have been reported when quercetin aglycone is consumed as an integral component of food (87). In a randomized crossover study, women ingested either quercetin aglycone-enriched cereal bars, or quercetin aglycone powder-filled hard capsules in combination with quercetin aglycone-free cereal bars (87). Ingestion of quercetin-enriched cereal bars increased C_{max} of quercetin (2.48 μM), isorhamnetin (0.19 μM) and tamarixetin (0.21 μM) by 3.8-10.5-times compared with the ingestion of quercetin-containing hard capsules. Additionally, quercetin AUC (1475.7 μM ● min) was 5-times greater after ingesting quercetin-enriched cereal bars compared with quercetin capsules. Therefore, bioavailability and absorption
of quercetin aglycone were enhanced by its ingestion as a part of cereal bar. Increases in quercetin aglycone absorption were likely due to manufacturing process of cereal bars, which homogenously mixed quercetin aglycone with other cereal components and then converted the whole mixture into a solid dispersion. Compared with hard capsules, solid dispersions provided a greater surface area, which may facilitate the dissolution of quercetin aglycone in the intestinal lumen, and its subsequent uptake by small intestine.

2.5.6 Nano-formulation of quercetin aglycone

Hydrophobicity of quercetin aglycone makes it an ideal candidate for encapsulation into lipid nanoparticles. Administration of quercetin as lipid nanoparticles is expected to improve its absorption efficiency, as lipid nanoparticles increase apparent water solubility of encapsulated lipophilic compounds in the gastrointestinal tract (88). Lipid nanoparticles also facilitate cellular uptake of encapsulated compounds, since their nano-scaled particle sizes (20-200 nm) enable them to efficiently permeate into tissues (89, 90). Several nano-formulation methods have been utilized to develop quercetin-containing lipid nanoparticles, including self-nanoemulsifying drug delivery system (SNEDDS) and solid lipid nanoparticle carrier system (91, 92).

Quercetin-containing SNEDDS (Q-SNEDDS) has been developed by incorporating quercetin aglycone into nanoparticles that consist of glycerol α-monodecanoate (Capmul MCM), Tween 20, and ethanol (91). Bioavailability of quercetin aglycone delivered as Q-SNEDDS was evaluated in rats that were orally administered Q-SNEDDS or quercetin aglycone suspension (50 mg quercetin aglycone equivalents/kg body weight) by gavage. Consistent with expectation, Q-SNEDDS improved absorption and bioavailability of quercetin aglycone, as evidenced by greater quercetin C\text{max} and AUC in the Q-SNEDDS group (C\text{max}: 4.6 μM and AUC: 101.4 μM ● h) compared with the control group (C\text{max}: 0.6 μM and AUC: 20.5 μM ● h). Quercetin T\text{max} was similar between two
groups, indicating that absorption rate of quercetin aglycone was unaffected by Q-SNEDDS. Elimination kinetics was not examined in this study, and therefore it remained unknown whether Q-SNEDDS affected quercetin elimination.

The quercetin-containing solid lipid nano-particle (QT-SLNs) consisted of quercetin aglycone, soy lecithin, glycercyl monostearate, PEG 400, and polysorbate 80 (Tween 80) (92). The influence of QT-SLNs on quercetin pharmacokinetics was examined in rats receiving the QT-SLNs suspension (50 mg quercetin aglycone equivalents/kg body weight), and rats in the control group received an equivalent oral dose of quercetin aglycone suspended in sodium carboxymethyl cellulose solution. Quercetin bioavailability was 5-times greater in rats receiving QT-SLNs (quercetin AUC: 1073.4 μM h) compared with the control rats. QT-SLNs also increased quercetin aglycone absorption, as evidenced by greater quercetin C_{max} in the QT-SLN group (C_{max}: 40.5 μM) compared with the control group (C_{max}: 19.5 μM). The elimination of quercetin was delayed following QT-SLNs administration, as reflected by a slower quercetin t_{1/2} in rats receiving QT-SLN (t_{1/2}: 18 h) compared with the control group (t_{1/2}: 5.8 h). Although explanations for slower elimination of quercetin in the QT-SLNs group remain unknown, it is possible that long-term QT-SLNs administration may enhance quercetin accumulation in tissues and potentiate its bioactivities.

Although administration of quercetin aglycone as lipid nano-particles improves its absorption and bioavailability, metabolic pathway and tissue distribution of quercetin-containing nanoparticles remain unclear. It is also remains unknown whether any adverse effects are induced following acute or chronic oral administration of quercetin nano-emulsions, which requires future investigation.
2.6 Implications of quercetin bioavailability for studying bioactivity

Findings from epidemiological studies suggest that participants with higher dietary intake of quercetin have lower CVD morbidity and mortality (3, 93, 94). However, studies examining potential cardioprotective activities of quercetin have shown inconsistent findings between \textit{in vitro} and \textit{in vivo} experimental systems. For example, quercetin at concentrations \(\geq 1 \text{ \(\mu\)M}\) decreased secretion of tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), an inflammatory cytokine, from palmitate-stimulated human umbilical endothelial cells (95). However, no changes in plasma TNF-\(\alpha\) were found in individuals with metabolic syndrome following 6 wk quercetin supplementation (150 mg/d or 1.6 mg/kg body weight/d), which was accompanied by increases in plasma quercetin from baseline concentrations of 71 nM to 269 nM (96). Therefore, it is speculated that plasma quercetin at concentrations of 275 nM is too low to induce any anti-inflammatory effects. Indeed, the effective concentrations reported in studies \textit{in vitro} are often greater than 10 \text{ \(\mu\)M}, whereas plasma quercetin concentrations are often significantly lower than 10 \text{ \(\mu\)M} in humans regardless of supplementation. Because of its poor bioavailability, it is possible that dietary quercetin intakes may result in tissue levels of quercetin that are not effective to achieve health-promoting effects or that observations from epidemiological studies suggesting an inverse relation between dietary quercetin intake and C-reactive protein may be attributed to other factors (97). Therefore, the claimed cardioprotective activities of quercetin and underlying mechanisms supported by \textit{in vitro} studies require validation in appropriate animal and clinical models of disease where quercetin is provided from foods or supplements.

There is disagreement in findings from clinical studies investigating beneficial effects of quercetin on CVD prevention. Some clinical studies support that quercetin supplementation decreases CVD risk as exemplified by inhibition of collagen-induced...
platelet aggregation in plasma collected from healthy participants who ingested quercetin-rich onion soups (13). Other clinical studies show no alterations in plasma C-reactive protein, a biomarker for CVD risk, in participants following quercetin supplementation (500 or 1000 mg/d for 12 wk) (98). Reasons for the discrepancy in cardioprotective activities of quercetin are difficult to explain because of different experimental designs, including supplement dose and supplementation duration, and different participants’ health status and their various baseline plasma quercetin concentrations. In addition, quercetin bioactivities are partly dependent on its bioavailability, and thus individual or population differences in quercetin bioavailability may be one reason that explains inconsistent results from studies examining cardioprotective activities of quercetin. Indeed, substantial individual differences are observed in quercetin bioavailability when participants ingest the same quercetin supplements with the same meals (16). The amount of absorbed quercetin may vary between study populations and between participants within the same study. Potential cardioprotective activities of quercetin are also likely to be diminished when participants absorb lower amounts of quercetin. Additional clinical studies that establish dose-response relationships between quercetin bioavailability, plasma quercetin and CVD risks biomarkers are also needed to better understand the cardioprotective activities of quercetin.

2.7 Conclusion

Although quercetin has poor bioavailability, intervention studies conducted in individuals at risk or having CVD support cardioprotective effects of quercetin. For example, quercetin supplementation decreases plasma oxidized LDL in individuals having metabolic syndrome and lowers blood pressure in hypertensive patients (7, 9, 96). Since quercetin bioavailability, at least in part, determines its cardioprotective activities,
these findings suggest that improving quercetin bioavailability may enhance its beneficial effects in individuals with CVD risks. Studies in animal models also show that co-administration of quercetin with fat or fiber increases its bioavailability (85, 86). Therefore, studies in humans, especially in those with CVD risk factors, are needed to validate bioavailability-enhancing effects of these food components. In addition, substantial inter-individual variability exists in absorption, metabolism and elimination of quercetin. However, few studies exist examining endogenous factors to explain individual differences in quercetin bioavailability. More complete information on factors contributing to inter-individual variability in quercetin bioavailability will better our understanding of its potential cardioprotective activities and its mechanisms of action.
2.8 Figures and tables

Figure 2.1 Backbone structures of benzoic acid (C1-C6), cinnamic acid (C3-C6) and flavonoids (C6-C3-C6).
**Figure 2.2** Biotransformation of quercetin. COMT, catechol-O-methyl transferase; LPH, lactase phlorizin hydrolase; SULT, sulfotransferase; UGT, uridine 5′-diphospho-glucuronosyltransferase.
Table 2.1 Quercetin contents in Food

<table>
<thead>
<tr>
<th>Food</th>
<th>Quercetin mg/100 g food weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lettuce (red)</td>
<td>40.27</td>
</tr>
<tr>
<td>Chili pepper</td>
<td>32.59</td>
</tr>
<tr>
<td>Cranberry</td>
<td>17.34</td>
</tr>
<tr>
<td>Onion (red)</td>
<td>17.22</td>
</tr>
<tr>
<td>Onion (yellow)</td>
<td>12.65</td>
</tr>
<tr>
<td>Black chokeberry</td>
<td>8.90</td>
</tr>
<tr>
<td>Tomato</td>
<td>4.56</td>
</tr>
<tr>
<td>Broccoli</td>
<td>4.25</td>
</tr>
<tr>
<td>Apple</td>
<td>2.47</td>
</tr>
</tbody>
</table>

a) Quercetin aglycone in foods is determined by HPLC after acid or alkaline hydrolysis.
CHAPTER 3
DIETARY FAT INCREASES QUERCETIN BIOAVAILABILITY IN OVERWEIGHT ADULTS

3.1 Abstract

Epidemiologic evidence supports that dietary quercetin reduces cardiovascular disease (CVD) risk, but its oral bioavailability is paradoxically low. The aim of this study was to determine whether dietary fat would improve quercetin bioavailability in adults at high-risk for CVD and to assess lipid-mediated micellarization of quercetin in vitro. In a randomized, cross-over study, overweight/obese men and postmenopausal women (n = 4M/5F; 55.9±2.1 y; 30.8±1.4 kg/m²) ingested 1095 mg of quercetin aglycone with a standardized breakfast that was fat-free (<0.5 g), low-fat (4.0 g), or high-fat (15.4 g). Plasma was obtained at timed intervals for 24 h to measure quercetin and its methylated metabolites isorhamnetin and tamarixetin. Compared to the fat-free trial, plasma quercetin maximum concentration (C_{max}) and area under curve (AUC_{0-24 h}) increased (p<0.05) by 45% and 32%, respectively, during the high-fat trial. During the high-fat trial, isorhamnetin C_{max} and AUC_{0-24 h} also increased by 40% and 19%, respectively, whereas C_{max} and AUC_{0-24 h} of tamarixetin increased by 46% and 43%, respectively. Dietary fat dose-dependently increased micellarization efficiency of quercetin aglycone in vitro. Dietary fat improves quercetin bioavailability by increasing its absorption, likely by enhancing its micellarization at the small intestine.
3.2 Introduction

Cardiovascular disease (CVD) is the leading cause of mortality in the US accounting for >2200 deaths/d and 16% of annual healthcare expenditures (2). Epidemiological studies indicate that overweight/obese men and postmenopausal women have greater CVD risk than normal weight men and premenopausal women, respectively (99, 100). Thus, safe and effective therapies are needed for CVD prevention in these high-risk populations. Bioactive food components derived from fruits and vegetables have been shown to improve indices of cardiovascular health (1). One such bioactive food component is quercetin, a major dietary flavonol that is ubiquitously found in edible portions of fruits and vegetables (8).

Epidemiological studies show that high dietary intakes of quercetin (3.3-18.7 mg/d) are associated with a 21-30% lower risk of CVD (3, 101). Although dietary supplementation of quercetin does not appear to exert beneficial effects in healthy individuals (98, 102), it decreases blood pressure in hypertensive patients (9) and lowers plasma oxidized LDL in obese individuals having characteristics of the metabolic syndrome (96). Our group also recently showed that acute supplementation of quercetin aglycone (1095 mg) decreases mean blood pressure by 5 mmHg in overweight hypertensive individuals despite having limited bioavailability (7).

At present, the available evidence regarding quercetin is too limited to recommend it as a strategy to reduce CVD risk. In particular, more information is needed to better understand its relatively poor and highly variable bioavailability as well as the dosage needed to elicit improvements in cardiovascular health. The estimated absorption of quercetin glucoside, the naturally occurring form of quercetin (103), ranges from 3-17% in healthy individuals receiving 100 mg (57). Likewise, the average bioavailability of quercetin aglycone, the form commonly found in dietary supplements (103), is 17% when administered orally to pigs (50 mg/kg body weight) (4). As with other polyphenols,
the relatively low bioavailability of quercetin may be attributed to its low absorption, extensive metabolism, and/or rapid elimination (14). 

Quercetin absorption is affected by differences in its glycosylation (25), the food matrix from which it is consumed (87), and the co-administration of dietary components such as fiber (104). Studies in pigs show that the bioavailability of quercetin aglycone increases with fat quantity (85) as well as the proportion of medium chain length fatty acids in the diet (105). The manner in which dietary fat improves quercetin bioavailability remains unclear, but is likely due to its hydrophobicity. Quercetin is a relatively nonpolar polyphenol compared to catechin (106) and has substantially lower solubility in water than in soybean oil (0.55 vs.100 μmol/L) (56). This suggests that its co-ingestion with nonpolar dietary agents, such as lipid, would improve its propensity to micellarize in the small intestine and increase its likelihood to be passively absorbed (85). Despite the predictable relation of dietary fat on quercetin bioavailability, no studies in humans have been conducted to confirm this expectation, which limits our ability to develop recommendations for quercetin consumption.

Improving quercetin bioavailability could potentiate its cardioprotective activities, especially in those who are at high-risk for CVD. The extent to which dietary fat regulates the bioavailability of quercetin in humans is unknown. We therefore conducted a 24 h cross-over pharmacokinetics study in obese/overweight men and postmenopausal women to define whether the co-ingestion of dietary fat increases the bioavailability of quercetin and affects its subsequent methylation to isorhamnetin and tamarixetin. We also performed a study in vitro to simulate digestion from the oral through the small intestinal phase to determine whether dietary fat affects the partitioning of quercetin into the aqueous micellar fraction.
3.3 Materials and methods

3.3.1 Materials

All solvents were of HPLC-grade and purchased from Fisher Scientific (Pittsburgh, PA) as were the following chemicals: ammonium acetate, ascorbic acid, citric acid, EDTA, diethylenetriaminepentaacetic acid (DTPA), perchloric acid and tamarixetin. α-Amylase, bile extract, β-glucuronidase/sulfatase (Helix pomatia; type HP-2S), isorhamnetin, lipase, morin, mucin, sodium phosphate monobasic, pancreatin, pepsin, quercetin, and 2-thiobarbituric acid were obtained from Sigma (St. Louis, MO). 2,2’-azobis-2-methyl-propanimidamide (AAPH) was obtained from Cayman Chemical (Ann Arbor, Michigan). Quercetin supplements containing quercetin aglycone were kindly provided by USANA Health Sciences (Salt Lake City, UT).

3.3.2 Subjects

The protocol for this study was approved by the Institutional Review Board at the University of Connecticut (Protocol #H09-164UCHC), and all participants provided written consent. Participants were recruited on the basis of age (40-70 y), BMI (26-40 kg/m²), fasting blood glucose (<6.1 mmol/L) and total cholesterol (<5.18 mmol/L), and resting blood pressure (<140/90 mmHg). Women were required to be postmenopausal for ≥1 y and not using hormone replacement therapy. Other inclusion criteria included nonsmoking status, alcohol use limited to ≤2 servings/d, non-use of dietary supplements (≥2 mo) and anti-inflammatory agents (≥2 wk), stable exercise patterns (≤5 h/wk), and no history of cancer, cardiovascular, renal, gastrointestinal or liver disease.

3.3.3 Study design

Participants completed a randomized, cross-over study separated by a 1 wk washout period. Participants were instructed to avoid quercetin-rich foods for 3 d prior to each visit to the study center. Specifically, we instructed participants to abstain from fruits, vegetables, and beverages containing ≥1 mg of quercetin per 100 g edible portion (e.g.
apples, onions, wine), which were identified from the USDA Database for the Flavonoid Content of Selected Foods (107). After an overnight fast (10-12 h), participants ingested quercetin aglycone (1095 mg) with a standardized breakfast that varied in fat content (Table 1). Breakfast consisted of water (500 mL) and a muffin made from all-purpose flour, unsalted butter, sugar, salt, water, vanilla extract, and baking powder. Muffins were formulated to be fat-free (<0.5 g; ~1% energy from fat), low-fat (4.0 g; 10% energy from fat), or high-fat (15.4 g; 30% energy from fat) whereas the amounts of carbohydrate and protein were kept constant. Blood samples were collected prior to (0 h; 0600-0700) and after consumption of the test meal and quercetin supplement at 1, 2, 3, 4, 6, 8, 10, 12, and 24 h. Participants were also provided a standardized low-quercetin lunch and dinner at 4 h and 12 h post-supplementation, respectively.

Quercetin was administered at 1095 mg based on our recent findings showing that this dietary supplementation level reduces blood pressure in hypertensive individuals (7). It was also provided as a dietary supplement rather than incorporating it into the muffin to avoid potential matrix effects of food (87) and to eliminate the possibility of heat-mediated degradation of quercetin, as suggested by studies *in vitro* indicating that quercetin degrades in a temperature-dependent manner (108). Quercetin aglycone, rather than quercetin glycoside, was administered to reduce between-individual variations in quercetin absorption because considerable inter-individual differences exist for the activity of small intestinal β-glucosidase, the enzyme responsible for the hydrolysis of quercetin glycosides (33). Quercetin aglycone is also the form commonly found in dietary supplements. Dietary fat of the high-fat meal was chosen consistent with current recommendations for fat (20-35% of energy from fat)(109) and lower intake levels were chosen to define potential dose-responses on quercetin bioavailability.
3.3.4 Dietary quercetin and isorhamnetin intakes

Participants were instructed to complete 3 d food records upon enrollment and before each pharmacokinetics trial. Dietary intakes of quercetin and isorhamnetin were assessed using Nutrition Data System for Research software (University of Minnesota) and the Nutrition Coordinating Center Flavonoid and Proanthocyanidin Provisional Table 2010 database. Dietary tamarixetin could not be assessed due to limitations in the food table database.

3.3.5 Sample handling

Blood was collected at timed intervals from a catheter placed in the antecubital vein. Plasma was separated by centrifugation (4°C, 15 min, 1500 x g), snap-frozen in liquid nitrogen, and stored at -80°C until analysis. For measurements of ascorbic acid and uric acid, an aliquot of freshly collected sodium heparinized plasma was mixed 1:1 with 10% perchloric acid containing 1 mmol/L DTPA. Following centrifugation (4°C, 5 min, 15000 x g), the supernatant was snap-frozen in liquid nitrogen, and stored at -80°C until analysis.

