Amorphous solid dispersion effects on *in vitro* solution concentrations of quercetin

by

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ABSTRACT [academic]

Quercetin is a flavonol with potential health benefits including activities against cardiovascular disease, obesity, and oxidative stress. However, the benefits of quercetin are likely limited by poor bioavailability, primarily attributed to its poor aqueous solubility (due to its hydrophobicity and crystallinity) and extensive phase-II metabolism. Improving the apparent solubility of quercetin has the potential to improve its *in vivo* bioavailability. Strategies to increase solution concentrations in the small intestinal lumen have the potential to substantially increase quercetin bioavailability, and efficacy. We aimed to achieve this by incorporating quercetin into amorphous solid dispersions (ASDs) with cellulose derivatives, eliminating crystallinity, and selectively releasing amorphous quercetin under simulated intestinal conditions (pH 6.8, 37°C). Amorphous quercetin was dispersed in cellulose esters including 6-carboxycellulose acetate butyrate (CCAB), hydroxypropylmethylcellulose acetate succinate (HPMCAS) and cellulose acetate suberate (CASub) to achieve stability and provide pH-triggered release. In addition, polyvinylpyrrolidone (PVP) containing CASub and CCAB blends were prepared to further promote enhanced dissolution. The ASD employing 10% quercetin in 20% PVP:70% CASub was most successful at enhancing the solution concentration of quercetin, providing an 18-fold increase in the area under the concentration/time curve (AUC) compared to quercetin alone. These results warrant *in vivo* assessment of quercetin-loaded ASDs formulated with CASub and its blend with PVP towards improving the bioavailability of quercetin.

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ABSTRACT [public]

Quercetin is a natural polyphenol found in many different foods of plant origin, most notably, fruits and vegetables. Quercetin has many documented health benefits, but as it does not dissolve well in water and is extensively metabolized in the body, it is not efficiently absorbed into the bloodstream. Improving the ability of quercetin to dissolve in water has great potential to increase the amount present in the bloodstream. One promising strategy that aims to increase the solubility of substances that do not dissolve well in water is amorphous solid dispersion (ASD). This strategy uses polymer matrices to keep a drug from becoming crystalline. We have employed polymers derived from natural cellulose in ASD with quercetin in hopes of improving its dissolution *in vitro*. Non-crystalline quercetin was dispersed in esters of cellulose including 6-carboxycellulose acetate butyrate (CCAB), hydroxypropylmethylcellulose acetate succinate (HPMCAS) and cellulose acetate suberate (CASub) to provide stability and afford pH-triggered release. In addition, polyvinylpyrrolidone (PVP) containing CASub and CCAB blends were prepared to further promote enhanced dissolution. The dispersion employing 10% quercetin in a blend of 20% PVP and 70% CASub (% w/w) was able to provide the highest amount of quercetin dissolved in water at intestinal pH, providing an 18-fold increase compared to quercetin alone. These results warrant further investigation into quercetin-loaded ASDs and their ability to increase the absorption of quercetin *in vivo*.

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Chapter 1: Introduction

There is tremendous interest in the potential beneficial health effects of flavonoids, including flavonols such as quercetin due to its dietary abundance and documented bioactivities. Nonetheless, major limitations of compounds like quercetin are low aqueous solubility, low bioavailability, and overall insufficient presence of the intact aglycone in the blood due to extensive metabolism *in vivo*, making it difficult for the positive health effects of consumption to be achieved. When positive health outcomes as a result of quercetin ingestion are observed, they aren't typically due to short-term doses [1]. Chronic dietary exposure of quercetin can lead to its sustained presence in the body, and it's those sustained concentrations that can have the most benefits [1]. Unfortunately, quercetin is both highly crystalline and hydrophobic. The combination of these two properties severely limits its solubility, which can be translated to poor absorption after consumption. This leads to overall poor systemic delivery, i.e. bioavailability, of quercetin, a detrimental quality to have for such a highly bioactive, abundant polyphenol in our diet.

Despite its potential benefits, as a xenobiotic, quercetin is actively metabolized and excreted [2-5]. The extensive modification and elimination of quercetin poses a challenge for achieving and maintaining the blood and tissue concentrations needed to obtain the proposed bioactivity. Aside from chronic consumption at extremely high doses, there have been many strategies proposed to help facilitate the absorption of quercetin and other polyphenolic compounds through increasing its apparent solubility. Amorphous solid dispersions (ASDs), cyclodextrins, self-emulsifying drug delivery systems (SEDDs), pro-drugs, liposomes, and solid-lipid nanoparticles (SLNs) are just a few of the options currently being evaluated (Table 2.2). All methods have their advantages and disadvantages, however, some methods, such as ASD, seem to be more favorable and are thus the focus of research moving forward. Specifically, ASDs

using cellulosic polymers are some of the more promising strategies being used [6]. Despite promising results *in vitro*, there are still questions on how successful this method will be *in vivo*, since ASD has not been tested thoroughly in animal models. As research advances, testing the best performing ASDs, SEDDs, pro-drugs, and other strategies *in vivo* will be a true evaluation of effectiveness, because *in vitro* dissolution is a model that predicts solubility but does not predict the full complexity of absorption and pharmacokinetics *in vivo*. There have been *in vitro* methodologies developed to try and simulate *in vivo* environments [7], however there will always be limitations when physiological conditions can not be exactly replicated [8].

Research Objectives and Specific Aims

The *long-term goal* of this research is to develop strategies to improve the health benefits from consumption of naturally occurring dietary phytochemicals. The *overall objective of this thesis research project* was to improve the dissolution of quercetin by incorporating it into cellulose-derived ASDs. The *central hypothesis* was that ASDs using cellulose derivatives will facilitate improved apparent solubility, i.e. solution concentrations, while also providing pH-responsive release selectively at near-neutral intestinal pH. The following research aims were carried out in order to reach the project objective:

- **Research Aim 1:** Determine the successful incorporation amorphous quercetin in ASD at different levels (% w/w).
    - *Hypothesis:* ASDs with 10%, 25%, and 50% quercetin will show amorphous qualities through XRD, DSC, FTIR, and SEM.
- **Research Aim 2:** Determine the impact of ASD on improving quercetin apparent solubility.

- *Hypothesis:* ASDs will significantly increase final quercetin solution concentration and its total dissolution at intestinal pH (6.8) as measured by the area under the concentration/time curve (AUC).

The future directions following of this research will be to assess the potential for identified ASD candidates to improve the bioavailability and bioactivity of quercetin *in vivo*. For example, as discussed in this literature review, quercetin is believed to possess anti-obesity and anti-diabetic bioactivities. Through a chronic feeding study of quercetin incorporated into ASDs, not only would we expect an enhancement in bioavailability of quercetin, but also enhancement of those two bioactivities as a result. This strategy to enhance dissolution as a means to improve bioavailability and bioactivity could be employed for many other flavonols (and flavonoids in general) that have health benefits but are limited by crystallinity, increasing the efficacy of these phytochemicals for prevention and amelioration of a number of diseases.

Chapter 2: Literature Review

Introduction

Polyphenols are naturally occurring compounds commonly found in foods of plant origin and are a major source of antioxidants consumed on a daily basis [9]. From the perspective of the plant's metabolism, compounds can be divided into two categories: primary and secondary metabolites. Primary metabolites are essential for growth, development, reproduction and the overall survival of a plant, whereas secondary metabolites serve additional functions not needed for immediate survival and growth [10]. Secondary plant metabolites provide non-essential benefits to the plant, such as deterring forage by insects and animals, attracting pollinators, deterring growth of competitor plants, etc. Polyphenols are secondary metabolites, and while not needed by the body, still have the potential to exhibit beneficial health effects when consumed. Flavonoids are commonly studied because of their abundance in nature, significant intake as part of the diet, and potential health benefits. Research has shown that these compounds contribute towards the prevention of diseases such as cancer, cardiovascular disease (CVD), and metabolic syndrome [11]. Polyphenols can be generally broken down into four major groups: phenolic acids, flavonoids, stilbenes, and lignans [11] (Fig. 2.1). Flavonoids are classified based on the presence of different substituents on the rings of their benzo-γ-pyrone ring structure [12] (Fig. 2.2).

