Oligomeric Cocoa Procyanidins Possess Enhanced Bioactivity Compared to Monomeric and Polymeric Cocoa Procyanidins for Preventing the Development of Obesity, Insulin Resistance, and Impaired Glucose Tolerance During High-Fat Feeding

Melanie R. Dorenkott

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
In
Food Science and Technology

Andrew Neilson, Chair
Matthew Hulver
Monica Ponder
Sean O'Keefe

April 15, 2014
Blacksburg, VA

Keywords: Cocoa procyanidins, cocoa extract, flavanols, degree of polymerization, obesity, insulin resistance, glucose tolerance, endotoxin.

Oligomeric Cocoa Procyanidins Possess Enhanced Bioactivity Compared to Monomeric and Polymeric Cocoa Procyanidins for Preventing the Development of Obesity, Insulin Resistance, and Impaired Glucose Tolerance During High-Fat Feeding

Melanie R. Dorenkott

Abstract

Flavanols are polyphenols that are known to have many beneficial effects on the body. Cocoa is a major source of these flavanols. However, research on the potential anti-obesity and anti-diabetic health benefits of cocoa flavanols is lacking in the literature. Furthermore, the effect that the size of these flavanols has on the extent of its beneficial properties is also unclear. The objective of this study was to evaluate the dietary effects of cocoa flavanols on the onset of obesity, insulin resistance and impaired glucose tolerance and to determine the impact that the size of these compounds has on the magnitude of this effect. Cocoa extract was fractionated into a monomer-, an oligomer-, and a polymer-rich fraction. Six groups ($n=9$) of C57BL/6J mice were fed either a control low-fat diet, a control high-fat diet, or a high-fat diet supplemented with 25 mg/kg*BW of cocoa extract or one of the three cocoa fractions. After 12 weeks on these diets, the oligomer-rich fraction proved to be most effective in preventing weight gain, fat mass accumulation, elevated fasting blood glucose and impaired glucose tolerance in diet-induced obese mice. This is the first long-term feeding study to examine the relative activities of cocoa constituents on diet-induced obesity and insulin resistance.

Acknowledgements

I would like to acknowledge the following individuals for their contributions and assistance with my graduate thesis work; Laura Griffin for her thiolysis and standard curve work, Katherine Goodrich for her reverse-phase HPLC work and for assisting me with parafilming SPE columns and weekly animal care and procedures, Katherine Thompson-Witrick for her thiolysis work, Gabrielle Fundaro for her assistance and guidance with the mouse procedures and with the endotoxin analysis, Liyun Ye for her normal-phase HPLC work, Joseph Stevens and Mostafa Ali for their help with animal blood collections during the sacrifices, my committee members Dr. Hulver, Dr. Ponder, and Dr. O'Keefe, for their advice and counsel along my graduate school journey, and my academic advisor and mentor Dr. Andrew Neilson for his wonderful guidance throughout my graduate career. I would also like to acknowledge the contributions of Karen Strat, Pamela Suroski, Charles Nwaihesie, Benjamin Furrow, and Julie Holdridge for their assistance with the animal experiments, and Dr. Hengjian Wang for assistance with the Folin-Ciocalteau assay. Furthermore, I would like to specially thank my labmate, Katherine Goodrich, for her friendship and advice both in and outside of graduate school over the past 2 years, as well as Steven Hawes for his unconditional love and support throughout my time in Blacksburg, Virginia.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

C, catechin; EC, epicatechin; PC, procyanidin; DP, degree of polymerization; mDP, mean degree of polymerization; CPC, cocoa procyanidin; T2D, type-2 diabetes; GLP-1, glucagon-like peptide; HF, high-fat; CE, cocoa extract; NFCS, nonfat cocoa solids; GC, gallocatechin; EGC, epigallacatechin; ND, not detectable; HPLC, high-performance liquid chromatography; MeOH, methanol; GAE, gallic acid equivalents; ECG, epicatechin gallate; EGCG, epigallocatechin gallate; GSE, grape seed extract; CDC, Centers for Disease Control and Prevention; BMI, body mass index; CVD, cardiovascular disease; TAG, triglyceride; FFA, free fatty acid; GLUT, glucose transporter; TNF-α, tumor-necrosis factor-α; NF-kB, nuclear factor-kB; IRS, insulin receptor substrate; IL, interleukin; LPS, lipopolysaccharide; TLR, toll-like receptor; ROS, reactive oxygen species; BW, body weight; LF, low-fat; GTT, glucose tolerance test; ITT, insulin tolerance test; STZ, streptozotocin; i.p., intraperitoneal; MS, mass spectrometry; SPE, solid phase extraction; ddH_20, distilled deionized water; ESI, electrospray ionization; TQD, triple quadrupole; MRM, multi-reaction monitoring; CID, collision-induced dissociation; DIO, diet-induced obesity; LAL, Limulus Amebocyte Lysate; AUC, area under the curve; EU, endotoxin units; IRβ, insulin receptor β; DPP4, dipeptidyl peptidase-IV.

Chapter 1:

Introduction

Flavanols are a structurally diverse class of compounds comprised of monomeric catechins [(±)-catechin (C), (−)-epicatechin (EC), etc.], as well as oligomers and polymers of these monomers known as the procyanidins (PCs). Flavanols are characterized by their degree of polymerization (DP)[1], and the mean DP (mDP), the average DP of all flavanols in a matrix. Cocoa products are one of the most flavanol-rich foods and display a wide range of PC DPs[2-4]. The main flavanol monomer in cocoa is (−)-EC, with lower levels of (−)-C and (+)-C[5-7]. In general, cocoa procyanidins (CPCs) have been identified up to DP of 10-12[1, 8, 9]. Although the cocoa bean is a rich source of flavanols, fermentation, processing and formulation greatly influence the final total flavanol concentration and the qualitative and quantitative PC profile[8-12].

There is increasing interest in the potential of flavanols to prevent and/or ameliorate obesity and type-2 diabetes (T2D). A large body of research exists on the anti-obesity and anti-diabetic activities of flavanols from foods such as grapes and tea, but comparatively little data exist for cocoa flavanols. The few human studies of the effects of chocolate and cocoa products on T2D and metabolism in humans have shown promising effects, including improved insulin sensitivity[13, 14] and increased skeletal muscle mitochondrial biogenesis[15]. The lack of human data on the effects of cocoa polyphenols on obesity and T2D is partially due to relatively few animal studies, the largely unknown mechanisms of action of cocoa, and a lack of data regarding the activities of specific cocoa flavanols. Diets supplemented with flavanol-rich cocoa or cocoa flavanols significantly reduce weight gain, adipose tissue mass, fasting blood glucose levels and glucose intolerance in various animal models of induced obesity and/or T2D[16-23]. Despite these findings, much remains to be understood regarding the beneficial effects of cocoa flavanols and the mechanisms by which cocoa flavanols inhibit obesity and T2D.

One major determinant of cocoa bioactivity is likely the nature and concentration of the constituent flavanols. Existing studies of cocoa have employed a wide variety of cocoa flavanols and concentrations due to differences in cocoa sources, processing (fermentation, roasting, Dutching, etc.), the use of extracts vs. cocoa products, extraction method, and type of cocoa product (liqueur, dark chocolate, etc.). Additionally, some studies lack definitive quantitative and

qualitative profiling of the exact composition of the flavanol dose administered. Consequently, definitive structure-activity relationships for cocoa flavanols, and the correlation between cocoa composition and efficacy, remain to be established. Of particular interest are the potential differences in bioactivities between the catechin monomers (C, EC) and the PCs (oligomers and polymers), all of which are present in cocoa. EC and CPCs have been shown to possess anti-obesity and anti-diabetic properties[24-26]. Emerging data suggest that these bioactivities may be highly correlated to flavanol DP[27-29]. Recently, Yamashita *et al.*[30] demonstrated that a tetrameric cocoa flavanol significantly improved insulin secretion in mice compared to monomeric, dimeric, and trimeric flavanols (EC, PC B_2, and PC C_1, respectively)[30]. Furthermore, cinnamtannin A2 significantly increased glucagon-like peptide-1 (GLP-1) secretion. These results suggest that insulin secretion and glucose homeostasis could be dependent on flavanol DP. Aside from this study, data on the anti-obesity and anti-diabetic activities of cocoa flavanols with distinct DPs *in vivo* are notably lacking. There has not been a long-term feeding study to examine the impact of chronic exposure to cocoa flavanols of distinct sizes on the onset of obesity, insulin resistance and impaired glucose tolerance.

The goal of this study was to compare the effects of long-term dietary exposure to cocoa flavanol monomers, oligomers and polymers on obesity and impaired glucose tolerance. This was done by fractionating cocoa flavanols into matrices with different mDP values and then evaluating the ability of each fraction to prevent obesity, insulin resistance and impaired glucose tolerance after chronic administration in mice fed a high-fat (HF) diet. It was hypothesized that the ability of cocoa flavanols to inhibit weight gain and the onset of insulin resistance and impaired glucose tolerance during an animal model of HF feeding was dependent upon flavanol DP.

In order to achieve the goal and test the central hypothesis, the following objectives were proposed:

1) Fractionate cocoa extract (CE) into three CPC fractions with distinct mDP's (monomer-, oligomer-, and polymer-rich fractions.)

2) Determine if CE and individual CPC fractions prevent the onset of obesity insulin resistance and impaired glucose tolerance during HF feeding in mice.

3) Determine the impact that mDP has on the anti-obesity and anti-diabetic activities of CPC fractions during HF feeding in mice.

Chapter 2:

Literature Review

Phenol Classification. Phenol is the collective term for compounds having one or more hydroxyl groups attached to an aromatic ring[31, 32]. Phenolic compounds exist naturally as secondary plant metabolites as they are not essential to plants growth and functioning and are thus not directly involved in the primary metabolism[33]. Nevertheless, phenolic compounds support and contribute to the maintenance of multiple metabolic pathways and can have invaluable significance to an organism. **Figure 2.1** displays the major categories of phenolic compounds (phenolic acids, coumarins, polyphenols) however, other subgroups do exist that are not shown.

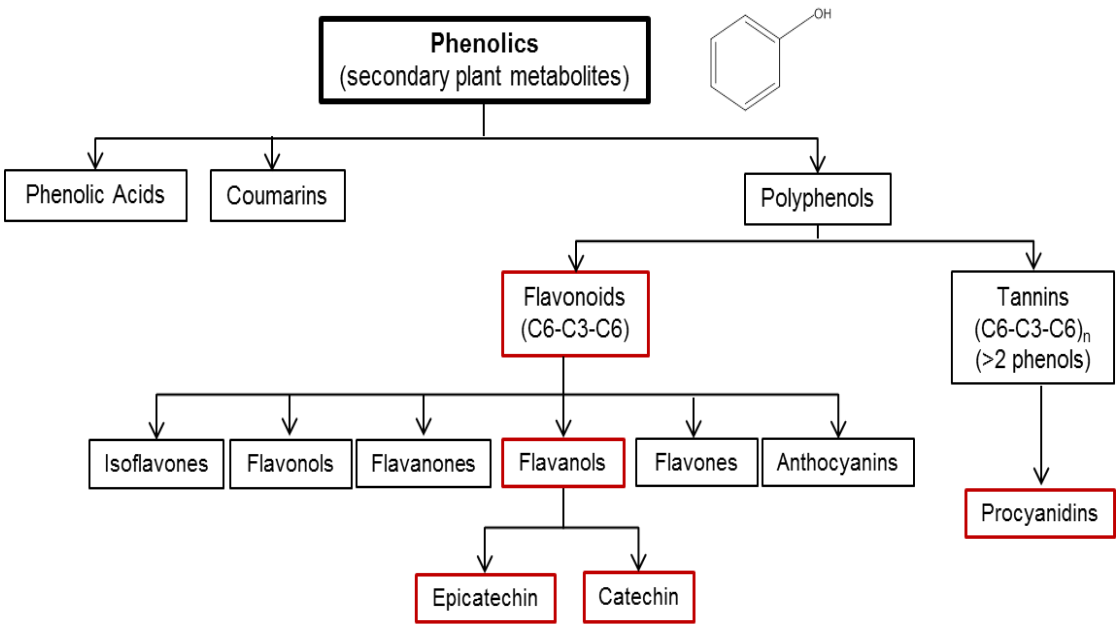


Figure 2.1. *Categorization of phenolic compounds.*

Polyphenols make up a very broad class of compounds found in many fruits and vegetables that are characterized by containing one or more aromatic rings with two or more hydroxyl groups on the same ring [8, 32, 34]. Flavanols (or flavan-3-ols) are among the polyphenol

subgroup known as flavonoids that are identified by their C6-C3-C6 backbone[2, 31, 34, 35]. Also among the flavonoid subgroup are thousands of known structures classified as isoflavones, flavonols, flavanones, flavones, and anthocyanins all of which contain three aromatic rings (15-carbon skeleton) and are often found naturally in plant products as glycosides[31, 36]. These subclasses of flavonoids are regarded as strong phytochemicals and exhibit a wide range of beneficial properties on both plant and human health[36].

Flavanols are present either as monomers or polymers sometimes referred to as condensed tannins (another term for PCs). The main flavanol monomers include (+)-C and (−)-EC[33, 34, 37, 38]. C and EC exist as isomers and are identified by the positions of the hydrogens on C_2 and C_3 of the C3 backbone fragment. The two hydrogens of (+)- and (−)-C are in the *trans* configuration while the hydrogens of (+)- and (−)-EC are in the *cis* configuration[31]. Oligomers (2-5 units) and polymers (6 or more units) are chains of these monomers connected via C-C linkages and can be expressed by their DP, which is equivalent to the number of monomeric units present in a given chain[1, 39-41]. **Figure 2.2** displays the structures of a flavanol monomer (EC), dimer, trimer and pentamer.

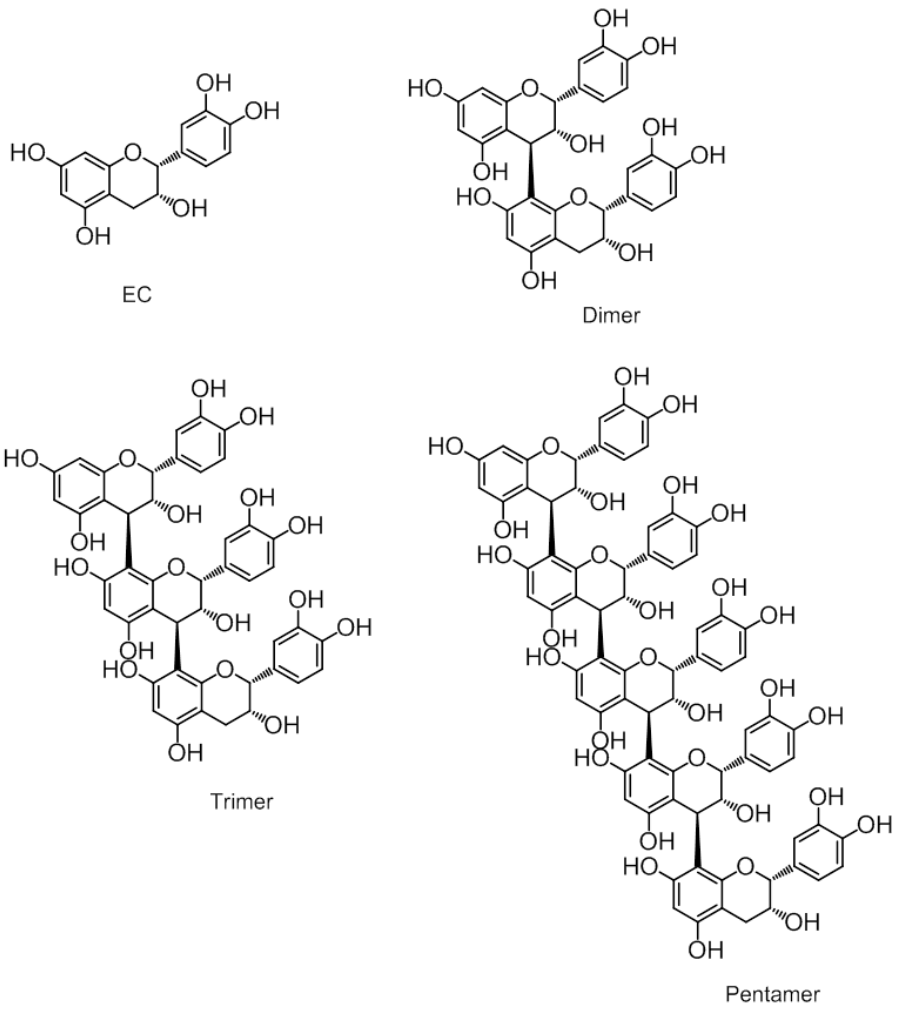


Figure 2.2. *Flavan-3-ol structures of EC monomer and select cocoa oligomers.*

Cocoa Flavanol Profile. Cocoa, along with tea, grapes, and wine, is known to be among the most flavanol-rich foods[2]. Lee *et al.* have demonstrated non-alkalized cocoa powder to have the greatest flavonoid content (mg per serving) compared to red wine, black tea, and green tea[3]. Another study found cocoa powder followed by dark chocolate to be the most abundant in total flavanols (mg/g) compared to other various fruit powders and retail fruit products[4]. Cocoa powder, cocoa liquor, and cocoa butter are derived from the cocoa bean (the seed of the fruit of the cocao tree, *Theobroma cacao* L.)[8, 42]. Forastero and Criollo are the two typically recognized varieties of cocao beans. The total polyphenol content of a finished cocoa product depends on the variety of cocao bean used. In general, the Forastero variety contains approximately one-third more polyphenols than the Criollo variety[8].

Cocoa powder, which is mostly made up of the nonfat cocoa solids (NFCS), contains the majority of the cacao bean polyphenols[9, 43]. According to Belščak *et al.* the amount of polyphenols in a cocoa product is strongly correlated to the amount of NFCS[5]. This is supported by Vinson *et al.* who found the concentration of monomeric flavanols to be 2.96-3.27 mg/g in cocoa powder, 0.48-1.37 mg/g in dark chocolate, and 0.15-0.16 mg/g in milk chocolate[12]. In a similar trend, a study determined the PC content of cocoa powder, chocolate liquor, and milk chocolate to be 40.8 mg/g, 22.3 mg/g and 2.7 mg/g respectively. This study attributed this decrease in PC content to a decrease in NFCS on % weight basis[9]. Miller *et al.* and Crozier *et al.* found related total flavanol contents in cocoa powder of 34.6 mg/g and 30.1 mg/g respectively[4, 43].

Flavanol monomers comprise 37% of the polyphenols found in the cocoa bean[8]. Adamson *et al.* observed monomer contents between 0.2-4.9 mg/g in defatted CE of chocolate liquor, dark chocolate and milk chocolate[44]. According to Hammerstone *et al.*, 1.08 mg/g monomer is present in dark chocolate[2]. The majority of these monomers in cocoa is EC and C[5] along with traces of gallocatechin (GC) and epigallocatechin (EGC)[45]. However, EC is typically found in cocoa in greater amounts compared to C[5-7]. Machonis *et al.* observed 68-91% of cocoa powder monomers to be EC[46]. Cooper *et al.* analyzed CEs from 68 different commercial chocolates (61 dark and 7 milk) and found a range of EC content of 0.071-1.94 mg/g and C content of 0.043-0.519 mg/g[10]. Similarly, Gu *et al.* observed EC contents of 0.18-2.29 mg/g and C contents of 0.05-1.17 mg/g in samples of non-alkalized and alkalized cocoa powder, dark chocolate, milk chocolate, and unsweetened chocolate[9]. Belščak *et al.* found EC contents of 0.1-1.87 mg/g and C contents of 0.02-0.5 mg/g in defatted CE of chocolate liquors, dark chocolates and milk chocolates[5].

In addition to monomers, PC oligomers (2-5 monomeric units) and polymers (6 or more units) are present in cocoa[7]. PC polymers exist in nature with DPs of up to 50 or more, however polymers in cocoa are typically detected in units up to ten[1, 8, 9]. The following individual PC's have been detected in cocoa, B1 (EC-(4β→8)-C), B2 (EC-(4β→8)-EC), B3 (C-(4α→8)-C), B4 (C-(4α→8)-EC), B5 (EC-(4β→6)-EC), C1 (EC-(4β→8)-EC-(4β→8)-EC), and D (EC-(4β→8)-EC-(4β→8)-EC-(4β→8)-EC)[8]. PC's make up 58% of the polyphenols found in cocoa beans[8]. PC dimers B2 and B5 have been reported in CE in the range of 0.041-1.17 mg/g and not detectable (ND)-0.236 mg/g respectively[10]. Adamson *et al.* reported dimer content of defatted

chocolate liquor, dark and milk chocolate extracts in the range of 0.2-4.2 mg/g[44]. Trimer C and tetramer D were reported in CE in the range ND-0.905 mg/g and ND-0.387 mg/g[10]. In addition, Adamson *et al.* found the contents of trimer at 0.1-2.8 mg/g, tetramer at 0.1-2.2 mg/g, pentamer at 0.1-1.7 mg/g, hexamer at 0.1-1.4 mg/g, heptamer at ND-0.7 mg/g, octamer at ND-0.6 mg/g, nonamer at ND-0.7 mg/g and decamer at ND-0.3 mg/g[44]. Lastly, according to Gu *et al.* the content of dimers-trimers can vary from 0.49-8.98 mg/g, tetramers-hexamers from 0.38-10.94 mg/g and heptamers-decamers from 0.17-7.46 mg/g in defatted commercial cocoa powder, unsweetened, dark and milk chocolates[9].

A considerable amount of research has been done to quantify the specific individual flavanols in cocoa and chocolate products. Unfortunately, depending on the origin of the cocoa bean and processing conditions, the composition of the cocoa or chocolate can vary as demonstrated in the content values above[8, 10, 11]. The fermentation parameters, roasting time and temperature, along with storage time and temperature all have a significant effect on the final flavanol content[8, 11, 35]. Specifically, the fermentation step can significantly reduce the polyphenol content via condensation and oxidation reactions[8]. In addition, the method and conditions used for analysis also account for a disparity among the reported cocoa flavanol compositions in the literature [8]. The process known as Dutching (or alkalizing) involves soaking the cacao beans in an alkaline solution to increase dispersability in water and reduce bitterness[43]. However, flavanols are pH-sensitive and may be degraded at elevated pH (pH > 5.5). Miller *et al.* demonstrated a steady decrease in flavanol content with increasing pH in cocoa powder[43]. As a result, Dutched chocolate typically exhibits low total flavanol content[11, 43]. A recent study demonstrated that Dutching decreases C and EC levels in cocoa powder by 38% and 67% respectively[6].

In addition to flavanol monomers and PCs, the cocao bean contains other compounds that have potential health-promoting capabilities. Anthocyanins are the water-soluble subclass of flavanoids responsible for the color of many plants and have been known to promote cardiovascular health[8, 31, 34]. Although these natural pigments have been reported to make up 4% of the total polyphenol content present in the cocoa bean, most is largely lost in the fermentation step during processing[8].

Other potentially biologically active compounds present in cocoa include the alkaloids theobromine and caffeine. Both compounds are methylxanthines and like flavanols, their

presence in cocoa is highly dependent on processing parameters and analysis techniques[5, 10]. For example, in a study evaluating the methylxanthines content across a broad range of different defatted cocoa products by high-performance liquid chromatography (HPLC) analysis using either water or MeOH as the solvent, the theobromine content ranged from 1.86 mg/g of milk chocolate in both water and MeOH extracts to 15.50 mg/g of cocoa powder in the water extract and the caffeine content ranged from 0.15 mg/g of defatted milk chocolate in the water extract to 1.42 mg/g of defatted chocolate with 88% cocoa solids in the MeOH extract [5]. Langer *et al.* observed a theobromine and caffeine content of 1.5 mg/g and 0.10 mg/g respectively in milk chocolate[7]. In this same study, the theobromine and caffeine contents of twelve dark chocolates ranged from 5.3-16.4 mg/g and 0.3-2.4 mg/g respectively[7]. Although discrepancies arise in theobromine and caffeine contents in cocoa samples due to lack of a standard method of analysis, it is well documented that theobromine is present in cocoa to a much greater degree compared to caffeine. Furthermore, it is also widely accepted that flavanols are present in cocoa in greater amounts than both theobromine and caffeine. In a study evaluating both the flavanol and methylxanthine content of two dark chocolates and nine milk chocolates, the monomer, theobromine and caffeine contents ranged from 2.00-14.89 mg/g, 1.17-1.96 mg/g, and 0.46-0.94 mg/g respectively[47].

Other Dietary Sources of Flavanols. Although cocoa has proven to be an exceptional source of flavanols, various other natural food stuff are also known to be rich sources of polyphenols. As a secondary plant metabolite, flavanols are ubiquitous in plants and carryout various functions. Most notably, is the content of flavanols in grape, tea, red wine, apples and berries. Lee *et al.* explored the total phenolic and flavonoid contents of such sources. Excluding cocoa powder which had the highest contents, the content of total phenols (expressed as gallic acid equivalents [GAE] per serving) and flavonoids (expressed as EC equivalents per serving) were detected in the following order red wine > green tea > black tea[3]. The typical flavanol profiles vary depending on the source compared to cocoa. For example, the major polyphenol in grape seed is epicatechin gallate [(−)-ECG][48] and in green tea is epigallocatechin gallate [(−)-(EGCG)] rather than EC as is in cocoa[3, 49].