3.3.6 HPLC analysis of quercetin and its methylated metabolites

Following absorption, quercetin is rapidly glucuronidated, sulfated and methylated, yielding metabolites including quercetin glucuronides, quercetin sulfates, and the methylated conjugates isorhamnetin glucuronide and tamarixetin glucuronide (41). Plasma total quercetin, total isorhamnetin, and total tamarixetin (i.e. the sum of free and glucuronidated/sulfated conjugates of each analyte) were measured by HPLC-Coularray (ESA, Inc., Chelmsford, MA) following enzymatic hydrolysis as described (110), with minor modifications. Plasma (200 µL) was mixed with 20 µL of 27.6 µmol/L morin (internal standard) dissolved in methanol, 900 U β-glucuronidase and 75 U of sulfatase prepared in 0.4 mol/L sodium phosphate monobasic (pH 5.0), and 20 µL of 6% ascorbic acid (w:v) prepared in 0.4 mol/L sodium phosphate monobasic and containing 2.5 mmol/L DTPA. Samples were incubated (37°C, 1 h), and then extracted 3-times with
diethyl ether, dried under nitrogen gas, and reconstituted in methanol. Injected samples were separated isocratically on a Kinetex pentafluorophenyl column (150 x 3.0 mm i.d., 3 μm; Phenomenex, Inc., Torrance, CA) at 0.4 mL/min. Mobile phase A was delivered at 30% and consisted of 75 mmol/L citric acid, 25 mmol/L ammonium acetate, and 0.27 mmol/L EDTA prepared in 20% methanol (v:v). Mobile phase B was delivered at 70% and consisted of 75 mmol/L citric acid, 25 mmol/L ammonium acetate, and 0.27 mmol/L EDTA prepared in 80% methanol (v:v). Analytes were detected using potential settings of -20, 225, 300, and 400 mV and quantified on their dominant channel using area ratios relative to the internal standard.

### 3.3.7 Clinical chemistries and biomarkers of antioxidants, oxidative stress, and inflammation

Plasma total cholesterol and glucose were determined spectrophotometrically using commercial assays (Point Scientific, Inc., Canton, MI). Total antioxidant capacity was measured using oxygen radical absorbance (ORAC) and ferric-reducing ability of plasma (FRAP) assays. ORAC was measured as described (111) and is based on the ability of plasma to scavenge reactive oxygen species formed by an AAPH-induced peroxyl radical-generating system. FRAP was measured as described (112) and is based on the ability of plasma antioxidants, especially those that are hydrophilic, to reduce ferric iron to ferrous iron. Ascorbic acid and uric acid were measured from perchloric acid-treated plasma by HPLC-Coularray as described (113). Plasma vitamin E (as α- and γ-tocopherol) and nitro-γ-tocopherol were measured by HPLC-Coularray following saponification, hexane extraction, and detected at potential settings of 350, 450, 525 and 600 mV (113). Nitro-γ-tocopherol standard was synthesized from γ-tocopherol as described (114). Plasma malondialdehyde, a marker of lipid peroxidation, was measured using a Shimadzu Prominence XR UFLC-FL system as described (115) following saponification, derivitization with 2-thiobarbituric acid, and detection at 520/553 nm.
Plasma C-reactive protein and myeloperoxidase were measured using separate commercially available ELISA kits (BioCheck, Inc., Foster City, CA).

3.3.8 In vitro digestion

Quercetin supplements and muffins were digested *in vitro* to assess micellarization efficiency of quercetin as described (116), with minor modifications. A portion of muffins (3.33 g) that were fat-free (0.01 g fat), low-fat (0.10 g fat), and high-fat (0.33 g fat) was mixed with 23.5 mg of quercetin and subjected to simulated oral, gastric, and small intestinal digestion using 4200 U of α-amylase, 0.3 mg of mucin, 20 mg of pepsin, 40 mg of pancreatin, 20 mg of lipase, and 240 mg of bile extract. Aqueous micellar fractions from the digesta were obtained by centrifugation (4°C, 1 h, 10,000 x g) and filtered through a 0.22 μm Steriflip-GP Filter Unit (Millipore, Billerica, MA). An additional high-fat muffin was subjected to simulated digestion as described above, but without bile extract to define the extent to which the transfer of quercetin aglycone to the aqueous fraction is dependent on micelle formation. Morin (internal standard) was added to the digesta and aqueous micellar fractions. Quercetin was then extracted and analyzed by HPLC-Couarray as described above. Micellarization efficiency was defined as the percent of quercetin transferred from the digesta to the aqueous fraction and was calculated as the concentration of quercetin in the aqueous micellar fraction relative to that in the digesta.

3.3.9 Statistical analysis

Pharmacokinetic parameters of plasma quercetin, isorhamnetin, and tamarixetin were determined for each participant during each trial by noncompartmental analysis using PK Solutions (Summit Research Service, Montrose, CO). All data are presented at means ± SEM and were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA) except that multiple linear regression was performed using SPSS 16.0 (SPSS Inc., Chicago, IL). Gender differences were initially evaluated using an unpaired Student’s t-test. No gender differences were observed and therefore all analyses were
performed on pooled data. Data were evaluated for equal variances using Bartlett’s test. Unequal variances were detected for maximum concentration ($C_{\text{max}}$) and absorption rate constant ($k_a$) of quercetin, $C_{\text{max}}$, elimination rate constant ($k_e$), and time to maximum concentration ($T_{\text{max}}$) of isorhamnetin, $C_{\text{max}}$, elimination half-life ($t_{1/2}$), and $T_{\text{max}}$ of tamarixetin. Statistical analyses were performed in parallel on log-transformed and untransformed data. No qualitative differences were observed between analyses. Untransformed data are therefore presented to permit more meaningful interpretations. Most data were analyzed using 1-way ANOVA with or without repeated measures as appropriate. Newman-Keuls post-test was performed to evaluate group mean differences. Multiple linear regression, controlling for within-subject repeated measures, was performed as described (117) to determine the relation between the $C_{\text{max}}$ of quercetin and the $C_{\text{max}}$ of isorhamnetin or tamarixetin. Simple linear regression was used to define the relation between fat quantity and micellarization efficiency of quercetin. All analyses were considered statistically significant at an $\alpha$-level of $p<0.05$. 
3.4 Results

3.4.1 Participants and diets

All participants completed each of the pharmacokinetics trials without any adverse events. Participants had a mean BMI of 30.8 ± 1.4 kg/m2 and borderline-high levels of resting systolic (132 ± 3 mm Hg) and diastolic (91 ± 3 mm Hg) blood pressure, borderline-high glucose (5.5 ± 0.2 mmol/L), and total cholesterol (4.8 ± 0.2 mmol/L) that was within normal clinical limits. Participants’ dietary intakes of quercetin (0.26 ± 0.05 mg/d) and isorhamnetin (0.01 ± 0.007 mg/d) were not different preceding each pharmacokinetic trial, but were lower (p<0.05) compared to habitual dietary intakes (15.6 ± 3.2 and 0.42 ± 0.14 mg/d, respectively), consistent with instructions to abstain from foods high in quercetin. Energy and macronutrient intakes during the pharmacokinetic trials were not different with the exception that dietary fat intake differed due to the varying levels of fat provided by the test muffins (Table 3.1).

3.4.2 Antioxidants and markers of oxidative stress and inflammation

Quercetin has been reported to possess antioxidant and anti-inflammatory properties (118), suggesting that alterations in antioxidant status, oxidative stress, and inflammation may affect its turnover. Therefore, for each trial, baseline (0 h) concentrations of antioxidants and biomarkers of oxidative stress and inflammation were measured. No differences between trials were observed for any of the markers measured (Table 3.2). C-reactive protein was related to myeloperoxidase (r = 0.52, p<0.05) and inversely related to vitamin C (r = -0.40, p<0.05), consistent with our findings that pro-inflammatory responses are associated with low vitamin C status (119).

3.4.3 Pharmacokinetics of quercetin

Baseline concentrations of plasma quercetin were not different between trials (Table 3.3). Following ingestion of quercetin with any of the test muffins, quercetin $C_{\text{max}}$ occurred at a $T_{\text{max}}$ of 6 h for all trials without any differences in $k_a$. Plasma quercetin
decreased thereafter until it returned to concentrations similar ($p > 0.05$) to baseline levels at 24 h (Figure 3.1A). Compared to the fat-free trial, quercetin $C_{\text{max}}$ increased by 12% in the low-fat trial without any statistical significance and by 45% in the high-fat trial ($p < 0.05$; Table 3.3). Elimination kinetics of plasma quercetin were unaffected by dietary fat as evidenced by no differences in $t_{1/2}$ or $k_e$ between trials. The area under curve (AUC$_{0-24\ h}$) of quercetin increased in a near dose-dependent manner in response to dietary fat, but only reached statistical significance during the high-fat trial compared to the fat-free trial. Thus, the lack of change in basal antioxidant status, oxidative stress, and inflammatory markers (Table 3.2) as well as pharmacokinetic parameters other than $C_{\text{max}}$ (Table 3.3) suggests that quercetin AUC$_{0-24\ h}$ increased in response to dietary fat due to greater absorption. Lastly, regardless of dietary treatment, high inter-individual variances in $C_{\text{max}}$ (32-55%), $T_{\text{max}}$ (18-34%), and $t_{1/2}$ (20-33%) were observed during each trial.

3.4.4 Methylated metabolites of quercetin

Similar to plasma quercetin, co-ingestion of quercetin with the high-fat muffin compared to the fat-free muffin resulted in 40% and 46% higher $C_{\text{max}}$ of isorhamnetin and tamarixetin, respectively (Table 3.3). The AUC$_{0-24\ h}$ of isorhamnetin and tamarixetin was also greater by 19% and 43%, respectively, during the high-fat trial compared to the fat-free trial. No other pharmacokinetic parameters of these methylated metabolites were affected by the dietary fat treatments. Both isorhamnetin $C_{\text{max}}$ ($r = 0.87$) and tamarixetin $C_{\text{max}}$ ($r = 0.94$) were highly correlated with quercetin $C_{\text{max}}$ ($p < 0.0001$), suggesting that greater availability of quercetin in response to dietary fat potentiates its biotransformation. High inter-individual responses for isorhamnetin were also observed for $C_{\text{max}}$ (27-47%), $T_{\text{max}}$ (22-58%), and $t_{1/2}$ (52-57%) regardless of dietary treatment. Similarly, inter-individual responses of tamarixetin were 30-56% for $C_{\text{max}}$, 11-29% for $T_{\text{max}}$, and 52-124% for $t_{1/2}$. 

48
Of additional interest is that isorhamnetin and tamarixetin are positional isomers, but had strikingly different pharmacokinetic profiles. Baseline isorhamnetin concentrations did not differ between trials whereas plasma tamarixetin was below detection limits (Table 3.3). Plasma isorhamnetin concentrations peaked at 8-10 h (Table 3.3) but did not return to baseline concentrations by 24 h (Figure 3.1B). This is in contrast to tamarixetin, which closely paralleled the absorption and elimination phases of quercetin (Figure 3.1C). Moreover, regardless of dietary fat treatment, plasma tamarixetin compared to isorhamnetin had greater $k_a$ and $k_e$, an earlier $T_{\text{max}}$, and a shorter $t_{1/2}$ ($p<0.05$; Table 3.3).

### 3.4.5 Micellarization efficiency of quercetin during in vitro digestion

To better define the manner in which dietary fat improved quercetin bioavailability, micellarization efficiency of quercetin was determined *in vitro* using a simulated digestion system. Recovery of quercetin following simulated digestion was >67% and was unaffected by the level of fat ($p>0.05$). Quercetin aglycone in the aqueous micellar fraction of the collected digesta increased in a dose-dependent manner up to 23% in response to greater levels of fat in the muffins (Figure 3.2A). In contrast, the omission of bile extract in the simulated digestion system resulted in only 1.3% of quercetin aglycone partitioning into the aqueous fraction when a high-fat muffin was subjected to digestion. Further corroborating was the strong relation of dietary fat quantity on micellarization efficiency ($r = 0.986$, $p<0.0001$; Figure 3.2B), supporting that quercetin absorption is highly mediated by its incorporation in mixed micelles.
3.5 Discussion

This study demonstrates for the first time in humans that dietary fat increases quercetin bioavailability by improving its absorption, and that its greater bioavailability increases its propensity to be biotransformed to methylated metabolites. By carefully increasing the fat content of a controlled test meal up to 15.4 g, we show that the bioavailability of quercetin increases by 32% independent of pharmacokinetic parameters other than $C_{\text{max}}$ or changes in basal oxidative stress. This was also accompanied by a significant increase in the formation of isorhamnetin and tamarixetin. We also provide new evidence that dietary fat dose-dependently increases quercetin incorporation into bile salt-containing micelles during a simulated digestion. Collectively, these findings indicate that the co-ingestion of reasonable quantities of dietary fat improves quercetin bioavailability by increasing its absorption, likely by enhancing its intestinal micellarization.

Considering the low bioavailability of quercetin, the present study provides important evidence that its co-ingestion with moderate amounts of dietary lipid is necessary to improve its absorption. By improving the bioavailability of quercetin, it may become possible to protect against CVD in high-risk populations. This is particularly important since supplementation of quercetin appears to be more efficacious among those at risk for CVD (96, 120) compared to healthy individuals (5, 121). In addition, the findings of this study improve our understanding, at least in part, of the variability in quercetin bioavailability and provide a practical strategy to standardize the administration of quercetin aglycone in future studies aiming to define its potential beneficial effects in humans.

The observed increases in quercetin bioavailability by dietary fat might be explained by fat-mediated decreases in gastric emptying (122). However, this is unlikely since $k_a$ and $T_{\text{max}}$ of quercetin were unaffected by the dietary fat treatments. We therefore
performed a simulated digestion in vitro to better define the manner in which dietary fat improved quercetin bioavailability. We show that the aqueous micellar fraction following simulated oral through small intestinal digestion contains higher levels of quercetin aglycone when the digestion was performed in the presence of both bile extract and fat compared to that completed in the absence of bile extract. This indicates that quercetin aglycone requires micelle incorporation to facilitate its absorption, which is consistent with the lipophilic properties of quercetin (106). Indeed, studies in rats estimate the absorption of quercetin at 0.2% when administered with water (56) whereas studies in pigs indicate that quercetin requires co-ingestion with lipid for it to be more bioavailable (85). We show that micellarization efficiency of quercetin increased to the greatest extent during simulated digestion of the high-fat muffin, consistent with greater quercetin absorption among participants during the high-fat muffin trial. Our findings in vitro therefore support that greater quercetin absorption in vivo, when ingested in the presence of dietary fat, is likely mediated through its micellarization in the small intestine. A separate line of evidence also suggests the possibility that dietary fat may enhance quercetin bioavailability at the large intestine by altering the gut microbiota. Indeed, chronic high-fat feeding fat decreases gut microbiota density without affecting the relative proportion of Clostridium cluster IV (123), a phylogenetic group that includes Clostridium orbiscindens, a microbiota known to metabolize quercetin (124). Whether compositional changes in microbiota occur acutely or in response to the modest levels of dietary fat used in the present study (0.5-15.4 g) remains unknown, and represent important areas for future investigation.

Although the primary objective of our study was to examine the pharmacokinetics of quercetin, we also provide evidence that dietary fat-mediated increases in quercetin absorption led to greater plasma accumulation of its methylated metabolites, specifically isorhamnetin and tamarixetin. Biotransformation of quercetin by small intestinal and
hepatic catechol-O-methyltransferase (COMT) yields the positional methylated isomers isorhamnetin and tamarixetin (103). Prior to quercetin administration, fasting isorhamnetin was <50 nmol/L whereas tamarixetin was below detection limits. Both of these metabolites increased following quercetin ingestion and increased to the greatest extent in the high-fat trial as evidenced by their higher $C_{\text{max}}$ and AUC$_{0-24 \text{ h}}$ (Table 3.3). Although no studies have examined whether dietary fat affects COMT, greater plasma accumulation of isorhamnetin and tamarixetin during the high-fat trial is likely due to the greater availability of quercetin for xenobiotic metabolism.

Despite isorhamnetin and tamarixetin having similar structures and metabolism, they had strikingly different plasma concentration-time curves. Consistent with studies in humans (87), we show that the $C_{\text{max}}$ of isorhamnetin was lower and its elimination ($k_e$) was slower than those of tamarixetin following the ingestion of quercetin. Regardless of our findings, few data exist demonstrating the bioactivities of these metabolites in vivo whereas studies in vitro show that isorhamnetin and tamarixetin at $\geq10$ μmol/L have vasodilatory and anti-platelet activity (125, 126). Clearly, these concentrations are substantially greater than those observed in the present study as well as in other studies following chronic supplementation of quercetin (5, 127). Thus, whether isorhamnetin and tamarixetin exert cardioprotective activities at physiologic levels is unclear and additional work is warranted to assess their bioactivities in vivo. In addition, future studies using COMT inhibitors are needed to better our understanding whether quercetin lowers the risk of CVD directly or indirectly through its methylated metabolites.

Despite participants following a low-quercetin diet for 3 d preceding each trial, which was verified from their food records, they had higher than expected plasma concentrations of quercetin (5). Nonetheless, because fasting plasma quercetin levels were not different between trials, and because all food and beverage during each 24 h pharmacokinetic trial were controlled in an identical manner, the observed changes in
plasma quercetin during each trial were attributed to the ingestion of supplemental quercetin. To further control pharmacokinetic responses, we also administered quercetin in a standardized manner in that our population was homogenous for age and body mass, participants were non-users of dietary supplements, and women were postmenopausal and not using estrogen therapy. Nonetheless, plasma responses of quercetin and its metabolites were highly variable between participants. This is consistent with others (5, 87, 128) and may reflect differences in gastric emptying (129) and/or polymorphisms in transporters and enzymes (e.g. COMT and multidrug resistance associated protein 2) involved in xenobiotic metabolism (130, 131). In contrast, our findings show that the $T_{\text{max}}$ and $t_{1/2}$ of quercetin regardless of dietary fat intake were 5.4-6.7 h and 8.4-8.9 h, respectively, whereas others report a $T_{\text{max}}$ of 2-3 h and $t_{1/2}$ of 14.1-16.8 h (5). This suggests that our participants absorbed quercetin slower and eliminated it more rapidly. The present study also shows that the proportion of isorhamnetin and tamarixetin (32-33%) relative to the total quercetin pool (i.e. the sum of quercetin, isorhamnetin, and tamarixetin), based on $C_{\text{max}}$ concentrations, was ~2-times greater than that observed previously (87). Because these earlier studies were conducted in younger cohorts having normal weight, these discrepancies could be explained by age-dependent impairments in digestive capacity (80), higher levels of oxidative stress (132), differences in the regulation of xenobiotic metabolizing pathways (133), and/or obesity-mediated increases in conjugation capacity of phase II enzymes and renal clearance (134).

The strength of our study was that it utilized a cross-over design to examine the dietary fat-mediated improvements in quercetin bioavailability in a population at high risk of CVD. These findings are therefore anticipated to lead to a practical strategy aimed at reducing CVD risk. Although we show that dietary fat improves quercetin absorption, the present work is limited in that the fat source of the muffins was primarily saturated fat.
provided from butter, a commonly used ingredient for formulating muffins. Also, the study was not designed to define the optimal dose of fat needed for maximal bioavailability of quercetin nor investigate potential differences in its bioavailability in response to saturated and unsaturated fatty acids. Additional work is warranted to not only define the quantity of fat, but also the fatty acid composition, that results in maximal bioavailability of quercetin consistent with recommendations to reduce saturated fat and total fat consumption (135). We also utilized an oral dose of quercetin provided as quercetin aglycone that is higher than that from food, which also typically contains quercetin glycosides. This approach was intentional in order to better understand our recently completed work demonstrating that the acute ingestion of quercetin aglycone (1095 mg) significantly reduces blood pressure in hypertensive individuals (7). Thus, our findings are not surprising in that the absorption of quercetin, calculated by multiplying the \( C_{\text{max}} \) of total quercetin (i.e. sum of quercetin, isorhamnetin and tamarixetin) by an estimate of plasma volume (136), was low (0.1-0.2% of administered dose regardless of fat intake) since this intake level does not recapitulate typical dietary patterns and could be due to the induction of xenobiotic metabolism at the small intestine that limits its absorption by enhancing its efflux to the intestinal lumen (137). Another strength of this study was that it simultaneously examined the pharmacokinetics of quercetin and its methylated metabolites in response to dietary fat. However, because we enzymatically hydrolyzed plasma flavonols using \( \beta \)-glucuronidase/sulfatase, our findings show pharmacokinetic responses of total quercetin, isorhamnetin, and tamarixetin. This precludes our understanding of whether increases in quercetin bioavailability alter the pharmacokinetic profiles of its conjugated metabolites (e.g. quercetin-3-glucuronide, quercetin glucuronide sulfate). Indeed, pharmacokinetics are known to differ between phase II metabolites of quercetin (41). Clearly, additional work is warranted to more fully
define potential alterations in pharmacokinetics of conjugated metabolites in response to greater quercetin bioavailability.