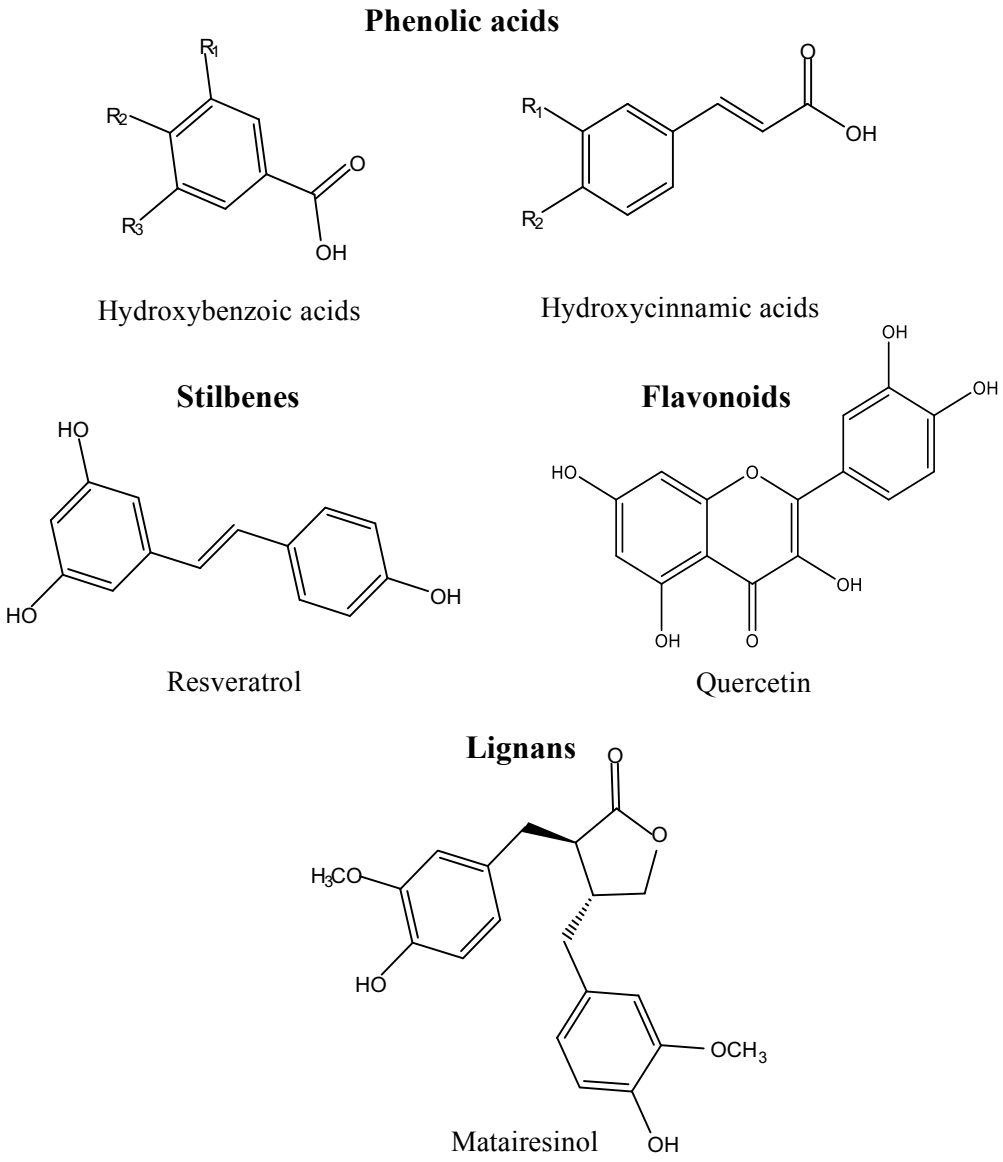


Figure 2.1. *Four main classes of polyphenols are displayed. Chemical structures of examples of polyphenols within each class are provided.*

Flavonols are some of the most common flavonoids found in foods [11]. Major dietary sources of flavonols are fruits and vegetables. Most health benefits associated with the consumption of flavonols have been believed to be due to their high antioxidant capacity, however recent research is favoring other mechanisms. For example certain flavonols may have the ability to upregulate GLUT4 receptors, the body's primary source of glucose removal from

circulation [13], in skeletal muscle, an effect that would have alleviating effects on metabolic syndrome [14]. Also, the flavonol quercetin has been shown to decrease muscle damage and body fat percentage in healthy males [15]. That being said, proposed mechanisms of flavonol bioactivities are still speculative despite some research regarding the topic.

Quercetin (Fig. 2.1), our flavonoid of interest, is a dietary flavonol consumed daily. It can be found in many fruits and vegetables, including apples, onions, broccoli, cranberries, apricots, and raw spinach. In addition, quercetin can be found in our diet as one of its common glycosides (glycoside refers to a compound that has a sugar moiety attached to the native form of, in this case, quercetin at one of its –OH groups), rutin (Fig. 2.3), but this is not the focus of the review. Some plant foods can contain as much as 500 mg polyphenols per 100 g of food [16]. Most vegetables contain < 10 mg/kg quercetin, with the exception of onions (up to 486 mg/kg), kale (110 mg/kg), broccoli (30 mg/kg), and French and slicing beans (~30 mg/kg). Fruits generally contain ~15 mg/kg quercetin, with only apples containing more (up to 72 mg/kg) [17]. In one review of dietary flavonols, quercetin content in beverages was provided in comparison to solid foods. Beverages such as juice (apple, grape, orange, tomato, grapefruit, lemon), tea (black, green, oolong), and wine all have < 2.5 mg/100 mL, while some raw fruits and vegetables have anywhere from 10-1,490 mg/kg [1]. Flavonol content in food is also affected by a variety of factors such as type of plant, seasonal influence, light and climate, degree of ripeness, and preparation/processing [1]. Different types of onions can range from 185 to 634 mg quercetin per kg fresh weight. Cherry tomatoes contain 17-203 mg quercetin per kg fresh weight compared to 2.2-11 mg per kg in normal beef tomatoes [18]. One study determined that sun exposure increased the amount of both quercetin aglycone and its glycosides in Pinot noir grapes. When compared to grapes that were shaded from the sunlight, glycosides were measured at a level three times

higher in moderately exposed grapes and seven times higher in highly exposed grapes [19]. Finally even during cooking of fruits and vegetables; high levels of heat normally reduce levels of flavonols in foods, most likely due to thermal degradation [1]. For example, bilberries that have been cooked (heated) have been shown to lose 40% of their quercetin content [20]. This trend is most likely seen with other flavonols in berries as well.