Cranberries are known to contain large amounts of flavonoids, namely anthocyanins which gives the fruit its color. White *et al.* investigated the flavonol and PC content in fresh and

freeze-dried cranberry pomace. The anthocyanin, oligomeric PC, and polymeric PC concentrations were 3.63, 3.33, and 26.3 mg/g in fresh pomace and 3.66, 5.57, and 18.6 mg/g in freeze-dried pomace[50]. The total PCs in a variety of apples, red wine, and cranberry juice was measured by HPLC analysis using PC standards (monomers-decamers)[2]. The average PC content of apples, red wine, and cranberry juice was 1.01 mg/g, 0.21 g/L, and 0.14 g/L respectively[2]. Lastly, the monomer and PC contents of freeze-dried cocoa bean, cranberry, and lowbush blueberry were quantified by reverse-phase HPLC analysis. Freeze-dried cocoa bean, cranberry and blueberry contained the following amounts of monomer; 14.2, 0.48, and 0.18 mg/g respectively, and PC's; 83.6, 32.22, and 19.8 mg/g respectively[40].

Most markedly in flavanol research, is the recent focus on grape seed extract (GSE) which comprises PC's extracted from the *Vitis vinifera* grape seeds. Results from multiple studies exploring the biological impact of GSE on different phenotypes demonstrate its ability to exhibit a wide range of therapeutic actions of which will be discussed later in this chapter. The flavanol composition of GSE contains approximately 80% EGCG and dimers-tetramers, 15% C and EC, and 5% pentamers-heptamers[48]. In the study done by Khanal *et al.* the total PC content of GSE was 26.5 mg/g. Additionally, the following amounts of monomers and PC constituents were detected in GSE, 4.23 mg/g (DP1), 1.37 mg/g (DP2), 1.96 m/g (DP3), 1.98 mg/g (DP4), 1.65 mg/g (DP5), 2.01 mg/g (DP6), 0.80 mg/g (DP7), 1.16 mg/g (DP8), 1.98 mg/g (DP9), 0.71 mg/g (DP10) and 8.59 mg/g (DP>10)[51]. This same study further evaluated grape pomace (stems, skins, and seeds) finding a significantly lower total PC content of 4.30 mg/g along with consistently lower concentrations of individual monomers and PCs[51].

Obesity and Type 2 Diabetes. In much of the recent literature, obesity has been repeatedly referred to as a growing worldwide epidemic. The Centers for Disease Control and Prevention (CDC) under the U.S. Department of Health and Human Services reported the adult obesity rate to be at 12% in 1999, 23% in 2005 and is now 34% in 2013[52, 53]. The CDC defines "overweight" as those with a body mass index (BMI) of ≥ 25 and obesity as a BMI of ≥ 30 as calculated by dividing weight (kg) by height (m^2)[54, 55].

In the most recent CDC 2013 estimated obesity rates, over 67% of adults are overweight or obese and 34% of adults are obese in America[52]. Thus, two-thirds of adult Americans are overweight or obese and one-third of adults are obese [55, 56]. In addition over 31% of adolescents

are overweight or obese and nearly 17% of adolescents are obese in America[52]. This is a significant increase in the prevalence rate of obesity over the past decade[57].

The unfavorable health consequences of obesity are well-documented in the literature affecting every major organ in the body. However most importantly, obesity can lead to hypertension, T2D, cardiovascular disease (CVD), cancer, and ultimately death[52, 55, 58]. In 2009, CVD accounted for one in every three deaths in the United States [52]. In a collaborative analysis including nearly 60 studies and 900,000 participants, it was found that for every 5 kg/m^2 over 25 kg/m^2 BMI, the risk of overall mortality increases 30%, and the risk of diabetic, renal, and hepatic mortality increases 60-120%[59].

The increasing prevalence of obesity in the U.S. population has been directly associated to the increasing rates of non-insulin dependent diabetes mellitus or T2D[52, 55, 56]. In many studies, elevated BMI is used as the main predictor of developing T2D[52]. Over 8% of the U.S. adult population had T2D in 2010, while it was estimated that 3.5% of the population had undiagnosed T2D and over 38% had pre-T2D[52].

T2D is associated with a multitude of risk factors including obesity, hyperinsulinemia, and elevated blood pressure, total triglycerides (TAGs) and low HDL cholesterol[60]. Complications associated with T2D are similar, if not more detrimental to one's health, than those of obesity. The risk of CVD is increased two to fourfold by T2D[60]. In one study involving 2,400 both non-diabetic and T2D participants over the course of 18 years, it was observed that those with T2D and no prior evidence of coronary heart disease were at a greater risk for coronary heart disease compared to those non-diabetics with prior evidence of coronary heart disease[61].

T2D is defined as a state of chronic hyperglycemia (elevated blood glucose concentrations),[60] and is associated with hyperinsulinemia (elevated blood insulin), and hyperlipidemia (elevated circulating free fatty acids [FFA] and TAG levels along with an accumulation of fat deposits on tissues and muscles). The pathogenesis of T2D involves either the state of insulin resistance or deficient insulin secretion, or a combination of the two[60].

Insulin Metabolism. In order for glucose to be used as an energy source, it must be transported from the blood, across the cell membrane, and into the cell via glucose transporters (GLUT)[62, 63]. The GLUT isoform, designated as GLUT-4, is responsible for the majority of glucose transport in skeletal muscle, and adipose tissue. The polypeptide hormone known as insulin is the main

regulator of GLUT-4[63-65]. Consequently, as the blood glucose level increases after a meal, pancreatic beta cells secrete insulin into the blood which then attach to insulin receptors on the cell membrane causing the translocation of the intracellular GLUT-4 transporters to come facilitate the entry of glucose into the cell[65, 66].

High blood glucose concentrations after a meal stimulate the release of insulin. This increase in insulin signals the liver to inhibit glucose production, or gluconeogenesis, and store any excess glucose as glycogen via glycogen synthase[67]. In contrast, when blood glucose concentrations are low due to fasting or exercise, the liver is responsible for the release of glucose via glycogen breakdown or glycogenolysis[65].

The body's constant ability to adjust for sharp increases and decreases in the blood glucose level through this glucose-insulin relationship is crucial to a person's health. Impaired maintenance of this glucose homeostasis and untreated chronic high blood sugars will lead to ketoacidosis and a multitude of complications dealing with the skin, eyes, and feet along with increased risk of neuropathy, nephropathy, stroke and CVD.

Approximately 85% of the insulin-mediated glucose uptake described above takes place in the skeletal muscle[63] along with a small portion occurring in adipose tissue[67]. However, both adipose tissue and the liver still play a crucial role in regulating metabolism[67, 68]. Many propose the basis of glucose homeostasis lies in the complex interactions between skeletal muscle and adipose tissue[63, 67, 68]. This hypothesis is supported by multiple knockout studies done in which one muscle-specific gene or insulin receptor was removed in mice and did not result in the development of T2D[69-71]. Consequently, development of complete T2D involves a culmination of events that lead to impaired functioning in skeletal muscle, adipose tissue, and liver and the interactions that occur between them[72]. This "cross-talk" between tissues involves various biomarkers and signaling pathways. The various roles of insulin are central to maintaining overall systemic metabolism. **Figure 2.3** displays a brief summary of these roles on the different tissues.

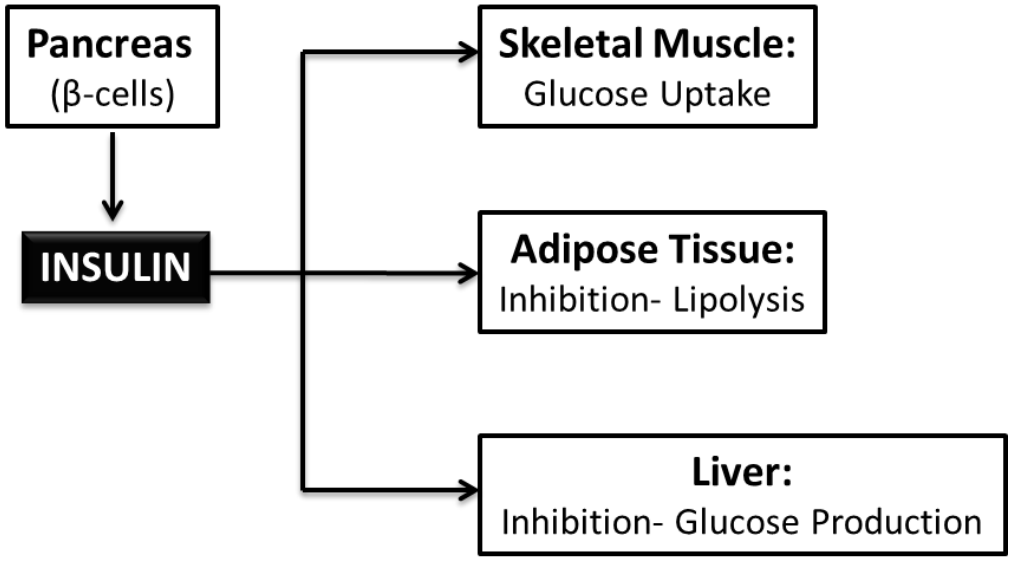


Figure 2.3. *Schematic representation of the roles insulin plays in muscle, fat, and the liver.*

Insulin promotes the uptake of glucose from the blood and into the skeletal muscle and adipose tissue. In adipocytes, insulin helps with the removal and storage of circulating fatty acids as well as inhibits lipolysis of stored TAGs via inhibition of lipase[67, 73]. Lastly, insulin signals the liver to stop glycogen breakdown that would otherwise result in increased blood glucose levels[73].

Insulin Resistance. In general, impaired glucose tolerance and insulin resistance are thought to be the primary indicators in the development of pre-T2D or T2D. Impaired glucose tolerance is defined by an elevated blood glucose level two hours after meal consumption[60]. Insulin resistance is a gradual progression of the bodies inability to effectively use insulin and is characterized by elevations in fasting and postprandial blood glucose and lipid levels[63, 66, 73] along with impaired hepatic glucose production[72]. Insulin resistance occurs in skeletal muscle when a greater amount of insulin is required to stimulate the uptake of a given amount of glucose from the blood. In other words, when the cell becomes less responsive to insulin resulting in less glucose uptake[65, 72]. This event leads to a greater amount of insulin secretion required for a given amount of glucose to be transported into the cell. Overtime, this necessary increase in demand for the production of insulin causes the beta cells to have impaired insulin secretion functioning. In extreme cases, this overproduction of insulin may eventually cause the beta cells to

completely lose the ability to secrete insulin or regulate glucose metabolism resulting in apoptosis and complete insulin-dependent diabetes[64].

Pathogenesis of Insulin Resistance. Despite extensive studies dealing with the matter, the exact underlying mechanisms of insulin resistance have yet to be elucidated however it is likely the result of various multifaceted interactions and factors. Skeletal muscle is the primary insulin-responsive tissue and thus constitutes major concern for insulin resistance. However, the roles played by adipose tissue and the liver are not to be underestimated. The principal risk factor in the development of T2D is obesity. Alterations, provoked by obesity, in skeletal muscle, adipose tissue and the liver ultimately lead to systemic insulin resistance[73]. In healthy individuals, insulin acts on adipose tissue by promoting the synthesis of lipids and inhibiting lipolysis[67]. However, in the obese, adipocytes are larger or inflamed and have the ability to block this insulin action thus increasing circulating FFA levels and exacerbating insulin resistance[65]. In addition to adipocytes, a cell type known as macrophages are also present predominantly in adipose tissue and are a major source of proinflammatory mediators[73]. Increased release of these mediators and FFA interfere with insulin signaling may decrease insulin sensitivity in skeletal muscle[65, 72, 73].

Adipocytes are responsible for the release of a group of hormones known as adipokines. Tumor-necrosis factor-α (TNF-α), adiponectin, and leptin are the main adipokines associated with insulin resistance[72]. The adverse effects of the well-known cytokine TNF-α have been extensively studied and will be described in detail later in this review. Expression of the adipocyte-specific protein adiponectin, helps to modulate FFA catabolism via stimulation of various nuclear receptors thus contributing to lower levels of circulating FFA and glucose[72, 74]. However, adiponectin secretion is down regulated in obese individual's thus promoting insulin resistance[67]. Leptin is a hormone expressed mainly by adipose tissue that is linked to hypothalamus brain function that signals the feeling of satiety in addition to increasing insulin sensitivity[72, 73].

Much evidence has shown insulin resistance underlies the activation of various proinflammatory pathways. Obesity causes an inflammation in the adipose tissue leading to an increased prevalence of macrophages. It is well known that both obesity-inflamed adipocytes and macrophages are highly responsible for an increase in the production of proinflammatory cytokines[65, 73]. These mediators interfere with a number of insulin signaling pathways thus

contributing to insulin resistance. TNF-α is released by both adipocytes and macrophages and is an important cytokine linked to insulin resistance[62, 73]. Secretion of TNF-α leads to an increase in circulating FFA[72]. The effects of TNF-α are mediated by nuclear factor-kB (NF-kB) activation[68] which stimulates various inflammatory pathway genes[73]. One widely accepted mechanism of TNF-α interference involves the phosphorylation of serine and threonine residues on the insulin receptor substrate (IRS) -1 protein that plays a crucial role in the intracellular insulin signaling pathway during insulin-mediated glucose uptake[62, 63]. Consequently, this IRS-1 phosphorylation obstructs the facilitation of glucose into the cell via GLUT-4 and increases blood glucose and insulin levels further propagating insulin resistance[63]. Interleukin (IL)-6 is another major factor secreted by adipocytes that leads to macrophage activation and further amplifies obesity-induced insulin resistance[72, 73, 75]. It functions similar to TNF-α and interferes with insulin signaling on the IRS level[65].

It has also been postulated that metabolic endotoxemia may play a role in the etiology of insulin resistance and impaired glucose tolerance via up-regulation of proinflammatory cytokines and mediators[76]. Lipopolysaccharide (LPS) or endotoxin is derived from the outer membrane of Gram-negative bacteria that is present in the gut. Typically, obese patients or those with HF diets have a greater colonization of this Gram-negative microbiota in the intestines[77]. In addition to an increase in these harmful bacteria, HF diets have also been shown to lower the number of good bacteria in the gut[78]. Circulating LPS also induces the expression of harsh cytokines such as TNF-α and IL-6 in skeletal muscle[79].

One proposed mechanism of systemic endotoxemia is an increase in intestinal permeability or gut barrier dysfunction[80-82] that is associated with a HF diet[83]. Farhadi *et al.* investigated this hypothesis *in vivo* and found a significant relationship between subject's increased gut leakiness and elevated serum endotoxin levels causing inflammation and abnormal lipid metabolism[80]. The study by Lambert *et al.* demonstrated that improved intestinal permeability was correlated to reduced plasma endotoxin levels in mice and ultimately prevented acute liver damage[84]. Maintaining the epithelial integrity is crucial to whole body homeostasis as it serves as a primary defense against the external environment. Normal intestinal barrier functioning involves selective absorption or transfer of bioactive nutrients or compounds while preventing the passage of harmful substances. However, an abnormal barrier function has less resilient selective control allowing for easier translocation of the damaging LPS across the intestinal epithelium[85]. This

defect is mostly likely the result of a number of complex interactions that disrupt the junction proteins that are responsible for the regulation of the endothelial membrane[86].

Once successfully across the membrane, the effects of LPS are largely due to interactions with the cell membrane protein, Toll-like receptor (TLR)-4. TLR-4 activates macrophages which stimulate NF-kB and subsequent expression of proinflammatory mediators that interfere with glucose metabolism and induce oxidative stress[76, 79]. Thus, increased induction of proinflammatory pathways further propagates diet-induced insulin resistance.

Obesity and T2D have largely been implicated with oxidative stress through various molecular mechanisms[87]. Oxidative stress is a state described as the body's inability to combat or neutralize an increase in the formation of harmful oxidants such as reactive oxygen species (ROS) or nitrogen species. Excessive accumulation of ROS has the ability to cause considerable damage to tissues, proteins, lipids, and DNA[87]. Furthermore, chronic oxidative stress may cause defects in insulin signaling and deterioration of pancreatic islet beta cells ultimately leading to apoptosis[88]. Increased adipose tissue mass and inflammation lead to altered mitochondrial lipid beta oxidation and an impaired oxidative capacity[65]. Elevated circulating FFA and glucose levels interfere with pancreatic beta cell function by inhibiting fatty acid and glucose oxidation in mitochondria thus impeding with ATP production[65, 72]. Hyperglycemia also enhances mitochondrial oxidative phosphorylation leading to the overproduction of superoxide, and decreased ATP production[88].

Insulin resistance and impaired glucose tolerance are associated with defects in both glucose and fatty acid metabolism in muscle, fat and liver tissues. However, the definite underlying mechanism cannot be resolved to one isolated event or phenomenon. Instead, the pathogenesis most likely involves various alterations and defects that manifest in multiple biochemical pathways, leading to abnormal genes expression and functioning.

Cocoa in the Prevention of Obesity, Insulin Resistance, and Impaired Glucose Tolerance. An extensive body of literature has demonstrated the various health-promoting capabilities of polyphenols. In the same respect, research specifically evaluating the beneficial attributes of polyphenols derived from materials such as GSE is also extensive. GSE-derived polyphenols have been shown to combat obesity and T2D by alleviating HF DIO, decreasing blood glucose and insulin resistance, and reducing the expression of obesity biomarkers[89-93]. In contrast, studies

of the anti-obesity and anti-diabetic effects of cocoa polyphenols are comparatively few. Refer to **Table 2.1** for previous studies of these effects in animal models. Diets supplemented with flavanol-rich cocoa or cocoa flavanols have been proven to significantly improve body weight (BW) gain[17, 21-23] and other biomarkers of obesity such as white adipose tissue mass[17, 22], total serum cholesterol[17, 18, 20, 23] and TAG levels[18, 20, 23] in various animal models of induced obesity and/or T2D. Multiple studies have also demonstrated the ability of diets rich in cocoa flavanols to reduce hyperglycemia as indicated by lower blood glucose levels in various animal models of induced obesity and/or T2D[16-21].

Despite these findings, much remains to be understood regarding the beneficial effects and mechanisms by which cocoa flavanols inhibit obesity and T2D. A major inconsistency among previous cocoa flavanol studies is the composition of the cocoa product used as a treatment. Depending on the starting material and extraction method, each cocoa product contained differing concentrations of EC, C, and oligomeric and polymeric CPCs. Although these varying cocoa products have demonstrated similar outcomes, these effects are reported at different magnitudes. Consequently, it remains to be determined how the mDP of cocoa flavanols correlates to efficacy in preventing the onset of obesity, insulin resistance and impaired glucose tolerance. One recent study has shown promising results in this area. Yamashita *et al.* orally administered individual doses (10 μm/kg*BW) of cocoa liquor-derived EC, PC-B1 (dimer), PC-C1 (trimer), and PC-A2 (tetramer) to ICR mice after 18h fasting and observed a significant increase in plasma insulin levels after 60 min without giving a glucose load in only the PC-A2 fed mice[30]. This study subsequently measured plasma GLP-1 levels in the PC-A2 treated mice and detected a significant increase in GLP-1 compared to the control[30]. These results suggest tetrameric PCs (and possibly larger PCs in general) may have greater potential to increase insulin secretion and regulate glucose metabolism compared to PCs with a lower DP.

Table 2.1. Review of Current Literature Exploring the Anti-Obesity and Anti-Diabetic Effects of Cocoa Flavanols in Animal Models.

Paper	Species	Model	Product Tested	Dose	Major Findings
Tomaru *et al.* 2007[16]	C57BL/KsJ Mice	db/db[a]	CLPr[b]	0.5% & 1% (w/w diet)	Both doses reduced resting blood glucose levels.
Yamashita *et al.* 2012[17]	C57BL/6 Mice	HF[c] diet (30% fat)	CLPr	0.5% & 2% (w/w diet)	Both doses reduced: • blood glucose levels after OGTT[d] • body weight • WAT[e] weight • total cholesterol
Matsui *et al.* 2005[22]	Wistar Rats	HF diet	Cocoa powder	12.5% (w/w diet)	Dose reduced: • body weight • mesenteric-WAT
Ruzaidi *et al.* 2005[18]	Wistar Rats	STZ[f]-induced diabetes	CE[g]	1, 2, & 3% (w/w diet)	Doses of 1 & 3% reduced: • glucose levels • total cholesterol All doses reduced TAG[h]
Jalil *et al.* 2008[19]	Sprague-Dawley Rats	STZ induced diabetes + HF diet (49% fat)	CE	600 mg/kg BW*d	Dose reduced: • blood glucose levels after OGTT • plasma FFA[i]
Jalil *et al.* 2009[20]	Sprague-Dawley Rats	STZ induced diabetes + HF diet (49% fat)	CE	600 mg/kg BW*d	Dose reduced: • blood glucose levels after OGTT • total cholesterol • LDL[j] cholesterol • TAG
Sanchez *et al.* 2010[21]	Zucker Fatty Rats	Standard diet (4% fat)	SCF[k]	5% (w/w diet)	Dose reduced: • body weight • plasma glucose levels • plasma insulin levels • plasma TAG

[a] Mice were induced with diabetes and obesity prior to study
[b] Cocoa liquor procyanidins
[c] High-fat diet
[d] Oral glucose tolerance test
[e] White adipose tissue
[f] Streptozotocin
[g] Cocoa extract
[h] Triglyceride
[i] Free fatty acid
[j] Low-density lipoprotein
[k] Soluble cocoa fiber

Very few studies evaluating the effects of chocolate and cocoa products in humans have also been done, but the studies that have been done have shown promising effects[94]. Grassi *et al.* demonstrated that a daily dose of dark chocolate containing 500 mg polyphenols decreased blood

pressure and improved insulin sensitivity after 15 days in healthy persons compared to those fed white chocolate (zero polyphenols)[13]. This same lab also observed a significant reduction in blood pressure and serum LDL cholesterol along with improved insulin sensitivity in hypertensive individuals after 15 days of dark chocolate consumption (88 mg flavanols per day)[14]. Additionally, impaired mitochondria function in the skeletal muscle is associated with insulin resistance and T2D[95]. Taub *et al.* reported an increase in biomarkers of skeletal muscle mitochondrial biogenesis in subjects with either T2D or stage II/III heart failure after consumption of dark chocolate (100 mg EC per day) for 3 months[15]. The lack of human data on the effects of cocoa polyphenols on obesity and T2D is partially due to relatively few animal studies, the largely unknown mechanisms of action of cocoa, and a lack of data regarding the activities of specific cocoa flavanols.

Previous studies give reason to believe that flavanols with different mDP's will exhibit significantly different biological activities. Karim *et al.* demonstrated a significant increase in endothelial nitric oxide synthase in rabbit aortic rings *in vitro* after treatment with CPC pentamer compared to monomer[27]. This same study also showed that CPC tetramers through decamers induced endothelium-dependent relaxation in rabbit aortic rings while monomers through trimers had no effect[27]. Kim *et al.* observed a significant increase of viability in HepG2 cells treated with a cytotoxic agent and treated with GSE-derived PC polymers compared to cells treated with an oligomeric fraction[28]. This same study also observed significantly greater inhibition of ROS generation in HepG2 cells treated with PC polymers compared to PC oligomers[28]. In another *in vitro* study, a treatment rich in PC polymers derived from pistachio nuts showed a significantly greater effect at lowering proinflammatory mediators (nitric oxide, TNF-α, prostaglandin E_2) from macrophages in RAW 264.7 macrophages compared to an oligomeric-rich PC fraction[29]. Aside from the study of Yamashita *et al.* discussed above[30], studies examining the impact of CPCs with distinct mDP *in vivo* are notably lacking.

Protective Effects of PC's From Dietary Sources Other Than Cocoa. PC's derived from dietary sources other than cocoa have proved to be powerful therapeutic agents towards the prevention of obesity and T2D and may serve as a potential insulin mimetic. The demonstrated health-promoting activities of these flavanols include, but are not limited to, ameliorating the deleterious effects of a HF diet, anti-hyperglycemic and anti-hyperinsulinemic agents, and

attenuating oxidative stress and systemic inflammation. In particular, the biological effects of GSE *in vivo* have been extensively studied and consistently yield favorable outcomes.