In conclusion, our work suggests that dietary fat intakes should be standardized in future studies aiming to define cardioprotective activities of quercetin. Given that the octanol-water partition coefficient of quercetin (1.82 ± 0.32) is similar to that of other flavonoids such as kaempferol (3.11 ± 0.54) and naringenin (2.60 ± 0.03) (63), the present findings may have broader application. Additional work is also needed to better define the mechanisms regulating quercetin metabolism and whether other dietary constituents affect its bioavailability. Our work is also specific to quercetin aglycone, thereby limiting whether these findings extend to conjugated forms of quercetin that are more common in fruits and vegetables. Furthermore, studies examining the cardioprotective activities of isorhamnetin and tamarixetin should be considered, consistent with observations that greater quercetin bioavailability increases its biotransformation to these methylated metabolites.

3.6 Acknowledgements

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All authors contributed to the editing and review of this manuscript. All authors read and approved the final manuscript. The authors have declared no conflict of interest.
3.7 Figures and tables

![Figure 3.1](image)

**Figure 3.1** Plasma concentration-time curves of quercetin (A), isorhamnetin (B), and tamarixetin (C) for 24 h following oral ingestion of 1095 mg of quercetin aglycone with a fat-free, low-fat, or high-fat meal. Data are means ± SEM (n = 9).
Figure 3.2 In vitro micellarization efficiency of quercetin (A) and its relation to fat content (B). Data are means ± SEM (n = 3) for each treatment. Means not sharing a superscript are different, p<0.05.
Table 3.1 Dietary intakes for 24 h of each trial\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Fat-Free Trial</th>
<th>Low-Fat Trial</th>
<th>High-Fat Trial</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breakfast</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy, <em>kcal</em></td>
<td>318</td>
<td>349</td>
<td>450</td>
</tr>
<tr>
<td>Carbohydrate, <em>g</em></td>
<td>72.5</td>
<td>72.5</td>
<td>72.5</td>
</tr>
<tr>
<td>Protein, <em>g</em></td>
<td>5.2</td>
<td>5.2</td>
<td>5.3</td>
</tr>
<tr>
<td>Fat, <em>g</em></td>
<td>0.5</td>
<td>4.0</td>
<td>15.4</td>
</tr>
<tr>
<td>Fat, % of <em>kcal</em></td>
<td>1.3</td>
<td>10.0</td>
<td>30.0</td>
</tr>
<tr>
<td><strong>Lunch, dinner, and snack(^b)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy, <em>kcal</em></td>
<td>2045 ± 74</td>
<td>1987 ± 73</td>
<td>2044 ± 71</td>
</tr>
<tr>
<td>Carbohydrate, <em>g</em></td>
<td>245 ± 8</td>
<td>239 ± 9</td>
<td>247 ± 8</td>
</tr>
<tr>
<td>Protein, <em>g</em></td>
<td>72.1 ± 4.6</td>
<td>71.4 ± 4.5</td>
<td>71.3 ± 4.3</td>
</tr>
<tr>
<td>Fat, <em>g</em></td>
<td>87.0 ± 2.7</td>
<td>83.5 ± 2.6</td>
<td>86.4 ± 2.4</td>
</tr>
</tbody>
</table>

\(^a\) All data are means ± SEM (\(n = 9\)).

\(^b\) No significant differences between trials were observed for energy or macronutrient intakes.
Table 3.2 Concentrations of plasma antioxidants and biomarkers of oxidative stress and inflammation at baseline in fat-free, low-fat and high-fat trials

<table>
<thead>
<tr>
<th></th>
<th>Fat-Free Trial</th>
<th>Low-Fat Trial</th>
<th>High-Fat Trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORAC, mmol/L Trolox eq.</td>
<td>9.97 ± 0.50</td>
<td>9.89 ± 0.34</td>
<td>8.81 ± 0.43</td>
</tr>
<tr>
<td>FRAP, mmol/L Trolox eq.</td>
<td>1.42 ± 0.09</td>
<td>1.45 ± 0.08</td>
<td>1.43 ± 0.07</td>
</tr>
<tr>
<td>Ascorbic Acid, µmol/L</td>
<td>33.5 ± 3.5</td>
<td>33.5 ± 2.9</td>
<td>34.5 ± 4.1</td>
</tr>
<tr>
<td>Uric Acid, mmol/L</td>
<td>0.30 ± 0.03</td>
<td>0.32 ± 0.03</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>α-Tocopherol, µmol/L</td>
<td>18.5 ± 1.3</td>
<td>18.0 ± 1.2</td>
<td>17.4 ± 0.8</td>
</tr>
<tr>
<td>γ-Tocopherol, µmol/L</td>
<td>2.09 ± 0.15</td>
<td>1.98 ± 0.16</td>
<td>1.97 ± 0.17</td>
</tr>
<tr>
<td>MDA b), µmol/L</td>
<td>0.96 ± 0.09</td>
<td>1.10 ± 0.06</td>
<td>1.04 ± 0.08</td>
</tr>
<tr>
<td>NGT, nmol/L</td>
<td>9.50 ± 1.33</td>
<td>9.37 ± 1.00</td>
<td>8.95 ± 1.49</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>4.03 ± 1.00</td>
<td>4.00 ± 1.26</td>
<td>4.01 ± 1.29</td>
</tr>
<tr>
<td>MPO, ng/mL</td>
<td>11.5 ± 1.0</td>
<td>11.8 ± 1.6</td>
<td>10.7 ± 1.4</td>
</tr>
</tbody>
</table>

a) Data are presented as mean ± SEM (n = 9). No significant differences were observed between trials for any of the markers measured.

b) CRP, C-reactive protein; MDA, malondialdehyde; MPO, myeloperoxidase; NGT, 5-nitro-γ-tocopherol.
Table 3.3 Plasma pharmacokinetic parameters of quercetin, isorhamnetin and tamarixetin in the fat-free, low-fat, and high-fat trials\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Fat-Free Trial</th>
<th>Low-Fat Trial</th>
<th>High-Fat Trial</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C_0)^b, (\mu mol/L)</td>
<td>0.19 ± 0.02</td>
<td>0.19 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>0.96</td>
</tr>
<tr>
<td>(C_{\text{max}}, \mu mol/L)</td>
<td>1.10 ± 0.13(^b)</td>
<td>1.24 ± 0.13(^{ab})</td>
<td>1.60 ± 0.29(^a)</td>
<td>0.03</td>
</tr>
<tr>
<td>(T_{\text{max}}, h)</td>
<td>5.66 ± 0.34</td>
<td>5.35 ± 0.55</td>
<td>6.66 ± 0.76</td>
<td>0.11</td>
</tr>
<tr>
<td>(k_a, \text{min}^{-1} \times 100^{-1})</td>
<td>0.50 ± 0.07</td>
<td>0.65 ± 0.16</td>
<td>0.53 ± 0.07</td>
<td>0.33</td>
</tr>
<tr>
<td>(t_{1/2}, h)</td>
<td>8.85 ± 0.93</td>
<td>8.45 ± 0.94</td>
<td>8.43 ± 0.55</td>
<td>0.89</td>
</tr>
<tr>
<td>(k_e, \text{min}^{-1} \times 100^{-1})</td>
<td>0.15 ± 0.02</td>
<td>0.15 ± 0.02</td>
<td>0.14 ± 0.01</td>
<td>0.92</td>
</tr>
<tr>
<td>(\text{AUC}_{0-24 h}, \mu mol \times \text{min})</td>
<td>693 ± 60.5(^b)</td>
<td>810 ± 83.1(^{ab})</td>
<td>913 ± 127(^a)</td>
<td>0.01</td>
</tr>
<tr>
<td>Bioavailability(^c)</td>
<td>100</td>
<td>117</td>
<td>132</td>
<td></td>
</tr>
</tbody>
</table>

| Isorhamnetin   |                |               |                |       |
| \(C_0, \mu mol/L\) | 0.04 ± 0.003   | 0.05 ± 0.005  | 0.04 ± 0.004   | 0.29  |
| \(C_{\text{max}}, \mu mol/L\) | 0.17 ± 0.02\(^b\) | 0.19 ± 0.02\(^b\) | 0.24 ± 0.04\(^a\) | 0.01  |
| \(T_{\text{max}}, h\) | 8.06 ± 0.59    | 10.0 ± 2.03   | 9.79 ± 1.97    | 0.51  |
| \(k_a, \text{min}^{-1} \times 100^{-1}\) | 0.34 ± 0.04    | 0.38 ± 0.08   | 0.40 ± 0.07    | 0.59  |
| \(t_{1/2}, h\) | 19.5 ± 3.36    | 22.0 ± 4.47   | 16.1 ± 3.27    | 0.55  |
| \(k_e, \text{min}^{-1} \times 100^{-1}\) | 0.08 ± 0.02    | 0.18 ± 0.13   | 0.12 ± 0.04    | 0.61  |
| \(\text{AUC}_{0-24 h}, \mu mol \times \text{min}\) | 176 ± 19.8\(^b\) | 178 ± 15.5\(^b\) | 208 ± 22.7\(^a\) | 0.04  |

| Tamarixetin    |                |               |                |       |
| \(C_0, \mu mol/L\) | ND             | ND            | ND             |       |
| \(C_{\text{max}}, \mu mol/L\) | 0.36 ± 0.04\(^b\) | 0.37 ± 0.04\(^b\) | 0.52 ± 0.10\(^a\) | 0.03  |
| \(T_{\text{max}}, h\) | 5.78 ± 0.22    | 6.61 ± 0.65   | 7.56 ± 0.74    | 0.12  |
| \(k_a, \text{min}^{-1} \times 100^{-1}\) | 0.67 ± 0.07    | 0.58 ± 0.08   | 0.75 ± 0.13    | 0.42  |
| \(t_{1/2}, h\) | 4.54 ± 0.79    | 5.03 ± 1.62   | 5.39 ± 2.23    | 0.84  |
| \(k_e, \text{min}^{-1} \times 100^{-1}\) | 0.36 ± 0.09    | 0.38 ± 0.08   | 0.40 ± 0.08    | 0.86  |
| \(\text{AUC}_{0-24 h}, \mu mol \times \text{min}\) | 181 ± 25.9\(^b\) | 205 ± 26.3\(^b\) | 259 ± 37.7\(^a\) | 0.01  |

\(^a\) Data are means ± SEM (n = 9). Means in a row without a common superscript differ, \(p<0.05\).

\(^b\) \(C_0\): baseline concentration; \(k_a\): absorption rate constant for quercetin or formation rate constant for isorhamnetin and tamarixetin.

\(^c\) Bioavailability (%) is calculated relative to the fat-free trial.
CHAPTER 4
QUERCETIN-CONTAINING SELF-NANOEMULSIFYING DRUG DELIVERY SYSTEM
FOR IMPROVING ORAL BIOAVAILABILITY

4.1 Abstract

Quercetin is a dietary flavonoid with potential chemoprotective effects, but has low bioavailability due to poor aqueous solubility and low intestinal absorption. A quercetin-containing self-nanoemulsifying drug delivery system (Q-SNEDDS) was developed to form oil-in-water nanoemulsions in situ for improving quercetin oral bioavailability. Based on quercetin solubility, emulsifying ability, and stability after dispersion in an aqueous phase, an optimal SNEDDS consisting of castor oil, Tween® 80, Cremophor® RH 40, and PEG 400 (20:16:34:30, w/w) was identified. Upon mixing with water, Q-SNEDDS formed a nanoemulsion having a droplet size of 208.8 ± 4.5 nm and zeta potential of -26.3 ± 1.2 mV. The presence of Tween® 80 and PEG 400 increased quercetin solubility and maintained supersaturated quercetin concentrations (5 mg/mL) for >1 month. The optimized Q-SNEDDS significantly improved quercetin transport across a human colon carcinoma (Caco-2) cell monolayer. Fluorescence imaging demonstrated rapid absorption of the Q-SNEDDS within 40 min of oral ingestion. Following oral administration of Q-SNEDDS in rats (15 mg/kg), the AUC and C\text{max} of plasma quercetin after 24 h increased by ~2- and 3-fold compared to quercetin control suspension. These data suggest that this Q-SNEDDS formulation can enhance the solubility and oral bioavailability of quercetin for appropriate clinical application.
4.2 Introduction

Cancer chemoprevention, using natural or synthetic molecules to prevent, inhibit or reverse carcinogenesis, is becoming increasingly common, especially at a time when the use of complementary and alternative medicine with natural health products has become more prevalent (138, 139). Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a dietary flavonoid present in vegetables, fruits, seeds, nuts, tea, and red wine. It exerts antioxidant activity by upregulating endogenous free radical defenses and suppressing oncogenesis and tumor progression signaling pathways, and has been studied as a potential chemoprevention agent (140, 141). However, clinical applications of quercetin for chemoprotection are limited due to its hydrophobicity, poor gastrointestinal absorption, and extensive xenobiotic metabolism at intestines and liver, which collectively contribute to its low oral bioavailability (142). Various approaches have been used to enhance the solubility, dissolution rate and hence, bioavailability of quercetin including solid dispersions, nanosuspensions, microemulsions, and solid lipid nanoparticles (92, 142-144). Despite successful preparations of stable solid dispersions and nanosuspensions, maximal solubility of a quercetin-containing nanosuspension was limited to only 0.4 mg/mL (143). Although solid lipid nanoparticles improve quercetin bioavailability in rats up to 5-times (92), these nanoparticles frequently have stability issues such that poorly soluble drugs are precipitated during storage. Furthermore, preparation of those formulations requires hazardous solvents (e.g. chloroform, acetone, and ethanol) (92, 143). Therefore, a critical need exists to develop alternative administrative vehicles to circumvent existing challenges of current formulations for quercetin.

Self-nanoemulsifying drug delivery systems (SNEDDS) have attracted increased attention as a mean to improve the oral bioavailability of lipophilic compounds due to their ability to improve drug solubility, membrane transport and absorption via the
lymphatic system, thus bypassing the liver and avoiding hepatic xenobiotic metabolism (145, 146). SNEDDS are anhydrous homogeneous liquid mixtures of oil, surfactant, co-surfactant, and lipophilic drug which spontaneously form transparent nanoemulsions upon aqueous dilution with gentle agitation (88, 147). Unlike thermodynamically unstable dispersion systems, such as emulsions and suspensions, SNEDDS are thermodynamically stable and have a high solubilizing capacity of lipophilic drugs. They also can be filled directly into gelatin capsules, which improves commercial viability and patient compliance (148). Several marketed self-emulsifying drug delivery system-based products, such as Sandimmune®, Neoral® (cyclosporine), Norvir® (ritonavir) and Fortovase® (saquinavir), have showed effectiveness of this delivery system (88).

Challenges exist to develop SNEDDS formulations that maintain the targeted dose in solution during gastrointestinal transit. This is attributed to many poorly soluble compounds having high solubility in SNEDDS, but being precipitated following aqueous dispersion of the formulation or during intestinal digestion (149). Persimmon leaf and *Hippophae rhamnoides* L. extracts containing quercetin have been incorporated into stable SNEDDS formulations that improved quercetin solubility and quercetin release *in vitro* with modest increases in bioavailability *in vivo* (150). Quercetin-containing SNEDDS have also been reported to improve quercetin bioavailability, but this formulation requires a large amount of ethanol (20%), which may limit the clinical application (91). Because a need exists to improve SNEDDS formulations that enhance quercetin bioavailability, the objective of this study was to develop and optimize a SNEDDS formulation that maintains supersaturated quercetin concentrations in nanoemulsions to enhance its oral bioavailability. We therefore developed an optimized quercetin-containing SNEDDS formulation and validated its effectiveness in enhancing quercetin permeability in cells and bioavailability in rats.
4.3 Materials and methods

4.3.1 Materials

Quercetin (aglycone), isorhamnetin, tamarixetin, morin, β-glucuronidase/sulfatase from *Helix pomatia* (S9626), Hank’s balanced salt solution (HBSS), Transwell® inserts, and lucifer yellow were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Ascorbic acid, diethylene triamine pentaacetic acid (DTPA), diethyl ether, methanol, nile red, sodium carboxymethyl cellulose (CMC-Na), sodium acetate, were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Brij® 78, Tween® 80, castor oil and PEG 400 were received as gifts from Croda (Columbus Circle Edison, NJ). Miglyol® 812 and Capmul® MCM were generously donated by Abitect Corporation (Janesville, WI). Capryol®, Labrasol®, Labrafil® M 1944 CS, Labrafil® M 2155 CS, and Transcutol® HP were kindly donated by Gattefosse (Saint-Priest Cedex, France). Cremophor® RH 40 was provided by BASF (Ludwigshafen, Germany). Caco-2 cells, Eagle’s minimum essential medium, and fetal bovine serum (FBS) were purchased from American Type Culture Collection (Manassas, VA). All other chemicals and solvents were of reagent grade and used without further purification.

4.3.2 Solubility assessment

An excess quantity of quercetin was added to 1 g of various oils (castor oil, soybean oil, Miglyol®812, Capryol®90, Capmul®C8, Capmul®MCM, Labrafil®1944, Labrafil®2155), surfactants (Brij®020, Brij®78, Brij®721, Tween®20, Tween®60, Tween®80, PEG-35-caster oil, Cremophor®RH40, Solutol®HS15) and co-surfactants (PEG 400 and Transcutol® HP). Mixtures of quercetin and excipients were first vortexed to facilitate mixing of quercetin with vehicles, and the mixtures were then allowed to reach equilibrium at 40 °C in a shaking water bath. After 48 h, mixtures were centrifuged at 10,000 g for 10 min to separate undissolved quercetin, followed by filtration through a 0.45 µm membrane filter. Filtrates were diluted with methanol and the concentration of
quercetin was determined using a UV/Vis spectrometer at 372 nm based on a standard curve of known quercetin concentrations.

4.4.3 Screening of surfactants for emulsifying activity

Surfactants (100 mg each of Labrasol®, Cremophor® RH 40, Solutol®, or Tween® 80) were added to 100 mg each of the oil phase (Capryol® 90, Capmul® MCM, Labrafil®, or castor oil). The mixtures were heated at 50 °C with mild stirring to achieve homogeneity. Each mixture (50 mg) was weighed and diluted with 15 mL distilled water to yield a fine emulsion. The formation of nanoemulsions was monitored by size measurement and observing the homogenous emulsion appearance without phase separation.

4.3.4 Construction of pseudo-ternary phase diagrams

Oil and surfactant/co-surfactant mixtures that could self-emulsify under dilution and gentle agitation were identified from pseudo-ternary phase diagrams of systems containing oil (20-70% w/w), surfactant (30-80% w/w), and co-surfactant (0-30% w/w). 300 mg of surfactant, co-surfactant, and oils with combination one (castor oil/Cremophor® RH 40/Transcutol® HP) and combination two (castor oil/Tween® 80/ Cremophor® RH 40/PEG 400) at various concentrations were mixed at 50 °C. Efficiency of nanoemulsion formation was assessed by adding 5 mg of each mixture to 2 mL of distilled water, followed by gentle agitation using a magnetic stirrer. The nanoemulsion formation was transparent as assessed by visual inspection and particle size of the resulting dispersions was determined by Dynamic Light Scattering (DLS) (Malvern Zetasizer). Dispersions having sizes <200 nm were deemed acceptable.