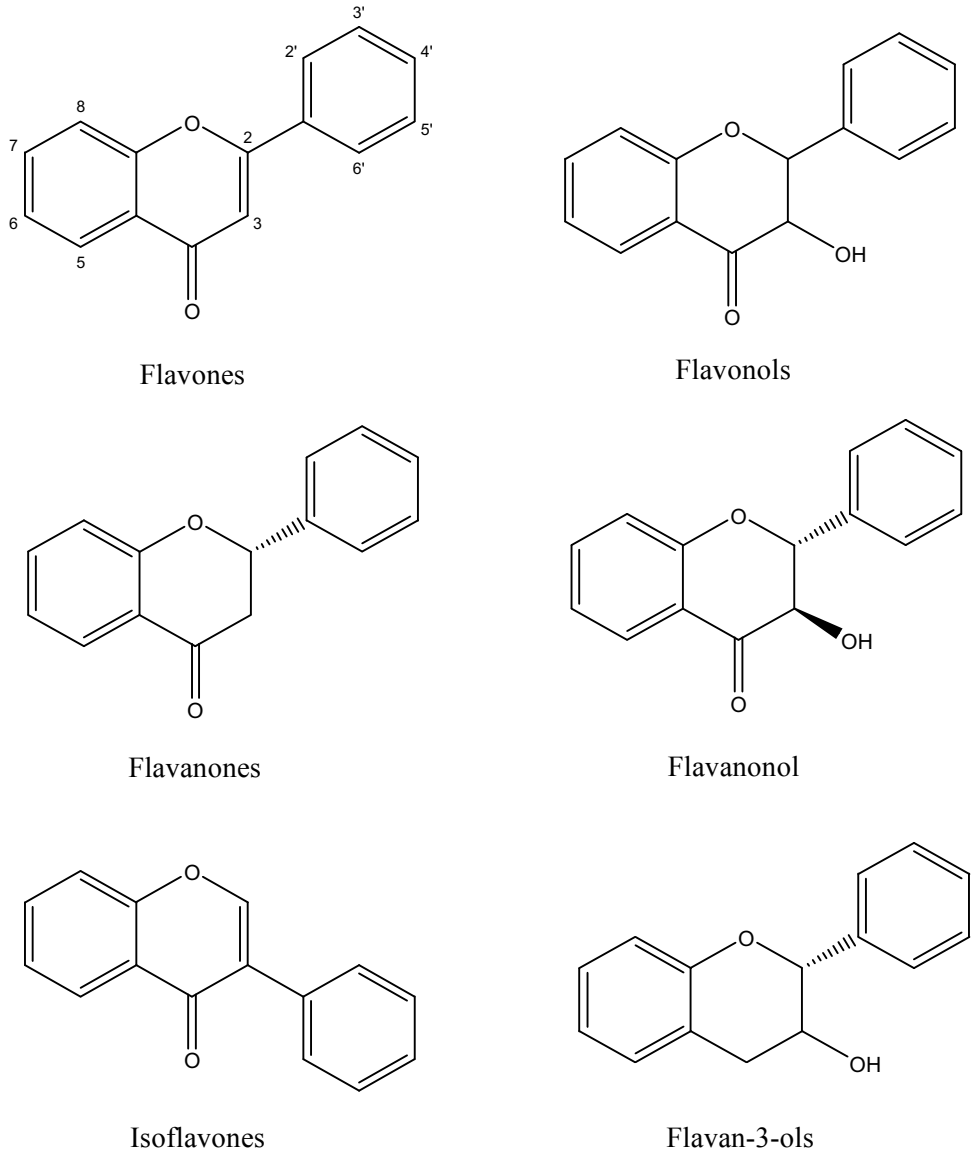


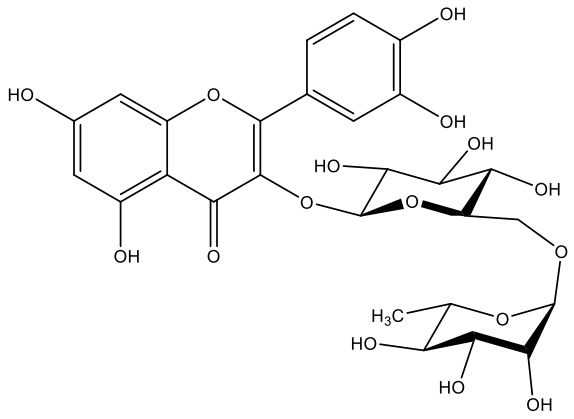
Figure 2.2. *Chemical structures of the different classes of flavonoids are shown above.*

Daily intake of flavonols has been reported as 20-35 mg/day, 65% of which has been determined to be quercetin and its glycosides, i.e. quercetin with sugar moieties attached through a phenolic –OH groups by glycosidic linkages [21] (Fig. 2.3 & 2.4). However, intake of quercetin aglycone from the diet has been reported to be as high as 29.4 mg/day [22]. Quercetin has also been hypothesized as a potential beneficial supplement to the normal Western diet [23]. If delivered in amounts ~300 mg, up to six times a day, intake could be close to 100 times higher than dietary intake levels and it is postulated that the positive health effects of quercetin would be significantly enhanced. However, quercetin can exhibit hormesis, a phenomenon that occurs when low doses of a toxin or xenobiotic prove to be more biologically beneficial than higher doses [24]. One review of quercetin stated that quercetin exhibits antioxidant behaviors at cellular concentrations below 40 μM, but is then a pro-oxidant when cellular concentrations are above 40 μM [24]. That being said, the supplement mentioned above may or may not be beneficial depending on the specific individual.

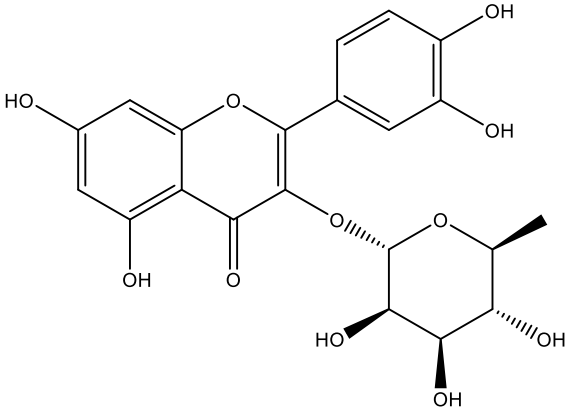
Dietary Source: Apples

According to Ceymann *et al*., the amount of quercetin and its derivatives found in four different types of apples (Braeburn, Fuji, Gala, and Golden Reinders) is 24-60 mg/kg fresh edible matter [25]. Based on the variety, polyphenolic content in apples can range from 211-926 mg gallic acid equivalents (GAE)/L in fresh pressed juice [26]. Nonetheless, a clear majority of quercetin exists in apples as conjugated derivatives. Common examples include rutin, quercetin rhamnoside, and glucuronidated quercetin (Fig. 2.3). Researchers employ hydrolysis to cleave the sugars from quercetin to gain an accurate quantification of total quercetin in apples. In apple peel, before hydrolysis, the free quercetin content is about 21 ± 2 mg/kg dry weight. After hydrolysis, that amount significantly increases to 250 ± 4 mg/kg dry weight [27]. Since apples

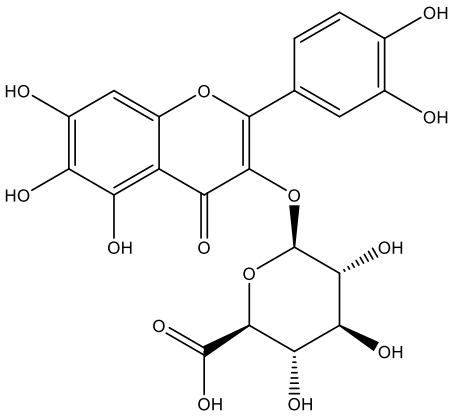
contain relatively large amounts of quercetin and other polyphenols, they have been the center of many studies relating consumption to proposed health benefits. For example, two quercetin metabolites found in apple peel extract, quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronic acid, have been shown to inhibit angiotensin converting enzyme (ACE) activity *in vitro*; ACE is an enzyme mainly responsible for hypertension [28]. In addition, apple flavonols, including quercetin and its glycosides, have demonstrated the ability to significantly improve blood lipid profiles (i.e. lower serum triacylglycerol and non-high density lipoprotein cholesterol concentrations) in rats with diet-induced hyperlipidemia [29].



Rutin



Quercetin rhamnoside



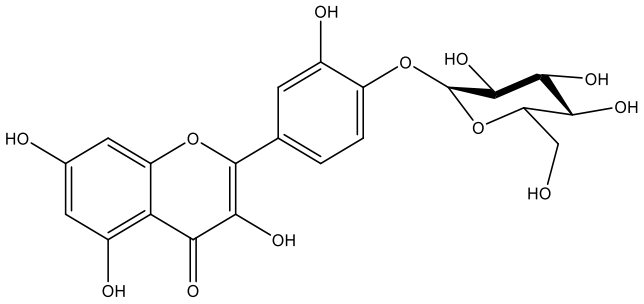
Quercetin 3-*O*-glucuronide

Figure 2.3. *The chemical structures of common conjugated derivatives of quercetin found in apples are shown.*

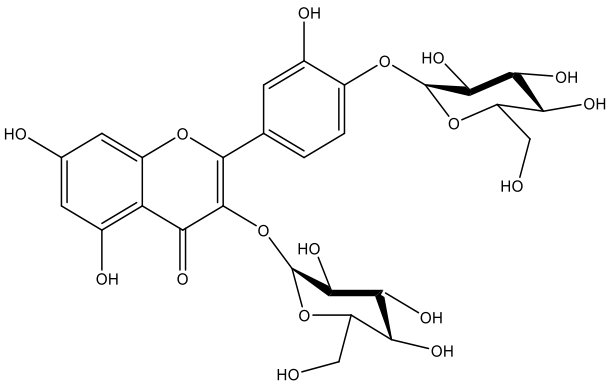
Even apple pomace has been investigated as a potential ingredient in new functional foods. The incorporation of apple pomace into extruded snacks would not change the proximate composition of the final products ($p < 0.05$), but could provide increased antioxidant activities upon consumption [30]. Finally, one preliminary study has shown that women who consumed greater than 1 apple/day had a 28% reduced risk of type 2 diabetes mellitus [31]. Although a limited number of examples have been discussed, it's easy to see that apples are a main focus when it comes to finding natural sources of antioxidants and disease prevention, among many potential health benefits.