Although the underlying mechanisms have yet to be definitively elucidated, flavanols derived from various food matrices have prevented or ameliorated symptoms of metabolic disease associated with a HF diet. This is demonstrated in the study done by Ohyama *et al.* that administered a HF diet [30% fat (w/w)] supplemented with 1% (w/w) monomer-rich GSE to mice for 12 weeks. A diet supplemented with 1% monomer-rich GSE normalized mice BW compared to the low-fat (LF) control group [7% fat (w/w)] and significantly lowered BW compared to the HF control[90]. The anti-obesity activity of GSE-derived flavanols was further investigated by Charradi *et al.* who did not observe a significant increase in BW compared to LF control [5% fat (w/w)] in Wistar rats after supplementation of 500 mg/kg*BW GSE in a HF diet [39% fat (w/w)] for 6 weeks. Furthermore, the rats plasma TAG, phospholipids, and cholesterol levels were also significantly reduced compared to the HF control group after 12 weeks of GSE supplementation[93]. Although, daily supplementation of 25 mg/kg*BW GSE to Wistar rats for 10 days that had previously been on a cafeteria diet for 13 weeks did not significantly reduce mice BW compared to HF controls, GSE treatment did prevent dyslipidemia in the mice that is associated with a HF diet as measured by normalization of mice liver weight, TAG, and cholesterol levels[96].

In another study examining flavanol anti-obesity effects, *ad libitum* water containing 0.52% (w/v) highly polymeric PC's (mDP's = 108) derived from shell seeds of Japanese horse chestnut significantly reduced BW gains in mice fed a HF diet [35% (w/w)] for 19 weeks compared to the HF control group[97]. It is important to note that prominent changes in BW were absent in mice given the polymeric PC treatment compared to the HF control group up until week 9 of the study[97]. This observation suggests that polymeric flavanols may have a greater effect on obesity when administered in chronic doses compared to acute doses. Furthermore, *Klaus* et al. investigated the anti-obesity effects of an EGCG-rich extract derived from green tea both acutely and chronically in diet-induced obese mice[98]. In the acute study, mice on standard diet were orally given a daily dose of 500 mg/kg*BW EGCG extract in water for 3 days[98]. This treatment had no effect on mouse BW, body temperature, or energy expenditure. In the chronic study, mice were on a HF diet for 4 weeks prior to supplementation of the HF diet with either 0.5 or 1% (w/w) EGCG extract for another 4 weeks[98]. Both EGCG treatments significantly reduced

BW and body fat (g) gains compared to the HF control group in a dose dependent-manner. Plasma TAG levels were significantly reduced in only the 0.5% EGCG treatment group compared to HF control[98]. The effects of long-term exposure to green tea catechins on a diet-induced obese model were further explored by Murase *et al*.[99]. After 11 months of exposure to diets, mice fed a HF diet [30% (w/w)] supplemented with both 0.2 and 0.5% tea catechins had significantly lower weight gains, visceral fat accumulation, and liver fat accumulation in a dose-dependent manner compared to the HF control group[99]. Moreover, both green tea catechin treatments suppressed elevation of plasma insulin levels in a dose-dependent manner compared to the HF control[99].

In addition to the anti-obesity activities of flavanols, the anti-diabetic activities have also been evaluated. Hwang *et al.* investigated the effects of GSE in genetically modified mice with T2D[89]. After a daily oral administration of 50 mg/kg*BW GSE, a significant reduction in blood glucose levels began to occur in mice at 4 weeks compared to the control group and this significance steadily increased in magnitude throughout the 8 week study[89]. Moreover, 8 weeks of this GSE treatment effectively reduced mice HbA1c levels to 5.7% compared to the control group level of 9.3%[89]. HbA1c is a standard measurement used to represent a subject's blood glucose levels from the previous 2-3 months and is typically 3.5-5.5% in non-diabetics. In a similar study, diabetic mice were given a daily gavaged dose of 200 mg/kg*BW of an oligomer-rich PC extract derived from a cinnamon species for 4 weeks[100]. After 4 weeks of treatment, a glucose tolerance test (GTT) and an insulin tolerance test (ITT) were performed. The oligomer-rich treatment significantly reduced mice fasting blood glucose levels, as well as significantly improved mice glucose and insulin tolerances compared to controls[100]. In another study, Wistar rats were administered a high-fructose [63% (w/w)] diet (to induced insulin resistance), and supplemented with either 0.5 or 1% (w/w) GSE for 8 weeks[74]. Both GSE treatments significantly increased plasma adiponectin levels and reduced plasma TAG levels compared to the high-fructose control group. Furthermore, a high-fructose diet supplemented with 1% GSE significantly inhibited suppression of GLUT-4 protein expression and increased glycogen accumulation in skeletal muscle compared to the high-fructose control group and both GSE treatments significantly restored mRNA expression of adiponectin levels compared to the positive control[74]. Chronic HF and high-fructose diets can reduce tissues responsiveness to insulin and impair glucose metabolism by down-regulating the expression of insulin-stimulated

proteins and mRNA genes involved in insulin signaling. This data suggests flavanols can ameliorate insulin resistance by improving the expression of proteins and mRNA genes that are essential to maintaining glucose homeostasis. The acute effects of GSE on type-1 diabetes was investigated by Pinent *et al.* using streptozotocin (STZ)-induced diabetic Wistar rats[101]. An orally gavaged dose of 250 mg/kg*BW GSE significantly reduced blood glucose levels over 270 min compared to the control group in STZ-rats both in the fasted and non-fasted states. Furthermore, this same GSE dose worked synergistically with a low-dose of insulin to significantly reduce fasting blood glucose levels compared to a low-insulin dose with no GSE[101].

Montagut *et al.* sought to clarify the mechanisms of flavanols on insulin metabolism at the molecular level and found no significant decrease in glucose uptake in 3T3-L1 adipocytes treated with 100 mg/L GSE vs. 100 nm insulin[102]. Moreover, this same dose of GSE proved to phosphorylate key intracellular mediators that activate signaling pathways involved in glucose uptake to a similar extent as insulin in 3T3-L1 adipocytes[102]. Pinent *et al.* further investigated the acute antihyperglycemic activities of GSE through *in vitro* experimentation with insulin-sensitive cell lines[101]. GSE significantly increased glucose uptake in L6E9 myotubes treated with 14 mg/L GSE, and in a dose-dependent manner in 3T3-L1 adipocytes treated with 35, 70, 105 and 140 mg/L GSE[101].

It has been proposed that the ability of flavanols to reduce the postprandial sharp rise in blood glucose levels is due to interference of carbohydrate or fat digestion and absorption into the blood stream via inhibition of the digestive enzymes α-amylase, α-glycosidase, and pancreatic lipase. One study explored this claim by performing either an oral starch tolerance test or an oral GTT on female 6-week old mice on standard chow[97]. For the starch tolerance tests, mice were fasted for 16 h, orally injected with various concentrations (250-1500 mg/kg*BW) of highly polymeric PC's derived from the seed shells of Japanese horse chestnut followed by an oral dose of starch. A similar method was used with the oral GTT however, PC's were only administered at concentrations of either 750 or 1500 mg/kg*BW. Polymeric PC doses of 500-1500 mg/kg*BW significantly reduced elevated blood glucose levels after 30 min and 1 h of starch administration however, both PC doses failed to have a significant effect on glucose levels after glucose administration[97]. This data supports the claim that flavanols inhibit digestive enzymes that facilitate the digestion and absorption of carbohydrates that cause an abrupt rise in glucose levels rather than inhibition of glucose absorption. It has also been believed that

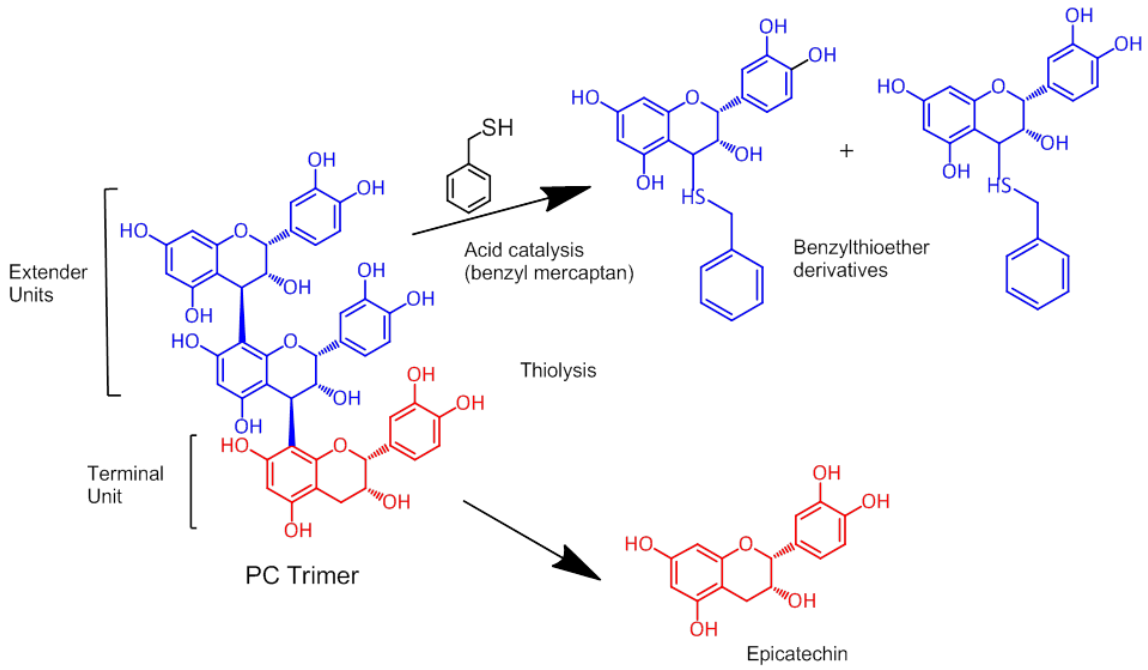
flavanols disturb fat digestion and absorption through inhibition of pancreatic lipase. Moreno *et al.* explored this claim *in vitro* using 3T3-L1 adipocytes and observed significant inhibition in lipase activity in cells treated with 0.1 and 1 mg/mL GSE in a dose-dependent manner compared to the control[103]. In the previously mentioned study by Klaus *et al.* involving the protective effects of green tea, both EGCG treatments significantly reduced mice food digestibility as indicated by an increase in feces energy content[98]. This observation also suggests that flavanols may act by altering carbohydrate and/or fat digestion and absorption in the gut.

A large amount of research has been done evaluating the protective effects of flavanols derived from dietary sources complemented by a wide range of proposed underlying modes of action. Most notably is the research done on GSE. Overall, *in vivo* supplementation of GSE in animals has been shown to alleviate DIO[90, 93, 104], reduce blood glucose levels[89, 101], improve , hyperinsulinemia[105] and mitochondrial functioning[106, 107], and modulate systemic inflammation brought about by DIO[91, 92]. It should be noted that although flavanols derived from dietary sources have consistently had positive biological effects in the literature, discrepancies still arise when attempting to compare outcomes across flavanol studies in the flavanol source and processing conditions, the animal model used, the dose administered, the route of flavanol administration [oral vs. intraperitoneal (i.p.)], and the length of flavanol exposure (acute vs. chronic).

Flavanol Separation and Characterization. There are various techniques used to evaluate the flavanol composition in a given sample. The Folin-Ciocalteu assay provides a colorimetric method of polyphenol analysis. With this method, the total polyphenol content is determined using a gallic acid standard curve. Consequently, the results are non-specific to the type of flavonoid present. For more accurate quantification or qualification results, chromatographic techniques are most often used.

Thiolysis offers a method to quantify the mDP or average chain length in a given sample of PCs through hydrolysis. Thiolysis involves an acid-catalyzed reaction in which the interflavan C-C bonds linking the flavanol monomers together are broken to release the terminal flavanol unit[39]. The nucleophilic benzyl mercaptan replaces this terminal unit with a benzylthioether adduct on the extender flavanol unit[108]. Consequently, this reaction allows the released flavanol

units to be quantified by reversed-phase chromatographic analysis[109]. **Figure 2.4** displays a schematic representation of this thiolysis reaction along with a brief sample mDP calculation.



Calculated mDP of PC trimer:

$$Calculated\ mDP\ (PCs\ only) = \frac{Net\ monomer + Net\ Derivative}{Net\ Monomer}$$

$$= \frac{1\ monomer + 2\ derivatives}{1\ monomer} = 3\ (trimer)$$

Figure 2.4. *Schematic representation of the thiolysis reaction; adapted from Meagher, L. P.; Lane, G.; Sivakumaran, S.; Tavendale, M. H.; Fraser, K., Characterization of condensed tannins from Lotus species by thiolytic degradation and electrospray mass spectrometry. Anim. Feed Sci. Technol. 2004, 117, 151-163 (with permission).*

Determination of a thiolysis product's mDP can be done via reverse-phase HPLC coupled with mass spectrometry (MS). Quantification of native monomers and PCs in an unknown sample can also be done using this system along with the respective monomer and PC standards. A large majority of studies have employed HPLC analysis for evaluating phenolic monomers or PCs derived from food matrices[5, 9, 10, 44]. HPLC is an efficient approach to resolving a solution containing multiple analytes of similar composition. HPLC involves injecting a liquid sample

into a column and passing a pressurized liquid mobile phase through the column, eluting the sample analytes at different times. Elution is based on the analytes ability to interact with the column which is determined by polarity. Various modes of HPLC are available, however reverse-phase HPLC is most commonly used with flavonoid compounds[110, 111]. Reverse-phase HPLC uses a hydrophobic stationary phase or column, and a hydrophilic mobile phase. Difficulties arise in analyzing samples with high mDP's due to a large variety of PCs and the increasing number of isomers that accompany more polymerized compounds[112]. Therefore, proper separation and detection of flavanols in a mixture is difficult, and sensitivity of the analysis method is crucial. Establishing the appropriate HPLC parameters and conditions is central to a clean elution and avoiding peak overlap.

Characterization of flavanols is greatly enhanced by coupling a HPLC system to a MS detector. Furthermore, quantification of compounds is most notably improved with tandem MS (MS-MS) detection. MS can provide information concerning both the quantity of a compound in a sample as well as its atomic structure[113]. The MS process involves first volatilization of the sample analytes and then converting the analytes into gas phase ions through ionization[113]. A mass analyser then sorts these ions using an electric and magnetic field allowing for quantification by a detector which then presents the raw data as the analytes mass-to-charge ratios[113].

Although it is not as specific as reverse-phase HPLC, separation of PCs by normal-phase HPLC is also commonly employed. Unlike reverse-phase, normal-phase HPLC uses an organic mobile phase and rather than separating specific PC compounds, it elutes PCs of the same DP[110, 112]. While this is an efficient method, it is unfavorable with highly polymerized samples. Clean isolation of compounds and the ability to produce distinct peaks decreases as the sample mDP increases[110]. Both reverse- and normal-phase HPLC have unreliable separation of DPs >12, however reverse-phase is the method of choice with DPs up to 10-12. Consequently, reverse-phase HPLC-MS/MS provides the best sensitivity and selectivity for separation and structural determination of cocoa monomers and PCs.

Conclusions

The need for agents possessing protective properties against the onset of obesity, insulin resistance and impaired glucose tolerance is evident from current incidence rates of obesity and T2D. Polyphenols make up a very broad category of aromatic compounds. Recently, results from studies involving the subgroup of polyphenols known as flavonoids, have warranted further investigation to their potential influence in the prevention and/or amelioration of various chronic and degenerative diseases. The exact mechanisms have yet to be determined by which flavanols can act on the body however, numerous routes have been postulated. These routes include but are not limited to partial restoration of glucose homeostasis via various alterations in insulin signaling pathways, inhibition or suppression of proinflammatory mediators, reduced circulating FFA and ROS, improvement in endothelial permeability, and amelioration of endotoxemia. Supplementation of flavanols derived from dietary sources have proven to partially restore or correct changes in glucose or lipid metabolism caused by a HF diet. Despite extensive research involving the health benefits of food polyphenols and specifically flavanol monomers and PCs, there still remains a large amount to be studied concerning the favorable effects of flavanols derived from cocoa on the onset of obesity, insulin resistance, and impaired glucose tolerance. Particularly, there is a lack of data regarding the impact of flavanols of different molecular weights (DP). The influence of flavanol chain length on the beneficial activities of flavanols is still largely unknown and should be explored further, as the complex nature of cocoa makes attributing activities to specific compounds difficult. Such studies are justified and necessary, but the difficulty in isolating sufficient quantities of these fractions has likely hindered research in this area. Furthermore, although multiple studies have evaluated the composition of cocoa proving it to be an abundant source of flavanols, inconsistencies arise between the source and concentration of the cocoa sample analyzed making it difficult to compare results and deciphering accuracy.

This study aimed to fractionate cocoa flavanols into three fractions with statistically different mDP's and then to evaluate the ability of each fraction to prevent the onset of obesity, insulin resistance and impaired glucose tolerance after chronic administration in mice fed a HF diet. Additionally, this study also aimed to determine the impact that different mDP's have on the extent of the inhibitory effect of cocoa flavanols in this model.

Chapter 3:

Cocoa Extraction and Fractionation

Introduction

Flavanols are one of the many subclasses of aromatic compounds that encompass the family of polyphenols known as flavonoids. Flavanols derived from food matrices such as grapes, wine, or tea have been shown to significantly ameliorate and/or improve various biological risk factors involved in the etiology of metabolic diseases. In addition, there have been multiple modes of action proposed by which these flavanols can act. Recent studies give reason to suspect that a flavanols size or DP may influence the extent to which it can positively affect the body. This study focuses specifically on the ability of PC's of different sizes derived from cocoa to act as anti-obesity or anti-diabetic agents in an animal model of HF feeding. In order to do so, sufficient quantities of CE and CPC fractions must be generated to complete a long-term feeding study. CE was extracted from commercial cocoa powder and fractionated into different flavanol fractions based on the average chain length or mDP of the cocoa constituents. Multiple types of analyses methods were needed to properly evaluate the mDP values and composition of the CE and individual CPC fractions as there is currently no standard method to definitely do so.

The *objective* of this chapter was as follows.

1) To produce a CE from commercial cocoa powder and to fractionate the CE into three significantly different molecular weights (a monomer-, oligomer- and polymer-rich fraction).

The *working hypothesis* of this chapter was as follows.

1) It was hypothesized that CE fractionation will result in three CPC fractions with statistically different mDP's (mDP of monomer-rich fraction < oligomer-rich fraction < polymer-rich fraction).

Materials and Methods

Standards. Catechin monomers (+)-C, (−)-EC, (−)-(EGC), (−)-ECG, and (−)-(EGCG) were obtained from Sigma (St. Louis, MO). PC dimer B_2 and trimer C_1 were obtained from ChromaDex (Irvine, CA). Purified PC dimer B_1, dimer B_2-gallate, dimer B_5, trimer T_2, tetramer A_2, and pentamers (DP5) through decamers (DP10) were obtained from Planta Analytica (Danbury, CT). Solvents for extraction, fractionation and normal-phase HPLC (hexane, acetone, acetic acid, MeOH, ethyl acetate, diethyl ether) were ACS grade or better and were obtained from Fisher (Pittsburg, PA) or VWR (Radnor, PA). Solvents and acids for UPLC-MS/MS (water, acetonitrile, and formic acid) were LC-MS grade (VWR).

Cocoa Flavanol Extraction. A flavanol-rich CE was produced following the solvent methods similar to Adamson *et al.*[44] and Robbins *et al.*[1]. The cocoa powder was first defatted to facilitate extraction of monomers and PCs. 100 g of non-alkalized Hershey cocoa powder (Hershey, PA) was then dispersed in 400 mL of hexane, sonicated for 10 min, stirred for 5 min and centrifuged (5 min, 20°C 5000 x *g*). The supernatant was discarded and the extraction was repeated 1-2 more times. The resulting defatted cocoa was then transferred to a large drying plate to allow residual hexane to evaporate at room temperature. The flavanols were then extracted from the defatted cocoa. The defatted cocoa was placed in a beaker with 400 mL of acetone:water:acetic acid (70:28:2) solution, sonicated for 10 min, stirred 5 min and centrifuged (5 min, 20°C 5000 x *g*). The supernatant was then deposited into a 500 mL RotaVap flask while the cocoa was scraped back into a beaker and underwent the previous steps with 400 mL 70:28:2 solution at least 1-2 more times. The acetone was then evaporated from the supernatant under vacuum by rotary evaporation (40-45°C). The remaining extract (predominantly water) was transferred into freeze dryer flasks and frozen for 20 min at -80°C. Once frozen, the extract was freeze dried for at least 2 days to remove any water. After freeze drying, the CE was crushed into a powder, weighed, and stored at −80°C. The average extraction yield from 100 g cocoa powder was ~12.8 g CE.

Cocoa Extract Fractionation. The CE was fractionated by SPE to produce three fractions with different mDP values (monomers, oligomers, and polymers) according to the method used by Sun *et al.*[114] with modifications. A tC_{18} Sep-Pak SPE column (20 cc, 5 g sorbent) (Waters,

Milford, MA) was attached on top of a C_{18} Sep-Pak SPE column (20 cc, 5 g sorbent). The columns were preconditioned with 10 mL MeOH followed by 10 mL distilled, deionized water (ddH_2O, pH 7.0) on a vacuum manifold. CE was dissolved in acetone: water: glacial acetic acid (70:28:2 v/v/v) to 0.1 g/mL and 1.5 mL of the CE solution was loaded onto the column. Highly polar compounds (primarily phenolic acids) were eluted with 10 mL ddH_2O and discarded. Monomers and oligomers were eluted together with 40 mL ethyl acetate, concentrated under vacuum by rotary evaporation, and set aside. The polymer fraction was then eluted from the columns with 40 mL MeOH, concentrated by rotary evaporation, dispersed in ddH_2O and freeze-dried as described in the extraction method until a dark brown powder remained. A new set of tC_{18} Sep-Pak + C_{18} Sep-Pak columns was then pre-conditioned, and the combined monomer/oligomer fraction (dissolved in 1.5 mL of the acetone: water: glacial acetic acid solution) was loaded onto the column. Monomers were eluted with 35 mL diethyl ether, concentrated by rotary evaporation, dispersed in ddH_2O and freeze-dried until a cream-colored powder remained. Oligomers were eluted with 40 mL MeOH, concentrated by rotary evaporation, dispersed in ddH_2O and freeze-dried until a light-brown powder remained. All three fractions were stored at −20°C for 2 d, weighed, and stored −80°C.

Folin-Ciocalteau to Quantify Total Polyphenols. The total The total polyphenol composition of the fractions was quantified by the Folin-Ciocalteau assay. CE and the three CPC fractions were diluted to 0.2 mg/mL in 40% ethanol. Samples (100 μL) were combined with 900 μL distilled ddH_2O and 2.5 mL 0.2 N Folin-Ciocalteau reagent (Sigma, St. Louis, MO) and vortexed. Sodium carbonate (2 mL, 7.5% w/v aqueous solution, Sigma) was added and the samples were vortexed. Samples were allowed to sit at room temperature for 2 h, and the absorbance was measured at 765 nm. Samples were analyzed in triplicate and quantified based on an external standard curve prepared with gallic acid standards (0-0.5 g/L) in 40% ethanol. Total polyphenol content was expressed as g of GAE/g of material.

Thiolysis to Estimate mDP. Thiolysis (see schematic, **Figure 2.4**) was employed to determine the average polymer chain length (mDP) that comprised each cocoa fraction. Thiolysis was carried out based on the method described by Guyot *et al.*[109] and Gu *et al.*[40] with modifications. The reaction substrates (CE or the monomer, oligomer, or polymer fractions) were first diluted to

0.5 mg/mL in MeOH. Then, 50 µL of the diluted reaction substrate, 50 µL HCl (3.3% w/v) and 100 µL benzyl mercaptan (5% w/v in MeOH) were combined. Controls (to estimate the monomer content in unthiolyzed samples) were prepared by combining 50 µL of diluted reaction substrate with 150 µL MeOH. Samples and controls were heated in a water bath (90°C for 5 min) and then cooled on ice for 5-10 min to stop the thiolysis reaction. Samples and controls were then prepared for UPLC/MS-MS analysis by combining 100 µL reaction product with 900 µL 0.1% formic acid in water/0.1% formic acid in acetonitrile (95:5) in an HPLC vial.

UPLC-MS/MS Method. Monomers (the PC terminal units) and benzylthioether derivatives (the PC extender units) from thiolysis reactions were quantified by UPLC-MS/MS. UPLC analysis was performed on Waters (Milford, MA) Acquity H-class separations module. A Waters Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 µm particle size) was used and maintained at 40°C. Binary gradient elution was carried out with 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B). Linear gradient elution was performed at a 0.6 mL/min solvent flow rate based on the following program: 95% A (0-0.5 min), 65% A (6.5 min), 20% A (7.5-8.6 min), 95% A (8.7–10.5 min). The sample temperature was kept at 10°C. UPLC effluent was analyzed by (−)-electrospray ionization (ESI) coupled to tandem mass spectrometry (MS/MS) on a Waters Acquity triple quadrupole (TQD) MS. Ionization was performed in (−) mode, with a capillary voltage of −4.25 kV, a cone voltage of 30.0 V, an extractor voltage of 3.0 V, and source and desolvation temperatures of 150 and 400°C respectively. Cone and desolvation gasses were N_2 with flows rates of 75 and 900 L/h respectively. MS/MS was performed using Ar as the collision gas at a flow rate of 0.25 mL/min in the collision cell. Data acquisition was carried out with MassLynx software (version 4.1, Waters). MS data collection was set to 10 points per peak with an average peak width of 6 s. The auto-dwell setting was used to automatically calculate dwell time based on an interscan delay time of 0.02 s. The Intellistart function of MassLynx was used to develop and optimize multi-reaction monitoring (MRM) parameters for each compound of interest. Compound solutions were directly infused into the ESI source (0.1 mg/mL in MeOH/0.1% formic acid at a flow rate of 50 µL/min) in combination with a background flow of 50% phase A/50% phase B at 0.6 mL min. Intellistart automatically selected the most abundant daughter ion, optimized the source cone voltage and MS/MS collision energy, and generated a single MRM transition for each compound. MRM was

performed on parent ions $[M-H]^-$ and signature daughter ions following collision-induced dissociation (CID). MRM mass span was 0.2 Da, the inter-channel delays and inter-scan times were both 1.0 sec. MRM and CID parameters for each compound of interest are listed in **Table 3.1**. All compound peaks were processed and quantified using the QuanLynx function of MassLynx software.