4.3.5 Preparation and characterization of quercetin-containing SNEDDS (Q-SNEDDS)

The selected SNEDDS formulations from phase-diagrams were used for solubility testing. SNEDDS formulations having the highest solubility of quercetin were used to prepare Q-SNEDDS. Quercetin (30 mg/g) was added to each formulation followed by
gently heating at 50 °C and mixing using a magnetic stirrer until a clear mixture was obtained. Formulations were examined for turbidity or phase separation prior to self-emulsification and particle size analysis. Water was then added to the Q-SNEDDS with gentle stirring to form a nanoemulsion (Q-SNEDDS nanoemulsion, 3 mg/mL). Particle size and zeta-potential of the nanoemulsion were measured using DLS instrument. The particle size was further confirmed by transmission electron microscopy (TEM). One drop of the nanoemulsion was placed on a formvar coated carbon grid for 3-5 min followed by a negative staining with phosphotungstic acid (PTA) solution (1% w/v, pH 7.4) for 1 min. The dried sample in the grid was imaged by TEM (FEI Tecnai Biotwin, Eindhoven, Netherlands).

4.3.6 Determination of quercetin encapsulation efficiency

Free quercetin was separated from Q-SNEDDS nanoemulsions by ultrafiltration using a 3000 Da molecular weight cutoff filter. Samples containing free quercetin and Q-SNEDDS nanoemulsion were diluted in methanol prior to HPLC analysis as described, with minor modifications (151), using a Ultimate 3000 HPLC-electrochemical system (Thermo Fisher Scientific, Inc., Sunnyvale, CA) equipped with a solvent delivery module (LPG-3400BM), a refrigerated autosampler (WPS-3000) maintained at 4 °C, and a dual-channel coulometric analytical cell (6011 RS ULTRA). Samples were separated isocratically at 0.45 mL/min on a Kinetex pentafluorophenyl column (150 x 3.0 mm i.d., 3 μm; Phenomenex, Inc., Torrance, CA) using a mobile phase consisting of 75 mmol/L citric acid, 25 mmol/L ammonium acetate, and 0.27 mmol/L EDTA prepared in 50% methanol (vol/vol). Detection was performed at potential settings of -100 and 225 mV and quercetin was quantified at 225 mV. The amount of quercetin encapsulated in SNEDDS was calculated by subtracting free quercetin from total quercetin in the Q-SNEDDS suspension. Encapsulation efficiency (EE) was calculated as: EE (%) = W x
100% / W_total, where W = quantity of quercetin loaded into Q-SNEDDS and W_total = total quantity of quercetin in the Q-SNEDDS suspension.

4.3.7 In vitro quercetin release study

*In vitro* release of quercetin from the nanoemulsions was performed using a dialysis technique. Briefly, Q-SNEDDS was diluted with simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.8) (USP 23 standard) to form Q-SNEDDS nanoemulsions at 3 mg/mL of quercetin. The quercetin-nanoemulsions (3 mL) were introduced into 5 mL-dialyzers (molecular weight cutoff: 10,000 Da) and immersed in 25 mL of simulated gastric and intestinal fluids containing 0.5% (w/v) Tween 80 at 37 °C in a shaking bath at 100 rpm. At selected time intervals, aliquots (1 mL) were removed from the dissolution medium and an equivalent volume of fresh medium was added. The concentration of quercetin was determined spectrophotometrically at a wavelength of 372 nm. The amount of quercetin released was calculated by comparing with standards of known quercetin concentrations.

4.3.8 Stability of the Q-SNEDDS

To determine the extent to which quercetin precipitated during dispersion and storage, Q-SNEDDS stability was assessed by storing Q-SNEDDS and Q-SNEDDS nanoemulsions at a quercetin concentration of 5 mg/mL in amber vials at room temperature. Size changes, drug precipitation, and phase separation were evaluated at predetermined time point. For quantification of quercetin content, Q-SNEDDS and Q-SNEDDS nanoemulsions were sampled at pre-determined time points and centrifuged at 10,000 g for 5 min to remove any precipitated quercetin. An aliquot of the supernatant was dissolved in methanol, followed by the dilution to an appropriate quercetin concentration prior to assay by UV/Vis spectrophotometry at 372 nm.
4.3.9 Transport of Q-SNEDDS in Caco-2 cells

Caco-2 cells were grown in MEM supplemented with 1% non-essential amino acids, 1% sodium pyruvate, 1% antibiotics, and 10% FBS at 37 °C in an atmosphere of 5% CO₂. When cells were confluent, they were trypsinized and seeded onto Transwell® polycarbonate inserts (12-well plate) at a density of 100,000 cells/well. Medium in the Transwell® plate was changed every 2 d and cells were allowed to differentiate for 21-28 d.

Prior to the experiment, cells were washed 3-times with pre-warmed transport medium (HBSS buffered with 0.35 g/L NaHCO₃ and 25 mM HEPES). Transwells® containing transport medium (0.4 mL in the apical side and 1.2 mL in the basolateral side) were then incubated at 37 °C for 15 min while shaking in an orbital shaking bath. Quercetin stock solution was prepared by dissolving quercetin in DMSO (3 mg/mL). Both the stock solution and Q-SNEDDS nanoemulsion were diluted using HBSS to achieve the same quercetin concentration of 50 µg/mL and filtered using a 0.2 µm sterile filter membrane immediately prior to initiating transport studies. Media from the apical side was removed and quercetin solution or Q-SNEDDS nanoemulsion (0.4 mL) was subsequently added to the apical side. At 0, 15, 30, 45, and 60 min, samples (0.5 mL) were collected from the receiver chamber, frozen at -80 °C until analysis, and an equivalent volume of HBSS was added to the apical side. Media were directly injected onto the aforementioned HPLC-electrochemical system (Thermo Fisher Scientific, Inc., Sunnyvale, CA) to determine quercetin concentration as described above. Integrity of the Caco-2 cell monolayer treated with free quercetin or Q-SNEDDS nanoemulsion were evaluated by adding lucifer yellow (100 µg/mL) to the donor chamber at the beginning of the transport study. At the end of the study, samples (200 µL) from the receiver chambers were transferred to a 96-well plate. The concentration of Lucifer yellow was
measured using a microplate reader \((E_s/E_m, 480 \text{ nm}/530 \text{ nm}; \text{Tecan group Ltd., Männedorf, Switzerland})\).

4.3.10 Visualization of intestinal Q-SNEDDS absorption in rats

The protocol for all studies in rats was approved by Institutional Animal Care and Use Committee at the University of Connecticut. Visualization of intestinal quercetin absorption delivered in rats was accomplished using the hydrophobic fluorescent dye nile red, which was encapsulated into Q-SNEDDS as described above. Free nile red (20 mg/mL) suspended in 0.5% (w/v) CMC-Na and Q-SNEDDS nanoemulsion containing nile red (20 μg/mL) were administered by oral gavage to male Sprague Dawley rats (300 ± 10 g; Charles River; Wilmington, MA) at 15 mg/kg BW of quercetin and then sacrificed after 40 min. Following a midline incision, the gastrointestinal tract was removed; individual segments (duodenum, jejunum, and ileum) were separated, and subsequently placed in cold phosphate buffered saline (PBS; pH 7.4). Isolated gastrointestinal segments were dissected along the mesenteric border and rinsed with cold PBS to remove luminal contents. A 0.5 cm piece of each segment was fixed overnight in 4% neutral buffered formalin. The gastrointestinal segments were washed with PBS and frozen in cryoembedding media (OCT) for subsequent cryostat sectioning at 20 μm per slice (CM3050S, Leica). The sections were applied to glass slides, rinsed with cold PBS, and visualized under an inverted fluorescence microscope at 10X magnification (Olympus, Japan).

4.3.11 Bioavailability of quercetin in rats

Male Sprague-Dawley rats (300 ± 10 g) having a jugular vein cannulation were purchased (Charles River; Wilmington, MA). For 1 wk prior to experimentation, rats had free access to water and a purified AIN-93G diet. Rats \((n = 5-6/group)\) were fasted overnight to assess quercetin bioavailability in response to oral administration of Q-SNEDDS and quercetin control suspension. Food was provided after 4 h of the study.
Control suspension of quercetin was prepared by suspending quercetin in 0.5% (w/v) CMC-Na, followed by sonication for 15 min. The quercetin control suspension or Q-SNEDDS nanoemulsion were provided by oral gavage (3 mg/mL; 15 mg/kg BW) at a dose consistent with our clinical bioavailability studies where participants ingested 1095 mg quercetin (~15 mg/kg BW) (151). Blood (0.4 mL) was collected into tubes containing sodium heparin prior to (0 h) and 1, 2, 4, 6, 8, 12, and 24 h after administration, and then plasma was separated by centrifugation (1200 g, 10 min, 4 °C) and stored at -80 °C. Liver and small intestine were collected at 24 h. Prior to freezing all tissues in liquid nitrogen and storing at -80 °C, intestinal contents were flushed with ice-cold PBS (pH 7.4) containing 2 g/L of albumin and 16.5 mmol/L sodium taurocholate.

Plasma throughout the 24 h pharmacokinetics study and tissues collected at 24 h were analyzed for quercetin and its methylated derivatives isorhamnetin (3'-O-methyl-quercetin) and tamarixetin (4'-O-methyl-quercetin) using the aforementioned HPLC-electrochemical system. Due to limited plasma availability at each time point, plasma quercetin analysis was limited to total quercetin (i.e. the sum of free quercetin and its glucuronidated and sulphates conjugates). For analysis, 100 μL of plasma was mixed with 10 μL of 27.6 μmol/L morin (internal standard) dissolved in methanol, 977 U β-glucuronidase and 33 U of sulfatase prepared in 1 mol/L sodium acetate buffer (pH 5.5), and 20 μL of 6% ascorbic acid (w/v) prepared in 1 mol/L sodium acetate buffer containing 2.5 mmol/L DTPA. Samples were incubated (37 °C, 2 h), extracted with diethyl ether, and then dried under nitrogen gas before being reconstituted in methanol and injected onto the HPLC. For the analysis of liver and small intestine, tissue (~1 g) was pulverized in liquid nitrogen and then mixed with 2 mL of 1 mol/L sodium acetate buffer (pH 5.5) containing 28 mmol/L ascorbic acid. Following centrifugation (600 g, 15 min, 4 °C), the supernatant was collected and two separate aliquots (600 μL) were mixed with 60 μL of 4.96 μmol/L morin and 1 mL of sodium acetate buffer containing 28 mmol/L
ascorbic acid. Samples were incubated (37 °C, 2 h) in the presence or absence of 977 U β-glucuronidase and 33 U of sulfatase to determine free and total (i.e. the sum of hydrolyzed conjugates and free) flavonoids, and extracted with diethyl ether, dried under nitrogen gas and reconstituted in methanol prior to HPLC analysis.

### 4.3.12 Statistical analysis

Plasma pharmacokinetics of quercetin, isorhamnetin, and tamarixetin, including area under the concentration curve (AUC\textsubscript{0-24 h}), maximum concentration (C\textsubscript{max}), time to maximum concentration (t\textsubscript{max}), elimination half-life (t\textsubscript{1/2}), and elimination rate constant (k\textsubscript{e}) were calculated using PK Solutions\textsuperscript{®} software (Summit Research Services, Inc., Montrose, CO, USA). Absorption or appearance rate constants were not calculated because of insufficient time points before T\textsubscript{max}. Data (means ± SEM) were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA) and were initially evaluated for normality using the Kolmogorov-Smirnov test. Treatment differences were analyzed using a Student's unpaired t-test for data having a normal distribution and a Mann-Whitney U-test was used for data lacking a normal distribution. All analyses were considered statistically significant at an α-level of P<0.05.
4.4 Results and discussion

4.4.1 Selection of components for SNEDDS

The selection of oil, surfactant, and co-surfactant are critical for improving solubility and achieving high payload in a SNEDDS formulation (152, 153). Components of SNEDDS were selected to maximize quercetin solubility while attaining high miscibility with each component to form a stable nanoemulsion spontaneously following dilution with water. The solubility of quercetin in different oils, surfactants and co-surfactants were determined (Figure 4.1). Quercetin showed higher solubility in Capryol®, Capmul® MCM, and castor oil compared to the solubility in soybean oil, miglyol 812, and labrafil and these oils were therefore investigated further. Quercetin also exhibited good solubility in each of the surfactants tested with Labrasol® (32.0 ± 1.3 mg/g), Cremophor® RH 40 (24.5 ± 0.9 mg/g), and Tween® 80 (17.9 ± 0.5 mg/g). The selection of surfactant was governed by their emulsification efficiency rather than their ability to solubilize quercetin, consistent with the approach of others (145, 154). Both Transcutol® HP and PEG 400 had great solubilizing capacity of quercetin, showing values of 73.8 ± 2.2 mg/g and 51.8 ± 0.5 mg/g, respectively. Thus, Transcutol® HP and PEG 400 were chosen as a co-surfactants to improve loading capacity of quercetin.

Various combinations of oils and surfactants were examined to determine their ability to form emulsions. Labrasol® emulsified the selected oils poorly as evidenced by the appearance of many oil droplets following dispersion of the Labrasol® mixture in aqueous solution. Cremophor® RH 40 showed the best emulsification efficiency, forming transparent solutions with oils after adding water. Although Tween® 80 was able to emulsify oils, it occurred with lower efficiency than Cremophor® RH 40. The three selected surfactants have HLB values ranged from 13-16, thus, the differences observed in their emulsifying ability may be attributed to the head group structure and chain length (147, 155). Cremophor® RH 40 and Tween® 80, which yielded clear nanoemulsions,
were selected for further study. The systems containing Capryol® or Capmul® MCM were unable to form stable nanoemulsions containing quercetin. Upon contact with aqueous media, these systems precipitated immediately. Although quercetin had lower solubility in castor oil compared with the medium chain length lipids (6-12 carbons), precipitation of quercetin did not occur in formulations containing castor oil, suggesting that lipid chain length regulates nanoemulsion stability. Long chain fatty acid lipids (13-21 carbons) such as castor oil have also been reported to form stable nanoemulsions without precipitation from SNEDDS containing indomethacin (153). Therefore, castor oil was deemed as the best oil for Q-SNEDDS preparation.

4.4.2 Construction of pseudo-ternary phase diagram and formulation optimization

Ternary phase behavior investigations aid in the selection of the optimal concentration of oil, surfactant, and co-surfactant in formulations to produce stable nanoemulsions. Formulations (n = 18) comprised of castor oil/Cremophor® RH 40/Transcutol® HP were prepared using the selected S_{mix} (surfactant:co-surfactant) as well as castor oil in the percentage range of 20-70% (w/w). Their self-emulsifying properties were evaluated. Figure 4.2A and 4.2B show the phase diagrams in which the shaded region indicates the nanoemulsion region. The relatively larger shaded region indicates better self-nanoemulsifying ability (154, 156). The phase diagram (Figure 4.2A) showed a large nanoemulsification region up to 60% (w/w) of castor oil. This may be due to the same carbon chain length of both Cremophor® RH 40 (polyoxy 40 hydrogenated castor oil) and castor oil, which could help Cremophor® RH 40 incorporate the oil droplet uniformly^{23}. In addition, Transcutol® HP contains a diethylene glycol structure that likely associates with polyethylene glycol structure contained in Cremophor® RH 40, which would be expected to further stabilize the nanoemulsions. Table 4.1 shows the average size and appearance of nanoemulsions in the phase diagram. Particle size and turbidity
of the nanoemulsions increased with increasing oil content. Formulations having ≤50% surfactant with transparent and bluish nanoemulsions and sizes ≤200 nm were selected for quercetin encapsulation.

Unexpectedly, quercetin (3% w/w) precipitated within 30 min after each of the selected formulations was diluted with distilled water regardless of the oil, surfactant and co-surfactant concentrations. However, when PEG 400 was substituted by Transcutol® HP, precipitation of quercetin was inhibited. Inhibiting quercetin precipitation by PEG 400 might be attributed to suppression in compound nucleation, the first step in crystallization of compounds intended for nanoemulsification (157). When Tween® 80 was combined with Cremophor® RH 40 and Transcutol® HP for preparation of Q-SNEDDS, stable nanoemulsions were generated. A combination of Tween® 80 with Cremophor EL has been also used to form stable nanoemulsions with Sefsol 218 oil and Oleanolic acid since Tween® 80 could not generate stable nanoemulsions when used alone (158). Therefore, a system comprised of castor oil, Cremophor® RH 40: Tween® 80, and PEG 400 was used to construct a new phase diagram in which the ratio of Tween® 80 and Cremophor® RH 40 was 2:1. This ratio was chosen based on the ability of the surfactant mixture to solubilize quercetin. Even though quercetin had higher solubility in Cremophor® RH 40 than Tween® 80, the presence of larger amount of Tween® 80 than Cremophor® RH 40 in the surfactant mixture resulted in higher solubility of quercetin (data not shown). The mechanism for this enhanced quercetin solubility is not completely clear. The combination of Cremophor® RH 40 and Tween® 80 could emulsify 40% (w/w) of castor oil (Figure 4.2B).

The droplet size of the nanoemulsions increased with increasing oil concentration and decreasing surfactant content (Table 4.2). Formulations 1-4 containing 20% (w/w) castor oil formed clear bluish nanoemulsions with droplet sizes less than 200 nm and were selected for further optimization. Increasing the proportion of PEG 400 resulted in
an increase in quercetin solubility in SNEDDS. Among these four formulations, those containing 20% oil, 50% surfactant and 30% co-surfactant had the greatest solubilizing ability for quercetin (49.2 ± 1.2 mg/g). Furthermore, no phase separation or precipitation of quercetin from the nanoemulsions was observed upon 72 h storage at ambient temperature. Therefore, this formulation was selected as the optimal SNEDDS formulation for quercetin.

4.4.3 Characterization of the optimized Q-SNEDDS

The optimal Q-SNEDDS spontaneously dispersed in aqueous media to form nanoemulsions at high encapsulation efficiency of quercetin (≥90%). The aqueous solubility of quercetin increased from 5 µg/mL to 5 mg/mL after incorporation into SNEDDS. Nanoemulsion droplet size is an important factor in formulating SNEDDS because it determines dissolution rate and absorption extent of compound (159). Size distribution and morphology of the resulting nanoemulsions were evaluated by DLS and TEM. Blank SNEDDS (containing no quercetin) resulted in nanoemulsions of 200 ± 10 nm. When quercetin was incorporated into the SNEDDS, the size was slightly increased to 215 ± 8 nm (Figure 4.3). TEM images of optimal blank SNEDDS and Q-SNEDDS 72 h post dilution in distilled water are shown in Figure 4.4. Spherical nanoemulsions were formed without change in morphology after quercetin incorporation. Furthermore, no sign of quercetin precipitation was observed in the TEM image inferring stability of the formed nanoemulsions. Quercetin-loaded nanoemulsions were negatively charged at their surface as reflected by their zeta potential of -26.3 ± 1.2 mV, likely due to the presence of castor oil. Zeta potential is an indicator of the stability of the nanoemulsion. A higher electrical charge (zeta potential >±30 mV) on the surface of the nano-droplets prevents aggregation due to the strong repellent forces among droplets (160). Thus, the optimized formulation with zeta potential value close to -30 mV is expected to be stable in solution.
SNEDDS nanoemulsions exhibited slow quercetin release at both pH 1.2 and pH 6.8 with about 8% release within 24 h (Figure. 4.5). Quercetin release from the nanoemulsions at pH 1.2 and pH 6.8 was not significantly different in the first 8 h ($P>0.05$). Quercetin-loaded nanoemulsions in both media showed no sign of precipitation, cloudiness or separation within 24 h. The results indicated that SNEDDS formulation improved quercetin solubility but retained quercetin within the nanoemulsions for an extended period of time.