Dietary Source: Onions

Onions are another source of quercetin in an every-day diet. Interestingly enough, worldwide onion consumption increased 25% from 1992 to 2002, making it the second most important horticultural crop after tomatoes [32]. The major metabolites of quercetin found in onions are quercetin-3,4'-diglucoside and quercetin-4'-monoglucoside (Fig. 2.4).



Quercetin- 4'-monoglucoside



Quercetin- 3,4'-diglucoside

Figure 2.4. *The chemical structures of quercetin-4'-monoglucoside and quercetin-3,4'–diglucoside are shown above.*

One study set out to quantify these two compounds in a few onion varieties and found significantly different levels between red, brown, and white onions (Table 2.1). Onions still prove to be a valuable source of quercetin with associated potential benefits of consumption. For example, Hubbard *et al*. have shown that ingestion of high-quercetin onion soup (69 mg total quercetin) could somewhat inhibit platelet aggregation [33]. Also, quercetin-enriched onion peel extract has been shown to have anti-obesity effects in high-fat fed rats. The suspected mechanism is inhibition of adipogenesis [34]. Onion (cv. Destiny) consumption in pigs reduced total blood cholesterol, low density lipoprotein, and triglycerides [35]. As we see this trend continue, the limited research on onions should be further investigated, presenting many potential opportunities for improved health.

Table 2.1. Quercetin content of three varieties of onion [36].

Onion Variety	Quercetin diglucoside content (mg/kg dry wt.)	Quercetin monoglucoside content (mg/kg dry wt.)
Redwing	1910	850
Cream Gold	1530	580
Spanish White	<10	<10

Dietary Source: Berries

A third dietary source of quercetin is berries, providing up to 240 mg quercetin per kg fresh weight upon consumption, as well as different types of quercetin conjugates (Table 2.2). Similar to apples and onions, there is ample research in the area of quercetin content in berries, the bioavailability of quercetin and its metabolites, as well as their bioactivities against many health complications and diseases. Two examples will be described in more detail. In a study of quercetin bioavailability, researchers measured serum quercetin concentrations in human subjects who ingested 100 g of berries a day, in addition to their normal diet, versus a group of subjects who consumed a normal diet without the addition of berries. After four weeks, a 42%

increase in serum quercetin concentration was seen in those subjects consuming the 100 g/day of berries. Four weeks later, the increase in serum quercetin concentration was up to 51% higher than the control [37]. In a separate bioavailability study, an average consumption of 160 g of berries per day (bilberries, ligonberries, black currants, and chokeberries) in middle-aged subjects led to an increase in plasma quercetin concentrations ranging from 51-84% throughout the eight week study [38]. Both of these studies show that berries are an excellent source of quercetin in the diet.

Table 2.2. Quercetin content in different species of berry [1, 39].

Berry Type	Quercetin content (mg/kg)
Bilberry	105-160
Blackberry	5-35
Blueberry	24-29
Cranberry	112-150
Elderberry	105-240
Raspberry	5-29
Strawberry	6-170

Bioactivity research regarding the quercetin found in different berries is extensive. Saw *et al.* have shown that three polyphenols found in berries (quercetin, kaempferol, and pterostilbene) exhibit potential cancer chemoprevention through synergistic antioxidant activity effects [40]. In a 2010 study, quercetin and quercetin 3-*O*-glycosides from *Vaccinium vitis idaea* (*V. vitis*) crude berry extracts were shown to increase the uptake of glucose, 37% and 38-59% respectively, in muscle cells by upregulating the activity of AMP-activated protein kinase (AMPK) [41]. This finding could be helpful for those individuals who have insulin resistance and/or other metabolic diseases, increasing overall efficiency of glucose uptake and reducing blood glucose levels. In another contrasting experiment, quercetin (6.5 μg/mL) in aronia berry extract inhibited

lipopolysaccharide (LPS)-stimulated expression of IL-10 [42], an anti-inflammatory cytokine that plays an important role in immune function. Decreased expression of IL-10 can lead to autoimmune disease susceptibility [43]. This suggests that the bioactivity of quercetin could also have deleterious effects *in vivo*. Research in the future on the benefits of consuming different species of berry could be important in showing the benefits of natural sources of quercetin and its health-protective effects.

Bioavailability

Overview of Bioavailability

Bioavailability can be described as the proportion of orally ingested compound that reaches the blood plasma over time, in its original form, and is readily available for the body to utilize and/or store or to exert its bioactivities[16]. This proportion represents the amount of ingested compound absorbed in the GI tract and transported into circulation [44]. Bioavailability is limited to a percentage of a compound's bioaccessibility, defined as the amount of a compound that is released from the food and solubilized in the GI tract and available for absorption [45]. Bioavailability is the result of many biochemical processes throughout the human body and it can be broken down into four main processes: absorption, distribution, metabolism, and excretion.

Before absorption can occur, a compound must be present in the body and be soluble in the gut region where it is absorbed. The main route of administration for drugs, flavonoids and other compounds is through ingestion, so the following processes will focus on compounds that have entered the body via that route. Other routes of administration include intravenous, subcutaneous, intramuscular, and inhalation [46]. Solubilization is the first step for a compound to be absorbed. During travel through the GI tract, ingested compounds will be presented to the

small intestine, an entirely aqueous environment, for potential absorption. Without solubilization, absorption is not possible. Absorption can be thought of as a function of the physical and chemical properties of a drug or polyphenol because in order for a compound to be considered "bioavailable" it must be absorbed into the bloodstream and reach its site of action [46]. Drugs and other compounds can be classified based on their solubility and permeability (Table 2.3). These parameters can give researchers a good idea of how a compound will perform *in vivo*, as well as during *in vtiro* dissolution. Absorption through the epithelial membrane is very limited for most compounds that have high molecular weight, large polar surface area, and a number of rotational bonds [47]. Some compounds with these characteristics may not be absorbed and pass through the digestive tract unchanged. In general, solubility and absorption are major obstacles some drugs and phytochemicals must face in order to have substantial bioavailability.

Table 2.3. Drug classification based on their solubility and permeability [48, 49].

Class	Characteristics
I	High solubility, high permeability; generally well absorbed
II	Low solubility, high permeability; dissolution-limited absorption
III	High solubility, low permeability; permeability limited absorption
IV	Low solubility, low permeability; poor oral bioavailability

Distribution refers to the passage of a drug to its intended target, and generally describes its transport throughout the body. Another major hurdle for high bioavailability is xenobiotic metabolism. The body's metabolism is incredibly extensive, with numerous biochemical pathways and enzymes involved, some of which will be discussed later. The body possesses a system of reactions designed to modify and excrete xenobiotics. This typically involves conversion to more polar forms, which eventually makes it more easily cleared from the body. This greatly affects bioavailability, reducing the amount of original (active) concentrations

reaching systemic circulation over time. Finally, elimination of compounds from the body is important when bioavailability is concerned. Elimination is another limiting factor because elimination and excretion lowers concentrations in the blood. Even when compounds overcome solubility and absorption limitations and enter into circulation, the liver, kidneys, and lungs filter virtually all of the body's blood and therefore are major organs where elimination is facilitated. In general, there are two types of transport during elimination – passive and active transport [47]. The primary goal of xenobiotic metabolism is to make compounds more polar, with the exception of methylation, and therefore easier to excrete in aqueous bodily fluids. This can be accomplished with (active) or without (passive) the consumption of energy, i.e. ATP. Passive diffusion of xenobiotics involves the movement of molecules through a concentration gradient, that is, from an environment of high concentration to one of low concentration. Xenobiotic metabolism can create an environment inside the cells of the liver, kidneys, or lungs that is highly concentrated with polar xenobiotic molecules, facilitating their excretion. Active transport of xenobiotics utilizes the body's ATP production to move compounds out of cells and towards elimination. Major sites of active transporters are the liver, kidney, brain, and intestine. Others include the adrenal gland, heart, and skeletal muscle [47].