Table 3.1. MRM Settings for MRM Detection of Flavanol Monomers and Thiolyzed Monomer Derivatives by UPLC-MS/MS.

Compound	t_R[a] (min)	Molecular Weight (g mol^{-1})	Parent Ion $[M-H]^-$ (m/z)	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
C/EC	2.5-4.1	290.142	288.98	245.05	40	14
ECG	4.2-5.0	441.952	440.92	169.00	38	16
C/EC benzylthioether derivative	6.6-8.5	412.031	410.94	124.97	30	18
ECG benzylthioether derivative	6.8-8.4	563.824	563.05	287.06	38	16

[a]retention time

Standard Curve for Thiolysis and Data Analysis. Monomers and benzylthioether derivatives measured in the thiolysis reaction product by UPLC-MS/MS were both quantified using external monomer standards due to the lack of available authentic flavanol benzylthioether standards. The mDP was calculated from the concentration data produced by the UPLC-MS/MS method using the following equations:

$$net\ monomers = \Sigma\ monomers_{thiolyzed\ sample} - \Sigma\ monomers_{unthiolyzed\ control}$$

$$calculated\ mDP\ (DP\ \geq\ 2\ only) = \frac{net\ monomers + \Sigma\ derivatives_{thiolyzed\ sample}}{net\ nonomers}$$

To calibrate the thiolysis values (calculated mDP values) obtained by this calculation, a standard curve (**Figure 3.1)** relating actual mDP vs. calculated mDP (thiolysis value) was produced by thiolyzing known standards covering DP 2-10 by the method described above (n=5 replicates per compound) at concentrations of 0.1 mg/mL in MeOH (lower concentrations are used for standards vs. 0.5 mg/mL for extracts, as the standards represent higher concentrations of individual compounds). Least-squares regression analysis was used to determine the relationship between actual mDP and measured mDP (calculated by thiolysis).

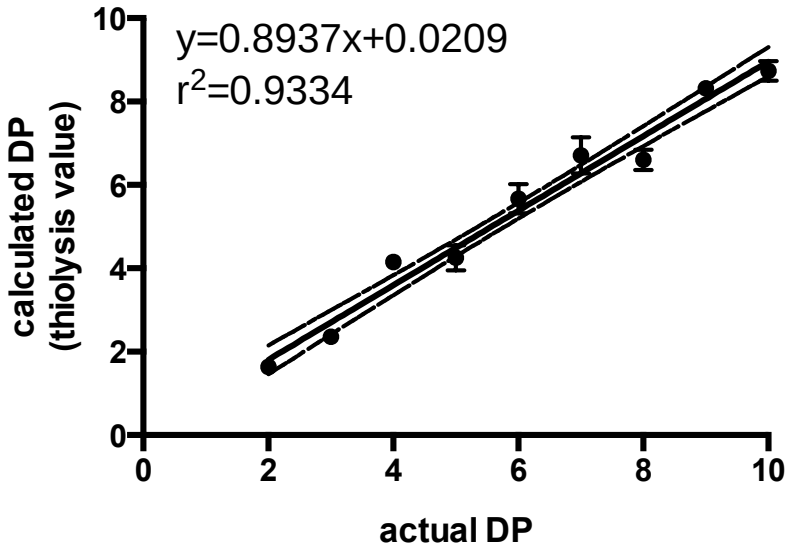


Figure 3.1. *Thiolysis standard curve relating the calculated mDP (thiolysis value) vs. actual mDP of authentic PC standards (plotted values are means ± SEM of n=5 replicates per compound). The least-squares regression line was plotted using each replicate value as an individual point. Dotted lines represent the 95% confidence interval of the regression line.*

Using this curve, actual mDP values were determined from the calculated mDP (thiolysis) values using the following equation:

$$actual\ mDP\ (DP \geq 2\ only) = \frac{calculated\ mDP - y\ intercept}{slope}$$

As monomer concentrations in the unthiolyzed sample are subtracted during the mDP calculation, the original mDP calculation only accounts for flavanol species with DP ≥ 2. To account for the monomers as well, the mDP was corrected by including the monomer concentrations in the unthiolyzed sample (with a defined DP of 1) in the mDP calculation as follows:

$$mDP\ (all\ flavanols) = \frac{\left[1 \times \Sigma\ \text{monomers}_{unthiolyzed\ control}\right] + \left[actual\ mDP\ (DP \geq 2\ only) \times\ net\ monomers\right]}{\Sigma\ \text{monomers}_{unthiolyzed\ control} + net\ monomers}$$

Normal-Phase HPLC Analysis. Cocoa fractionation was further evaluated by normal-phase HPLC profiling[115]. Analyses were performed on an Agilent Technologies (Santa Clara, CA) 1260 Infinity HPLC equipped with a solvent degasser, quaternary pump, an autosampler with temperature control, a thermostat column compartment, and a fluorescence detector. Separations were carried out using a Develosil Diol column (100 Å, 250 × 4.6 mm, 5 μm particle size) equipped with a Luna HILIC guard column (4 × 3.0 mm ID SecurityGuard cartridge and cartridge holder) (both from Phenomenex, Torrance, CA). The column temperature was 35°C. Binary gradient elution employing 2% acetic acid (v/v) in acetonitrile (phase A) and 2% acetic acid (v/v) and 3% ddH_2O (v/v) in MeOH (phase B) was performed at a flow rate of 1 mL/min. The gradient was as follows: 93% A at 0 min, 93% A at 3 min, 62.4% A at 60 min, 0.0% A at 63 min, 0.0% A at 70 min, 93.0% A at 76 min, 7.0% B at 0 min, 7.0% B at 3 min, 37.6% B at 60 min, 100.0% B at 63 min, 100.0% B at 70 min, and 7.0% B at 76 min. Fluorescence detector excitation and emission wavelengths were 230 nm and 321 nm, respectively. CE and cocoa fractions were prepared at 10 mg/mL in acetone: water: acetic acid (70:28:2, v/v/v) immediately prior to analysis. All the samples and standards were held at 5°C in the autosampler before injection. Injection volume was 5 μL. Mixtures of authentic standards consisting of monomers (DP 1: C, EC, ECG), PC oligomers (dimers-hexamers), and PC polymers (heptamers-decamers) were prepared and used as a reference for comparison of elution profiles.

Reverse-Phase UPLC-MS/MS Analysis. CE and cocoa fractions were analyzed by UPLC-MS/MS (*n*=3) to quantify individual monomer and PC species up to decamers. CE and cocoa fractions were prepared by diluting to 0.05 mg/mL in 0.1% formic acid in water/0.1% formic acid in acetonitrile (95:5). UPLC separations and MS/MS analyses were performed on the Acquity UPLC-TQD and UPLC HSS T3 column described above (2.1 mm × 100 mm, 1.8 μm particle size). Column temperature, sample temperature, mobile phases, flow rate, gradient, ESI parameters, MS/MS CID settings, data collection parameters, and Intellistart tuning of authentic standards to generate MRM functions were the same as described above. MRM parameters for monomers and PCs are listed in **Table 3.2**.

Table 3.2. MS/MS Settings for MRM Detection of Monomers and PCs[a].

Compound	t_R[b] (min)	MW (g mol^{-1})	[M – H]$^{-}$[c] (m/z)	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
PC dimer B_1	2.68	578.136	577.136	289.105	38	24
(−)-epigallocatechin	2.76	306.038	305.038	124.977	40	22
unknown dimer 1[d]	2.92	578.136	577.136	425.102	36	16
(±)-catechin	2.99	290.028	289.028	245.057	36	14
PC trimer T_2	3.09	866.218	865.218	289.102	36	48
unknown dimer 2[d]	3.29	578.136	577.136	425.102	36	16
PC dimer B_2	3.34	578.136	577.136	425.102	36	16
(−)-epicatechin	3.63	290.092	289.092	245.056	42	12
(−)-epigallocatechin gallate	3.67	458.038	457.038	168.982	34	16
PC trimer C_1	3.82	866.218	865.218	287.085	46	32
cinnamtannin tetramer A_2	3.97	1154.808	576.404	125.020	26	34
PC dimer B_2 gallate	3.99	730.164	729.164	407.129	42	32
PC octamers	4.04	2307.17	1152.58	125.17	48	68
unknown dimer 3[d]	4.07	578.136	577.136	425.102	36	16
PC pentamers	4.10	1442.820	720.410	125.022	26	44
PC hexamers	4.23	1731.038	864.519	125.020	32	56
PC nonamers	4.33	2586.36	864.12	125.17	28	46
PC heptamers	4.41	2018.80	1008.40	125.17	36	56
(−)-epicatechin gallate	4.60	442.076	441.076	168.968	38	18
PC decamers	4.60	2883.55	960.18	125.17	30	52
PC dimer B_5	4.64	578.136	577.136	289.107	30	26

[a]procyanidin
[b]retention time
[c]all MRMs used singly-charged parent ions except for cinnamtannin tetramer A_2, pentamers, hexamers, heptamers, octamers, which were doubly-charged ($[M - 2H]^{2-}$), and nonamers and decamers, which were triply-charged ($[M - 3H]^{3-}$)
[d]likely PC dimers B_3, B_4, and either B_6, B_7 or B_8

Statistical Analysis. Statistical significance of the mDP values obtained for the CPC fractions were tested by 1-way ANOVA with Fischer's LSD post-hoc test using GraphPad Prism v.6 (La Jolla, CA). Values were considered significantly different at the level of $P<0.05$. Outliers within each treatment were identified and removed using Dixon's Q-test at the same level however no outliers were detected across all tests performed. A sample size calculation was performed to determine the number of thiolysis replicates (n) needed to detect significant differences between the mDP of the individual CPC fractions. The n size was calculated based on the mDP values of the CPC fractions from preliminary data. The mDP of monomer, oligomer and polymer CPC fractions were 1.47±0.15, 3.53±0.11, and 6.85±0.12 (mean ± SD). The sample size (n=5/fraction) was calculated based on detecting the preliminary data obtained for monomer mDP compared to oligomer mDP as significantly different (α=0.05, β=30%, power=70%). The study was powered based on monomer and oligomer mDP values, as this pair of CPC fractions displayed the greater

standard deviation values compared to oligomer vs. polymer (and powering for monomer vs. polymer would likely not facilitate detection of monomer vs. oligomer and oligomer vs. polymer as significantly different).

Results

Extraction and Fractionation. The cocoa extraction method was performed repeatedly and resulted in the production of approximately 87.96 g CE. The extraction method yielded approximately 12 g CE from ~100 g cocoa powder. Each CE batch that was produced, was stored separately from one another. The fractionation method was then performed repeatedly using a total of approximately 14.7 g CE from 2 separate CE batches. The total amounts and yields of CE and cocoa fractions produced for this study are displayed in **Table 3.3**. An average of ~155 mg monomer, ~282 mg oligomer, and ~502 mg polymer were produced from each batch of 10 replicate fractionations (a total of 15 mL containing 1.5 g CE, or 1.5 mL containing 0.15 g CE per replicate). The monomer, oligomer, and polymer fractions produced from separate fractionation batches were combined to produce a single uniform lot of each fraction. Sufficient amounts of CE and individual CPC fractions were produced to account for the mouse diets across the total length of the animal study.

Table 3.3. Total amounts of CE and Individual Cocoa Fractions Produced for Mice Feed.

Fraction	Total Amount Obtained (g)	Yield (mg fraction/g extract)
CE	87.96	----
Monomer	1.42	78.23
Oligomer	2.68	181.21
Polymer	4.77	316.32

Totally Polyphenol Content and Thiolysis. The Folin-Ciocalteau method was employed to quantify the total polyphenol content of the CE and CPC fractions. As shown in **Figure 3.2**, the total polyphenol contents (on a w/w basis) of the CE and the CPC fractions were polymer > oligomer > monomer = CE. Thiolysis was performed on the CE and individual CPC fractions (n=5) to estimate the mDP of the constituent flavanols.

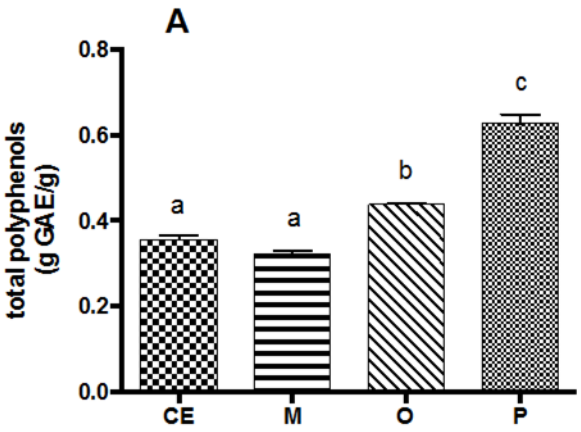


Figure 3.2. *Total polyphenols content [expressed as g gallic acid equivalents (GAE)/g of material] of CE and individual cocoa fractions as quantified by the Folin-Ciocalteau assay. Values are mean ± SEM of n=3 replicate analyses. Statistical comparisons were performed using one-way ANOVA with Fisher's LSD post-hoc test (P<0.05). Treatments with different superscripts are significantly different.*

Figure 3.3 illustrates mDP values for CE and each fraction. Refer to **Figure 3.1** for the standard curve used to determine actual mDP values from calculated mDP (thiolysis) values. The actual mDP values of the PCs (species with DP ≥ 2) for CE and cocoa monomer, oligomer, and polymer fractions were 4.38±0.16, −6.03±2.62, 2.80±0.09, and 3.81±0.07, respectively. A negative mDP value for the monomer fraction is due to the fact that thiolysis does not characterize monomer species present prior to thiolysis since these are subtracted prior to calculation of mDP in order to quantify monomers released from PCs during thiolysis. Therefore, thiolysis is poorly suited to characterize matrices that contain primarily monomeric flavanols. Consequently, a negative value is expected for this fraction and the actual mDP is treated as essentially zero. As shown in **Figure 3.3**, the mean mDP values of the PCs for each fraction were significantly different from each other (CE > polymer > oligomer > monomer). As thiolysis only measures mDP of dimers and above, an additional calculation was required to factor in the monomer content (DP =1). The actual mDP values accounting for both monomers and PCs for CE and cocoa monomer, oligomer, and polymer fractions were 2.99±0.05, 1.22±0.01, 2.73±0.09, and 3.78±0.072 respectively. As shown in **Figure 3.3**, the mean mDP values including monomers for each fraction were significantly different from each other (polymer > CE > oligomer > monomer). It should be noted that thiolysis is a highly qualitative

procedure that has not been fully validated for many species of PCs due to the lack of appropriate authentic standards. Therefore, thiolysis results are most likely approximations of the relative differences between fractions as opposed to representing absolute mDP values that account for all species present. The mDP value that accounts for the monomers is the best overall representation of the average size of the flavanols in each fraction. This measure indicates that the CE and three CPC fractions had significantly different average flavanol sizes from each other. The polymer fraction was enriched for the larger species and therefore had a larger mDP than CE, while the monomer and oligomer fractions were enriched for distinct subsets of the smaller species and had smaller mDP values compared to CE.

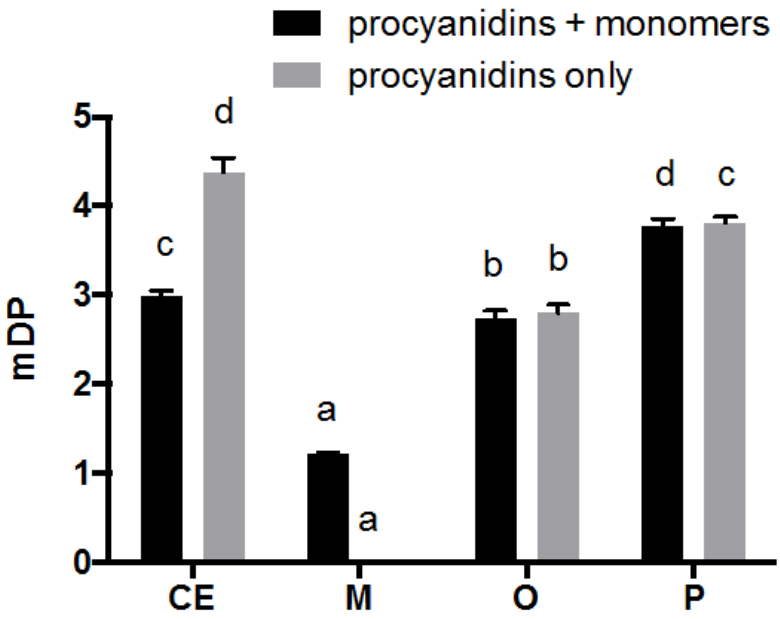


Figure 3.3. *Actual mDP values of CE and individual cocoa fractions as determined by thiolysis. Values are presented based on including procyanidins only (DP ≥ 2, i.e. the thiolysis value) or procyanidins + monomers (all flavanols) in the calculation. Values are mean ± SEM of n=5 replicate analyses. Statistical comparisons between fractions were performed separately for procyanidin values and values including both procyanidins + monomers values using a one-way ANOVA with Fisher's LSD post-hoc test (P<0.05). Treatments with different superscripts are significantly different within each value type. Note that for the monomer (M) fraction, the procyanidin-only value is presented as 0 as the thiolysis value for this fraction was negative due to the poor suitability of thiolysis for characterizing monomer-rich matrices. CE: cocoa extract, M: monomer-rich fraction, O: oligomer-rich fraction, P: polymer-rich fraction.*

Normal-Phase HPLC Analysis. In order to further assess the flavanol profiles of the 4 fractions, normal-phase HPLC with fluorescence detection was performed to resolve larger PC oligomers and some polymers. **Figure 3.4** displays the normal-phase HPLC flavanol profiles of CE and CPC fractions. Analysis of known standards showed that retention time roughly correlated to DP (larger species eluted later, top 3 chromatograms in **Figure 3.4**). CE appeared to have a wide range of both early- and late-eluting (i.e. small and large) flavanol species. By comparing relative peak heights within each chromatogram (bottom 4 chromatograms in **Figure 3.4**), the monomer, oligomer, and polymer fractions appeared to be enriched for early-, mid- and late-eluting flavanol species, respectively. It should be noted fluorescence chromatograms do not accurately represent relative PC composition, as relative response factors for fluorescence detection decrease rapidly with increasing DP [1]. Therefore, larger PCs are relatively underrepresented by simple comparison of peak heights of crude chromatograms.

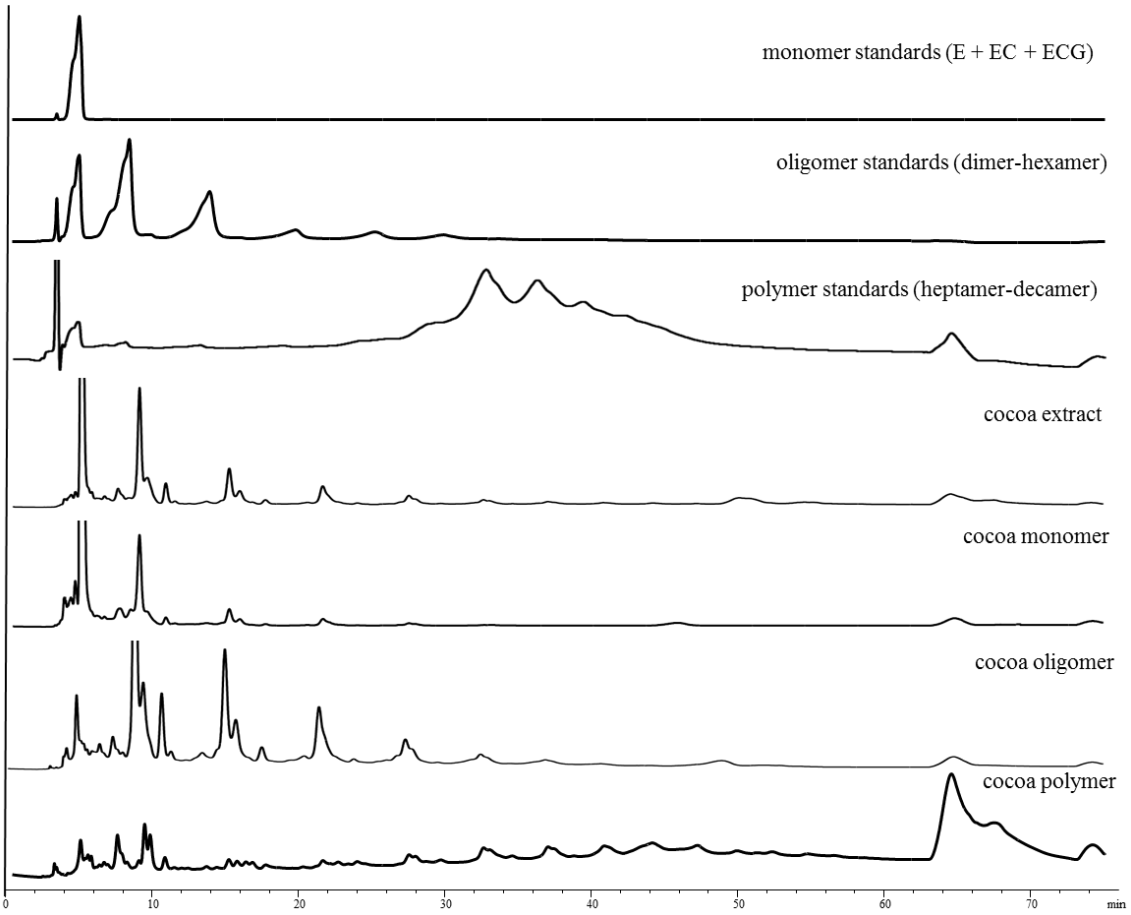


Figure 3.4. *Fluorescence profiles of normal-phase HPLC chromatograms of authentic standards [monomers, oligomers (DP 2-6), and polymers (DP 7-10)] and the profiles of the cocoa extract, monomer, oligomer, and polymer fractions used in the mouse diets. Note that the scale is different for each chromatogram (chromatograms were scaled/cropped to emphasize relative peak heights within each trace). The scale for each chromatogram in fluorescence units (LU) is as follows: monomer standards, 740 LU; oligomer standards, 72 LU; polymer standards, 46 LU; CE, 230 LU; monomer-rich fraction, 160 LU; oligomer-rich fraction, 150 LU; polymer-rich fraction, 40 LU.*

Reverse-Phase UPLC-MS/MS Analysis. Finally, reverse-phase UPLC-MS/MS analysis was performed to quantify native monomers through decamers in each fraction. **Table 3.4** displays the levels of native monomers and specific PCs that were detected in the CE and cocoa monomer, oligomer, and polymer fractions. As can be seen from this table, CE was composed of a wide variety of flavanol species, including large amounts of monomers, dimers, and PCs with DP 3-6 and 7-10. The monomer fraction was highly enriched for monomer species (5.3-fold increase) compared to CE, and was slightly enriched for dimers (1.8-fold increase) compared to CE, but was depleted for PCs of DP 3-6 and DP 7-10 (1.5-fold and 12.8-fold decrease,

respectively) compared to CE. The oligomer fraction was highly depleted of monomers (9.4-fold decrease) compared to CE, but was greatly enriched for dimers and PCs DP 3-6 (2.9-fold and 2.8-fold increase, respectively) compared to CE and was slightly enriched for DP 7-10 (1.5-fold increase) compared to CE. Finally, the polymer fraction was highly depleted for monomers, dimers and PCs DP 3-6 (84.4-fold, 24.4-fold, and 3.6-fold decrease, respectively) compared to CE and was slightly enriched for PCs DP 7-10 (1.3-fold increase) compared to CE. As the reverse-phase UPLC analysis only quantified PCs up to DP 10, the composition of larger PCs that likely make up a large portion of CE and the majority of the polymer fraction (see **Figure 3.4**) are not reflected in the total composition values in **Table 3.4**.

Table 3.4. Levels of Native Monomers and PCs in CE and Individual Cocoa Fractions.