4.4.4 Stability of Q-SNEDDS

To evaluate storage stability of Q-SNEDDS, we monitored quercetin content and phase separation of the Q-SNEDDS as well as changes in particle size and quercetin content of Q-SNEDDS after dilution with water upon storage at ambient temperature. After 1 month, phase separation with settling of the quercetin-containing layer on the bottom was observed in the system comprised of quercetin-castor oil/ Cremophor® RH 40/ Transcutol® HP. In contrast, there was no change in quercetin content or phase separation from the optimal Q-SNEDDS formulation (inset of Figure. 4.6A). While there were insignificant changes in particle size and quercetin content of the optimal Q-SNEDDS nanoemulsion, nanoemulsions of quercetin-castor oil/Cremophor® RH 40/ Transcutol® HP precipitated with a dramatic decrease in quercetin content and increase in particle size within 10 d (Figure. 4.6). These findings indicate that the inclusion of PEG 400 and Tween® 80 in the optimal Q-SNEDDS formulation prevented quercetin precipitation and maintained it at supersaturated levels for an extended period of time.

4.4.5 Transport of the Q-SNEDDS across Caco-2 cell monolayer

Human Caco-2 cell monolayers are frequently utilized as a model of intestinal absorption (161). Changes in intestinal permeability of quercetin by the SNEDDS formulation were assessed by measuring quercetin transport across epithelial cell layers. Quercetin concentration in both DMSO/HBSS solution (control solution) and SNEDDS
was kept the same (50 µg/mL) to eliminate the concentration effect on quercetin transport. Quercetin is known to be unstable after exposure to cell culture conditions longer than 60 min (65). Thus, the transport study was performed at 37 °C for 60 min. Quercetin transport across the Caco-2 cell monolayer increased time-dependently (Figure 4.7). At 45 and 60 min, the transport amount of quercetin administered as Q-SNEDDS was significantly greater than that from quercetin control solution (p<0.05). By 60 min, the cumulative amount of transported quercetin delivered from Q-SNEDDS was ~2-times greater than that of quercetin control solution (159.7 ng/mL for the Q-SNEDDS vs. 85.1 ng/mL for quercetin control solution). Permeability of lucifer yellow though the Caco-2 cell monolayer was utilized as an indicator of tight junction integrity (162). Transport of lucifer yellow after 1 h was 1.8 ± 0.2 % in all samples tested, indicating good integrity of the Caco-2 cell monolayer at the end of the study. However, it was reported that the width of Caco-2 cell tight junction increased largely shown on TEM images after exposure to a self-emulsifying nanoemulsion while the integrity value was about 90% at 15 min and 95% at 2 h time point (163). The test is time-dependent and may only reflect some changes of the integrity of cell monolayer since the tight junction opening is a reversible process. Tight junction opening after being treated with self-emulsifying systems and certain surfactants has been observed (164), which presents a possible mechanism for the enhanced absorption in terms of paracellular transport. During the transport study, quercetin control suspension precipitated from solution, which attenuated its transport compared to quercetin solubilized in Q-SNEDDS. It is also likely that the presence of Cremophor® RH 40 and Tween® 80 in Q-SNEDDS increases the permeability of quercetin by facilitating transcellular absorption (156, 165). The enhanced quercetin transport across the intestinal cell monolayer suggests the potential of bioavailability enhancement from the Q-SNEDDS.
4.4.6 Histological evaluation

To visualize quercetin absorption in rat intestines in response to Q-SNEDDS, the hydrophobic fluorescent dye (nile red) was incorporated into Q-SNEDDS and quercetin suspended in CMC-Na. The fluorescent signal may indicate quercetin due to its similar physicochemical properties to nile red. Average particle size and zeta potential of the Q-SNEDDS containing nile red were unchanged compared to the formulation without the dye (data not shown). Figure 4.8 shows fluorescence images of different segments of rat intestine 40 min after oral administration of quercetin control suspension (control) and Q-SNEDDS, and 1 h and 2 h post-administration of Q-SNEDDS containing nile red. After 40 min, the red fluorescent signal originating from nile red was minimally visible in intestinal segments of rats treated with the control, whereas a strong red signal appeared in all intestinal segments of rats treated with the Q-SNEDDS. The dim signal in controls was likely due to the existence of nile red in its insoluble form which was washed out at the end of the study, while the solubilized form of the dye in the nanoemulsions emitted a strong fluorescence signal. Moreover, stronger red signals were visible outside than inside the intestine villi, which may be attributed to the fact that the Q-SNEDDS remained intact and became attached to the intestine villi. The red signal of the Q-SNEDDS outside the intestine villi decreased and no red signal was visible inside the villi after 1 h and 2 h, indicating that the dye/quercetin were absorbed rapidly from the intestinal lumen. The intensity of the red signal was not significantly different in duodenum, jejunum and ileum, suggesting that these three sites of the rat intestine were all important for Q-SNEDDS absorption. This result is supported by prior studies showing that the absorption in duodenum, jejunum, and ileum of microemulsions containing quercetin was not significantly different and ranged from 34-41% (92).
4.4.7 Pharmacokinetics of quercetin in rats

Plasma pharmacokinetics of quercetin was determined to examine the extent to which Q-SNEDDS increased quercetin bioavailability. Plasma quercetin concentrations increased following oral administration of either formulation and decreased to concentrations no different from baseline by 24 h regardless of formulation administered (Figure 4.9A). However, plasma quercetin $C_{\text{max}}$ increased by 3-times and AUC$_{0-24}$ increased by 2-times when quercetin delivered from Q-SNEDDS was administered compared to quercetin control suspension (Table 4.3), and these observations occurred without any differences in $T_{\text{max}}$ or $k_e$ between treatments. Collectively, Q-SNEDDS increased quercetin $C_{\text{max}}$ and AUC$_{0-24}$ without affecting its elimination kinetics, suggesting that Q-SNEDDS improved quercetin bioavailability by enhancing its absorption.

Similar to quercetin, plasma isorhamnetin and tamarixetin (methylated metabolites of quercetin) increased after ingestion of either formulation and returned to baseline levels by 24 h (Figure 4.9B-C). The $C_{\text{max}}$ of isorhamnetin and tamarixetin increased by 3-times following Q-SNEDDS administration compared to quercetin control suspension, which was in agreement with 2-times greater AUC$_{0-24}$ from Q-SNEDDS (Table 4.3). The $T_{\text{max}}$ of isorhamnetin and tamarixetin was 2.3-3.4 h earlier following Q-SNEDDS administration compared to quercetin control suspension, but this occurred without any statistical significance. In addition, no other pharmacokinetic parameters differed between groups (Table 4.3). These findings indicate that the extent and rate of methylation of quercetin increased in response to SNEDDS administration.

Isorhamnetin and tamarixetin are positional methylated isomers generated from quercetin in a catechol-O-methyltransferase (COMT)-dependent manner (166). Tamarixetin has been reported to show greater potency in inhibiting platelet aggregation in vitro than quercetin itself (167). Therefore, Q-SNEDDS increases tamarixetin to a
greater extent than quercetin control suspension, which may have important benefits to human health. Despite the similar mechanism of generation of tamarixetin and isorhamnetin, they exhibited distinct pharmacokinetic responses. Regardless of the quercetin formulation administered, plasma pharmacokinetics of isorhamnetin was biphasic (Figure 4.9C), suggesting the possibility of enterohepatic recirculation of isorhamnetin, a common route of disposition of quercetin and its methylated metabolites (66). Isorhamnetin C_{\text{max}} was also greater than that of tamarixetin regardless of the quercetin formulation administered \((P<0.05)\), which is in agreement with earlier studies showing that isorhamnetin is the predominant methylated metabolite of quercetin in rats fed a quercetin-containing diet (168). Consistent with our clinical findings from a quercetin pharmacokinetics study (151), plasma isorhamnetin was eliminated slower \((P<0.05)\) and its half-life was longer \((6.0 \pm 1.8 \text{ h}; P<0.05)\) than that of tamarixetin (Table 3). Thus, isorhamnetin pharmacokinetics differed from tamarixetin, suggesting that the positional methylation of quercetin regulates its disposition.

Accumulation of quercetin occurs in the intestines and liver at 24 h following an acute, single-dose administration of quercetin-4’-glucoside in rats (48). In the present study, accumulation of quercetin was evaluated in small intestines and liver at 24 h when the pharmacokinetic study was terminated. Small intestinal accumulation of quercetin did not differ between treatments whereas concentrations of total isorhamnetin were greater in response to Q-SNEDDS administration (Table 4). In contrast, quercetin and isorhamnetin at liver, whether free or total quercetin or isorhamnetin, accumulated to a greater extent in response to Q-SNEDDS, indicating that the SNEDDS formulation improved quercetin absorption. Greater persistence of free quercetin at liver compared to small intestines likely reflects tissue-specific differences in xenobiotic metabolism. Our findings also suggest that the greater accumulation of free quercetin and free isorhamnetin at liver in response to Q-SNEDDS might extend their bioactivity, but clearly
such work was beyond the scope of this study. Likewise, additional work is needed to assess time-dependent differences in tissue accumulation of quercetin.

In contrast to quercetin, isorhamnetin was detected only as its conjugated metabolites in small intestine regardless of formulation administered whereas total isorhamnetin increased by 1.6 times in small intestine in response to Q-SNEDDS administration. In liver, Q-SNEDDS increased total isorhamnetin and free isorhamnetin accumulation by 3- and 6- times, respectively, compared to quercetin control suspension. Consistent with plasma pharmacokinetics indicating faster tamarixetin elimination, tamarixetin was not detectable in small intestine or liver regardless of formulation administered.

Collectively, these findings demonstrate that Q-SNEDDS increases quercetin absorption, thereby enhancing its bioavailability. Additional studies are needed to identify the mechanisms for the enhanced bioavailability of quercetin by Q-SNEDDS. According to the available reports, we speculate that it may be associated with the following factors. Poor absorption of quercetin is partly attributed to its limited water solubility (31). The optimal SNEDDS formulation used in this study increases the apparent water solubility of quercetin and maintains quercetin at supersaturated concentrations in gastrointestinal tract, thereby the large surface area provided by the fine droplets of the nanoemulsion increases dissolution which is one of the rate limiting factors for quercetin absorption. The presence of surfactants in the SNEDDS formulation may increase intestinal epithelial permeability by disturbing the cell membrane and partitioning into the cell membrane where they can form polar defects in the lipid bilayer. SNEDDS formulation may also produce an increased reversible effect on the opening of tight junction so that improve the permeability (163). Since quercetin is transported by chylomicrons (169, 170), Q-SNEDDS may increase lymphatic transport of quercetin in ileum. It was reported that long-chain fatty acids of castor oil enhance lymphatic transport of quercetin by
increasing chylomicron synthesis in rats (44). Tween® 80 also enhances chylomicron secretion in Caco-2 cells (171). Thus, administration of Q-SNEDDS is expected to increase chylomicron-dependent transport of quercetin into the lymphatic system. Cremophor® RH 40 was reported to decrease the efflux activity of multidrug resistance-associated protein 2 (MRP2) (172). This suggests that SNEDDS formulation may reduce intestinal excretion of quercetin at the present of Cremophor® RH 40 to favor its absorption. The developed SNEDDS formulation is biocompatible and free of alcohol, which potentiated commercialization and clinical application compared to another newly developed self-emulsifying formulation (91).

4.5 Conclusion

In this study, Q-SNEDDS was successfully prepared and optimized. The optimal Q-SNEDDS comprised of castor oil, Tween® 80, Cremophor® RH 40, and PEG 400 (20:16:34:30) spontaneously self-emulsified in water to form nanoemulsions that significantly increased quercetin solubility (5 mg/mL) and are stable for 1 month at ambient temperature. Our findings demonstrate that this Q-SNEDDS formulation increases the transport of quercetin across Caco-2 cell monolayer and enhances absorption and oral bioavailability of quercetin in rats. Further studies are needed to investigate the mechanisms of this improvement in bioavailability and whether SNEDDS formulation reinforces chemoprotection activity of quercetin.

4.6 Acknowledgement

The authors are grateful for financial support from the Diet and Health Initiative at the University of Connecticut.
4.7 Figures and tables

Figure 4.1 Solubility of quercetin in various oils, surfactants, and co-surfactants.
Figure 4.2 Pseudo-ternary phase diagrams of formulations composed of castor oil/Cremophor® RH 40/Transcutol® HP (A), and castor oil/ Tween® 80/ Cremophor® RH 40/PEG 400 (B).
Figure 4.3 Particle size distribution of optimal nanoemulsions without quercetin (A), and optimal quercetin-loaded nanoemulsions (B).
Figure 4.4 TEM images of optimal nanoemulsions without quercetin (A), and optimal Q-SNEDDS nanoemulsions at 72 h post-dilution of Q-SNEDDS (B).
Figure 4.5 Release profiles of Q-SNEDDS nanoemulsion in simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.8) containing 0.5% (w/v) Tween 80 at 100 rpm and 37 °C.
Figure 4.6 Stability of Q-SNEDDS nanoemulsions from two systems (castor oil/Cremophor® RH 40/Transcutol® HP and castor oil/ Tween® 80/ Cremophor® RH 40/PEG 400) stored at room temperature for 1 month (A) average particle size, and (B) quercetin content remained in the nanoemulsions.
Figure 4.7 The mean cumulative transport versus time of quercetin solution and Q-SNEDDS nanoemulsions across Caco-2 cell monolayer at 37 °C for 1 h using initial quercetin concentration of 50 μg/mL. *Significantly different from quercetin solution.
Figure 4.8 Fluorescence images of rat intestine treated with Q-SNEDDS nanoemulsions and quercetin control suspension (control) containing nile red.
Figure 4.9 Plasma concentration-time profiles of quercetin (A), tamarixetin (B) and isorhamnetin (C) in Spraque-Dawley rats followed by an oral administration of 15 mg/kg quercetin control suspension or 15 mg/kg Q-SNEDDS nanoemulsion.
Table 4.1 Formulations, droplet size and appearance of nanoemulsions composed of castor oil/Cremophor® RH 40/Transcutol® HP

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Oil: Smix Castor oil (%)</th>
<th>Cremophor® RH 40 (%)</th>
<th>Transcutol® HP (%)</th>
<th>Average droplet size (nm)</th>
<th>Appearance of nanoemulsion</th>
</tr>
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<tr>
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<tr>
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<td>0</td>
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</tr>
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<td>0</td>
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</tr>
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<td>6:4</td>
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</tr>
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<td>14</td>
<td>10:0</td>
<td>100</td>
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<td>0</td>
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</table>

**Note:** ND indicates not determined.
Table 4.2 Formulations, droplet size and appearance of nanoemulsions composed of castor oil/Tween® 80/ Cremophor® RH 40/PEG 400

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Oil: Smix Castor oil (%)</th>
<th>Tween® 80: Cremophor® RH 40 (2:1) (%)</th>
<th>PEG 400 (%)</th>
<th>Average droplet size (nm)</th>
<th>Appearance of nanoemulsion</th>
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<td>2:8</td>
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<td>159.5 Clear, bluish</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>10</td>
<td>80</td>
<td>0</td>
<td>157.1 Clear, bluish</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>20</td>
<td>80</td>
<td>0</td>
<td>188.4 Clear, bluish</td>
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<tr>
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<td>50</td>
<td>30</td>
<td>80</td>
<td>0</td>
<td>200.8 Clear, bluish</td>
</tr>
<tr>
<td>5</td>
<td>3:7</td>
<td>30</td>
<td>70</td>
<td>0</td>
<td>266.3 Translucent</td>
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<tr>
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<td>10</td>
<td>80</td>
<td>0</td>
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<td>20</td>
<td>80</td>
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<tr>
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<td>30</td>
<td>80</td>
<td>0</td>
<td>261.6 Milky-like</td>
</tr>
<tr>
<td>9</td>
<td>4:6</td>
<td>40</td>
<td>60</td>
<td>0</td>
<td>251.5 Milky-like</td>
</tr>
<tr>
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<td>80</td>
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<td>7:3</td>
<td>70</td>
<td>30</td>
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### Table 4.3 Pharmacokinetic parameters of quercetin in rats after oral administration of quercetin control suspension and Q-SNEDDS (15 mg/kg)a)

<table>
<thead>
<tr>
<th></th>
<th>Quercetin control suspension</th>
<th>Q-SNEDDS</th>
<th>Quercetin control suspension</th>
<th>Q-SNEDDS</th>
<th>Quercetin control suspension</th>
<th>Q-SNEDDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ b) (mg/L)</td>
<td>1.20 ± 0.17</td>
<td>3.75 ± 0.96*</td>
<td>0.04 ± 0.01</td>
<td>0.12 ± 0.01*</td>
<td>0.11 ± 0.03</td>
<td>0.35 ± 0.07*</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>1.8 ± 0.6</td>
<td>1.2 ± 0.2</td>
<td>3.8 ± 1.2</td>
<td>1.5 ± 0.2</td>
<td>5.4 ± 2.0</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>$k_e$ (h$^{-1}$)</td>
<td>0.19 ± 0.05</td>
<td>0.15 ± 0.04</td>
<td>0.21 ± 0.05</td>
<td>0.28 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.09 ± 0.02</td>
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<tr>
<td>$t_{1/2}$ (h)</td>
<td>5.1 ± 1.6</td>
<td>7.4 ± 2.4</td>
<td>4.5 ± 1.4</td>
<td>2.5 ± 0.1</td>
<td>10.4 ± 1.9</td>
<td>8.6 ± 1.1</td>
</tr>
<tr>
<td>$\text{AUC}_{0-24 \text{h}}$ (mg/L x h)</td>
<td>6.7 ± 1.4</td>
<td>14.0 ± 2.8*</td>
<td>0.3 ± 0.1</td>
<td>0.6 ± 0.1*</td>
<td>1.4 ± 0.5</td>
<td>3.2 ± 0.7*</td>
</tr>
</tbody>
</table>

a) Data are means ± SEM (n=5-6 rats/group). * Significantly different from quercetin control suspension, $P<0.05$

b) Abbreviations: $C_{\text{max}}$: plasma maximum concentration; $T_{\text{max}}$: time to maximum concentration; $k_e$: elimination rate constant; $t_{1/2}$: elimination half-life; $\text{AUC}_{0-24 \text{h}}$: area under the concentration curve.
Table 4.4 Tissue concentrations of quercetin metabolites after oral administration of quercetin control suspension and Q-SNEDDS to rats at doses of 15 mg/kg\textsuperscript{a})

<table>
<thead>
<tr>
<th></th>
<th>Small Intestine</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quercetin</td>
<td>Quercetin</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>control</td>
</tr>
<tr>
<td>Free quercetin (ng/g tissue)</td>
<td>0.37 ± 0.14</td>
<td>0.36 ± 0.11</td>
</tr>
<tr>
<td>Total quercetin (ng/g tissue)</td>
<td>4.04 ± 1.06</td>
<td>5.46 ± 0.52</td>
</tr>
<tr>
<td>Free isorhamnetin (ng/g tissue)</td>
<td>ND\textsuperscript{b)}</td>
<td>ND</td>
</tr>
<tr>
<td>Total isorhamnetin (ng/g tissue)</td>
<td>2.24 ± 0.53</td>
<td>3.64 ± 0.39\textsuperscript{*}</td>
</tr>
</tbody>
</table>

\textsuperscript{a)} Data are means ± SEM (n=5-6 rats/group). * Significantly different from quercetin control suspension, P<0.05

\textsuperscript{b)} Not detectable.
CHAPTER 5
QUERCETIN BIOAVAILABILITY IS ASSOCIATED WITH INADEQUATE PLASMA VITAMIN C STATUS AND GREATER PLASMA ENDOTOXIN IN ADULTS

5.1 Abstract

Quercetin bioavailability exhibits high inter-individual variations for reasons that remain unclear. We conducted a 24 h pharmacokinetic study to investigate whether individual differences in circulating antioxidants, oxidative stress and inflammation, and intestinal permeability affect quercetin bioavailability. Healthy adults (n = 9M/7F; 34.3 ± 4.5 y; 27.0 ± 1.7 kg/m²) ingested 1095 mg quercetin aglycone with a standardized meal. Plasma antioxidants, biomarkers of oxidative stress and inflammation, and endotoxin were measured at baseline (0 h), and quercetin and its methylated metabolites isorhamnetin and tamarixetin were measured at timed intervals for 24 h. Plasma pharmacokinetics of quercetin, isorhamnetin and tamarixetin were highly variable between participants (CV_inter = 37-96%). Plasma vitamin C concentrations (34.6 ± 2.5 μmol/L), but no other antioxidants, were inversely correlated to the Cmax and AUC0-24 h of total quercetin (Qtotal; sum of quercetin, isorhamnetin and tamarixetin; r = -0.52 to -0.53; P<0.05). Plasma endotoxin (0.13 ± 0.01 EU/mL), a surrogate marker of intestinal permeability, was correlated to Qtotal Cmax (r = 0.45; P<0.05) and tended to be correlated to Qtotal AUC0-24 h (r = 0.38; P = 0.07). Additionally, vitamin C was inversely related to C-reactive protein, myeloperoxidase, and endotoxin (r = -0.46 to -0.55; P<0.05), whereas endotoxin was positively correlated to C-reactive protein (r = 0.73; P<0.05). These findings suggest that vitamin C status and plasma endotoxin may be associated with
inter-individual variations in quercetin bioavailability. Greater quercetin absorption and bioavailability may be associated with poor vitamin C status and increased intestinal permeability in healthy adults.
5.2 Introduction

Quercetin is one of the most abundant dietary flavonoids (8), and its biological activities are under active investigation, especially in relation to the prevention of cardiovascular disease (CVD). Although some clinical studies suggest that quercetin decreases CVD risk (13), others fail to support its cardioprotective activities (98). The explanation for these discrepancies remains unclear, but the bioactivities of quercetin are related to its bioavailability. Unfortunately, marked inter-individual variability exists in humans for the bioavailability of quercetin and other polyphenols (16, 173, 174). For example, the coefficient of variation (CV) for plasma maximum concentrations (C_{max}) of quercetin (CV = 61-92\%) as well as that of anthocyanins and catechins (CV = 18-177\%) vary greatly between individuals following their oral ingestion (16, 173, 174). Likewise, cardioprotective activities of quercetin occur in a concentration-dependent manner (13), suggesting that its highly variable bioavailability may help to explain the discrepancies observed in evaluating its beneficial health effects. Thus, a need exists to more thoroughly understand the factors regulating quercetin bioavailability to better define its putative cardioprotective activities.