Factors Affecting Bioavailability of Flavonoids

In many cases, the bioavailability of flavonoids is highly variable, often a consequence of the type of food it is in as well as the matrix from which they are delivered. For example, it has been found that quercetin glycosides from onions are more bioavailable than the glycosides and rutin found in apples and tea [50]. Generally speaking, an increase or decrease in the concentration of flavonoid or its metabolites in blood plasma after consumption of polyphenol enriched foods can be directly related to 1) the amount absorbed, 2) solubility/release in the gut, and 3) the gut's

ability to absorb these compounds [51]. As a result, many researchers focus closely on the factors that affect polyphenol absorption. A few key factors will be described [52]. One important factor is the size of the flavonol, which has an enormous effect on absorption. Typically, lower absorption levels are seen as the size of the flavonol increases. In fact, there is a set of predictive "rules" for drug bioavailability developed based on drug behavior that can be related to flavonols – "Lipinksi's Rule of 5" [53]. According to the rules, compounds are more likely to exhibit poor membrane permeation and absorption if 1) there are more than five H-bond donors, 2) more than ten H-bond acceptors, 3) the compound has a molecular weight > 500, and 4) the calculated LogP is greater than five [53], where LogP refers to the logarithm of octanol/water partition coefficient [54]. Drugs, polyphenols, and other compounds that enter our body will typically exhibit low permeation (absorption through the epithelial cell membrane) and hence poor bioavailability if one or more of these conditions is met.

Components within the GI tract can also have an effect on absorption. For example, dietary fat (lipid) has been shown to increase the bioavailability of quercetin through the formation of micelles, allowing easier diffusion into the bloodstream [55]. Polyphenol binding to dietary lipids may also help decrease lipase activity and fat absorption [56]. One recent study was able to show a relationship between poor vitamin C status in adults and increased quercetin absorption [57]. Another factor is that flavonols are lipophilic in nature, posing a challenge for solubility in water need to cross the unstirred water and access the absorptive mucosal layer of the intestine. This also severely decreases the amount of passive diffusion of flavonols through the epithelium. Crystallinity is another key factor that restricts solubility. It refers to the structural order of a compound's molecules so that they are in the lowest, often most stable, energy state. This often leads to crystalline compounds having low aqueous solubility, due to the

high amount of energy needed to break the bonds present in their crystal lattice structures. The opposite of crystalline is amorphous, or disorder of a compound's molecules, a state where aqueous solubility of most compounds, especially quercetin, is much higher than that of the crystalline form [58-60]. Both states can be measured through differential scanning calorimetry (DSC), where the energy changes associated with crystallization and fusion can be calculated [61]. Two of the most important parameters measured by DSC are a compound's melting point and glass transition temperature (T_g). Amorphous compounds are changed from a solid to a "rubbery", disordered state at their T_g. This high internal energy state enables an amorphous compound to possess enhanced thermodynamic properties, most important being solubility [62]. Important to note, however, T_g is not the same as melting point (T_m), where compounds are changed from a solid to liquid. In DSC of a solid dispersion, a drug will prove to be amorphous if there is the absence of a melting peak in the thermogram [61].

Quercetin is very crystalline in its native form, existing as packages of dimers of itself held together by extensive networking of hydrogen bonds, with the crux of the matrices being the double bonded oxygen at C4 (Fig. 2.1) [63]. In addition, quercetin achieves crystallinity with the help of water molecules, which also exhibit hydrogen bonding with the hydroxyl groups located at C7, C3', and/or C4' (Fig. 2.2.). On top of those crystalline properties, the hydroxyl groups at C3 and C5 provide extra stability through intramolecular bonding with the double bonded oxygen, quercetin's only hydrogen bond acceptor (Fig. 2.1) [63]. Due to its extensive crystallinity and high hydrophobicity, quercetin possesses extremely low aqueous solubility, ranging from 0.00215 g/L to 0.0077 g/L at 25°C [64, 65]. Solubility is important when discussing bioavailability. It is imperative that quercetin, and many other flavonols, be soluble during digestion and metabolism so that they can be efficiently absorbed by the body. Before reaching the epithelium,

compounds must first be released from the food into the gut milieu and then pass through an unstirred water layer (~40 μm in humans and ~100 μm in rats) and the mucosal (~123 μm) layer in the jejunum, both of which act as barriers [66-68]. Without substantially increased solubility, quercetin and other flavonols are unlikely to be in circulation because they will not be able to reach the unstirred water layer (poor release from food) and will have trouble diffusing through the unstirred water layer (due to poor solubility) of the enterocyte apical surface in the lumen. Furthermore, what little amount is absorbed is extensively metabolized into more polar, soluble constituents which can be excreted easier through the urine.

Upon reaching the enterocyte surface, quercetin is present in an abundance of conjugated glycoside forms, making it more difficult for absorption into the enterocyte to occur (see Lipinski's Rule of 5 above). The aglycone needs to be present for maximum absorption. This can be facilitated by one particular brush border enzyme, lactase phlorizin hydrolase (LPH), located on the luminal epithelium. It has been studied and shown to act on some dietary glycosides before they are absorbed, cleaving the conjugate and releasing the aglycone [69]. Once the aglycone is present, absorption can happen in a few different ways. The most simple absorption mechanism is passive diffusion, where quercetin is in high concentration in the lumen and low inside the enterocytes and that concentration gradient drives molecules of quercetin into the cell. There are two other main routes of absorption; paracellular and transcellular transport. Paracellular transport of quercetin occurs in the tight junction spaces between enterocytes. Transcellular transport involves quercetin actually passing through the membranes of the enterocytes, usually via membrane transporters [70, 71].

Even after absorption into epithelial cells, quercetin is subject to extensive modification, which significantly reduces the amount of circulating aglycone, i.e. bioavailability (Table 2.4).

This is a major concern because the epithelium of the small intestine is the single most absorptive region of the GI tract. About 93% of quercetin is metabolized in this region, compared to only 3.1% in the liver; a 30-fold difference [72], and such low absorption correlates with reduced bioavailability.

Table 2.4. A summary of literature on the bioavailability of quercetin in both human and animal models is provided.

Model	Source	Dose	AUC[a]	C_{MAX}[b]	Ref.
Human	Onions	68	7.7	0.74	[50]
	Apples	107	3.5	0.3	
	Pure rutin	100	3.3	0.3	
	Q-4'-glucoside	150	18.8	3.5	
	Q-3-glucoside	156	19.1	5	
	Q-4'-glucoside	160	17.5	4.5	
	Onions	100	32.1	7.6	
	Q-4'-glucoside	100	27.8	7.0	
	Onion	139	N/A	1.34	[51]
	Aglycone	500	0.207	0.051	[73]
	Aglycone	40.5[c]	17.71	1.43	[57]
	Aglycone	35.6[c]	13.5	1.24	[55]
Rat	Aglycone	10	7.5	N/A	[74]
	Aglycone	50	48.435	4.9×10^{-3}	[75]
	Aglycone	100	80.3	9.5×10^{-3}	
	Aglycone	50	187.85	19.536	[76]
	Aglycone	100	0.414	0.9437	[77]
	Buckwheat extract	2.892	18	1.821	[78]
	Buckwheat extract	5.784	33.7	3.642	
	Buckwheat extract	11.568	79.6	6.788	
Cow	Aglycone	50	0.182	0.09	[79]

[a]AUC expressed as μmol·h/L, [b]C_{MAX} expressed as μmol/L, [c]Doses provided in mg per kg/m^2

Metabolism

Xenobiotic Metabolism

The possible health benefits of most flavonoids may be strongly influenced by its chemical modification during xenobiotic metabolism and the activities of the resulting metabolites. Benefits from metabolites, however, are still speculative. In addition, the body absorbs the flavonoid aglycone relatively poorly so the observed benefits are limited. This phenomenon is present because flavonoids are xenobiotic compounds, that is, they are extrinsic to the normal metabolism of our bodies [47]. Xenobiotic metabolism acts upon compounds that have potential to be toxic, yet could also be harmless or even beneficial, but due to the body's inability to distinguish each compound's effects, the processes that take place are performed to reduce their entrance into circulation, reduce their activity and enhance water solubility through metabolism, and enhance their excretion [80]. Major routes of excretion are through urine and feces. When xenobiotic metabolism is studied, these two types of samples are most commonly examined. Minor routes include tears, sweat, and hair [47].