Compound	Composition[ab] (mg compound/g dried fraction)			
	CE[c]	Monomer	Oligomer	Polymer
(±)-catechin	1.67±0.018 b	9.52±0.180 c	0.119±0.002 a	0.0129±0.003 a
(−)-epicatechin	5.90±0.064 b	30.6±0.530 c	0.667±0.015 a	0.0726±0.009 a
(−)-epigallocatechin	0.0151±0.000 b	0.0237±0.003 b	0.0199±0.001 b	0.0132±0.004 a
PC[d] dimer B_1	0.356±0.013 b	0.476±0.010 c	1.12±0.008 d	0.0135±0.001 a
PC dimer B_2	4.88±0.149 b	6.12±0.125 c	15.4±0.201 d	0.250 ±0.003 a
PC dimer B_5	3.10±0.030 b	7.52±0.246 c	7.32±0.206 c	0.0838±0.042 a
unknown PC dimer 1[e]	0.0360±0.006 b	0.0474±0.011 b	0.114±0.009 c	0 a
unknown PC dimer 2[e]	0.116±0.010 b	0.157±0.014 b	0.433±0.022 c	0 a
unknown PC dimer 3[e]	0 a	0.678±0.012 c	0.311±0.156 b	0 a
PC trimer C_1	8.12±0.197 c	6.31±0.276 b	26.3±0.427 d	0.932±0.033 a
cinnamtannin tetramer A_2	3.52±0.296 c	2.29±0.290 b	10.6±0.209 d	0.534±0.049 a
PC pentamers	3.69±0.261 b	1.73±0.094 a	9.77±0.438 c	1.40±0.073 a
PC hexamers	2.70±0.164 b	0 a	3.65±0.193 c	2.18±0.216 b
PC heptamers	2.14±0.116 b	0 a	1.93±0.187 b	2.72±0.255 c
PC octamers	0.736±0.076 c	0.196±0.012 b	1.88±0.060 d	0a
PC nonamers	1.78±0.292 b	0.142±0.142 a	3.03±0.186 c	1.87±0.123 b
PC decamers	0 a	0 a	0 a	3.68±1.228 a
Total monomers	7.59±0.081 b	40.2±0.682 c	0.806±0.013 a	0.0899±0.015 a
Total dimers	8.48±0.155 b	15.0±0.394 c	24.7±0.267 d	0.347±0.041 a
Total PC DP 3-6	18.0±0.763 c	10.3±0.422 b	50.4±1.08 d	5.04±0.231 a
Total PC DP 7-10	4.65±0.208 b	0.338±0.153 a	6.83±0.141 b	5.82±1.45 b

[a]Treatments with different letters for the same compound (or sum of compounds) are significantly different
[b]Data are reported as mean ± SEM from *n*=3 replicate analyses
[c]Cocoa extract
[d]Procyanidin
[e]Likely procyanidin dimers B_3, B_4, and either B_6, B_7 or B_8

Discussion

A CE was produced from cocoa powder through solvent extraction methods and then fractionated by SPE. The fractionation method generated three CPC fractions of statistically different sizes. Multiple types of analysis techniques were employed to evauluate the compositions of the CE and CPC fractions. The Folin-Ciocalteau data (**Figure 3.2**) shows that the CE and the CPC fractions were predominantly composed of polyphenols, as the GAE were >30%. Although there were differences in the total polyphenol contents as measured by GAE between the fractions, structural differences may account for some of these differences. After accounting for flavanol monomers, the thiolysis data (**Figure 3.3)** demonstrates the increase in average CPC chain length from monomer, oligomer, CE, and polymer fractions, respectively. However, given that the thiolysis method does not accurately measure monomers and has not been properly validated for estimation of flavanol chain length, these results should be seen more as approximations of mDP values rather than definitive quantitative data. Thus, of the two thiolysis values presented, the value accounting for monomers is the best representation of the true chain length of the CE and CPC fractions. Furthermore, as will be described in Chapter 4, attempting to formulate the mouse diets on an equivalent mol/kg*BW basis would have been problematic due to the fact that thiolysis is only an estimation of molecular weight, and there may be components that are not accounted for by the assays used to characterize the CE and cocoa fractions (normal-phase HPLC, which was qualitative, and UPLC, which only measured up to DP 10). Therefore, formulation of the mouse diets on a mg/kg*BW basis was determined to be the preferred approach.

Comparison of the chromatograms of the CE and CPC fractions to the chromatograms of known PC standard generated via normal-phase HPLC (**Figure 3.4**) provides evidence of the flavanol profiles present in each fraction. Retention time is correlated to PC size as the larger PCs were retained longer and eluted last. However, this method most likely underrepresents larger sized flavanols. Quantification of the native monomers through decamers present in CE and CPC fractions determined via reverse-phase HPLC analysis (**Table 3.4**) further supported the claim of each fraction being primarily enriched with its respected monomers or PC species. It should be noted that **Table 3.4** only reflects quantities of flavanol compounds for which authentic standards were available. Taken together, four separate measures of CPC composition

provide evidence to support that the fractions designated as “monomer”, “oligomer” and “polymer” were indeed relatively enriched for flavanol monomers, oligomers, and polymers, respectively, compared to CE and compared to each other.

Conclusions

The objective of this chapter was to produce a CE from commercially available cocoa powder and then further fractionate this CE into three flavanol fractions with significantly different mDP's. The extraction and fractionation methods employed in the study were capable of producing gram quantities of flavanol fractions from complex matrices for long-term feeding studies, thus overcoming a major limitation in experimental design. Furthermore, the fractionation method also successfully produced three statistically distinct CPC fractions (a monomer-, oligomer-, and polymer-rich fraction). The compositions of the CE and individual CPC fractions were evaluated using the Folin-Ciocalteau assay, an enzyme-catalyzed thiolysis reaction, normal-phase HPLC, and reverse-Phase UPLC. Each method of analysis demonstrated a significant difference in flavanol composition between CE, monomer-, oligomer-, and polymer-rich fraction thus the objective of this chapter was successfully achieved.

Chapter 4:

Animal Study

Introduction

Previous studies have demonstrated the powerful health benefits of flavanols derived from dietary sources on the body however few studies have investigated these claims in regards to cocoa flavanols. Even fewer are studies that have sought to define the structure-function relationship of these flavanols on the body. In this chapter, a mouse model was used in which lean mice were fed a HF diet containing 60% kcal from fat to induce obesity, insulin resistance, and impaired glucose tolerance[116, 117]. The CE and individual CPC fractions (monomer, oligomer, and polymer) that were generated in the previous chapter were incorporated into the HF diet, and the onset of obesity and T2D biomarkers were compared to a negative control (LF diet only), and a positive control (HF diet only). **Figure 4.1** displays the schematic representation of this mouse study.

The *objectives* of this chapter were as follows.

1) Determine if CE and individual CPC fractions prevent the onset of obesity, insulin resistance and impaired glucose tolerance in a mouse model of HF feeding.

2) Determine the impact that mDP has on the anti-obesity and anti-diabetic activities of CPC fractions.

The *working hypotheses* of this chapter were as follows.

1) Chronic administration of CE and CPC fractions will significantly prevent the development of obesity, insulin resistance and impaired glucose tolerance in mice fed a HF diet.

2) The extent to which CPC fractions will prevent the onset of obesity, insulin resistance and impaired glucose tolerance will be directly correlated to each fraction's mDP, i.e. fractions with greater mDP will exert greater inhibition.

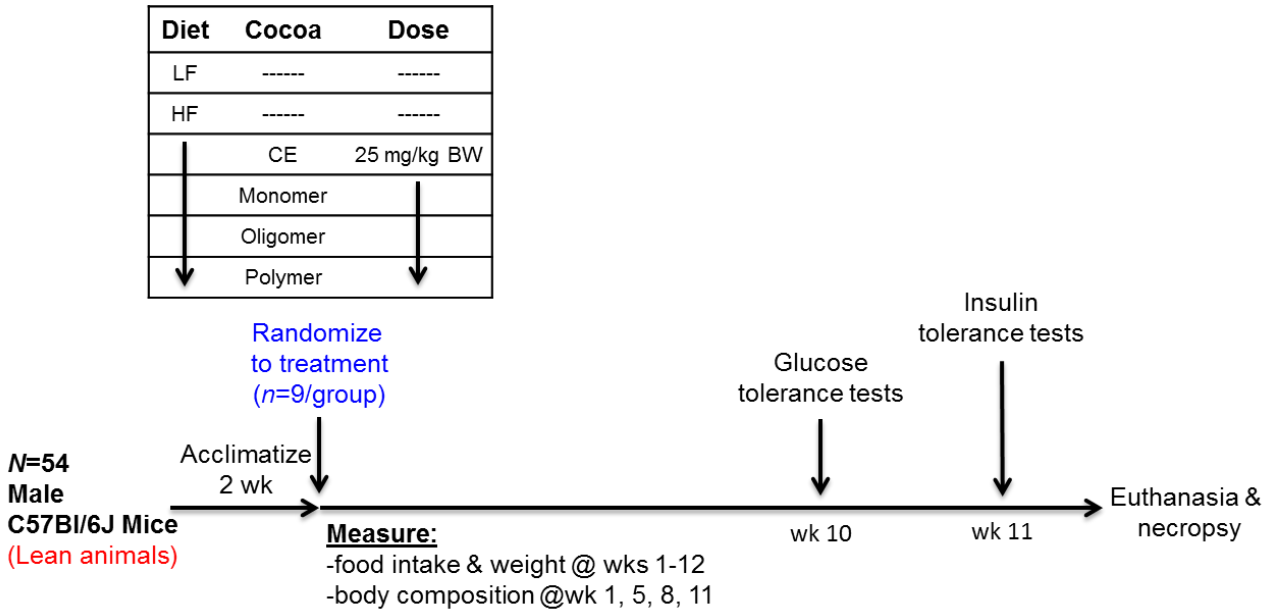


Figure 4.1. *Mouse study schematic diagram.*

Materials and Methods

Animals. Animal experiments were approved by the Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University (protocol 13-099-FST). Male C57BL/6J mice (*N*=54, ~25 g) were obtained from Jackson (Bar Harbor, ME). Following arrival, mice were maintained on standard rodent chow for 2 weeks under standard housing conditions (3 mice/cage, 12 h light/dark cycle, 30-70% relative humidity, 20-26 °C) to acclimatize to the vivarium environment. To reduce coprophagy, bedding was changed twice per week throughout the study. Mice were provided food and water *ad libitum* unless otherwise specified.

Diets and Treatments. Following acclimatization, mice were assigned to one of six diet/treatment combinations displayed in **Table 4.1** (*n*=9/group, 3 mice/cage).

Table 4.1. Description of Mouse Diets and Treatment Groups.

Abbreviation	Diet	Treatment	*N*
LF	LF	LF control	9
HF	HF	HF control	9
HF-CE	HF	Cocoa extract	9
HF-M	HF	CPC monomer	9
HF-O	HF	CPC oligomer	9
HF-P	HF	CPC polymer	9

Animals were fed one of two diets from the diet-induced obesity (DIO) model series (Research Diets, Inc., New Brunswick, NJ): a LF diet (Research Diets D12450J) with 10% kcal from fat, or a HF diet (Research Diets D12492) with 60% kcal from fat. Both diets contain equal amounts of sucrose. LF and HF diet formulas are displayed in **Table 4.2.**

Table 4.2. Composition of Basal Mouse Diets from the Research Diets Diet-Induced Obesity (DIO) Model Series.

Component	LF Diet[a]		HF Diet[bc]	
	g%	kcal%	g%	kcal%
Protein	19.2	20.0	26.2	20.0
Carbohydrate	67.3	70.0	26.3	20.0
Fat	4.3	10.0	34.0	60.0
total		100.0		100.0
kcal/g	3.9		5.2	
Ingredient	**g**	**kcal**	**g**	**kcal**
casein, 80 mesh	200.0	800.0	200.0	800.0
L-cystine	3.0	12.0	3.0	12.0
corn starch	506.2	2024.8	0.0	0.0
maltodextrin 10	125.0	500.0	125.0	500.0
Sucrose	68.8	275.2	68.8	275.2
cellulose, BW200	50.0	0.0	50.0	0.0
soybean oil	25.0	225.0	25.0	225.0
lard[d]	20.0	180.0	245.0	2205.0
mineral mix s10026	10.0	0.0	10.0	0.0
dicalcium phosphate	13.0	0.0	13.0	0.0
calcium carbonate	5.5	0.0	5.5	0.0
potassium citrate, 1 H20	16.5	0.0	16.5	0.0
vitamin mix v10001	10.0	40.0	10.0	40.0
choline bitartrate	2.0	0.0	2.0	0.0
FD&C yellow dye #5	0.0	0.0	0.0	0.0
FD&C blue dye #1	0.0	0.0	0.1	0.0
Total	1055.1	4057.0	773.9	4057.0

[a]Research Diets D12450J
[b]Research Diets D12492
[c]Cocoa extract or the monomer-, oligomer-, or polymer-rich fractions were added to the HF diet at 262.53 mg/Kg
[d]Lard contributed 72 mg cholesterol/100 g lard. The cholesterol contents of the LF and HF diets were 51.6 and 279.6 mg/kg, respectively

Each diet/treatment combination (**Table 4.1**) was produced by thoroughly mixing the appropriate combination of HF diet with the corresponding CE or cocoa fraction using a standard kitchen blender. Diets were maintained at −20°C throughout the study to prevent degradation of lipids or cocoa compounds. In order to maintain freshness and prevent degradation of fat or cocoa compounds, food was replaced twice per week for all cages. Animals were maintained on diets for 12 wk. The doses of CE or CPC fractions (mg/kg mouse BW) are listed in **Table 4.3**.

The CE and CPC fractions were incorporated into the animal diets based on estimated daily food consumption to deliver the dose listed in **Table 4.3**.

Table 4.3. Composition of Experimental High-Fat Diet Containing Either CE or Individual Cocoa Fractions.

Diet	Total Dose (mg/kg*BW/d)	Food Dose[a] (mg CE/kg food)	Food Formulation (mg/3 kg diet)
HF[b]	25	262.53	787.59

[a]Based on an estimated food intake of 95.23 g food/kg*BW/d from previous studies lab studies.
[b]High-fat.

Although previous cocoa studies have administered diets supplemented with flavanols greater than the chosen dose of 25 mg/kg*BW/d (**Table 2.1**), the cocoa treatments of this study are highly concentrated and were administered over a longer period of time. Additionally, data from a previous study indicates that 10 mg GSE/kg*BW/d is a sufficient and effective dose of compounds similar to the cocoa oligomer fraction to improve glucose tolerance in this model system of HF feeding (**Figure 4.2**). Furthermore, higher doses (50 and 100 mg/kg*BW/d) were not as effective at improving glucose tolerance. Despite the similarity in flavanol composition between GSE and cocoa oligomer fractions, a dose of cocoa (25 mg/kg*BW/d) was used that is 2.5 times as great as the effective GSE dose in order to account for the broader range of compounds that make up the oligomer fraction and the greater % fat in the proposed study (60% vs. 45%).

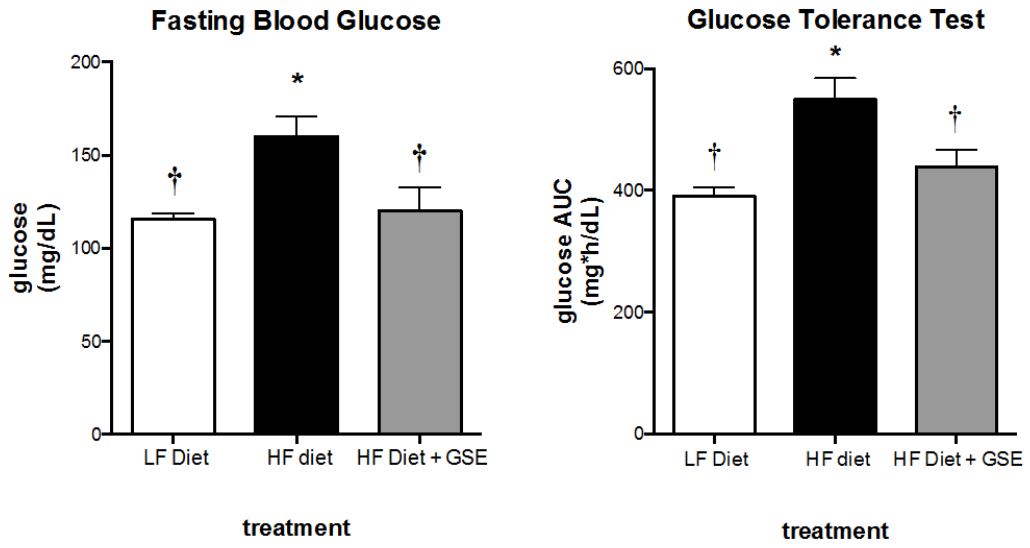


Figure 4.2. *Preliminary study done with C57BL/6 mice (n=8/treatment). LF diet (10% kcal fat), HF diet (45% kcal fat), HF diet + GSE (10 mg GSE/kg*BW/d). GTT was done as described previously at 14 weeks. Statistical analysis was done using Student's t test (α=0.05), *significantly different from LF control diet, †significantly different from HF control diet.*

Food and Calorie Intake. Animals were allowed food and water *ad libitum* throughout the study and were not pair-fed. Food intake for each cage was measured twice per week by weighing the food provided to the cage and then weighing the leftover food prior to replacing with fresh food. Food intake for each cage was calculated as the difference between fresh food and leftover food weights, and then divided by the number of animals per cage. Calorie intake for each cage was also calculated using the food intake data and the kcal/g food present in the LF and HF diets (**Table 4.2**).

Body Weight and Body Composition. Mouse BW was measured weekly. Mice were weighed individually using an empty cage lid placed on a tarred lab balance. Mouse body composition (g and % of fat, lean, and free fluid mass) was measured during weeks 1, 5, 8, and 11 of the study. Body composition analysis was performed in duplicate using a Bruker LF90 NMR analyzer (Billerica, MA) as described in the manufacturer's instructions.

Glucose Tolerance Test and Insulin Tolerance Test. GTTs were performed during week 10. Mice were fasted for 12 h (overnight) prior to GTTs. Mice were then administered glucose

(USP-grade, Hospira, Inc., Lake Forest, IL) via i.p. injection (1 g glucose/kg*BW in saline). Blood was collected from the tail at 0 min (prior to glucose administration) and at 30, 60, 90 and 120 min following glucose administration. For blood collection, the tail tip was removed with sharp surgical scissors, and a droplet of blood was obtained for glucose measurement. Glucose was measured using One Touch Ultra Blue test strips (LifeScan, Inc., Milpitas, CA) and a standard glucometer. During week 11, mice underwent ITTs. Mice were fasted for 4 h prior to ITTs, and then injected with insulin i.p. (Humulin R, Cardinal Health, Dublin, OH, 0.65 U insulin/kg*BW in saline,) and a droplet of blood was expressed from the tail at 0 min (prior to insulin administration) and after 0, 15, 30, 45, 60 min. Tail blood collection and blood glucose measurement were performed as described above for GTT.

Euthanasia and Necropsy. At the end of week 12, mice were fasted overnight and euthanized by CO_2 (following AVMA Guidelines on Euthanasia) followed by bilateral pneumothorax. Blood was immediately collected by cardiac puncture (with 1cc tuberculin syringes and ½" 26 gauge needles), clotted for 30 min at room temperature in BD Vacutainer® serum separation tubes with Clot Activator. Clotted blood was centrifuged (15 min, 4°C, 1,500 x *g*), and the resulting serum was stored at −80°C.

Fasting Serum Insulin and Endotoxin. Insulin levels in fasting serum samples collected immediately following euthanasia were measured using a murine insulin ELISA assay kit (EZRMI-13K, Millipore, Billerica, MA) in duplicate according to manufacturer's instructions. Endotoxin levels in the serum were determined using PyroGene™ Recombinant Factor C Endotoxin Detection fluorescence assay kits (Lonza Walkersville, Inc., Walkersville, MD) [118]. Limulus Amebocyte Lysate (LAL) assay plates, LAL reagent water, and pyrogen-free tubes (all from Lonza) were employed to minimize exogenous endotoxin contamination. Assays were performed in triplicate on 100 µL diluted serum (50 µL serum + 50 µL LAL water). Fluorescence was quantified using a BioTek Synergy 2 plate reader (Winooski, VT), and data were processed using Gen 5 software (v1.08, BioTek).

Statistical Analysis. The sample size for each treatment group was calculated based on weight gains observed in a previous study of C57BL/6 mice fed a diet containing 60% kcal from fat.

Based on average final weights of 38.69±4.61 g following 10 weeks of feeding (compared to average initial weights of 21.50±1.51 g), the sample size (n=9/group) was calculated based on detecting a 15% decrease in final weight for a theoretical treatment group compared to a 60% fat control group as significantly different (α=0.05, β=20%, power=80%). The study was powered based on weight gain, as obesity is a primary endpoint of the study and also based on previous lab experience that revealed the effects on weight gains are typically smaller than effects on GTT/ITT outcomes. Additionally, the study was conservatively powered (to detect a small difference in weight, 15%) to account for factors that would decrease power such as group housing and animal loss due to illness or fighting. Dixon's Q-test (α<0.05) was performed to identify outliers however only one was identified in the HF-M treatment group and was removed during week 10 of the study. One-way ANOVA was performed to determine overall significance of treatment effects (significance defined as P<0.05). If significance of treatments effects was detected with the ANOVA, a Fisher's LSD post-hoc test was then performed to compare all treatment means (P<0.05).

Results

Food and Calorie Intake. Food intake of each cage was monitored throughout the study. Intake data for weeks 1-2 were likely influenced by adjustment from the standard rodent chow to the semi-purified experimental diets, and fasting required for GTTs and ITTs influenced data from weeks 10-11. Subsequently, food intake data from weeks 3-9 were considered the most representative of the mice food intake throughout the 12 week study. Calculated food intakes at week 3, 6, and 9 of the feeding study are presented in **Figure 4.3**. The average weekly food intake per animal was consistent across all groups in the HF diet (one-way ANOVA indicated no overall effect of treatment on food intake within the HF diet). This supports the conclusion that supplementation of the HF diet with CE or cocoa fractions did not affect food intake, which excludes reduced food intake as the mechanism by which cocoa fractions modulated weight gain, obesity, or glucose homeostasis in this experiment. The differences in measured food intake between the LF and HF diets may be due to differences in caloric density[119] (**Table 4.2**) and/or differences in consistency (the LF diet was a powder whereas the HF diet was mealy/doughy) which may have affected diet wasting due to the ability of the mice to remove feed from the feed cups without eating it. Lack of apparent increases in food intake with weight gain may be due to the inherent difficulties in accurately measuring intake of powdered diets.

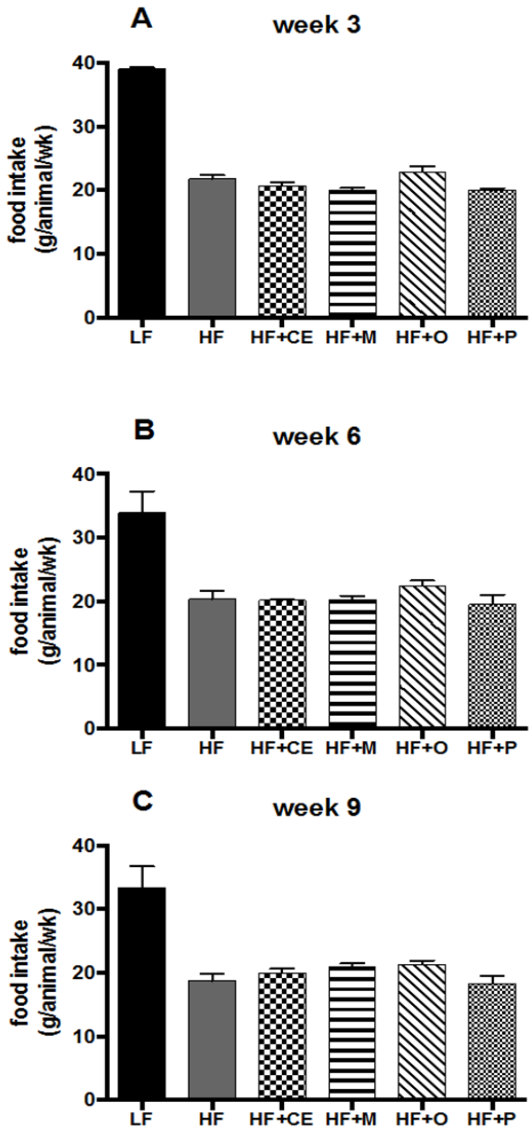


Figure 4.3. *Calculated food intake values at weeks 3 (***A***), 6 (***B***), and 9 (***C***) of the feeding study. Data shown are mean ± SEM of n=3 replicate cages/treatment (n=3 mice/cage). One-way ANOVA indicated no overall effect of dietary treatment on food intake (P=0.1439, 0.0792, and 0.0541 for overall diet effect at 3, 6, and 9 week, respectively). Therefore, post-hoc test for significance between treatment means were not performed. LF: low-fat control diet, HF: high-fat control diet, HF+M: high-fat control diet + monomer-rich fraction, HF+O: high-fat control diet + oligomer-rich fraction, HF+P: high-fat control diet + polymer-rich fraction.*

The average calorie intakes of the mice during weeks 3, 6, and 9 of the feeding study are displayed in **Figure 4.4**. Mice on the LF control diet consumed significantly more calories

compared to all other diets during week 3 (**Figure 4.4A**). However, the LF group consumed statistically the same amount of calories as the HF+O group during weeks 6 (**Figure 4.4B**) and 9 (**Figure 4.4C**).