The bioavailability of quercetin in healthy individuals is relatively poor as evidenced by limited increases in its circulating concentrations following oral ingestion of 100 mg quercetin glucoside (57). Further complicating is evidence from controlled studies in humans showing large inter-individual variations in quercetin pharmacokinetics, but for reasons that are not fully understood (5, 108). For example, these variations are not attributed to demographic or lifestyle factors (e.g. age, gender, BMI, tobacco use, or physical activity) (79). However, quercetin undergoes small intestinal and hepatic xenobiotic metabolism and is subject to degradation by intestinal microbiota (175). Thus, inter-individual variations in quercetin bioavailability could be due to polymorphisms in proteins responsible for xenobiotic metabolism and/or intestinal microbiota composition.
(176). Alternatively, inter-individual variations in quercetin pharmacokinetics could be mediated by differences in antioxidant status and oxidative stress. Indeed, quercetin has antioxidant activities consistent with its scavenging of reactive oxygen species *in vitro* (177).

The one-electron reduction potential (330 mV) of quercetin is similar to that of α-tocopherol (500 mV) and vitamin C (280 mV) (178, 179), suggesting that antioxidant interactions and oxidative stress may differentially regulate its bioavailability. For example, studies *in vitro* show that the susceptibility of quercetin to oxidation by hydrogen peroxide and horseradish peroxidase is decreased by its co-incubation with vitamin C (180), supporting that vitamin C spares quercetin from oxidation or that it regenerates oxidized quercetin to its native form. Quercetin aglycone also passively diffuses into enterocytes during absorption (28), suggesting that intestinal permeability, which is regulated by inflammation and oxidative stress (181), may affect quercetin absorption.

Individual differences in quercetin bioavailability could have important implications for health-related outcomes such that its limited bioavailability would preclude it from exerting cardioprotective benefits. Thus, a greater understanding of the determinants explaining the high inter-individual variations in quercetin bioavailability would better support the development of dietary recommendations for quercetin in relation to CVD prevention. In this study, we hypothesized that inter-individual variations in quercetin bioavailability would be explained by between-individual differences in biomarkers of antioxidant status, inflammation, and intestinal permeability. We conducted a controlled pharmacokinetics study in healthy individuals to examine the extent to which quercetin pharmacokinetic parameters of absorption and elimination were affected by antioxidant status, and biomarkers of oxidative stress, inflammation, and intestinal permeability.
5.3  Materials and methods

5.3.1  Participants

This protocol was approved by the Institutional Review Board at the University of Connecticut, and all participants provided written consent. Healthy adults were recruited based on age (18-65 y), BMI (19-40 kg/m²), non-smoking status, non-use of dietary supplements (>2 mo), fasting blood glucose ≤5.6 mmol/L, fasting blood cholesterol ≤5.2 mmol/L, and resting blood pressure ≤140/90 mmHg. Individuals having any history of vascular, renal, or gastrointestinal disease, metabolic abnormalities, cancer, or using any medications were excluded.

5.3.2  Study design

Participants abstained from alcohol and exercise for 24 h and quercetin-rich foods for 3 d prior to completing a 24 h pharmacokinetics study. On a single occasion, participants arrived to the study center at 0700 h in the fasted state (10-12 h) to ingest 1095 mg quercetin aglycone (USANA Health Sciences, Salt Lake City, UT, USA) with a standardized breakfast consisting of a muffin (450 kcal; 15.4 g fat, 72.5 g carbohydrate, 5.3 g protein) and water (500 mL). The dose of quercetin and breakfast composition were provided consistent with our prior studies in overweight adults showing that a 30% fat-containing meal, compared to a fat-free meal, increases the bioavailability of quercetin aglycone by 32% by increasing its absorption (151). Quercetin aglycone rather than quercetin glycoside was provided to minimize between-subject variations in absorption resulting from known inter-individual differences in the activity of small intestinal β-glucosidase, the enzyme that hydrolyzes quercetin glycosides (33). Blood samples were collected from an in-dwelling catheter immediately prior to (0 h, baseline) and after ingestion of quercetin at 1, 2, 3, 4, 6, 8, 10, 12, and 24 h. To control for between-individual variations in meal timing and dietary quercetin, participants were
provided standardized meals that were devoid of quercetin at 4 h and 12 h after quercetin administration.

5.3.3 Dietary quercetin and isorhamnetin intakes

Participants were instructed by a registered dietitian to complete a food diary for 3 d prior to the study to assess compliance to a low-quercetin diet. Dietary intakes of quercetin and isorhamnetin were analyzed using Nutrition Data System for Research® (University of Minnesota, Minneapolis, MN, USA) and “Nutrition Coordinating Center Flavonoid and Proanthocyanidin Provisional Table 2010” database as described previously (151).

5.3.4 Blood processing and clinical chemistries

Blood samples were collected from the antecubital vein into tubes containing ethylenediaminetetraacetic acid or sodium heparin. Plasma was obtained by centrifugation, snap-frozen in liquid nitrogen, and stored at -80°C. For measurements of vitamin C and uric acid, sodium heparinized plasma was mixed 1:1 with 10% perchloric acid containing 1 mmol L⁻¹ diethylenetriaminepentaacetic acid, centrifuged (15,000 x g, 5 min, 4°C), and the supernatant was snap-frozen in liquid nitrogen before being stored at -80°C. Fasting plasma glucose and cholesterol were measured using spectrophotometric clinical assays in accordance with the manufacturer’s instructions (Point Scientific, Inc., Canton, MI, USA).

5.3.5 Plasma quercetin and methylated metabolites

Quercetin undergoes rapid phase II xenobiotic metabolism resulting in glucuronidated and sulfated metabolites of quercetin as well as glucuronidated and sulfated metabolites of its catechol-O-methyltransferase-derived methylated forms, isorhamnetin and tamarixetin. Plasma total quercetin, total isorhamnetin, and total tamarixetin (i.e., the sum of free and glucuronidated/sulfated conjugates of each analyte) were therefore measured by HPLC-Couarray (ESA Inc., Chelmsford, MA, USA).
following enzymatic hydrolysis using β-glucuronidase and sulfatase and diethyl ether extraction as we described previously (151).

5.3.6 Biomarkers of antioxidant status, oxidative stress, and inflammation

Plasma biomarkers of antioxidants, oxidative stress, and inflammation were measured at baseline (0 h, i.e., immediately prior to quercetin ingestion) to define their associations with quercetin pharmacokinetic parameters. Vitamin C (reduced form) and uric acid were measured from perchloric acid-treated plasma by HPLC-Couarray as described (113). Vitamin E was extracted using hexane following saponification and then measured by HPLC-Couarray as described (113). Carotenoids were extracted from deproteinated plasma using hexane and then measured by HPLC at 450 nm as described (182). Plasma malondialdehyde, a biomarker of lipid peroxidation, was measured by HPLC-FL following derivitization with thiobarbituric acid and extraction with butanol as we described (115). Plasma C-reactive protein (CRP) and myeloperoxidase were measured using separate ELISA kits according to the manufacturer's instructions (BioCheck, Inc., Foster City, CA, USA).

5.3.7 Plasma endotoxin

Plasma endotoxin is associated with gut permeability (183) and was assessed at baseline as a surrogate marker of gut integrity. Plasma endotoxin was measured using a chromogenic limulus amebocyte lysate kit according to manufacturer's instructions (Lonza, Inc., Walkersville, MD, USA). In brief, plasma was incubated (10 min, 37 ºC) with a limulus amebocyte lysate reagent that contains a proenzyme that is activated proportional to the concentration of endotoxin. The sample was then incubated (6 min, 37ºC) in the presence of a p-nitroaniline-containing substrate. p-Nitroaniline becomes hydrolyzed during this reaction resulting in the ability to measure its absorbance at 410 nm. Plasma endotoxin concentrations were then determined by linear regression from a standard curve prepared in parallel using known concentrations of endotoxin standards.
5.3.8 Statistical analyses

Plasma pharmacokinetics of quercetin, isorhamnetin and tamarixetin, including plasma maximum concentration (C\text{max}), area under the concentration curve (AUC\text{0-24 h}), time to maximum concentration (t\text{max}), elimination half-life (t\text{1/2}), appearance rate constant (k\text{a}), and elimination rate constant (k\text{e}) were calculated using PK Solutions® (Summit Research Services, Inc., Montrose, CO, USA). Inter-individual coefficients of variation (CV\text{inter}) for each pharmacokinetic parameter were calculated by dividing the standard deviation by the mean. Data were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and all data are reported as means ±SEM. Data were evaluated for normality using the D’Agostino and Pearson omnibus normality test and those lacking a normal distribution were log transformed to achieve normality. Gender differences were initially evaluated using an unpaired Student’s t-test. Because no gender differences existed, data were pooled for all analyses. Linear regression was used to determine correlation coefficients between study variables. All analyses were considered significant at \( P \leq 0.05 \).
5.4 Results

5.4.1 Participants and dietary intakes of quercetin and isorhamnetin

Sixteen participants (n = 9M/7F; 34.3 ± 4.5 y; 27.0 ± 1.7 kg/m²) completed the study without any adverse events. Their fasting concentrations of plasma glucose (5.2 ± 0.1 mmol/L) and total cholesterol (4.3 ± 0.2 mmol/L) were within normal clinical limits, and resting systolic (116 ± 4 mmHg) and diastolic (78 ± 3 mmHg) blood pressure were indicative of normotension. Participants’ dietary quercetin intakes for the 3 d preceding the pharmacokinetics trial were 0.40 ± 0.13 mg/d and no dietary isorhamnetin was consumed. Typical intakes of quercetin are 15.6-16.8 mg/d (184), supporting good compliance of participants in following a low-quercetin diet.

5.4.2 Plasma pharmacokinetics of quercetin, isorhamnetin and tamarixetin

Ingestion of quercetin resulted in its plasma concentrations increasing from baseline levels of 0.28 ± 0.03 μmol/L to a Cₘₐₓ of 1.43 ± 0.18 μmol/L at a tₘₐₓ of 6.1 ± 0.9 h (Table 5.1). Despite standardized administration of quercetin, substantial inter-individual variations occurred for quercetin pharmacokinetic parameters. Plasma quercetin Cₘₐₓ in the lowest responder (0.43 μmol/L) was 7-times lower than that from the highest responder (3.03 μmol/L), and the CVₐₜₐ of quercetin Cₘₐₓ was 51%. Similarly, kₐ and tₘₐₓ were highly variable with a CVₐₜₐ of 78% and 56%, respectively. Although plasma quercetin decreased at 24 h to concentrations no different from baseline (P>0.05; Figure 5.1A), the elimination kinetic parameters kₑ and t¹/₂ were highly variable with CVₐₜₐ of 39% and 53%, respectively. Quercetin bioavailability, determined on the basis of its AUC₀-24 h, had a CVₐₜₐ of 45% consistent with the highest responder (32.8 μmol/L x h) having 6-times higher AUC₀-24 h than the lowest responder (5.1 μmol/L x h; Table 5.1).

Isorhamnetin and tamarixetin are methylated metabolites generated in a catechol-O-methyltransferase-dependent manner from quercetin (166). In response to quercetin
administration, plasma isorhamnetin increased from baseline concentrations of 0.15 ± 0.03 μmol/L to a C\text{max} of 0.41 ± 0.06 μmol/L whereas plasma tamarixetin was below detection limits at baseline but increased to a C\text{max} of 0.48 ± 0.07 μmol/L (Table 5.1). The C\text{max} of isorhamnetin (r = 0.62) and tamarixetin (r = 0.92) were each correlated to quercetin C\text{max} (P<0.05), supporting that quercetin methylation is dependent upon the quantity of quercetin absorbed. Accordingly, the C\text{max} of isorhamnetin and tamarixetin exhibited a high CV\text{inter} of 55-61% (Table 5.1). All other pharmacokinetic parameters of isorhamnetin and tamarixetin were also variable (CV\text{inter} = 37-96%; Table 5.1). Lastly, isorhamnetin was the major circulating methylated metabolite for 6 participants whereas tamarixetin predominated for 10 participants, supporting that inter-individual differences also exist in quercetin biotransformation.

### 5.4.3 Relations between quercetin pharmacokinetics and biomarkers of oxidative stress

To better define plasma biochemical factors regulating quercetin status, we calculated total quercetin bioavailability as the sum of plasma concentrations of quercetin, isorhamnetin and tamarixetin (abbreviated herein as Q\text{total}) and examined relations between pharmacokinetic responses of Q\text{total} with plasma antioxidants, and biomarkers of oxidative stress and inflammation. Neither plasma antioxidants, including α-tocopherol, γ-tocopherol, uric acid, or select carotenoids (lutein, zeaxanthin, β-carotene, and lycopene), nor oxidative stress and inflammatory biomarkers (malondialdehyde, CRP, myeloperoxidase), were correlated to Q\text{total} AUC\text{0-24 h} or C\text{max} (Table 5.2) or other pharmacokinetic parameters. In contrast, plasma vitamin C concentrations were inversely correlated to Q\text{total} AUC\text{0-24 h} and C\text{max} (Figure 5.2), and explained 27-28% of the variance in these pharmacokinetic parameters, but did not correlate with any other pharmacokinetic parameters of Q\text{total}. Additionally, participants' plasma vitamin C concentrations (34.6 ± 2.5 μmol/L) were lower than the mean vitamin
C concentration (51.4 μmol/L) of American adults (185), and a third of our participants had suboptimal vitamin C status (<28 μmol/L) (185). These data suggest that low plasma vitamin C status may be associated with greater absorption and bioavailability of quercetin, and that vitamin C status may partly explain inter-individual differences observed in quercetin bioavailability, at least in our participants.

5.4.4 Relations between quercetin pharmacokinetics, endotoxin, and vitamin C

Plasma endotoxin was measured as a surrogate marker of intestinal permeability (183). Participants’ plasma endotoxin concentrations were 0.13 ± 0.01 EU/mL, which is similar those reported previously in healthy individuals (0.15-0.35 EU/mL) (186). We further observed that plasma endotoxin was correlated ($P<0.05$) to $Q_{\text{total Cmax}}$ and tended to correlate to $Q_{\text{total AUC0-24 h}}$ ($P = 0.07$; Figure 5.3), supporting the concept that quercetin absorption is higher in individuals having greater intestinal permeability. We also evaluated inter-relations between plasma vitamin C, endotoxin, and biomarkers of oxidative stress and inflammation. Plasma vitamin C concentrations were inversely related to plasma endotoxin (Figure 5.4A), suggesting that inadequate vitamin C status is associated with greater intestinal permeability. Plasma vitamin C concentrations were also inversely related to myeloperoxidase (Figure 5.4B) and CRP ($r = -0.54$; $P<0.05$), and endotoxin was positively correlated to CRP ($r = 0.73$; $P<0.05$), suggesting that poor vitamin C status and greater intestinal permeability are associated with inflammatory responses.
5.5 Discussion

The findings of this study suggest that inter-individual variations in quercetin bioavailability are explained, in part, by poor plasma vitamin C status that is associated with greater intestinal permeability and inflammation. Quercetin absorption, metabolism, and elimination were highly variable as evidenced by the 36-96% $CV_{\text{inter}}$ in these pharmacokinetic parameters. Of the plasma antioxidants measured, none were associated with any quercetin pharmacokinetic parameters except that low vitamin C concentrations were correlated to greater quercetin absorption and bioavailability. An inverse relation between vitamin C and endotoxin, and a positive relation between endotoxin and $Q_{\text{total}} C_{\text{max}}$, suggest that poor vitamin C status is related to greater intestinal permeability, which may contribute to greater quercetin absorption. Inter-relations between vitamin C, CRP, and endotoxin, and an inverse relation between vitamin C and myeloperoxidase, suggest that pro-inflammatory responses are associated with low vitamin C status and greater intestinal permeability. Collectively, these observations suggest a quercetin/vitamin C interaction such that poor vitamin C status may increase quercetin absorption, possibly by inflammation-induced impairments in gut barrier function, and supports the need for controlled interventions that directly test this hypothesis.

Vitamin C supplementation in individuals with suboptimal vitamin C status (<28 µmol/L) improves circulating vitamin C level and lowers plasma endotoxin concentration (187). Vitamin C administration in rats with intestinal obstruction, a model of impaired intestinal barrier function, decreases bacteria translocation to the lymph (188). Thus, we hypothesized that our participants had increased intestinal permeability due to their suboptimal vitamin C levels (34.6 ± 2.5 µmol/L) (185). In support, the inverse correlation observed between plasma vitamin C and endotoxin suggests that suboptimal vitamin C status is associated with impaired intestinal barrier integrity. Greater intestinal
permeability would be expected to increase intestinal quercetin absorption, consistent with our observation that endotoxin positively correlates with $Q_{\text{total}} C_{\text{max}}$. No significant relations were observed between endotoxin and $Q_{\text{total}}$ pharmacokinetic parameters of elimination, supporting that intestinal permeability affects quercetin absorption, but not its rate of elimination. This would also explain why $Q_{\text{total}} AUC_{0-24}$ only tended to correlate with plasma endotoxin since this estimate of bioavailability reflects not only the extent of absorption of quercetin but also its extent of elimination.

The mechanism by which vitamin C regulates intestinal integrity is unclear. Vitamin C inhibits the growth of *Helicobacter pylori*, a gram-negative bacteria involved in gastric disease (189). This suggests a possibility that poor vitamin C status may increase bacteria overgrowth and/or endotoxin production and additional study is warranted to define whether vitamin C affects intestinal integrity by regulating microflora composition. In addition, media concentrations of vitamin C at 60 µmol/L insufficiently inhibit free radical generation in stimulated neutrophils (190), suggesting that suboptimal vitamin C status increases inflammatory responses contributing to oxidative stress. Low vitamin C status is associated with inflammation and oxidative damage in humans (119). These findings are consistent with our observations of inverse correlations between plasma vitamin C with myeloperoxidase and CRP. We also observed a positive relation between plasma endotoxin and CRP. Although the mechanism by which CRP diminishes intestinal integrity cannot be determined from our study, plasma CRP predicts gut permeability in pancreatitis patients (191).