Drug metabolism has been extensively studied and is considered part of the body's xenobiotic metabolism, so it can be used to describe the challenges that both they and flavonols face upon consumption. Barriers to xenobiotics reaching their target include the extremely acidic stomach and enzyme-rich intestinal environments, as well as the physical barrier of the epithelium. After passing through the epithelium, the compound is subject to metabolic enzymes and transporters, provided it is a substrate, which work to efflux the drug out of the enterocytes and back into the lumen for excretion or into portal circulation for further metabolism by the liver. From there, drugs may be transported back into the intestine via bile that originates in the liver and is excreted by the gall bladder, which can eventually be reabsorbed end up back in

portal circulation. This is known as enterohepatic recycling [81]. Enterohepatic recycling is usually the cause of longer apparent half-life, multiple peaks in circulating levels, and increased drug plasma concentration hours after administration [82]. The liver, lungs, and brain are also some major sites of xenobiotic transformation [47], each with significant transformation of foreign compounds. Finally, drugs not absorbed in the small intestine are subject to further modification by the colonic microflora, which can both positively and negatively influence bioavailability [83].

Xenobiotic Metabolism in the Small Intestine, Liver, and Kidneys

Xenobiotic metabolism is a complex process in response to the vast and varied array of unknown compounds that may enter the body. It can be affected by diet – mainly through varying levels of intake of macronutrients, but is also affected by factors such as alcohol consumption or micronutrient intake. For example, lipids and carbohydrates have been shown to be dietary effectors for the CYP450 2E1 enzyme [84]. This specific CYP450 enzyme plays an important role in carcinogen and hepatotoxin metabolism, as well as a potential role in lipid peroxidation [85]. Xenobiotic metabolism can be thought about as three different phases: I, II, and III. In phase I, xenobiotics are converted into more polar substances to make them more reactive species. The most notable pathway of modification involves the cytochrome P450s (CYPs). The CYPs are most commonly found in the intestinal enterocytes and liver, and their main function is to modify xenobiotic compounds, making them more reactive through aromatic hydroxylation, deamination, N- and O- dealkylation, and other reactions [2]. Interestingly enough, quercetin is typically not a substrate for the CYPs and does not undergo metabolic transformations in phase I due to the presence of five hydroxyl groups on the native compound (Fig. 2.1). Although, in terms of metabolic activity, the CYPs are highly concentrated in the liver, they are also present in the kidneys and do exhibit Phase I modification of drugs and other xenobiotics. Other Phase I

enzymes shown to have biotransformation activity in the kidneys include flavin-containing monooxygenases (FMOs), prostaglandin H synthases (PHSs), and carboxylesterases [47, 86]. More recent research has shown that the kidneys have significant metabolic capacity, specifically in activities of CYPs [87], however there is speculation towards other enzymes and their significant contribution to Phase I xenobiotic metabolism.

Phase II of xenobiotic metabolism also takes place in the intestinal enterocytes, liver, and kidneys and involves a number of transferase enzyme-mediated reactions that further modify the majority of phase I constituents into glucuronidated, sulfated, and methylated conjugates of the original compound. Less frequently, conjugation reactions result in compounds that are acetylated, glutathione conjugated, or amino acid conjugated [2]. Nemeth *et al.* have proven this notion, showing that metabolized forms of flavonoids reaching blood circulation are in fact mostly glucuronidated, sulfated, and methylated derivatives [88]. In addition, Birt *et al.* have also reviewed some metabolic properties of flavonoids and isoflavonoids and reached similar conclusions. They have shown that glucuronidated and sulfated conjugates of the parent flavonoid compound are more readily available in the blood than the parent flavonoid itself [44]. Overall, the goal of phase II of xenobiotic metabolism is to 1) modify xenobiotics by changing their chemical structure, therefore decreasing activity, and 2) prepare certain compounds for enhanced excretion by making them more hydrophilic through conjugation. The only exceptions are the methyltransferases, which typically convert xenobiotics into less polar conjugates. Methylation reactions are still considered detoxification reactions, however [47]. Unlike Phase I, quercetin is extensively involved in Phase II xenobiotic metabolism, being modified into a number of methylated, sulfated, and glucuronidated conjugates post-consumption. Common

metabolites include 3-O-glucuronosyl quercetin, 3'-O-glucuronosyl quercetin, 7-O-glucuronosyl quercetin, 3'-O-methyl-quercetin, and 4'-O-methyl-quercetin [89].

Phase III of xenobiotic metabolism involves the active transport of drugs and other modified compounds across the cellular membrane. Unlike the other two phases of xenobiotic metabolism, phase III does not involve modification reactions, rather the involvement of transporters working to efflux the xenobiotic back out of the enterocyte and into the intestinal lumen. Another fate involves efflux into the interstitial space or circulation, most likely leading the xenobiotic through portal circulation to the liver for metabolic detoxification. The most notable transporter in the intestine is P-glycoprotein (permeability glycoprotein), a member of the large ATP-binding cassette (ABC) family of proteins [2, 5]. These transport proteins rely on the hydrolysis of ATP in order to facilitate the expulsion of xenobiotics from the cell. Other ABC drug transporters located in the intestine include MRP1, MRP2, and ABC-P [5]. Phase III metabolism of xenobiotics results in many excreted compounds and is a crucial part of the body's defense mechanisms against certain substances. For quercetin, other flavonols, and all polyphenols for that matter, the phases of xenobiotic metabolism are huge challenges to overcome, which explains the low bioavailability and reduced bioactivities *in vivo*. A schematic overview of the three phases of xenobiotic metabolism of quercetin is provided in Figure 2.5

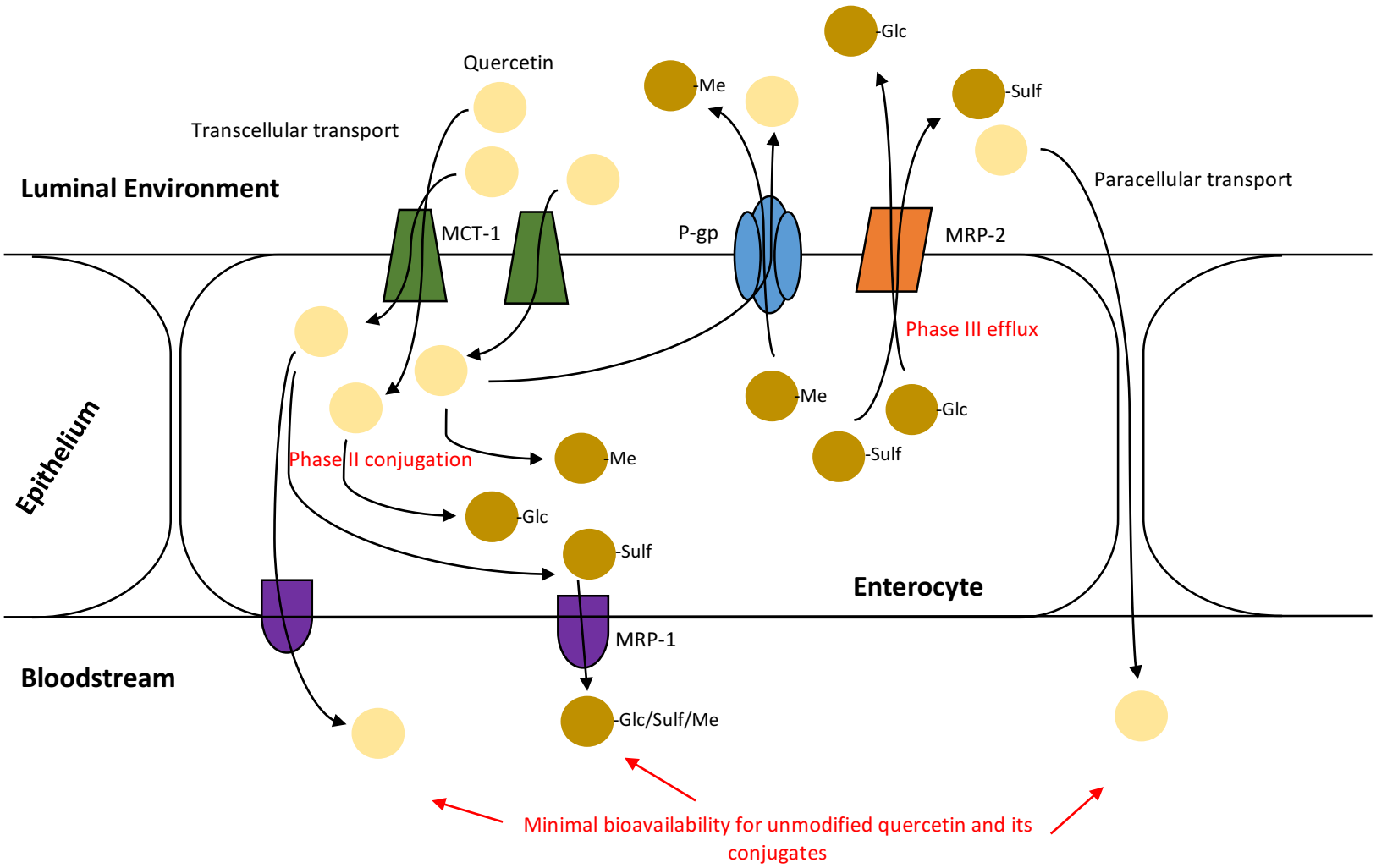


Figure 2.5. *An overview of how xenobiotic metabolism takes place at the cellular level of the small intestine is shown. MCT-1 (monocarboxylate transporter 1); P-gp (P-glycoprotein); MRP-1 (multidrug resistance protein 1; Glc/Sulf/Me (glucuronidated, sulfated, and methylated quercetin conjugates).*