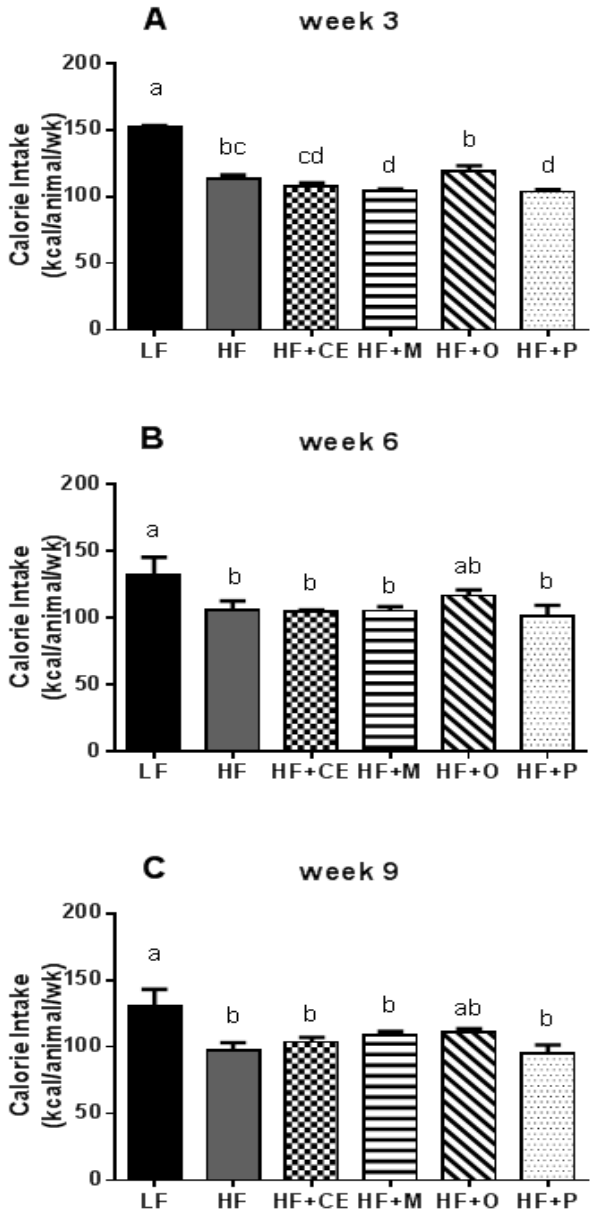


Figure 4.4. *Calculated calorie intake values at weeks 3 (***A***), 6 (***B***), and 9 (***C***) of the feeding study. Data shown are mean ± SEM of n=3 replicate cages/treatment (n=3 mice/cage). Significance between treatments was determined with a one-way ANOVA and Fisher's LSD post-hoc test (P<0.05). Treatments with different superscripts are significantly different. LF: low-fat control diet, HF: high-fat control diet, HF+M: high-fat control diet + monomer-rich fraction, HF+O: high-fat control diet + oligomer-rich fraction, HF+P: high-fat control diet + polymer-rich fraction.*

Weight Gain and Body Composition. Weight gains during the feeding study are displayed in **Figure 4.5A**. 12 weeks of HF (60% kcal from fat) feeding caused a significantly greater weight gain compared to the LF (10% kcal from fat) control group (final weights were 172±6 vs. 142±4% of initial weight, respectively). Mice fed HF diets supplemented with cocoa monomers and oligomers did not gain a significantly different amount of weight compared to mice fed a LF control diet (157±5, 152±7 and142±4% of initial weight, respectively). However, within the HF diet, only the HF + oligomer group was significantly different from the HF control. Changes in fat mass during the feeding study are displayed in **Figure 4.5B.** Mice from the HF control group gained significantly more fat mass than did mice in the LF control group (8.08±0.72 vs. 3.26±0.72 g, respectively). Supplementation of the HF diet with cocoa oligomers inhibited a significant increase in fat mass compared to the LF control (5.19±1.16 vs. 3.26±0.72 g, respectively), whereas supplementation of the HF diet with CE, monomers and polymers did not significantly inhibit increases in fat mass compared to the LF control. Within the HF diet, supplementation with cocoa oligomers significantly inhibited increases in fat mass compared to CE (5.19±1.16 vs. 7.88±1.68 g, respectively) but not compared to monomers or polymers.

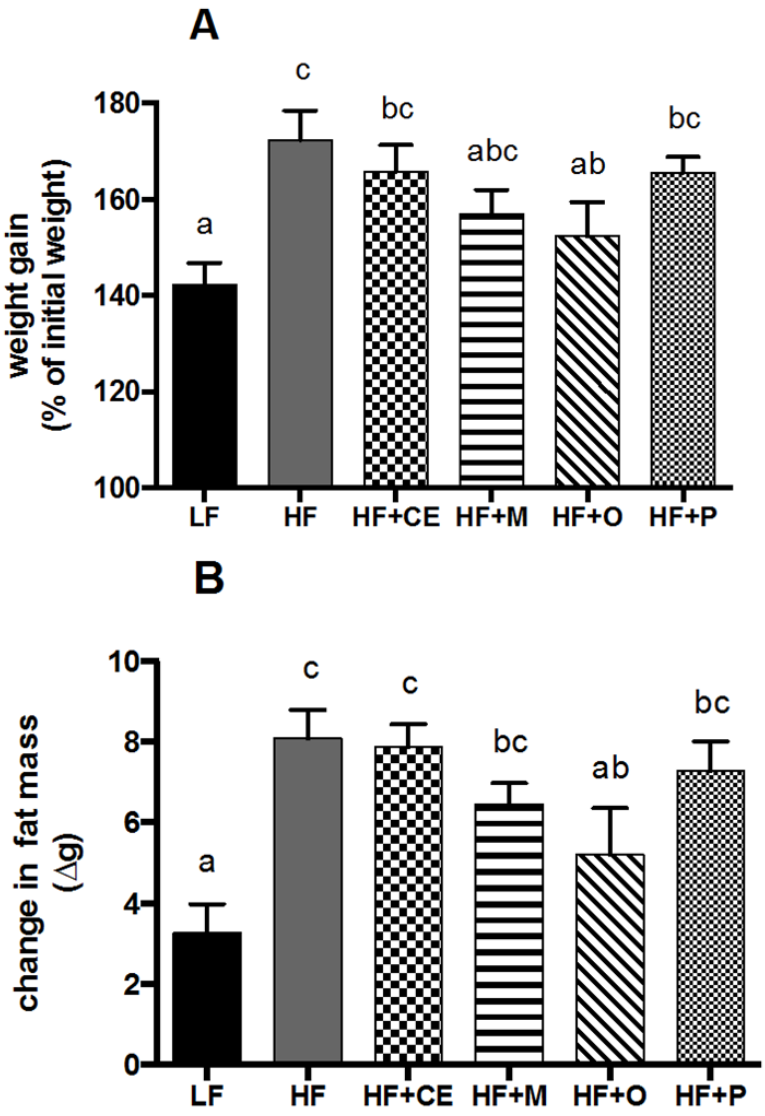


Figure 4.5A. *Weight gains (final weights expressed as a percentage of initial weight) after 12 weeks of feeding.* **B.** *Changes in total body fat mass (difference between final and initial fat masses) after 11 weeks of feeding. Data shown are mean ± SEM of n=9 animals/treatment (n=8 for the HF+M group due to loss of one mouse from this group during the feeding study). Significance between treatments was determined with a one-way ANOVA and Fisher's LSD post-hoc test (P<0.05). Treatments with different superscripts are significantly different. LF: low-fat control diet, HF: high-fat control diet, HF+M: high-fat control diet + monomer-rich fraction, HF+O: high-fat control diet + oligomer-rich fraction, HF+P: high-fat control diet + polymer-rich fraction.*

Glucose and Insulin Tolerance. 12 h fasting blood glucose levels at week 10 of the feeding study are displayed in **Figure 4.6A**. Mice fed a HF control diet had significantly elevated 12 h fasting glucose levels compared to mice in the LF control group (180±16 vs. 130±12 mg/dL, respectively). The 12 h fasting glucose levels of mice from the HF + CE and HF + monomer groups (175±7 and 174±6 mg/dL, respectively) were also significantly elevated compared to the LF control group. Supplementation of the HF diets with cocoa oligomers and polymers resulted in intermediate 12 h fasting glucose levels (158±15 and 150±9 mg/dL, respectively) that were not statistically different from either the LF or HF control groups. **Figure 4.6B** displays the GTT data as measured by the area under the curve (AUC) of the blood glucose levels over time following i.p. glucose administration. The HF control exhibited significantly higher AUC (i.e. impaired glucose tolerance) compared to a LF diet (34,400±2,500 vs. 22,000±1,400 mg*min/dL, respectively). Supplementation of the HF diet with oligomers resulted in an intermediate GTT AUC (27,800±1,500 mg*min/dL) that was significantly lower than the HF control but also significantly higher than the LF control. Supplementation of the HF diet with CE, monomers, and polymers did not lower GTT AUC compared to the HF control.

4 h fasting blood glucose levels at week 11 of the feeding study are shown in **Figure 4.6C**. Mice fed the HF control diet had significantly elevated 4 h fasting blood glucose levels compared to mice fed the LF control diet (184±7 vs. 145±9 mg/dL, respectively). Supplementation of the HF diet with cocoa oligomers inhibited increased 4 h fasting blood glucose compared to LF control (147±10 vs. 145±9 mg/dL, respectively). Supplementation of the HF diet with CE, cocoa monomers or polymers did not inhibit increased 4 h fasting blood glucose levels, as these treatments had levels significantly higher than the LF control and were not significantly different from the HF control. **Figure 4.6D** displays the ITT data as measured by the blood glucose AUC over time following i.p. insulin administration. The ITT data closely mirrored the 4 h fasting glucose data. Mice fed the HF control diet had significantly elevated ITT AUC values compared to mice fed the LF control diet (9,680±420 vs. 7,090±710 mg*min/dL, respectively). Supplementation of the HF diet with cocoa oligomers inhibited increased ITT AUC compared to LF control (7,800±450 vs. 7,090±710 mg*min/dL, respectively). Supplementation of the HF diet with CE, cocoa monomers or polymers did not inhibit increased ITT AUC values, as these treatments had values significantly higher than the LF control and were not significantly different from the HF control.

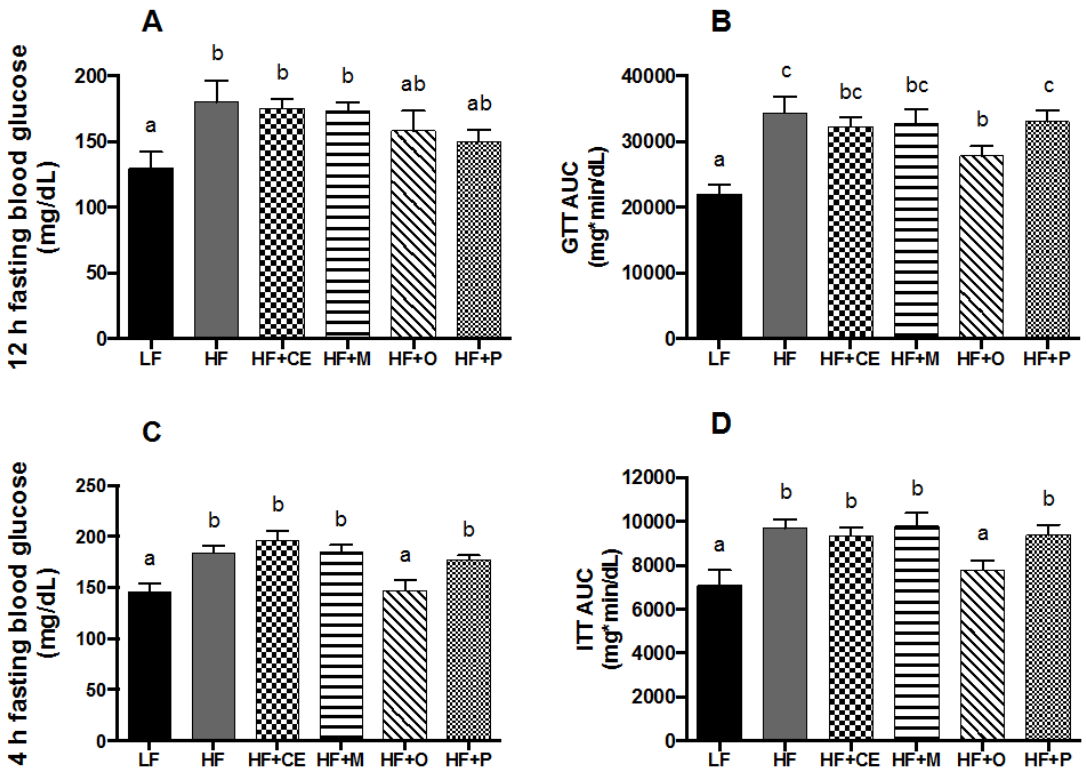


Figure 4.6A. *Fasting (12 h) blood glucose levels at week 10.* **B.** *Glucose tolerance test (GTT) area under the blood glucose concentration curve (AUC) at week 10.* **C.** *Fasting (4 h) blood glucose levels at week 11.* **D.** *Insulin tolerance test (ITT) area under the blood glucose concentration curve (AUC) at week 11. Data shown are mean ± SEM of n=9 animals/treatment (n=8 for the HF+M group due to loss of one mouse from this group during the feeding study). Significance between treatments was determined with a one-way ANOVA and Fisher's LSD post-hoc test (P<0.05). Treatments with different superscripts are significantly different. LF: low-fat control diet, HF: high-fat control diet, HF+M: high-fat control diet + monomer-rich fraction, HF+O: high-fat control diet + oligomer-rich fraction, HF+P: high-fat control diet + polymer-rich fraction.*

Fasting Insulin. Fasting serum levels of insulin at the end of the 12-week feeding study are presented in **Figure 4.7.** The HF diet significantly elevated fasting serum insulin levels compared to the LF control (0.813±0.121 vs. 2.64±0.55 ng/mL, respectively). Supplementation of the HF diet with CE did not significantly inhibit increases in fasting serum insulin, as this treatment was not significantly different compared to the HF diet alone (2.19±0.56 vs. 2.64±0.55 ng/mL, respectively) but was significantly higher than the control LF diet. Supplementation of

the HF diet with monomers, oligomers, and polymers resulted in fasting insulin levels (1.19±0.23, 1.23±0.42 and 1.43±0.21 ng/mL, respectively) that were significantly lower than the HF diet and not significantly different from the control LF diet.

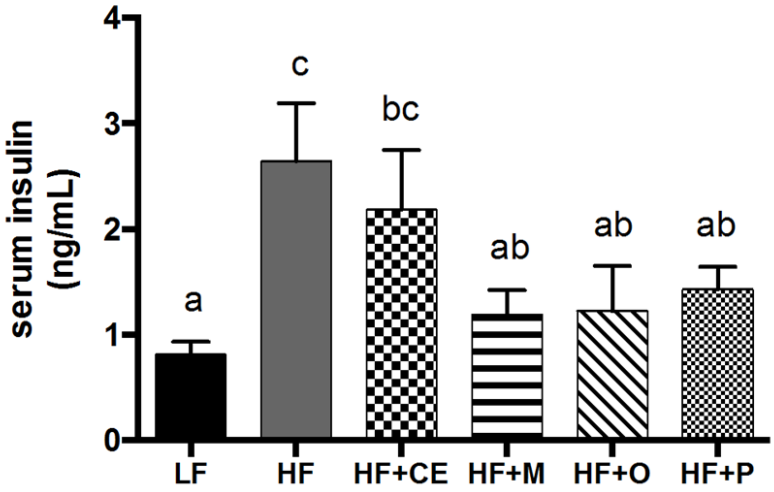


Figure 4.7. *Fasting serum insulin levels following the 12-week feeding study. Data shown are mean ± SEM of n=9 animals/treatment (n=8 for the HF+M group due to loss of one mouse from this group during the feeding study; n=6 for the HF+M group and n=7 for the HF+P group due to loss of samples during preparation for the ELISA assay). Significance between treatments was determined with a one-way ANOVA and Fisher's LSD post-hoc test (P<0.05). Treatments with different superscripts are significantly different. LF: low-fat control diet, HF: high-fat control diet, HF+M: high-fat control diet + monomer-rich fraction, HF+O: high-fat control diet + oligomer-rich fraction, HF+P: high-fat control diet + polymer-rich fraction.*

Endotoxin. Fasting serum levels of endotoxin at the end of the 12-week feeding study are presented in **Figure 4.8.** HF feeding did not significantly increase fasting serum endotoxin concentrations compared to the LF diet [7.95±0.28 vs. 7.73±0.64 endotoxin units (EU)/mL, respectively]. Supplementation of the HF diet with CE, however, significantly increased endotoxin (9.51±0.61 EU/mL) compared to the LF and HF controls. Conversely, supplementation of the HF diet with monomers and oligomers significantly decreased endotoxin levels (4.98±0.15 and 5.76±0.47 EU/mL respectively) compared to LF and HF controls.

Supplementation of the HF diet with polymers did not significantly affect endotoxin levels (7.83 ±0.38 EU/mL) compared to the LF and HF controls.

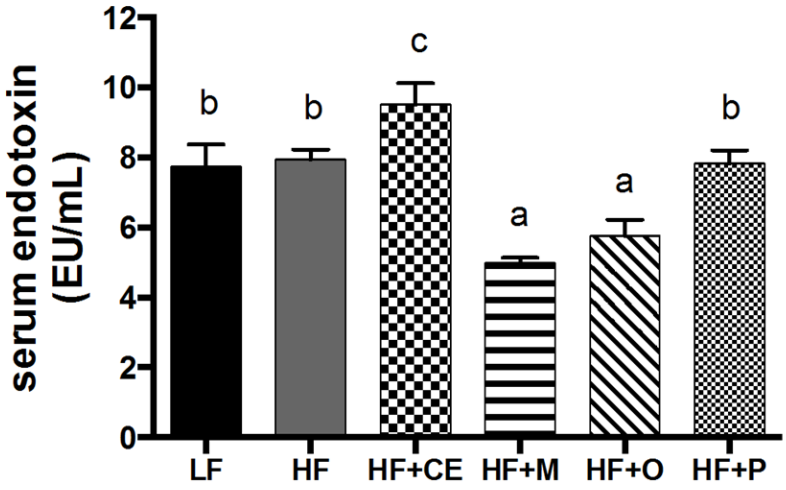


Figure 4.8. *Fasting serum endotoxin levels following the 12-week feeding study. Data shown are mean ± SEM of n=9 animals/treatment (n=8 for the HF+M group due to loss of one mouse from this group during the feeding study). Significance between treatments was determined with a one-way ANOVA and Fisher's LSD post-hoc test (P<0.05). Treatments with different superscripts are significantly different. LF: low-fat control diet, HF: high-fat control diet, HF+M: high-fat control diet + monomer-rich fraction, HF+O: high-fat control diet + oligomer-rich fraction, HF+P: high-fat control diet + polymer-rich fraction.*

Discussion

This is believed to be the first long-term feeding study to examine the impact of size (individual species DP and overall mDP of the matrix) on the ability of monomeric flavanols and PCs of various size to inhibit the onset of obesity and impaired glucose tolerance. These results indicate that a cocoa fraction enriched in oligomeric PCs with an overall mDP value of approximately 2.73 likely are the most effective flavanol constituents of cocoa for prevention of weight gain, fat mass accumulation, elevated fasting blood glucose and impaired glucose tolerance. The fraction enriched in oligomers appeared to be more effective than the monomer-enriched or polymer-enriched fractions as well as the composite CE itself.

The results from the present study are generally in agreement with the few previous studies of the impact of flavanol DP on biological processes related to obesity and glucose homeostasis. Gu *et al.*[120] demonstrated that PC DP was generally proportional to inhibitory activity against digestive enzymes (pancreatic α-amylase, pancreatic lipase, and secreted phospholipase A_2), suggesting that larger PCs may more effectively control nutrient digestion and release of glucose into the bloodstream. Yamashita *et al.*[121] showed that larger PCs (DP≥4) more effectively inhibited intestinal α-glucosidase than smaller PCs (DP≤3), although smaller PCs more effectively stimulated glucose uptake and AMP-activated protein kinase phosphorylation in rat skeletal muscle cells. Yamashita *et al.*[30] further demonstrated that the CPC oligomer cinnamtannin A_2 (a PC tetramer) more effectively stimulates insulin secretion and activation of insulin signaling in skeletal muscle [via stimulating phosphorylation of insulin receptor β (IRβ) and IRS-1 than smaller PCs and EC. Montagut et al.[102] also demonstrated that PC oligomers activate insulin signaling and stimulate glucose uptake in Chinese hamster ovary cells overexpressing the human insulin receptor[102]. While these studies suggested various relationships between PC size and specific bioactivities, this data suggests that the overall effect on DIO and impaired glucose tolerance is optimal for PCs of intermediate sizes.

The observation that cocoa flavanol fractions inhibited the development of obesity-induced hyperinsulinemia is consistent with previous reports[105, 122]. These studies suggest that flavanols decrease lipid accumulation in β-cells and subsequent glucose-stimulated insulin production under hyperlipidemic conditions. However, studies in healthy animals suggest that flavanols may actually increase insulin secretion by increasing levels of GLP-1, which is a

hormone secreted from gut L-cells in response to carbohydrate ingestion that stimulates insulin secretion[30]. *In vitro* and animal studies also suggest that PCs improve insulin sensitivity in skeletal muscle and adipocytes, thus decreasing the need for insulin secretion[121, 123]. These studies and the present data highlight the fact that the effects of flavanols on mechanisms related to glucose homeostasis are highly context dependent. Distinct mechanisms of action may be involved in the prevention of obesity and insulin resistance vs. the amelioration of existing obesity and insulin resistance[124]. Furthermore, flavanols may improve insulin and blood glucose levels by distinct mechanisms in the acute post-prandial (gut mechanisms such as inhibiting macronutrient digestion/absorption, increased GLP-1 and insulin secretion)[30, 120] vs. chronic effects in the fasted state (peripheral tissue mechanisms such as decreased insulin secretion, improved insulin sensitivity, etc.). It should also be noted that, while all three flavanol fractions effectively inhibited hyperinsulinemia (**Figure 4.7**), only the oligomer-rich fraction effectively improved glucose tolerance compared to the HF diet (**Figure 4.6B**), inhibited increases in 4 h fasting glucose compared to the LF control (**Figure 4.6C**), and inhibited the development of insulin resistance (**Figure 4.6D**). Therefore, only the oligomer-rich fraction effectively prevented development of a pre-diabetic phenotype.

The finding that an oligomer-rich fraction was generally more effective than a monomer-rich fraction is intriguing, as the epithelial absorption and systemic bioavailability of PCs is generally regarded to be inversely proportional to size[125-127], consistent with "Lipinski's Rule of 5" for drug bioavailability. This suggests that oligomers, which are largely unabsorbed in their native form, likely act primarily through a mechanism located in the gut. This hypothesis is supported by the fact that PCs appear to improve glucose tolerance and increase insulin secretion when glucose is administered orally but not by i.p. injection[128]. Potential mechanisms by which unabsorbed PCs may act in the gut to inhibit the onset of obesity and impaired glucose tolerance include inhibition of macronutrient digestion and absorption[120, 121], stimulation of GLP-1 secretion and subsequent GLP-1 driven insulin secretion[30], and inhibition of GLP-1 degradation by dipeptidyl peptidase-IV (DPP4)[128]. In the present study, cocoa oligomers appear to more effectively control 4 h fasting glucose levels vs. 12 h fasting glucose levels (**Figure 4.6A, C**). This data indicates that oligomers more effectively modulate postprandial blood glucose control as opposed to frank fasting blood glucose levels, suggesting that oligomers may primarily exert

acute post-meal effects while the compounds are still in the upper GI tract, as opposed to chronic systemic effects.