This study in healthy adults exemplifies that, regardless of standardized oral administration of quercetin aglycone, similar circulating plasma quercetin concentrations are not achieved. Heterogeneous responses in quercetin bioavailability are in agreement with clinical studies investigating the pharmacokinetics of quercetin (67) and related flavonoids (e.g. catechin and anthocyanin) (173, 174). Understanding these
inconsistencies is of significance because quercetin bioavailability is an important determinant of its health-promoting bioactivities. Notably, studies in vitro show that quercetin-mediated vasorelaxation is concentration-dependent (192). Individual differences in quercetin bioavailability result in variable circulating or tissue quercetin concentrations, which may help to explain the observed discrepancies in clinical studies conducted in similar cohorts that have examined the anti-hypertensive activity of quercetin (9, 96). Likewise, no cross-over studies have determined the potential dose-response of plasma quercetin and/or quercetin bioavailability in relation to its bioactivity, which represents an important area of future investigation. Similarly, quercetin supplementation (500 or 1000 mg/d, 12 wk) does not improve oxidative stress biomarkers (121). This could be attributed to variable quercetin bioavailability as demonstrated by highly inconsistent changes in plasma quercetin ranging from 0-11.6 µmol/L regardless of the dose of quercetin ingested (79). Variable responses in plasma quercetin also occurred independent of participants’ age or BMI (79). Thus, it is critically important to fully define determinants of inter-individual variations in quercetin bioavailability in order to better inform future studies aiming to evaluate its cardioprotective activities.

Our findings imply that factors regulating quercetin bioavailability may be complex. In support, the observed correlations suggest that vitamin C status and gut health may be potential mediators of quercetin bioavailability such that low vitamin C status and high intestinal permeability are associated with greater quercetin absorption, at least in our participants. Intestinal permeability, consistent with others (183), was assessed indirectly by measuring plasma endotoxin. In agreement with 44% of our participants being overweight (BMI >25 kg/m²), obesity increases intestinal permeability and translocation of gut-derived endotoxin, likely by altering gut microbiota composition and decreasing tight junction resistance (183, 193, 194). Paradoxically, our data suggest that quercetin
supplementation in individuals having compromised vitamin C status and intestinal barrier integrity might have targeted cardioprotective benefits due to its greater bioavailability (195).

Antioxidant activities of quercetin would be expected to enable redox interactions with other antioxidants as part of the antioxidant network (196). Indeed, quercetin increases plasma and hepatic vitamin E in rats, possibly by scavenging free radicals and reducing vitamin E peroxyl radicals (197). Vitamin C also protects quercetin against nitrate-dependent oxidation in saliva (198). Because studies support that vitamin C preserves quercetin in vitro (199), quercetin bioavailability would be hypothesized to be enhanced by greater vitamin C status. Contradicting this hypothesis, we show that plasma vitamin C was inversely related to pharmacokinetic parameters of quercetin absorption and bioavailability, suggesting vitamin C-dependent redox recycling of quercetin does not occur, at least not in this population.

Limited information exists to explain inter-individual variations in quercetin bioavailability. Our observations suggest a relation between vitamin C status and quercetin absorption. This finding is important in light of evidence indicating that more than 20% of Americans have suboptimal vitamin C status (185), which in itself is associated with greater CVD risk (200). Future studies should consider participants' vitamin C status when investigating the cardioprotective activities of quercetin.

Although our work suggests a central involvement of vitamin C in regulating quercetin absorption, heterogeneous responses in quercetin bioavailability are also potentially due to individual differences in xenobiotic metabolism of quercetin resulting from polymorphisms in phase II metabolism proteins. For example, 10 hepatic uridine 5'-diphospho-glucuronosyl transferases catalyze quercetin glucuronidation in humans (201), and each has its own conjugating efficiency (72). Polymorphisms in uridine 5'-diphospho-glucuronosyl transferases, which are known to occur (202), would be
expected to differentially affect circulating quercetin levels and its half-life. To better define the contribution of polymorphisms in xenobiotic metabolism on quercetin bioavailability, future studies should examine the pharmacokinetics of quercetin aglycone and its phase II metabolites. In addition, multi-drug associated resistance protein 2 is an intestinal efflux transporter of quercetin and its activity decreases in rats with intestinal inflammation (28, 203). Inhibition of multi-drug associated resistance protein 2 activity could potentially enhance quercetin absorption by limiting its enterocyte efflux. Thus, inflammation mediated by poor vitamin C status may increase quercetin absorption by inhibiting multi-drug associated resistance protein 2 activity. However, whether vitamin C status directly or indirectly alters the activities of enzymes and transporters involved in quercetin metabolism and uptake requires further investigation. Likewise, because this investigation was performed in a small cohort, larger scale investigations involving vitamin C intervention are warranted to confirm the observed relationships. Lastly, although plasma endotoxin is associated with gut permeability (183, 204), future studies should also consider using sugar probes (e.g. lactulose, mannitol) to better define gut barrier function (205) and its relationship to quercetin absorption.

5.6 Conclusion

Our findings, that quercetin bioavailability in healthy individuals may be inversely associated with plasma vitamin C status, provide a critical but early step towards fully defining dietary factors regulating quercetin bioavailability. Additional work is needed to define the causal relation between vitamin C status and quercetin bioavailability in healthy adults.

5.7 Acknowledgements

This work was supported by a grant from the Donaghue Nutrition Research Program.
Figure 5.1 Plasma concentrations (means ± SEM) of quercetin (A), isorhamnetin (B), tamarixetin (C), and $Q_{\text{total}}$ (D) for 24 h following oral ingestion of quercetin aglycone (1095 mg) by healthy adults ($n = 16$). $Q_{\text{total}}$ represents the sum of plasma quercetin, isorhamnetin, and tamarixetin.
Figure 5.2 Correlations between plasma vitamin C and (A) AUC\textsubscript{0-24 h} and (B) C\textsubscript{max} of Q\textsubscript{total}. Q\textsubscript{total} represents the sum of plasma quercetin, isorhamnetin, and tamarixetin following oral ingestion of quercetin aglycone (1095 mg) in healthy adults (n = 16). AUC\textsubscript{0-24 h} is the 24 h area under the concentration curve of Q\textsubscript{total} and C\textsubscript{max} is the maximal plasma concentration of Q\textsubscript{total}. 

\[ r = -0.53 \quad P < 0.05 \]

\[ r = -0.52 \quad P < 0.05 \]
Figure 5.3 Correlations between plasma endotoxin and (A) AUC$_{0-24\ h}$ and (B) C$_{\text{max}}$ of Q$_{\text{total}}$. Q$_{\text{total}}$ represents the sum of plasma quercetin, isorhamnetin, and tamarixetin following oral ingestion of quercetin aglycone (1095 mg) in healthy adults ($n = 16$). AUC$_{0-24\ h}$ is the 24 h area under the concentration curve of Q$_{\text{total}}$ and C$_{\text{max}}$ is the maximal plasma concentration of Q$_{\text{total}}$. 
Figure 5.4 Correlations between plasma vitamin C and (A) plasma endotoxin and (B) plasma myeloperoxidase.
<table>
<thead>
<tr>
<th></th>
<th>Quercetin</th>
<th>Isorhamnetin</th>
<th>Tamarixetin</th>
<th>Q_total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Range</td>
<td>CV&lt;sub&gt;inter&lt;/sub&gt;</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>C&lt;sub&gt;0&lt;/sub&gt; (μmol/L)</td>
<td>0.28 ± 0.03</td>
<td>0.11-0.64</td>
<td>44%</td>
<td>0.15 ± 0.03</td>
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<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (μmol/L)</td>
<td>1.43 ± 0.18</td>
<td>0.43-3.03</td>
<td>51%</td>
<td>0.41 ± 0.06</td>
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<tr>
<td>t&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>6.1 ± 0.9</td>
<td>1.0-12.1</td>
<td>56%</td>
<td>12.0 ± 1.6</td>
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<tr>
<td>AUC&lt;sub&gt;0-24 h&lt;/sub&gt; (μmol/L x h)</td>
<td>17.71 ± 1.98</td>
<td>5.13-32.83</td>
<td>45%</td>
<td>6.52 ± 0.74</td>
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<tr>
<td>k&lt;sub&gt;a&lt;/sub&gt; (h&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.49 ± 0.10</td>
<td>0.09-1.53</td>
<td>78%</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>11.4 ± 1.5</td>
<td>4.7-28.6</td>
<td>53%</td>
<td>31.4 ± 6.1</td>
</tr>
<tr>
<td>k&lt;sub&gt;e&lt;/sub&gt; (h&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.07 ± 0.01</td>
<td>0.02-0.15</td>
<td>39%</td>
<td>0.04 ± 0.01</td>
</tr>
</tbody>
</table>

a) AUC<sub>0-24 h</sub>: 24 h area under concentration curve; C<sub>0</sub>, fasting concentration; C<sub>max</sub>, plasma maximum concentration; CV<sub>inter</sub>, inter-individual coefficients of variation; k<sub>a</sub>: appearance rate constant; k<sub>e</sub>, elimination rate constant; Q<sub>total</sub>, sum of plasma quercetin, isorhamnetin and tamarixetin following enzymatic hydrolysis of glucuronidated and sulfated conjugates; t<sub>max</sub>, time to maximum concentration; t<sub>1/2</sub>, elimination half-life.
**Table 5.2** Correlations between plasma antioxidants, biomarkers of oxidative stress and inflammation, and AUC$_{0-24}$ h and C$_{\text{max}}$ of Q$_{\text{total}}$\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SEM</th>
<th>Q$<em>{\text{total AUC}}$$</em>{0-24}$ h</th>
<th>r</th>
<th>P</th>
<th>Q$<em>{\text{total C}</em>{\text{max}}}$</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C (μmol/L)</td>
<td>34.6 ± 2.5</td>
<td>-0.53</td>
<td>0.03</td>
<td></td>
<td>-0.52</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>α-Tocopherol (μmol/L)</td>
<td>14.2 ± 0.8</td>
<td>0.12</td>
<td>0.66</td>
<td>0.05</td>
<td>0.05</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>γ-Tocopherol (μmol/L)</td>
<td>2.03 ± 0.13</td>
<td>-0.15</td>
<td>0.59</td>
<td></td>
<td>-0.13</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>Uric acid (μmol/L)</td>
<td>303.4 ± 17.5</td>
<td>-0.26</td>
<td>0.34</td>
<td></td>
<td>-0.13</td>
<td>0.61</td>
<td></td>
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<tr>
<td>Lutein (μmol/L)</td>
<td>0.24 ± 0.02</td>
<td>-0.05</td>
<td>0.89</td>
<td></td>
<td>-0.19</td>
<td>0.61</td>
<td></td>
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<tr>
<td>Zeaxanthin (μmol/L)</td>
<td>0.029 ± 0.004</td>
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<td>0.66</td>
<td></td>
<td>-0.30</td>
<td>0.39</td>
<td></td>
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<tr>
<td>β-Carotene (μmol/L)</td>
<td>0.24 ± 0.06</td>
<td>-0.19</td>
<td>0.59</td>
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<td>-0.30</td>
<td>0.40</td>
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<tr>
<td>Lycopene (μmol/L)</td>
<td>0.29 ± 0.04</td>
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<td>0.77</td>
<td></td>
<td>-0.21</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>Malondialdehyde (μmol/L)</td>
<td>1.04 ± 0.05</td>
<td>0.37</td>
<td>0.16</td>
<td></td>
<td>0.22</td>
<td>0.41</td>
<td></td>
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<tr>
<td>C-reactive protein (mg/L)</td>
<td>2.53 ± 0.68</td>
<td>-0.31</td>
<td>0.24</td>
<td></td>
<td>-0.26</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Myeloperoxidase (ng/mL)</td>
<td>16.5 ± 1.80</td>
<td>0.02</td>
<td>0.95</td>
<td></td>
<td>-0.01</td>
<td>0.99</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) AUC$_{0-24}$h: area under concentration curve; C$_{\text{max}}$: plasma maximum concentration; Q$_{\text{total}}$: sum of plasma quercetin, isorhamnetin and tamarixetin following enzymatic hydrolysis of glucuronidated and sulfated conjugates.
CHAPTER 6
DISCUSSION

6.1 Overview

Quercetin has low bioavailability and elicits highly variable inter-individual responses following its ingestion. Since bioavailability partly determines beneficial effects of quercetin on cardiovascular health, elucidating factors regulating its bioavailability will better our understanding of its cardioprotective activities. Accordingly, the overall goal of this dissertation was to investigate the influence of dietary fat, nano-formulation, and antioxidant status on quercetin aglycone bioavailability in humans and rats.

The central hypothesis of this dissertation was that adequate antioxidant status would improve quercetin aglycone bioavailability and that its bioavailability would be enhanced by co-ingestion with dietary fat or administration as nano-emulsion. Studies in this dissertation demonstrated that dietary fat improved quercetin aglycone bioavailability by increasing its absorption in obese adults (151). Separate studies in rats demonstrated that a quercetin aglycone-containing nanoemulsion enhanced quercetin aglycone bioavailability by enhancing its absorption, resulting in its greater intestinal and hepatic accumulation (206). Lastly, contrary to our hypothesis, data from healthy adults completing quercetin pharmacokinetics studies showed that greater quercetin bioavailability was associated with inadequate plasma vitamin C status and greater plasma endotoxin in healthy adults (207). The work presented in this dissertation demonstrates that dietary fat, nano-emulsion, plasma vitamin C status and intestinal permeability have pivotal roles in regulating quercetin bioavailability.
6.2 Dietary fat improves quercetin bioavailability in humans

Studies *in vitro* routinely support the health-promoting activities of quercetin and its methylated metabolites when treatments are administered at concentrations ≥10 μM (126, 208). For example, pre-treatment of macrophages with 10 μM of quercetin aglycone or 10 μM of isorhamnetin aglycone decreased lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α) secretion by >50% compared to macrophages stimulated by LPS only (208). Pre-treatment with 10 μM of tamarixetin also inhibited collagen-stimulated platelet aggregation by 20% (126). In contrast to these *in vitro* studies, plasma quercetin concentrations are much lower in humans following its chronic supplementation (≥2 wk) (Table 6.1). Although plasma quercetin concentrations increase dose-dependently following its long-term supplementation (Figure 6.1), the highest plasma quercetin only reached 2.3 μM during 12 wk supplementation of 1000 mg/d in individuals (79). Plasma concentrations of isorhamnetin and tamarixetin are even lower, as exemplified by their concentrations ranging 0.3-33.6 nM following supplementation of 150 mg/d of quercetin aglycone for 2 wk in healthy individuals (5). Based on *in vitro* studies (126, 208), plasma quercetin and its metabolites at concentrations ≤2 μM may not be effective to elicit potential cardioprotective activities. Indeed, a 6 wk supplementation of quercetin aglycone (150 mg/d) in individuals with metabolic syndrome had no effect on plasma TNF-α, even though plasma quercetin was increased from baseline concentrations of 71 nM to 269 nM (96). It is possible that enhancing quercetin bioavailability may potentiate its cardioprotective activities. Therefore, studies in this dissertation focused on investigating strategies for improving quercetin bioavailability in order to better inform future clinical trials aiming to define cardioprotective activities of quercetin, consistent with epidemiological studies showing that individuals with higher dietary intakes of quercetin (≥3.9 mg/d) have lower CVD mortality (3).
Studies in Chapter 3 demonstrated that co-ingestion of quercetin aglycone with dietary fat improved quercetin bioavailability in obese adults, as evidenced by a 32% increase in plasma quercetin AUC following ingestion of a meal containing 15.4 g fat (30% energy from fat) compared to a meal providing <0.5 g fat (1% energy from fat) (151). These findings are consistent with studies in pigs showing that a diet containing 32% fat (weight%) increased quercetin aglycone bioavailability by 50% compared to a 3% fat diet (weight%) (85). Improvements in quercetin aglycone bioavailability in our study were accompanied by increases in its absorption, as indicated by a 45% increase in quercetin $C_{\text{max}}$ after participants ingested quercetin aglycone with the high-fat meal (15.4 g fat) compared to the fat-free meal (<0.5 g fat). However, dietary fat ingestion did not affect other pharmacokinetics parameters, including $T_{\text{max}}$ and $t_{1/2}$, supporting that enhanced quercetin aglycone bioavailability in obese adults was attributed to increases in its absorption. Findings from an in vitro digestion study in Chapter 3 further support that dietary fat may enhance quercetin aglycone absorption by enhancing its micellarization efficiency. Collectively, studies in Chapter 3 demonstrated that dietary fat increased absorption of quercetin aglycone, thereby improving its bioavailability in obese adults.

The study examining the influence of dietary fat on quercetin bioavailability in humans is important because they provide supportive evidence for utilizing dietary fat to improve quercetin bioavailability in clinical studies. It will also facilitate studies that aim to define cardioprotective activities of quercetin. For example, a 12 wk supplementation of quercetin aglycone (1000 mg/d) in men and women had no effects on biomarkers of CVD risk, including TNF-$\alpha$, C-reactive protein (CRP) and monocyte chemoattractant protein-1 (98). In this study, participants ingested quercetin supplements without meals (98), which likely contributed to its poor quercetin bioavailability and its limited effect in improving CVD risk factors. Therefore, it is possible that co-ingestion of quercetin
aglycone with meals providing fat may improve quercetin bioavailability, and in turn, promote its cardioprotective activities.

Although studies in Chapter 3 demonstrate that dietary fat content improves quercetin aglycone bioavailability, it remains unclear whether dietary fat source or fatty acid type regulate quercetin aglycone bioavailability in humans. Bioavailability and absorption of quercetin aglycone in rats were enhanced following its co-administration with long-chain fatty acids compared to a glucose solution, whereas co-ingestion of medium-chain fatty acids did not affect quercetin aglycone bioavailability (44). Improved quercetin absorption mediated by long-chain fatty acids occurred with concomitant increases in lymphatic concentrations of total quercetin and its metabolites (i.e. the sum of aglycone, glucuronidated and sulfated form of quercetin and isorhamnetin) (44). This suggested that long-chain fatty acids promoted transport of quercetin from the small intestine into lymph, which, in turn, may decrease its intestinal efflux, resulting in increased quercetin absorption. Mechanisms by which long-chain fatty acids enhanced lymphatic transport of quercetin remained unclear, but lymphatic concentrations of quercetin and its metabolites were unaffected when lipoproteins were removed from lymph collected from rats co-administered quercetin aglycone with long-chain fatty acids (44). This ruled out the possibility that improved lymphatic transport of quercetin and its metabolites was resulted from their incorporation into chylomicrons. Collectively, long-chain fatty acids improved quercetin aglycone absorption by increasing its transport into lymph in rats. It remains unknown whether long-chain fatty acids improve bioavailability and absorption of quercetin aglycone in humans through a similar manner, and future studies are needed.