Pharmacokinetics of Quercetin

One way to assess flavonoid bioavailability is by studying its pharmacokinetic behavior. Pharmacokinetics (PK) can be defined as the study of xenobiotic compound movement through the body. A PK study follows the course of a xenobiotic through administration, absorption, distribution, metabolism, and excretion (ADME) [90]. In order for the PK behavior of a compound to be measured, it must be absorbed across the intestinal lumen and reach portal circulation where it will undergo first pass metabolism in the liver [90]. PK behavior is measured after administration of a compound and subsequent blood draws throughout a given time period. Common PK parameters include AUC, C_{MAX}, T_{MAX}, and $t_{1/2}$. AUC stands for area under the concentration/time curve and it represents the total amount of absorbed compound into circulation over the span of administration to excretion. Observing a high AUC suggests that the compound of interest possesses high bioavailability. C_{MAX} refers to the maximum concentration of drug that is obtained through a blood sample. Increasing C_{MAX} will likely increase the bioavailability of a compound. Additionally, T_{MAX} can be calculated from the pharmacokinetic data. T_{MAX} is the time at which C_{MAX} is met. A low T_{MAX} will indicate quick absorption of a compound into circulation. Finally, $t_{1/2}$ represents a compound's half-life, that is, the amount of time it takes for the C_{MAX} of drug to be reduced by one half.

Some recent studies of quercetin pharmacokinetics are discussed here. According to Russo *et al*., a normal diet provides circulating concentrations of quercetin less than 1 μM [91], suggesting 1) poor aqueous solubility, 2 poor permeability, and/or 3) extensive metabolism of quercetin *in vivo*, all resulting in poor bioavailability of the aglycone. Another study found that in rats, only 6.7% of a 10 mg/kg oral dose of quercetin was absorbed into the portal vein in its unmodified form, while its conjugates were proven to be much more readily available [72]. A third

group of researchers found that after IV injection (no barrier to absorption), 93.8% of an administered quercetin dose was present as sulfated and glucuronidated conjugates. After oral administration, only 53% of the dose was circulating and all of it was sulfated and glucuronidated conjugates; the quercetin aglycone was not present [74]. Moon *et al*. have analyzed the pharmacokinetic response of humans administered 500 mg quercetin capsules, 3 times a day, over a 7-day period. They found that C_{MAX} levels of 0.0154 μg/mL and 0.448 μg/mL for quercetin aglycone and its total sulfated or glucuronidated conjugates, respectively [73]. Another study assessed the pharmacokinetics of orally administered quercetin (10 mg/kg) in rats. For unmodified quercetin, the T_{MAX}, C_{MAX}, and AUC were 0.078 h, 0.21 μg/mL, and 0.06 μg*h/mL, respectively. For total sulfated or glucuronidated conjugates of quercetin the T_{MAX}, C_{MAX}, and AUC were 0.42 hours, 2.53 μg/mL, and 15.1 μg*h/mL, respectively [72].

Metabolism of quercetin and other flavonoids is tremendously intricate, emphasizing the importance of research in this area of not only quercetin and other flavonols, but the entire class of flavonoids and polyphenols. That being said, solubility is a vital determinant of overall metabolism. Strategies to increase the apparent solubility of quercetin and other flavonols are important in understanding and making improvements upon their bioavailability and metabolism. Increasing the achievable solution concentrations of quercetin has the potential to also increase its bioavailability and saturate its metabolic transformations, which is a very important factor that limits its bioavailability.

Improving the Solubility & Bioavailability of Quercetin

Improving upon the bioavailability of quercetin could be an important way to facilitate realization of its potential health benefits across the population. The same can be said for other flavonols and polyphenols in general. A summary of strategies employed to increase the

solubility of hydrophobic and/or crystalline compounds, and by extension potentially increase bioavailability, is provided in Table 2.5. With an increase in bioavailability (through achievement of enhanced trans-membrane and trans-cellular concentration gradients to drive absorption and potential saturation of xenobiotic metabolizing enzymes), quercetin has the potential to promote normal health, prevent disease, and act in a therapeutic manner for numerous health problems. Improvements in biomarkers of cancer, CVD, and metabolic syndrome have all be documented as bioactivities of quercetin [11]. These three diseases currently affect a large proportion of the population of the United States [92, 93] and need to be addressed. Numerous strategies have been proposed, yet not one method is completely ideal, reinforcing the need for research on increasing the bioavailability of polyphenols, phytochemicals, and poorly absorbed drugs. These strategies are focused on increasing bioavailability through increasing the amount of quercetin that can be dissolved in solution *in vivo*. Some strategies used are self-emulsifying drug delivery systems (SEDDS), cyclodextrins, liposomes, and solid lipid nanoparticles (SLNs). SEDDS are defined as isotropic mixtures of oils, surfactants, solvents, and co-solvents/surfactants with certain drugs in order to improve oral bioavailability [94]. Once administered and in the presence of the GI tract, self-emulsification of these systems provide highly lipophilic compounds better dissolution opportunities than would be observed without the presence of a SEDD system. Cyclodextrins employ cyclic oligosaccharides to physically entrap hydrophobic drugs in a hydrophobic interior pocket, reducing degradation and increasing potential absorption [95]. Liposomes are effective at improving drug delivery because they consist of both hydrophobic and hydrophilic components, forming a small artificial vesicle for drugs [96]. Finally, through high-pressure homogenization, SLNs can be prepared to increase the stability and controlled release of drugs. Using solid lipids (triglycerides, fatty acids, steroids, and waxes),

emulsifiers, and water, SLNs can be prepared from physiological lipids which decreases acute and chronic toxicity [97]. Even though these strategies have proven to be successful, however, there is still room for improvement. ASD has promise to become a leading strategy to enhance the bioavailability and overall effectiveness of natural compounds such as quercetin.

In addition, there are even patents published that address the potential solubility enhancement of quercetin. Some highlighted patents will follow. One example would be the use of cellulosic polymers to inhibit crystallization of quercetin (and other polyphenols or drugs) [98]. This is in fact ASD and will be discussed in the next section, as it is the method used throughout this research. Another patent involved the solubilization of quercetin aglycone with one of its conjugated derivatives, quercetin-3-*O*-glycoside, followed by drying to impart improved solubility in water of the dried mixture [99]. This is a good example of an early technique used to modify quercetin, break up its crystallinity through that modification, with the end result being improved apparent solubility. Along the same lines, a separate patent was published regarding the conjugation process of bioactive components and how that may positively influence solubility and bioavailability [100]. Additionally, a type of high-pressure processing of "nutritional supplements" has also been patented [101]. This strategy proposed compressing both a long-chain polysaccharide with one or more supplements (quercetin included), allowing the prevention of immediate release into solution. The thought process behind this strategy appears similar to the strategy that ASD employs and that is delayed release *in vivo*, increasing potential solubility and absorption. Finally, one researcher even found and patented the discovery that the apparent solubility of flavonols, encompassing quercetin, could be increased by providing a mixture of flavonol-composited product and anthocyanin-composited product [102]. Due to the potential

health benefits, its obvious that improving the dissolution and subsequent bioavailability of quercetin, and other natural polyphenols, is tremendously important.

Table 2.5. Summary of literature on bioavailability improving (through improving apparent solubility) techniques for drugs and other xenobiotics.