Another possible mechanism for PC action in the gut is the inhibition of inflammation and metabolic endotoxemia. Low-grade inflammation in skeletal muscle has been shown to impair insulin signaling and induce metabolic derangements in skeletal muscle[77, 129]. A key mediator of this inflammatory state is endotoxin (LPS), the major glycolipid component of the outer membrane of gram-negative commensal bacteria in the gut. Endotoxin is continuously transported from the gut into circulation by paracellular diffusion following bacterial cell lysis. Circulating endotoxin activates the toll-like family of pathogen-associated molecular pattern recognition receptors that initiate an inflammatory signaling response via NF-κB activation[130]. Typically, post-prandial endotoxin levels rise acutely and then return to baseline within 4- 6 h in healthy individuals. HF feeding, however, increases postprandial endotoxin levels and can also induce a chronically elevated endotoxin state[131]. Research in mice and humans has linked chronically elevated endotoxin to development of obesity and insulin resistance via activation of TLR-4 in skeletal muscle. PCs are well known to reduce inflammation[91], and control of the chronic low-grade inflammatory state resulting from HF feeding may represent a mechanism by which dietary PCs prevent and/or ameliorate metabolic syndrome. PCs may inhibit metabolic endotoxemia and the resulting metabolic derangements (including insulin resistance) by reducing epithelial permeability to endotoxin[132, 133], or by blunting TLR-4 stimulation[134] and induction of inflammation by circulating endotoxin[135, 136]. Although these activities have all been demonstrated separately for PCs, inhibition of endotoxemia resulting in improved skeletal muscle insulin signaling and systemic glucose tolerance have yet to be mechanistically linked for PCs. Data supporting the hypothesis that improved regulation of inflammatory tone ameliorates insulin resistance would represent a novel mechanism by which PCs prevent metabolic syndrome. The present data indicated that HF deeding did not significantly elevate fasting serum endotoxin compared to the LF diet (**Figure 4.8**). However, this lack of difference may be due to the prolonged (12 h) fast, as increases in circulating endotoxin are generally observed in the postprandial (fed) state[131]. Nevertheless, supplementation of the HF diet with both monomers and oligomers significantly reduced fasting endotoxin concentrations. This suggests that these fractions warrant further consideration for prevention of metabolic endotoxemia. The potential mechanisms by which monomers and oligomers reduced serum endotoxin concentrations include

reduced endotoxin production in the gut (by gut microflora) and/or reduced paracellular permeability of the gut to endotoxin (by increased gut junction protein expression). Additional studies are required to determine the impact of cocoa monomers and oligomers on postprandial serum endotoxin profiles.

Potential anti-obesity and anti-diabetic activities of cocoa flavanols that require absorption and distribution of PCs to target tissues, such as direct stimulation of pancreatic β-cell function[122, 137] and insulin signaling in skeletal muscle[102], likely play a complimentary role to the gut-mediated activities that have been identified due to the poor bioavailability of PCs. Further research is required to determine the relative importance of the gut activities of unabsorbed PCs vs. the systemic activities (in the pancreas, liver, adipose tissue, skeletal muscle etc.) of absorbed PCs. Additionally, the microbial metabolites of these compounds may play a role in systemic activities of poorly absorbed native compounds.

It is important to note the low cocoa flavanol doses employed in the present study. CE and cocoa fractions were employed at 25 mg/kg*BW/d [262.5 mg/kg diet, or ~0.02625% (w/w) in the diet]. This is considerably lower than doses used in the majority of previous feeding studies of CEs or cocoa flavanols (**Table 2.1**). Previous studies have employed doses up to 600 mg/kg*BW/d or 0.5-12.5% (w/w) in the diet (although the higher % compositions in the diet were cocoa powder, which is ~2-5% flavan-3-ols by weight)[16-22]. The doses employed in previous studies may be on the extreme end of what is feasible for translation to human dietary dosing. However, it was demonstrated in this chapter that CEs or CPC fractions can be effective at lower doses, which translate to more reasonable human doses [25 mg cocoa flavanols/kg*BW/d in a 20 g mouse is roughly equivalent to 0.122 g cocoa flavanols in a 60 kg human[138], which is ~2.43 g cocoa powder, or ~1/2 tablespoon]. Therefore, data from this study suggest that moderate doses of cocoa flavanols or cocoa powder have the potential to be more effective in human clinical trials than previously thought. This concept was proposed previously by Castell-Auvi *et al.*. [105], who demonstrated that high doses had no effect on hyperinsulinemia and lower doses (5-15 mg/kg*BW) actually worsened hyperinsulinemia, while 25 mg/kg*BW (the same dose employed in this study) improved hyperinsulinemia. It is also important to note that this dose did not alter food intake in the current model, whereas the higher doses in previous studies may have inhibited the onset of DIO and insulin resistance by significantly altering food

intake (and hence, macronutrient intake) due to poor palatability or other factors (however, this is unknown as food intake is rarely reported).

One confounding factor in this study design was that the mice were fed equivalent doses of the CE and cocoa fractions on a mg/kg*BW basis. Due to the differences in average molecular weight of the species in each treatment, mice were therefore fed different concentrations on a mol/kg*BW basis. However, the oligomer-enriched fraction was still the most effective of the four cocoa treatments in spite of the fact that the mice on the monomer treatment received significantly greater doses than mice on the oligomer treatment, on a mol/kg*BW basis. This fact further reinforces the conclusion that cocoa oligomers possess enhanced anti-obesity and anti-diabetic bioactivities compared to CE, monomeric, and polymeric cocoa flavan-3-ols.

Conclusions

Flavanols have demonstrated their ability to reduce various risk factors in the development of metabolic diseases. The present study aimed to determine the effects of chronic exposure to cocoa-derived flavanols on the onset of obesity, insulin resistance, and impaired glucose tolerance during HF feeding in mice. 12 weeks of feeding on a HF diet (60% kcal fat), induced obesity, insulin resistance and impaired glucose tolerance in mice compared to mice on a LF diet (10% kcal fat), as evident from weight gains, fasting glucose levels, glucose tolerance, insulin tolerance, and fasting plasma insulin levels. Oligomeric PCs appear to possess the greatest anti-obesity and anti-diabetic bioactivities of the flavanols in cocoa, particularly at the low doses employed for the present study. The oligomer-rich fraction significantly prevented mice weight gain, fat mass accumulation, elevated 4 h fasting blood glucose and insulin resistance compared to LF control as well as improved mice glucose tolerance compared to HF control. However, all CPC fractions had some effect *in vivo*. All three CPC fractions improved hyperinsulinemia and both the monomer- and oligomer-rich fractions reduced fasting endotoxin levels compared to control groups. Since this was an observational study done to explore the biological impact of cocoa flavanols during HF feeding, the underlying mechanisms of cocoa flavanols still have yet to be determined. However, the insignificant food intakes of mice across all HF treatment groups is evidence that an alteration in food intake is not a potential mechanism of action for this study.

Although previous animal studies have used higher flavanol concentrations, a dose of 25 mg/kg*BW was sufficient enough to induce noticeable biological changes across all CPC treatment groups. Additional studies of prolonged feeding of flavanol fractions *in vivo* are needed to further identify the fractions with the highest bioactivities and therefore the greatest potential for translation to human clinical applications at reasonable doses.

Chapter 5:

Conclusions and Future Work

The objectives of this study were completed through separation of CE into individual CPC fractions (monomers, oligomers, and polymers) followed by chronic administration of these fractions to mice during an obese, insulin resistant and impaired glucose tolerant model (HF feeding). To test the impact of CE and CPC fractions on the onset of obesity, insulin resistance, and impaired glucose tolerance, mouse BW, body fat %, insulin resistance and glucose tolerance were monitored and compared to control animals. To test the impact that the cocoa flavanol mDP has on the extent of their preventative power, statistical analysis was done on the mouse data between each of the individual CPC fractions. The oligomer-rich cocoa fraction demonstrated the greatest preventative power in a HF diet-induced obese model compared to CE, monomer- and polymer-rich fractions.

The promising results of this study suggest several future directions. First, further fractionation of cocoa and isolation of individual compounds in the oligomer fraction for long-term feeding studies are needed to further refine understanding of which cocoa flavanols possess the greatest potential for anti-obesity and anti-diabetic activities in humans. Included in this is a more refined SPE fractionation method that can improve the amount of flavanols lost during the process and generate greater fraction yields. Second, additional studies are needed to determine the relative importance of the gut activities of unabsorbed PCs vs. the systemic activities of absorbed PCs (or absorbed microbial metabolites) in the oligomer fraction. A critical strategy will be to compare the anti-obesity and anti-diabetic effects of PCs administered orally vs. by i.p. injection or catheter infusion to mice in the context of HF feeding. Identification and localization of the mechanistic target will guide design of the most effective delivery strategies for human interventions. Third, specific mechanistic studies are needed to identify the primary mechanism(s) by which PC oligomers inhibit the onset of obesity and impaired glucose tolerance during HF feeding. The majority of studies (including the present study) generally observe biomarkers that are adversely modulated by HF feeding and improve during supplementation with PCs. While these experiments are useful for hypothesis generation, mechanistic experiments are needed which specifically inhibit and/or stimulate putative mechanistic targets

in the context of HF feeding or PC exposure in order to isolate and definitively identify the specific mechanisms of action. Identification of specific mechanisms that are essential for PC bioactivities will facilitate the design of human interventions with appropriate mechanistic targets and outcomes. The mechanisms which warrant the most consideration appear to be those located in the gut, specifically: inhibition of macronutrient digestion and absorption, increased GLP-1 secretion, DPP4 inhibition, and inhibition of endotoxin production and/or uptake. Finally, studies are required to determine whether the anti-obesity and anti-diabetic effects of PC oligomers are primarily acute postprandial activities (altered macronutrient absorption and/or GLP-1 secretion due to the presence of oligomers in the upper GI tract), or systemic effects (in skeletal muscle, etc.) chronically induced by low levels of circulating PCs.

References

1. Robbins, R. J.; Leonczak, J.; Li, J.; Johnson, J. C.; Collins, T.; Kwik-Uribe, C.; Schmitz, H. H., Determination of flavanol and procyanidin (by degree of polymerization 1-10) content of chocolate, cocoa liquors, powder(s), and cocoa flavanol extracts by normal phase high-performance liquid chromatography: collaborative study. *J. AOAC Int.* 2012, *95*, 1153-1160.
2. Hammerstone, J. F., Procyanidin content and variation in some commonly consumed foods. *J. Nutr.* 2000, *130*, 2086S-2092S.
3. Lee, K. W., Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. *J. Agr. Food Chem.* 2003, *51*, 7292-7295.
4. Crozier, S. J.; Preston, A. G.; Hurst, J. W.; Payne, M. J.; Mann, J.; Hainly, L.; Miller, D. L., Cacao seeds are a "Super Fruit": A comparative analysis of various fruit powders and products. *Chem. Cent. J.* 2011, *5*.
5. Belščak, A.; Komes, D.; Horžić, D.; Ganić, K. K., Comparative study of commercially available cocoa products in terms of their bioactive composition. *Food Res. Int.* 2009, *42*, 707-716.
6. Andres-Lacueva, C.; Monagas, M.; Khan, N.; Izquierdo-Pulido, M.; Urpi-Sarda, M.; Permanyer, J.; Lamuela-Raventos, R. M., Flavanol and flavonol contents of cocoa powder products: Influence of the manufacturing process. *J. Agr. Food Chem.* 2008, *56*, 3111-3117.
7. Langer, S., Flavanols and methylxanthines in commercially available dark chocolate: a study of the correlation with nonfat cocoa solids. *J. Agr. Food Chem.* 2011, *59*, 8435-8441.
8. Wollgast, J.; Anklam, E., Review on polyphenols in Theobroma cacao: changes in composition during the manufacture of chocolate and methodology for identification and quantification. *Food Res. Int.* 2000, *33*, 423-447.
9. Gu, L., Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products. *J. Agr. Food Chem.* 2006, *54*, 4057-4061.
10. Cooper, K. A.; Campos-Gimenez, E.; Alvarez, D. J.; Nagy, K.; Donovan, J. L.; Williamson, G., Rapid reversed phase ultra-performance liquid chromatography analysis of the major cocoa polyphenols and inter-relationships of their concentrations in chocolate. *J. Agr. Food Chem.* 2007, *55*, 2841-2847.
11. Payne, M. J.; Hurst, W. J.; Miller, K. B.; Rank, C.; Stuart, D. A., Impact of fermentation, drying, roasting, and Dutch processing on epicatechin and catechin content of cacao beans and cocoa ingredients. *J. Agr. Food Chem.* 2010, *58*, 10518-10527.
12. Vinson, J. A., Phenol antioxidant quantity and quality in foods: cocoa, dark chocolate, and milk chocolate. *J. Agr. Food Chem.* 1999, *47*, 4821-4824.
13. Grassi, D., Short-term administration of dark chocolate is followed by a significant increase in insulin sensitivity and a decrease in blood pressure in healthy persons. *Am J. Clin. Nutr.* 2005, *81*, 611-614.
14. Grassi, D., Cocoa reduces blood pressure and insulin resistance and improves endothelium-dependent vasodilation in hypertensives. *Hypertension (Dallas, Tex. 1979)* 2005, *46*, 398-405.
15. Taub, P. R.; Ramirez-Sanchez, I.; Ciaraldi, T. P.; Perkins, G.; Murphy, A. N.; Naviaux, R.; Hogan, M.; Maisel, A. S.; Henry, R. R.; Ceballos, G.; Villarreal, F., Alterations in skeletal muscle indicators of mitochondrial structure and biogenesis in patients with type 2 diabetes and heart failure: effects of epicatechin rich cocoa. *Clin. Transl. Sci.* 2012, *5*, 43-47.

16. Tomaru, M.; Takano, H.; Osakabe, N.; Yasuda, A.; Inoue, K.; Yanagisawa, R.; Ohwatari, T.; Uematsu, H., Dietary supplementation with cacao liquor proanthocyanidins prevents elevation of blood glucose levels in diabetic obese mice. *Nutrition* 2007, *23*, 351-355.
17. Yamashita, Y., Prevention mechanisms of glucose intolerance and obesity by cacao liquor procyanidin extract in high-fat diet-fed C57BL/6 mice. *Arch. Biochem. Biophys.* 2012, *527*, 95-104.
18. Ruzaidi, A.; Amin, I.; Nawalyah, A. G.; Hamid, M., The effect of Malaysian cocoa extract on glucose levels and lipid profiles in diabetic rats. *J. Ethnopharmacol.* 2005, *98*, 55-60.
19. Jalil, A. M. M.; Ismail, A.; Pei, C. P.; Hamid, M.; Kamaruddin, S. H. S., Effects of cocoa extract on glucometabolism, oxidative stress, and antioxidant enzymes in obese-diabetic (Ob-db) rats. *J. Agr. Food Chem.* 2008, *56*, 7877-7884.
20. Jalil, A. M. M.; Ismail, A.; Chong, P. P.; Hamid, M.; Kamaruddin, S. H. S., Effects of cocoa extract containing polyphenols and methylxanthines on biochemical parameters of obese-diabetic rats. *J. Sci. Food Agr.* 2009, *89*, 130-137.
21. Sanchez, D.; Moulay, L.; Muguerza, B.; Quinones, M.; Miguel, M.; Aleixandre, A., Effect of a soluble cocoa fiber-enriched diet in Zucker fatty rats. *J. Med. Food* 2010, *13*, 621-628.
22. Matsui, N.; Ito, R.; Nishimura, E.; Yoshikawa, M.; Kato, M.; Kamei, M.; Shibata, H.; Matsumoto, I.; Abe, K.; Hashizume, S., Ingested cocoa can prevent high-fat diet-induced obesity by regulating the expression of genes for fatty acid metabolism. *Nutrition* 2005, *21*, 594-601.
23. Eteng, M. U.; Ibekwe, H. A.; Umoh, U. I.; Ebong, P. E.; Umoh, I. B.; Eyong, E. U., Theobromine rich cocoa powder induces weight loss and changes in lipid profile of obese Wistar rats. *Discov. Innovat.* 2006, *18*, 191-196.
24. Cordero-Herrera, I.; Martín, M. Á.; Goya, L.; Ramos, S., Cocoa flavonoids attenuate high glucose-induced insulin signalling blockade and modulate glucose uptake and production in human HepG2 cells. *Food Chem. Toxicol.* 2014, *64*, 10-19.
25. Cordero-Herrera, I.; Martín, M. A.; Bravo, L.; Goya, L.; Ramos, S., Cocoa flavonoids improve insulin signalling and modulate glucose production via AKT and AMPK in HepG2 cells. *Mol. Nutr. Food Res.* 2013, 974-985.
26. Martín, M. Á.; Fernández-Millán, E.; Ramos, S.; Bravo, L.; Goya, L., Cocoa flavonoid epicatechin protects pancreatic beta cell viability and function against oxidative stress. *Mol. Nutr. Food Res.* 2013, 1-10.
27. Karim, M., Effects of cocoa extracts on endothelium-dependent relaxation. *J. Nutr.* 2000, *130*, 2105S-2108S.
28. Kim, Y., Protective effects of oligomeric and polymeric procyanidin fractions from defatted grape seeds on tert-butyl hydroperoxide-induced oxidative damage in HepG2 cells. *Food Chem.* 2013, *137*, 136-141.
29. Gentile, C.; Allegra, M.; Angileri, F.; Pintaudi, A. M.; Livrea, M. A.; Tesoriere, L., Polymeric proanthocyanidins from Sicilian pistachio (Pistacia vera L.) nut extract inhibit lipopolysaccharide-induced inflammatory response in RAW 264.7 cells. *Eur. J. Nutr.* 2012, *51*, 353-363.
30. Yamashita, Y.; Okabe, M.; Natsume, M.; Ashida, H., Cinnamtannin A2, a tetrameric procyanidin, increases GLP-1 and insulin secretion in mice. *Bio. Sci. Biotech. Bioch.* 2013, 888-891.
31. Daniel, M., *Medicinal plants : chemistry and properties*. Science Publishers: Enfield, NH, 2006.

32. Vermerris, W. N. R. L., *Phenolic compound biochemistry*. Springer: Dordrecht, the Netherlands, 2006.
33. Tsao, R., Chemistry and biochemistry of dietary polyphenols. *Nutrients* 2010, *2*, 1231-1246.
34. de Pascual-Teresa, S., Flavanols and anthocyanins in cardiovascular health: a review of current evidence. *Int. J. Mol. Sci.* 2010, *11*, 1679-1703.
35. Hackman, R.; Polagruto, J.; Zhu, Q.; Sun, B.; Fujii, H.; Keen, C., Flavanols: digestion, absorption and bioactivity. *Phytochem Rev* 2008, *7*, 195-208.
36. Martens, S.; Mithöfer, A., Flavones and flavone synthases. *Phytochemistry* 2005, *66*, 2399-2407.
37. Mehrinfar, R.; Frishman, W. H., Flavanol-rich cocoa a cardioprotective nutriceutical. *Cardiol. Rev.* 2008, *16*, 109-115.
38. Rimbach, G., Polyphenols from cocoa and vascular health—a critical review. *Int. J. Mol. Sci.* 2009, *10*, 4290-4309.
39. Cheynier, V.; Labarbe, B.; Moutounet, M., Estimation of procyanidin chain length. *Method. Enzymol.* 2001, *335*, 82-94.
40. Gu, L., Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC−MS fluorescent detection method. *J. Agr. Food Chem.* 2002, *50*, 4852-4860.
41. He, F., Chemical synthesis of proanthocyanidins in vitro and their reactions in aging wines. *Molecules (Basel, Switzerland)* 2008, *13*, 3007-3032.
42. Selmi, C.; Mao, T. K.; Keen, C. L.; Schmitz, H. H., The anti-inflammatory properties of cocoa flavanols. *J. Cardiovasc. Pharm.* 2006, *47 Suppl 2*, S163-71; discussion S172-6.
43. Miller, K. B., Impact of Alkalization on the Antioxidant and Flavanol Content of Commercial Cocoa Powders. *Journal of Agricultural and Food Chemistry* 2008, *56*, 8527-8533.
44. Adamson, G. E., HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *J. Agr. Food Chem.* 1999, *47*, 4184-4188.
45. Bravo, L., Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* 1998, *56*, 317-333.
46. Machonis, P. R.; Machonis, P. R.; Jones, M. A.; Schaneberg, B. T.; Kwik-Uribe, C. L., Method for the determination of catechin and epicatechin enantiomers in cocoa-based ingredients and products by high-Performance liquid chromatography: single-laboratory validation. *J. AOAC Int.* 2012, *95*, 500-507.
47. Tokusoglu; x000D; zlem; Tokusoglu, O.; Unal, M. K., Optimized method for simultaneous determination of catechin, gallic acid, and methylxanthine compounds in chocolate using RP-HPLC. *Z. Lebensm. Unters. F. A.* 2002, *215*, 340-346.
48. Gabetta, B.; Fuzzati, N.; Griffini, A.; Lolla, E.; Pace, R.; Ruffilli, T.; Peterlongo, F., Characterization of proanthocyanidins from grape seeds. *Fitoterapia* 2000, *71*, 162-175.
49. Bose, M.; Lambert, J. D.; Ju, J.; Reuhl, K. R., The major green tea polyphenol, (-)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat-fed mice. *J. Nutr.* 2008, *138*, 1677-83.
50. White, B. L.; Howard, L. R.; Prior, R. L., Impact of Different Stages of Juice Processing on the Anthocyanin, Flavonol, and Procyanidin Contents of Cranberries. *J. Agr. Food Chem.* 2011, *59*, 4692-4698.

51. Khanal, R. C.; Howard, L. R.; Prior, R. L., Procyanidin Content of Grape Seed and Pomace, and Total Anthocyanin Content of Grape Pomace as Affected by Extrusion Processing. *J. Food Sci.* 2009, *74*, H174-H182.
52. Go, A. S., Heart Disease and Stroke Statistics--2013 Update: A Report From the American Heart Association. *Circulation* 2013, *127*, e6-e245.
53. Mokdad, A. H.; Serdula, M. K.; Dietz, W. H.; Bowman, B. A.; Marks, J. S.; Koplan, J. P., THe spread of the obesity epidemic in the united states, 1991-1998. *Jama- J. Am. Med. Assoc.* 1999, *282*, 1519-1522.
54. Ogden, C. L.; Carroll, M. D.; Kit, B. K.; Flegal, K. M., Prevalence of obesity in the United States, 2009-2010. In US Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Health Statistics: 2012.
55. Landsberg, L.; Aronne, L. J.; Beilin, L. J.; Burke, V.; Igel, L. I.; Lloyd-Jones, D.; Sowers, J., Obesity-related hypertension: Pathogenesis, cardiovascular risk, and treatment—A position paper of the The Obesity Society and the American Society of Hypertension. *Obesity* 2013, *21*, 8-24.
56. Hildreth, K. L.; Van Pelt, R. E.; Schwartz, R. S., Obesity, Insulin Resistance, and Alzheimer's Disease. *Obesity* 2012, *20*, 1549-1557.
57. Ogden, C. L., Prevalence of Obesity and Trends in Body Mass Index Among US Children and Adolescents, 1999-2010. *Jama- J. Am. Med. Assoc.* 2012, *307*, 483.
58. Ionut, V.; Burch, M.; Youdim, A.; Bergman, R. N., Gastrointestinal hormones and bariatric surgery-induced weight loss. *Obesity* 2013, *21*, 1093-1103.
59. Prospective Studies, C., Body-mass index and cause-specific mortality in 900 000 adults: collaborative analyses of 57 prospective studies. *Lancet 373*, 1083-1096.
60. Laakso, M., Cardiovascular disease in type 2 diabetes: challenge for treatment and prevention. *J. Intern. Med.* 2001, *249*, 225-235.
61. Juutilainen, A., Type 2 Diabetes as a "Coronary Heart Disease Equivalent": An 18-year prospective population-based study in Finnish subjects. *Diabetes Care* 2005, *28*, 2901-2907.
62. Pirola, L.; Johnston, A. M.; Obberghen, E., Modulation of insulin action. *Diabetologia* 2004, *47*, 170-184.
63. Havekes, B.; Sauerwein, H. P., Adipocyte-myocyte crosstalk in skeletal muscle insulin resistance; is there a role for thyroid hormone? *Curr. Opin. Clin. Nutr.* 2010, *13*, 641-6.
64. Alsahli, M.; Gerich, J. E., Pathogenesis of type 2 diabetes. In Springer US: 2012; pp 149-166.
65. Stumvoll, M.; Goldstein, B. J.; van Haeften, T. W., Pathogenesis of type 2 diabetes. *Endocr. Res.* 2007, *32*, 19-37.
66. Kahn, S. E., The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of type 2 diabetes. *Diabetologia* 2003, *46*, 3.
67. Saltiel, A. R.; Kahn, C. R., Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 2001, *414*, 799-806.
68. Argilés, J. M.; López-Soriano, J.; Almendro, V.; Busquets, S., Cross-talk between skeletal muscle and adipose tissue: A link with obesity? *Medicinal research reviews* 2005, *25*, 49-65.
69. Kulkarni, R. N.; Brüning, J. C.; Winnay, J. N.; Postic, C.; Magnuson, M. A.; Kahn, C. R., Tissue-specific knockout of the insulin receptor in pancreatic β cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell* 1999, *96*, 329-339.