It also remains unknown whether dietary fat improves bioavailability of quercetin glycosides, the major form of quercetin in plant foods (18), in humans. Using quercetin-3-glucoside as an example, its bioavailability in pigs was improved by dietary fat (85).
Quercetin AUC was increased by 22% following co-administration of quercetin-3-glucoside with a diet containing 32% fat (weight%) compared with a diet containing 3% fat (weight%) (85). In addition, the 32% fat diet decreased the extent of quercetin-3-glucoside absorption, as evidenced by a 29% decrease in quercetin C\text{max} compared with the 3% fat diet. The 32% fat diet also decreased quercetin T\text{max} by 36% compared with the 3% fat diet, indicating that the rate of quercetin-3-glucoside absorption was increased by the 32% fat diet. The 32% fat diet also delayed plasma quercetin elimination, as evidenced by higher plasma quercetin at 12 h (0.09 μM) compared with the 3% fat diet (0.03 μM). Therefore, dietary fat improved quercetin-3-glucoside bioavailability in pigs by delaying its elimination. Mechanisms explaining the influence of dietary fat on absorption and elimination of quercetin-3-glucoside remain unknown. Intestinal absorption of quercetin-3-glucoside is mediated through lactose phlorizin hydrolase and/or sodium-dependent glucose transporter 1 (33, 84), but no evidence demonstrates that their activities are affected following acute ingestion of dietary fat. Dietary fat may delay quercetin elimination by increasing its reabsorption during enterohepatic recirculation (85), but additional studies in bile duct- and duodenum-cannulated rodents are needed to examine this possibility. Nonetheless, future studies in humans are needed to investigate whether dietary fat improves bioavailability of quercetin-3-glucoside and other quercetin glycosides.

### 6.3 Self nano-emulsifying drug delivery system improves quercetin bioavailability

Although studies in Chapter 3 demonstrate that dietary lipid enhances quercetin aglycone bioavailability in obese adults, quercetin bioavailability is only improved marginally following its co-ingestion with 15.4 g fat (151). Indeed, this approach only increased the amount of absorbed quercetin from 1 mg in the fat-free trial (<0.5 g fat) to 1.5 mg in the high-fat trial (15.4 g fat), as estimated by multiplying quercetin C\text{max} by
plasma volume (estimated as 3 L for adults; (60, 61)). Only 0.13% of the dose of quercetin aglycone (1095 mg) was estimated to be absorbed when it was co-ingested with the high-fat meal (15.4 g fat). More concerning was that plasma quercetin C\textsubscript{max} only reached 1.6 μM, which was more than 5-times lower than concentrations shown to be effective in promoting cardioprotective activities in studies \textit{in vitro} (10-200 μM) (126, 208-210). For example, co-incubating 10 μM of quercetin with rat aortic rings for 6 h significantly mitigated vasoconstriction induced by angiotensin II, whereas vasoconstriction was unaffected following co-incubation of 1 μM of quercetin with rat aortic rings (210). Pretreatment of quercetin (10-200 μM) with macrophages dose-dependently inhibited mRNA expression of pro-inflammatory cytokines, including TNF-α and interleukin-6, that were otherwise induced by LPS (209). Significant inhibitory effects were found when macrophages were pretreated with 100 or 200 μM of quercetin (209).

Therefore, while this dissertation supports dietary fat to improve quercetin bioavailability, more effective or complementary approaches may be needed to improve its bioavailability more substantially in order to observe cardioprotective activities.

Poor absorption of quercetin aglycone is clearly a significant factor limiting its bioavailability. To circumvent this problem, studies in Chapter 4 were directed at encapsulating quercetin aglycone into lipid nano-particles formulated using self nano-emulsifying drug delivery system (SNEDDS) to promote its absorption and bioavailability. A rat pharmacokinetics model where quercetin aglycone-containing SNEDDS (Q-SNEDDS) was orally administered was utilized to examine the influence of Q-SNEDDS on quercetin aglycone bioavailability (206). Q-SNEDDS enhanced quercetin aglycone absorption, as evidenced by 3-times higher quercetin C\textsubscript{max} in rats receiving Q-SNEDDS compared to quercetin aglycone suspended in sodium carboxymethylcellulose solution. Q-SNEDDS also increased quercetin AUC by 2-times compared to quercetin aglycone suspension, whereas Q-SNEDDS did not affect other pharmacokinetics parameters.
Therefore, Q-SNEDDS improved quercetin aglycone absorption, thereby enhancing its bioavailability. In addition, greater quercetin aglycone bioavailability mediated by Q-SNEDDS was paralleled by a 3-times increase in quercetin concentrations in liver collected at 24 h compared to quercetin aglycone suspension. Collectively, Q-SNEDDS improved quercetin aglycone bioavailability and its hepatic accumulation in rats.

These findings provide evidence that Q-SNEDDS more effectively improves quercetin bioavailability compared to its oral administration concurrent with dietary lipid. Similar doses of quercetin aglycone (15 mg/ kg BW quercetin aglycone) were utilized in studies in Chapter 3 and Chapter 4, though these studies were conducted in different species (i.e. rats vs. humans) (151, 206). Rats were chosen to characterize bioavailability of quercetin aglycone delivered as Q-SNEDDS, because the safety of Q-SNEDDS following oral ingestion remains unknown. Nonetheless, plasma quercetin $C_{\text{max}}$ was 12 μM and represented 1% of the administered dose absorbed after rats received Q-SNEDDS (206). The estimated absorption was calculated by multiplying quercetin $C_{\text{max}}$ by plasma volume of rats (estimated as 4.12 mL/100 g body weight for a 300 g Sprague Dawley rat (211)) and then dividing the ingested dose. Since only 0.13% of ingested quercetin was absorbed in obese adults following its co-ingestion with a meal providing 15.4 g fat (151), quercetin bioavailability was enhanced to a greater extent by Q-SNEDDS compared to quercetin aglycone administration with dietary lipid. Another study in rats where a higher dose of quercetin aglycone (45 mg/kg BW quercetin aglycone) is administered with dietary lipid also supports that Q-SNEDDS more effectively improves quercetin bioavailability (56). After rats were orally administered quercetin aglycone with a 20% soybean oil solution (weight%), 0.2% of administered quercetin was absorbed, as estimated based on a plasma quercetin $C_{\text{max}}$ of 8 μM, and a plasma volume of 3.92 mL/100 g body weight for a 200 g Wistar rat (56, 212). In addition, quercetin aglycone administration as Q-SNEDDS is an important approach that not only
improves quercetin bioavailability, but also increases plasma quercetin concentrations to levels consistent with concentrations used in studies in vitro showing cardioprotective activities of quercetin. Indeed, a quercetin $C_{\text{max}}$ of 12 μM was achieved after rats received Q-SNEDDS (15 mg/kg BW quercetin aglycone) (206). This quercetin $C_{\text{max}}$ level was similar to the concentration of quercetin aglycone (10 μM) that inhibited TNF-α secretion from LPS-stimulated macrophages (208). This suggests a possibility that cardioprotective activities of quercetin may be enhanced following Q-SNEDDS administration, which represents a future area of research.

Although Q-SNEDDS was evaluated in this dissertation, solid lipid nano-particles have also been developed to improve quercetin bioavailability (92). This quercetin-containing solid lipid nano-particle (QT-SLNs) consisted of quercetin aglycone, soy lecithin, glyceryl monostearate, PEG 400, and Tween® 80 (polysorbate 80) (92). After rats were administered QT-SLNs orally (50 mg/kg BW quercetin aglycone), quercetin absorption was estimated at 0.9%, which was calculated based on a quercetin $C_{\text{max}}$ of 40 μM, and a plasma volume of 3.92 mL/100 g body weight for a 250 g Wistar rat (92, 212). This suggests that QT-SLNs appears to improve quercetin aglycone bioavailability to a similar extent as Q-SNEDDS on the basis that 1.7% of quercetin aglycone was absorbed following Q-SNEDDS administration (15 mg/kg BW quercetin aglycone) (206).

Absorptive and metabolic pathways of Q-SNEDDS remain unknown, which precludes our better understanding of the mechanisms explaining how Q-SNEDDS improves quercetin absorption. No clear evidence depicts whether Q-SNEDDS remains intact in the gastrointestinal lumen. Quercetin may be absorbed along with intact Q-SNEDDS through endocytosis, since Q-SNEDDS has a particle size of 215 nm, and particles having sizes <500 nm are internalized by endocytosis (213). However, cellular metabolism of intact Q-SNEDDS is unknown. Although isorhamnetin and tamarixetin at 0.3 μM were detected in rat plasma collected at 15 min following oral administration of
Q-SNEDDS (30 mg/kg BW quercetin aglycone) (Bruno Lab, unpublished observations), it remains unclear whether quercetin is released from intact Q-SNEDDS in the small intestine or in the liver for its phase II metabolism.

Q-SNEDDS may lose its structural integrity in the gastrointestinal lumen, and Q-SNEDDS components will be released and absorbed separately. Q-SNEDDS is composed of castor oil, Tween® 80 (polysorbate 80), Cremophor® RH 40 (polyoxyl 40 hydrogenated castor oil), and PEG 400 (polyethylene glycol 400). PEG 400, a commonly used intestinal permeability marker, can be directly absorbed and excreted without further metabolic conversion through urine (214). Castor oil and Cremophor® RH 40 consist of long-chain fatty acids, primarily ricinoleic acid (C18:1 (n-9)) (215), whereas Tween® 80 mainly contains oleic acids (216). Although there are no studies elucidating absorptive pathways of castor oil, Tween® 80 and Cremophor® RH 40, these Q-SNEDDS components are likely to be absorbed in a manner similar to long-chain fatty acids. This is supported by a study in vitro showing that 6-14% of fatty acids equivalents were released from Tween® 80 and Cremophor® RH 40 following their incubation with pancreatic enzymes for 90 min (217). Intestinal uptake of these oil phase and surfactants may also improve quercetin aglycone absorption, consistent with previous studies showing that quercetin aglycone absorption is enhanced following co-ingestion of dietary fat and long-chain fatty acids in obese adults and rats (44, 206). Collectively, future studies are needed to elucidate absorptive and metabolic pathway of Q-SNEDDS, which will help to explain mechanisms underlying enhanced quercetin absorption by Q-SNEDDS.

Since toxicity is a significant concern that currently precludes the utilization of nanoparticles in humans (90), the acute toxicity Q-SNEDDS was evaluated in rats at 24 h following oral administration of Q-SNEDDS at doses of 30 mg/kg BW quercetin aglycone. There were no significant differences in plasma biomarkers of liver and kidney
functions (i.e., aspartate transaminase, alanine transaminase, creatinine and urea nitrogen) in rats receiving Q-SNEDDS compared with quercetin aglycone suspension (Table 6.2). However, it remains unknown whether any toxic effects will be induced by a higher dose of Q-SNEDDS (>30 mg/kg BW quercetin aglycone) or its chronic administration. Therefore, additional studies are needed to assess the safety of Q-SNEDDS following its long-term administration and to determine the dose of Q-SNEDDS that may cause toxicity.

6.4 Vitamin C and intestinal permeability may mediate quercetin bioavailability

Consistent with findings from previous clinical studies examining pharmacokinetics of quercetin and other polyphenols (67, 173, 174), studies in Chapter 3 showed that quercetin bioavailability varied significantly between individuals following ingestion of the same dose and form of quercetin (151). For example, after obese adults ingested quercetin aglycone supplements with a meal providing 15.4 g fat, quercetin AUC ranged 478-1577 μM \cdot min and inter-individual variation was 42%. Although marked inter-individual variability is common in examining quercetin bioavailability, factors that determine this inter-individual variation remain unclear. Therefore, another objective of this dissertation was to investigate factors responsible for inter-individual variation in quercetin bioavailability.

Studies in Chapter 5 provided novel evidence that plasma vitamin C status and intestinal permeability may be endogenous factors contributing to inter-individual variability in quercetin bioavailability in humans (207). Participants in our studies were healthy but had inadequate plasma vitamin C status. Their average plasma vitamin C concentration (mean ± SEM; 34.6 ± 2.5 μM) was lower than the average vitamin C concentration (51 μM) of American adults (185) and a third of our participants had suboptimal vitamin C status (<28 μmol/L) (185). In these participants, quercetin aglycone bioavailability was inversely related to plasma vitamin C, but positively related to plasma...
endotoxin, a surrogate marker of intestinal permeability. These findings suggest that individual differences in plasma vitamin C status and intestinal permeability may be associated with inter-individual variability in quercetin bioavailability. Further, inflammation may be associated with sub-average plasma vitamin C status and greater intestinal permeability, as suggested by inverse associations between vitamin C, endotoxin and CRP, and a positive association between endotoxin and CRP. Taken together, although these correlative relationships do not demonstrate causality, these findings support a possibility that inadequate vitamin C status may increase quercetin bioavailability, through inflammation-induced increases in intestinal permeability.

Findings shown in Chapter 5 imply that individuals who have suboptimal plasma vitamin C status and greater intestinal permeability may absorb a greater amount of quercetin aglycone, and thus may benefit from quercetin supplementation. This hypothesis can be tested using guinea pig, since they lack the ability to synthesize vitamin C, and compromised vitamin C status can be induced by vitamin C-deficient diet. Subsequently, quercetin supplementation is provided to examine its bioavailability and potentially cardioprotective effects. On the other hand, these findings suggest that individuals who have sufficient plasma vitamin C status may absorb a lower amount of quercetin aglycone, and thus quercetin aglycone supplements may not exert beneficial effects in these individuals. This notion provides a speculative explanation for no improvements in biomarkers of inflammation in young healthy adults following quercetin supplementation (5), but additional studies are needed to investigate whether vitamin C supplementation decreases quercetin bioavailability in a dose-dependent manner. Collectively, the observational results in Chapter 5 imply that plasma vitamin C status and intestinal permeability may be determinants for inter-individual variability in quercetin bioavailability. More importantly, these results suggest that assessment of plasma
vitamin C and intestinal permeability may facilitate future clinical studies that aim to investigate individual responses to quercetin treatment in relation to CVD prevention.

6.5 Pharmacokinetics of isorhamnetin and tamarixetin

In addition to quercetin pharmacokinetics, this dissertation also characterized pharmacokinetics of isorhamnetin and tamarixetin in both humans and rats by measuring plasma total isorhamnetin and total tamarixetin using HPLC-Couarray following enzymatic hydrolysis and diethyl ether extraction (151, 206). Findings from this dissertation provide novel evidence that isorhamnetin pharmacokinetics differ from tamarixetin in humans and rats regardless of the manner to which quercetin is administered, though they are positional isomers and produced by catechol-O-methyltransferase-catalyzed methylation of quercetin. For example, isorhamnetin $t_{1/2}$ is 19 h in obese adults, whereas $t_{1/2}$ of tamarixetin is 5 h (151). In rats, isorhamnetin $t_{1/2}$ is 10 h, whereas tamarixetin $t_{1/2}$ is 4 h (206). In addition, there are specie differences in the extent of formation of these two methylated metabolites. Our studies in healthy adults showed that isorhamnetin was the major circulating methylated metabolite for 6 individuals, whereas tamarixetin predominated for 10 individuals (207). This supports the presence of inter-individual variability in formation of methylated metabolites of quercetin in humans. In contrast, isorhamnetin is the predominant methylated metabolite in rats, as evidenced by $>2$-times greater isorhamnetin $C_{\text{max}}$ compared with tamarixetin $C_{\text{max}}$ regardless whether rats were orally administered quercetin aglycone suspension or Q-SNEDDS (15 mg/BW quercetin aglycone) (206). Further, isorhamnetin concentrations were 13 pmol/g tissue in the liver collected at 24 h after rats were orally administered quercetin aglycone suspension, whereas tamarixetin was not detectable (206). This finding suggests that isorhamnetin may accumulate in the liver to a greater extent than tamarixetin following long-term supplementation of quercetin. This is supported by a 11 wk quercetin supplementation study in rats showing that isorhamnetin accounted for $>57\%$
of total flavonol (i.e. the sum of quercetin, isorhamnetin and tamarixetin) that were detected in plasma and tissues such as lung, kidney and liver (168). Collectively, differences in pharmacokinetics between isorhamnetin and tamarixetin suggest that the positional methylation of quercetin may regulate its disposition.

6.6 Limitations and future direction

Studies in Chapter 3 and Chapter 4 demonstrated that absorption and bioavailability of quercetin aglycone were improved in a dietary fat- and Q-SNEDDS-dependent manner (151, 206). However, quercetin exists mainly as quercetin glycosides in foods (18). Therefore, future studies are needed to investigate the influence of dietary fat or nano-formulation on quercetin glycosides bioavailability. In addition, because of its poor bioavailability, no adverse health effects of quercetin were found in rats receiving 0.5 g/kg BW quercetin aglycone for 410 d (103). However, it is possible that quercetin may induce toxicity when its plasma and tissue concentrations are significantly increased along with improved bioavailability. Therefore, additional studies are needed to investigate whether improvements in quercetin bioavailability mediated by dietary fat or Q-SNEDDS result in any toxic effects, especially following chronic quercetin administration. Studies in Chapter 5 suggest that plasma vitamin C status and intestinal permeability may be factors contributing to inter-individual variability in healthy individuals such that quercetin bioavailability is greater in individuals who have sub-average vitamin C status and greater intestinal permeability. Additional studies in a larger cohorts are needed to examine the observed associations among quercetin bioavailability, vitamin C and intestinal permeability. More studies are also warranted to establish causality between quercetin bioavailability and vitamin C using experimental models where quercetin is co-administered with varying levels of vitamin C.
6.7 Conclusion

This dissertation demonstrated that greater quercetin aglycone bioavailability could be achieved when it was ingested with dietary fat or administered orally as nano-emulsion, or when sub-average plasma vitamin C status and greater intestinal permeability were present. The findings described herein are of significance in that they contribute to a better understanding of both exogenous and endogenous determinants for quercetin bioavailability. These findings provide the foundational basis for development of effective and feasible strategies to improve quercetin bioavailability in humans. These findings will also facilitate intervention studies aiming to evaluate putative cardioprotective activities of quercetin and development of dietary recommendation for quercetin in effort to mitigate CVD risks.
6.8 Figures and tables

![Graph showing the relationship between dose of quercetin aglycone supplements and plasma quercetin response in chronic clinical studies shown in table 6.1.](image)

**Figure 6.1** Relationship between dose of quercetin aglycone supplements and plasma quercetin response in chronic clinical studies shown in table 6.1
<table>
<thead>
<tr>
<th>Dose and duration$^a$</th>
<th>Plasma quercetin concentration (nM)</th>
<th>Ref</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Post-supplementation</td>
</tr>
<tr>
<td>50 mg/d, 2 wk</td>
<td>52.5 (44.4, 58.9)</td>
<td>145 (110, 190)</td>
</tr>
<tr>
<td>100 mg/d, 2 wk</td>
<td>44.9 (34.3, 74.9)</td>
<td>217 (176, 252)</td>
</tr>
<tr>
<td>150 mg/d, 2 wk</td>
<td>64.2 (51.6, 70.9)</td>
<td>380 (331.4, 636.3)</td>
</tr>
<tr>
<td>150 mg/d, 6 wk</td>
<td>71</td>
<td>269</td>
</tr>
<tr>
<td>500 mg/d, 12 wk</td>
<td>285 (253)</td>
<td>1380 (1285)</td>
</tr>
<tr>
<td>1000 mg/d, 4 wk</td>
<td>100 (255)</td>
<td>1500 (794)</td>
</tr>
<tr>
<td>1000 mg/d, 12 wk</td>
<td>300 (246)</td>
<td>2000 (1838)</td>
</tr>
</tbody>
</table>

a) Quercetin aglycone supplements were provided in all the listed studies.

b) Values are medians (25th, 75th percentile) in this study, whereas values are means (SD) in other studies.

c) Increases in plasma quercetin concentration is calculated as concentration after supplementation subtracts baseline concentration.
Table 6.2. Plasma biomarkers of liver and kidney injury in rats prior to and 24 h after oral administration of quercetin aglycone in sodium carboxymethyl cellulose (Q) or Q-SNEDDS (30 mg/kg BW quercetin aglycone)

<table>
<thead>
<tr>
<th></th>
<th>Q</th>
<th>Q-SNEDDS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>24 h</td>
</tr>
<tr>
<td>ALTa) activity (U/L)</td>
<td>31.6 ± 1.7</td>
<td>33.1 ± 2.0</td>
</tr>
<tr>
<td>AST activity (U/L)</td>
<td>90.4 ± 7.1</td>
<td>84.3 ± 10.1</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>11.2 ± 0.6</td>
<td>13.8 ± 1.2</td>
</tr>
</tbody>
</table>

a) ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen.
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145


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