70. Brüning, J. C.; Michael, M. D.; Winnay, J. N.; Hayashi, T.; Hörsch, D.; Accili, D.; Goodyear, L. J.; Kahn, C. R., A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol. Cell* 1998, *2*, 559-569.
71. Blüher, M.; Kahn, B. B.; Kahn, C. R., Extended longevity in mice lacking the insulin receptor in adipose tissue. *Science* 2003, *299*, 572-574.
72. Peppa, M.; Koliaki, C.; Nikolopoulos, P.; Raptis, S. A., Skeletal muscle insulin resistance in endocrine disease. *J. Biomed. Biotechnol.* 2010, *2010*, 13.
73. Olefsky, J. M., Macrophages, inflammation, and insulin resistance. *Annu. Rev. Physiol.* 2010, *72*, 219-246.
74. Meeprom, A.; Sompong, W.; Suwannaphet, W.; Yibchok-anun, S., Grape seed extract supplementation prevents high-fructose diet-induced insulin resistance in rats by improving insulin and adiponectin signalling pathways. *Brit. J. Nutr.* 2011, *106*, 1173-1181.
75. Bruce, C. R., Cytokine regulation of skeletal muscle fatty acid metabolism: effect of interleukin-6 and tumor necrosis factor- *Am. J. Physiol-Endoc. M.* 2004, *287*, E616-E621.
76. de Kort, S.; Keszthelyi, D.; Masclee, A. M., Leaky gut and diabetes mellitus: what is the link? *Obes. Rev.* 2011, *12*, 449-458.
77. Creely, S. J.; McTernan, P. G.; Kusminski, C. M.; Fisher, F. M.; Da Silva, N. F.; Khanolkar, M.; Evans, M.; Harte, A. L.; Kumar, S., Lipopolysaccharide activates an innate immune system response in human adipose tissue in obesity and type 2 diabetes. *Am. J. Physiol-Endoc. M.* 2007, *292*, E740-E747.
78. Ley, R. E.; Bäckhed, F.; Turnbaugh, P.; Lozupone, C. A.; Knight, R. D.; Gordon, J. I., Obesity alters gut microbial ecology. *P. Natl. Acad. Sci. USA.* 2005, *102*, 11070-11075.
79. Lang, C. H., Endotoxin stimulates in vivo expression of inflammatory cytokines tumor necrosis factor alpha, interleukin-1 [beta],-6, and high-mobility-group protein-1 in skeletal muscle. *Shock* 2003, *19*, 538.
80. Farhadi, A.; Gundlapalli, S.; Shaikh, M.; Frantzides, C.; Harrell, L.; Kwasny, M. M.; Keshavarzian, A., Susceptibility to gut leakiness: a possible mechanism for endotoxaemia in non-alcoholic steatohepatitis. *Liver Int.* 2008, *28*, 1026-1033.
81. Hsueh, W. A., Role of endothelial dysfunction in insulin resistance. *Am. J. Cardiol.* 2003, *92*, 10-17.
82. Tamai, H.; Horie, Y.; Kato, S.; Yokoyama, H.; Ishii, H., Long-term ethanol feeding enhances susceptibility of the liver to orally administered lipopolysaccharides in rats. *Alcohol Clin. Exp. Res.* 2002, *26*, 75s-80s.
83. Cani, P. D.; Bibiloni, R.; Knauf, C.; Neyrinck, A. M.; Delzenne, N. M.; Burcelin, R., Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 2008, *57*, 1470-1481.
84. Lambert, J. C.; Zhou, Z.; Wang, L.; Song, Z.; Mcclain, C. J.; Kang, Y. J., Prevention of alterations in intestinal permeability is involved in zinc inhibition of acute ethanol-induced liver damage in mice. *J. Pharmacol. Exp. Ther.* 2003, *305*, 880-886.
85. Groschwitz, K. R., Intestinal barrier function: Molecular regulation and disease pathogenesis. *J. Allergy Clin. Immun.* 2009, *124*, 3-20.
86. Arrieta, M. C., Alterations in intestinal permeability. *Gut* 2006, *55*, 1512-1520.
87. Robertson, R. P., Oxidative stress and impaired insulin secretion in type 2 diabetes. *Curr. Opin. Pharmacol.* 2006, *6*, 615-619.

88. Robertson, R. P., Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. *J. Biol. Chem.* 2004, *279*, 42351-42354.
89. Hwang, I. K., Effects of grape seed extract and its ethylacetate/ethanol fraction on blood glucose levels in a model of type 2 diabetes. *Phytother. Res.* 2009, *23*, 1182-1185.
90. Ohyama, K., Catechin-rich grape seed extract supplementation attenuates diet-induced obesity in C57BL/6J mice. *Ann. Nutr. Metab.* 2011, *58*, 250.
91. Terra, X.; Montagut, G.; Bustos, M.; Llopiz, N.; Ardevol, A.; Blade, C.; Fernandez-Larrea, J.; Pujadas, G.; Salvado, J.; Arola, L.; Blay, M., Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. *J. Nutr. Biochem.* 2009, *20*, 210-218.
92. Terra, X., Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. *J. Nutr. Biochem.* 2011, *22*, 380-387.
93. Charradi, K., Grape seed and skin extract prevents high-fat diet-induced brain lipotoxicity in rat. *Neurochem. Res.* 2012, *37*, 2004-2013.
94. Corti, R., Cocoa and cardiovascular health. *Circulation* 2009, *119*, 1433-1441.
95. Patti, M. E., Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *P. Natl. Acad. Sci. USA.* 2003, *100*, 8466-8471.
96. Quesada, H.; del Bas, J. M.; Pajuelo, D.; Diaz, S.; Fernandez-Larrea, J.; Pinent, M.; Arola, L.; Salvado, M. J.; Blade, C., Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver. *Int. J. Obesity* 2009, *33*, 1007-1012.
97. Kimura, H.; Ogawa, S.; Sugiyama, A.; Jisaka, M.; Takeuchi, T.; Yokota, K., Anti-obesity effects of highly polymeric proanthocyanidins from seed shells of Japanese horse chestnut (Aesculus turbinata Blume). *Food Res. Int.* 2011, *44*, 121-126.
98. Klaus, S.; Pültz, S.; Thöne-Reineke, C.; Wolfram, S., Epigallocatechin gallate attenuates diet-induced obesity in mice by decreasing energy absorption and increasing fat oxidation. *Int. J. Obesity* 2005, *29*, 615-623.
99. Murase, T.; Nagasawa, A.; Suzuki, J.; Hase, T.; Tokimitsu, I., Beneficial effects of tea catechins on diet-induced obesity: stimulation of lipid catabolism in the liver. *Int. J. Obes. Relat. Metab. Disord.* 2002, *26*.
100. Chen, L.; Sun, P.; Wang, T.; Chen, K.; Jia, Q.; Wang, H.; Li, Y., Diverse mechanisms of antidiabetic effects of the different procyanidin oligomer types of two different cinnamon species on db/db mice. *J. Agr. Food Chem.* 2012, *60*, 9144-9150.
101. Pinent, M., Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines. *Endocrinology (Philadelphia)* 2004, *145*, 4985-4990.
102. Montagut, G.; Onnockx, S.; Vaque, M.; Blade, C.; Blay, M.; Fernandez-Larrea, J.; Pujadas, G.; Salvado, M. J.; Arola, L.; Pirson, I.; Ardevol, A.; Pinent, M., Oligomers of grape-seed procyanidin extract activate the insulin receptor and key targets of the insulin signaling pathway differently from insulin. *J. Nutr. Biochem.* 2010, *21*, 476-481.
103. Moreno, D. A.; Ilic, N.; Poulev, A.; Brasaemle, D. L.; Fried, S. K.; Raskin, I., Inhibitory effects of grape seed extract on lipases. *Nutrition* 2003, *19*, 876-879.
104. Decorde, K.; Teissedre, P. L.; Sutra, T.; Ventura, E.; Cristol, J. P.; Rouanet, J. M., Chardonnay grape seed procyanidin extract supplementation prevents high-fat diet-induced

obesity in hamsters by improving adipokine imbalance and oxidative stress markers. *Mol. Nutr. Food Res.* 2009, *53*, 659-666.
105. Castell-Auvi, A.; Cedo, L.; Pallares, V.; Blay, M. T.; Pinent, M.; Motilva, M. J.; Garcia-Vallve, S.; Pujadas, G.; Maechler, P.; Ardevol, A., Procyanidins modify insulinemia by affecting insulin production and degradation. *J. Nutr. Biochem.* 2012, *23*, 1565-1572.
106. Pajuelo, D.; Fernández-Iglesias, A.; Díaz, S.; Quesada, H.; Arola-Arnal, A.; Bladé, C.; Salvadó, J.; Arola, L. s., Improvement of mitochondrial function in muscle of genetically obese rats after chronic supplementation with proanthocyanidins. *J. Agr. Food Chem.* 2011, *59*, 8491-8498.
107. Pajuelo, D., Chronic dietary supplementation of proanthocyanidins corrects the mitochondrial dysfunction of brown adipose tissue caused by diet-induced obesity in Wistar rats. *Brit. J. Nutr.* 2011, *1*, 1.
108. Meagher, L. P., Characterization of condensed tannins from Lotus species by thiolytic degradation and electrospray mass spectrometry. *Animal feed science and technology* 2004, *117*, 151-163.
109. Guyot, S., Reversed-phase HPLC following thiolysis for quantitative estimation and characterization of the four main classes of phenolic compounds in different tissue zones of a french cider apple variety (Var. Kermerrien). *J. Agr. Food Chem.* 1998, *46*, 1698-1705.
110. Kelm, M. A., Identification and quantitation of flavanols and proanthocyanidins in foods: How good are the datas? *Clin. Dev. Immunol.* 2005, *12*, 35.
111. Brunetto, M. a. d. R.; Gutiérrez, L.; Delgado, Y.; Gallignani, M.; Zambrano, A.; Gómez, Á.; Ramos, G.; Romero, C., Determination of theobromine, theophylline and caffeine in cocoa samples by a high-performance liquid chromatographic method with on-line sample cleanup in a switching-column system. *Food Chem.* 2007, *100*, 459-467.
112. Valls, J.; Millán, S.; Martí, M. P.; Borràs, E., Advanced separation methods of food anthocyanins, isoflavones and flavanols. *J. Chromatogr.* 2009, *1216*, 7143-7172.
113. Glish, G. L., The basics of mass spectrometry in the twenty-first century. *Nat. Rev. Drug Discov.* 2003, *2*, 140-150.
114. Sun, B., Separation of grape and wine proanthocyanidins according to their degree of polymerization. *J. Agr. Food Chem.* 1998, *46*, 1390-1396.
115. Robbins, R. J., Method performance and multi-laboratory assessment of a normal phase high pressure liquid chromatography–fluorescence detection method for the quantitation of flavanols and procyanidins in cocoa and chocolate containing samples. *J. Chromatogr.* 2009, *1216*, 4831-4840.
116. Tsunoda, N., High-monounsaturated fat diet—induced obesity and diabetes in C57BL/6J mice. *Metabolism* 1998, *47*, 724-730.
117. Shortreed, K. E., Muscle-specific adaptations, impaired oxidative capacity and maintenance of contractile function characterize diet-induced obese mouse skeletal muscle. *PloS one* 2009, *4*, e7293.
118. Reheman, A.; Tasneem, S.; Ni, H.; Hayward, C. P., Mice with deleted multimerin 1 and alpha-synuclein genes have impaired platelet adhesion and impaired thrombus formation that is corrected by multimerin 1. *Thromb. Res.* 2010, *125*, e177-e-183.
119. Lin, S., Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice. *Int. J. Obes. Relat. Metab. Disord.* 2000, *24*, 639-646.
120. Gu, Y. Y.; Hurst, W. J.; Stuart, D. A.; Lambert, J. D., Inhibition of key digestive enzymes by cocoa extracts and procyanidins. *J. Agr. Food Chem.* 2011, *59*, 5305-5311.

121. Yamashita, Y.; Okabe, M.; Natsume, M.; Ashida, H., Comparison of anti-hyperglycemic activities between low- and high-degree of polymerization procyanidin fractions from cacao liquor extract. *J. Food Drug Anal.* 2012, *20*, 283-287.
122. Castell-Auvi, A.; Cedo, L.; Pallares, V.; Blay, M.; Pinent, M.; Ardevol, A., Grape seed procyanidins improve beta-cell functionality under lipotoxic conditions due to their lipid-lowering effect. *J. Nutr. Biochem.* 2013, *24*, 948-953.
123. Montagut, G.; Blade, C.; Blay, M.; Fernandez-Larrea, J.; Pujadas, G.; Salvado, M. J.; Arola, L.; Pinent, M.; Ardevol, A., Effects of a grapeseed procyanidin extract (GSPE) on insulin resistance. *J. Nutr. Biochem.* 2010, *21*, 961-967.
124. Pinent, M.; Cedo, L.; Montagut, G.; Blay, M.; Ardevol, A., Procyanidins improve some disrupted glucose homoeostatic situations: an analysis of doses and treatments according to different animal models. *CRC. Cr. Rev. Food Sci.* 2012, *52*, 569-584.
125. Zumdick, S.; Deters, A.; Hensel, A., In vitro intestinal transport of oligomeric procyanidins (DP 2 to 4) across monolayers of Caco-2 cells. *Fitoterapia 83*, 1210-1217.
126. Deprez, S.; Mila, I.; Huneau, J. F.; Tome, D.; Scalbert, A., Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial Caco-2 cells. *Antioxid. Redox Sign.* 2001, *3*, 957-967.
127. Prasain, J. K.; Peng, N.; Dai, Y. Y.; Moore, R.; Arabshahi, A.; Wilson, L.; Barnes, S.; Wyss, J. M.; Kim, H.; Watts, R. L., Liquid chromatography tandem mass spectrometry identification of proanthocyanidins in rat plasma after oral administration of grape seed extract. *Phytomedicine* 2009, *16*, 233-243.
128. Gonzalez-Abuin, N.; Martinez-Micaelo, N.; Blay, M.; Pujadas, G.; Garcia-Vallve, S.; Pinent, M.; Ardevol, A., Grape seed-derived procyanidins decrease dipeptidyl-peptidase 4 activity and expression. *J. Agr. Food Chem.* 2012, *60*, 9055-9061.
129. Shi, H.; Kokoeva, M. V.; Inouye, K.; Tzameli, I.; Yin, H.; Flier, J. S., TLR4 links innate immunity and fatty acid-induced insulin resistance. *J. Clin. Invest.* 2006, *116*, 3015-3025.
130. Frisard, M. I.; McMillan, R. P.; Marchand, J.; Wahlberg, K. A.; Wu, Y.; Voelker, K. A.; Heilbronn, L.; Haynie, K.; Muoio, B.; Li, L.; Hulver, M. W., Toll-like receptor 4 modulates skeletal muscle substrate metabolism. *Am. J. Physiol-Endoc. M.* 2010, *298*, E988-E998.
131. Cani, P. D.; Amar, J.; Iglesias, M. A.; Poggi, M.; Knauf, C.; Bastelica, D.; Neyrinck, A. M.; Fava, F.; Tuohy, K. M.; Chabo, C.; Waget, A.; Delmee, E.; Cousin, B.; Sulpice, T.; Chamontin, B.; Ferrieres, J.; Tanti, J. F.; Gibson, G. R.; Casteilla, L.; Delzenne, N. M.; Alessi, M. C.; Burcelin, R., Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 2007, *56*, 1761-1772.
132. Song, P.; Zhang, R.; Wang, X.; He, P.; Tan, L.; Ma, X., Dietary grape-seed procyanidins decreased postweaning diarrhea by modulating intestinal permeability and suppressing oxidative stress in rats. *J. Agr. Food Chem.* 2011, *59*, 6227-6232.
133. Goodrich, K. M.; Fundaro, G.; Griffin, L. E.; Grant, A.; Hulver, M. W.; Ponder, M. A.; Neilson, A. P., Chronic administration of dietary grape seed extract increases colonic expression of gut tight junction protein occludin and reduces fecal calprotectin in a secondary analysis of healthy Wistar Furth rats. *Nutrition Research* 2012, *32*, 787-794.
134. Sung, N. Y.; Yang, M. S.; Song, D. S.; Kim, J. K.; Park, J. H.; Song, B. S.; Park, S. H.; Lee, J. W.; Park, H. J.; Kim, J. H.; Byun, E. B.; Byun, E. H., Procyanidin dimer B2-mediated IRAK-M induction negatively regulates TLR4 signaling in macrophages. *Biochem. Bioph. Res. Co.* 2013, *438*, 122-128.

135. Terra, X.; Valls, J.; Vitrac, X.; Mérrillon, J.-M.; Arola, L.; Ardèvol, A.; Bladé, C.; Fernández-Larrea, J.; Pujadas, G.; Salvadó, J.; Blay, M., Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. *J. Agr. Food Chem.* 2007, *55*, 4357-4365.
136. Pallares, V.; Fernandez-Iglesias, A.; Cedo, L.; Castell-Auvi, A.; Pinent, M.; Ardevol, A.; Salvado, M. J.; Garcia-Vallve, S.; Blay, M., Grape seed procyanidin extract reduces the endotoxic effects induced by lipopolysaccharide in rats. *Free Radical Bio. Med.* 2013, *60*, 107-114.
137. Ding, Y.; Zhang, Z. F.; Dai, X. Q.; Jiang, Y. F.; Bao, L.; Li, Y. J.; Li, Y., Grape seed proanthocyanidins ameliorate pancreatic beta-cell dysfunction and death in low-dose streptozotocin- and high-carbohydrate/high-fat diet-induced diabetic rats partially by regulating endoplasmic reticulum stress. *Nutr. Metabolism* 2013, *10*, 51.
138. Reagan-Shaw, S.; Nihal, M.; Ahmad, N., Dose translation from animal to human studies revisited. *FASEB J.* 2008, *22*, 659-661.

Appendices

Appendix A: Cocoa Extraction Protocol

Reagents

1. Cocoa powder
2. Hexane
3. 70:28:2 Acetone:MilliQ Water:Acetic Acid solution

Materials

1. Gloves, goggles,
2. 100 mL graduated cylinder
3. 1000 mL, 500 mL, 250 mL beakers
4. Sonicator
5. Hot plate, stir bar
6. 250 mL centrifuge tubes
7. Avanti J-E Centrifuge
8. Large drying plate
9. 500 mL RotoVap flask
10. IKA RV 10 Basic (RotoVap)
11. Freeze dryer
12. Freeze dryer flask
13. Freeze dryer filters

Preparation

1. Measure out ~100 g of cocoa. This will give a yield of ~10 grams of cocoa extract (CE).
2. Pre-freeze 250 mL beaker and freeze dryer flasks for freeze dryer in -20°C freezer.
3. Tape/staple filters to the lids of the freeze dryer flasks.
4. Turn on the RotoVap.

Method

De-fat Cocoa & Remove Fiber

1. Place measured cocoa and 400 mL of hexane in a 1000 mL beaker.
2. Sonicate the mixture for 10 min, ~22°C.

3. Add a stir bar to the beaker and stir on a hot plate for 5 min.
4. Pour mixture into 4 250 mL centrifuge tubes.
5. Centrifuge for 5 min at 5000 x g using the Beckman Coulter Avanti J-E Centrifuge, Rotor 14JA.
6. Discard the supernatant and transfer the cocoa back into the 1000 mL beaker.
7. Repeat steps 1-5.
8. Discard the supernatant and add 10-20 ml hexane in each centrifuge tube and shake well before pouring all the contents to the silver drying plate. Allow the remaining hexane to evaporate. The cocoa should turn from a muddy sludge back into a dry, light brown powder.

Extract Polyphenols

1. Place the de-fatted cocoa in another 1000 mL beaker.
2. Add ~400 mL of 70:28:2 Acetone:MilliQ Water:Acetic Acid solution.
3. Sonicate the mixture for 10 min, ~22°C.
4. Add a stir bar to the beaker and stir on a hot plate for 5 min.
5. Pour the mixture into 2 250 mL centrifuge tubes.
6. Centrifuge for 5 min at 5000 x g using the Beckman Coulter Avanti J-E Centrifuge.
7. Pour the supernatant into a 500-1000 ml beaker.
8. Scrape the cocoa back into the 1000 mL beaker.
9. Repeat steps 2-8 at least 2 more times.
10. Allow the acetone to evaporate out of the solution. This may need to be done in batches; RotaVap no more that ~200 mL at one time. If the solution stops boiling, increase the vacuum. If the vacuum cannot be turned up anymore and the solution is not boiling, then it is done.
11. Turn on the freeze dryer to pre-cool.
12. Pour the CE into pre-cooled 250 mL beakers and then place beaker in pre-cooled freeze drying flasks. The liquid should make only a thin layer on the bottom. Use multiple beakers/flasks if necessary.
13. Place the freeze drying beakers/flasks in the -80°C freezer for 20 minutes, or until the CE freezes.
14. Place frozen freeze drying flasks with beaker holding the CE onto the freeze dryer with a bin of dry ice underneath flask. Surround/cover the flask as much as possible with dry ice to assure CE stays frozen for as long as possible.
15. Freeze dry the cocoa CE for at least 2 days.
16. After freeze drying:
    a. Crush the CE into a powder with a spatula.
    b. Measure and record the yield.
    c. Transfer the CE to a 50 mL centrifuge tube and store in the -80°C freezer.

Appendix B: Cocoa SPE Fractionation Protocol

Reagents

1. Cocoa extract (CE)
2. Solvents (MQwater (pH=7), MeOH, ethyl acetate, 70:28:2 Acetone:MilliQ Water:Acetic Acid solution

Materials

1. Gloves, goggles
2. 1 Waters Sep-pak column tC18 & C18 (20 cc, 5 g)
3. 20 mL glass culture test tubes
4. 500mL RotaVap flask
5. IKA RV 10 Basic RotaVap
6. Freeze dryer
7. Freeze dryer flask

Preparation

1. Attach tC18 column on top of C18 column using strips of parafilm.
2. Precondition columns by running 10 mL MeOH in each followed by 10 mL MQwater.
3. Discard eluents under each column.
4. Place freeze dryer flasks in freezer and turn on freeze dryer.

Note: the following method is described per one column, ie. If using ten columns, multiple all quantities by ten.

Method

Monomer & Oligomer Removal

1. Dissolve 150 mg CE in 1.5 mL acetone:water:acetic acid 70:28:2 (100 mg/mL), load 1.5 mL of mixture in each column.
2. Elute phenolic acids with 10 mL water in each column, turn vacuum on.
3. Discard eluents.
4. Add 40 mL ethyl acetate to each column, turn vacuum on.
5. Collect eluents from column in RotaVap flask.

6. RotaVap monomer and oligomer eluent.
7. Once all ethyl acetate is removed, add ~1.0-1.5 mL 70:28:2 mix to the RotaVap flask containing the monomer and oligomer.
8. Dissolve monomer and oligomer in mix using the sonicator. Be sure to remove any monomer/oligomer residues from all sides of the RotaVap. we

Polymer Removal

1. Place 25 mL MeOH in the same column eluted with ethyl acetate in step #4, turn vacuum on.
2. Continue eluting with MeOH until no color is left in column.
3. Collect all polymer eluents from column in RotaVap flask.
4. RotaVap polymer eluent to remove MeOH.
5. Freeze dry polymer concentrate and store in -80°C.
6. For all fractions, refer to Steps 13-15 of Appendix A for proper freeze drying method.

Monomer & Oligomer Separation

1. Precondition new column as in Prep Step #1.
2. Deposit ~1.0-1.5 mL of mix from Step #8 into column (be sure that all of the mix from Step #8 is deposited onto a column), discard eluent.
3. Add 35 mL diethyl ether to each column, turn vacuum on.
4. Collect monomer eluents from column into RotaVap flask
5. RotaVap monomer eluent to removed diethyl ether.
6. Freeze dry monomer concentrate and store in -80°C.
7. Add 40 mL MeOH to each column, turn vacuum on.
8. Collect oligomer eluents from column in RotaVap flask.
9. RotaVap oligomer eluent to remove MeOH.
10. Freeze dry oligomer concentrate and store in -80°C.

Appendix C: Cocoa Thiolysis Protocol

Reagents

1. Reaction substrate (cocoa extract or other procyanidin-rich product)
2. MeOH
3. 0.05% benzyl mercaptan in MeOH
4. 3.39% HCl in MeOH
5. 0.1% formic acid in water (phase A)/0.1% formic acid in acetonitrile (phase B)(95:5)

Materials

1. Gloves, glasses
2. 50-100 µL micropipette and tips
3. Microcentrifuge tubes with screw caps
4. Parafilm
5. HPCL vials and caps

Preparation

1. Heat water on hot plate to 90°C.
2. Defrost reaction substrate to room temperature.

Method

1. Dilute reaction substrate to 0.5 mg/mL in MeOH.
2. Prepare to perform the reaction under a hood with proper PPE.
3. In a microcentrifuge tube, combine:
    a. 50 µL of diluted reaction substrate.
    b. 50 µL of HCl reagent.
    c. 100 µL of benzyl mercaptan reagent.
4. To prepare a control sample, in a microcentrifuge tube, combine:
    a. 50 µL of diluted reaction substrate.
    b. 150 µL of MeOH.
5. Parafilm the lids of the microcentrifuge tubes tightly and invert each tube ~3 times.
6. Place the microcentrifuge tubes in the 90°C water for 5 min.
7. Remove samples from water and place immediately in ice for 5-10 min.
8. To prepare the samples for LC/MS analysis:

a. Transfer 100 µL from the centrifuge tube to an HPLC vial.
b. Add 900 µL of 95:5 phase A/phase B.
c. Invert gently to mix.

9. Analyze using LC/MS method. Inject 20 µL quantities.