Strategy	Advantages	Disadvantages
Pro-drug formulations	Simplicity in formulation and improved pharmacokinetics [103]; stabilizes drugs against metabolism [104]	Ineffective for targeting specific sites [103]; pro-drug metabolites are inactive [104]
Self-emulsifying drug delivery systems (SEDDs)	Improved oral absorption and bioavailability [94, 105]; numerous options for formulating [106]	Use of surfactants can be toxic [94, 105]; may affect drug stability [105]; higher particle size [106]
Cyclodextrins	Increase solubility, dissolution rate, and drug stability [107]; substitute for surfactants and organic co-solvents[108]	Precipitation of solid drug-cyclodextrin complexes [109], 2-3% absorption after oral administration [95]; renal toxicity [108]
Liposomes	Carries both hydrophobic and hydrophilic compounds [110]; protects against GI tract environment [110, 111]; increased bioavailability [111]	Lacks stability [111]; low encapsulation yield/leakage, rapidly eliminated after administration, and high production cost [96]
Chemical modification	Disrupts intermolecular attraction forces and lowers glass transition temperature [112], improves aqueous solubility [113]	Decreases decomposition temperature [112], inherent risk of reducing bioactivity with structural modification
Solid lipid nanoparticles (SLNs)	Increased drug stability and controlled drug release [97]; able to be produced industrially [97, 114]	Drug degradation during processing and lipid modification during storage [97]; potential toxicity[115]
Amorphous solid dispersions (ASDs)	Increased bioavailability; drugs in amorphous state (vs. crystalline) [59, 60, 116]; non-toxic and prevents drug recrystallization [59, 61, 116]	Drug recrystallization during storage and poorly translatable to industrial production [59]; some formulations are damaging to the environment[61]

Amorphous Solid Dispersion

Introduction to ASD

Commonly used in the pharmaceutical industry, ASD (Fig. 2.6) is one technique used to improve oral bioavailability of numerous drugs by using polymer matrices to overcome the energy that would normally be needed to remove a drug from its low energy, crystalline state into solution. In ASD, drugs are removed from a crystalline state through hydrogen bond formation with functional groups of different polymers. This is known as an amorphous state. Although this form is a less favorable, high-energy state, an amorphous drug dispersion will become thermodynamically stable when polymers are introduced. Upon introduction to an aqueous environment, drugs in the amorphous state can appear to be more soluble than their thermodynamically established solubility and can subsequently be absorbed at a higher rate than normal by the GI tract upon oral consumption. The mechanism of release for ASDs is discussed in more detail later on. Substituted polymers are often used in ASDs because they possess functional groups that are able to interact with and stabilize the functional groups of the dispersed drug. One example would be hydrogen bonding between a carboxyl (-COOH) group on a polymer and a hydroxyl (-OH) group on a drug molecule. This can happen in repetition between multiple subunits of the polymer and with multiple molecules of the drug, creating a matrix where the amorphous form of a drug can be considered "dispersed" with the polymer. In addition, substituted polymers are advantageous to use because, if properly designed, they are both hydrophilic (substituted functional groups) and hydrophobic (cellulose ring) and will act accordingly in solution, which allows selective release of the dispersed drug into solution in its amorphous form. Highly amorphous compounds have been shown to significantly increase drug solution concentration, which would be biologically relevant, even if the compound is only partially amorphous [117].

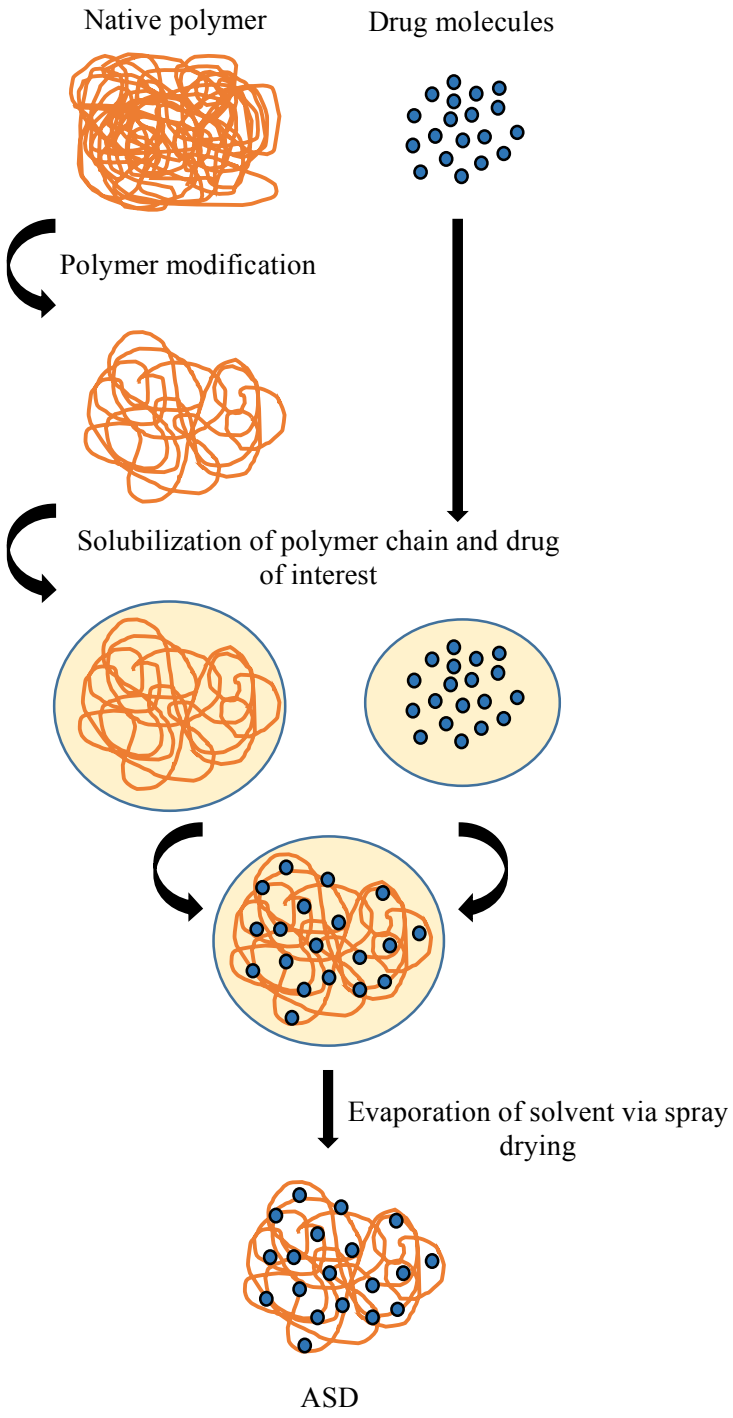
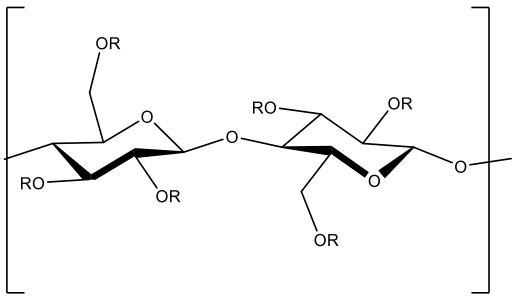


Figure 2.6. *A schematic diagram is provided to show the formulation of an amorphous solid dispersion (ASD).*

Typical polymers utilized in ASD applications include poly(vinylpyrrolidone) (PVP), hydroxypropylmethyl cellulose (HPMC), and hydroxypropylmethyl cellulose acetate succinate (HPMCAS) [8, 91] (Fig. 2.7). Preparation methods of ASDs include spray-drying, co-precipitation, and hot melt-extrusion [58]. Spray drying involves the solubilization of both the drug and carrier polymer in suspension, followed by atomization and immediate drying [118]. Atomization significantly reduces particle size of the dispersion and the instantaneous drying prevents phase

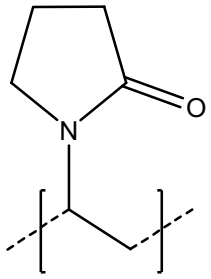


HPMC

R = -H, $-CH_3$, or $-CH_2CH(OH)CH_3$

HPMCAS

R = -H, $-CH_3$, $-COCH_3$, $-CH_2CH(OH)CH_3$
$-COCH_2CH_2COOH$, $CH_2CH(CH_3)OCOCH_3$
$-CH_2CH(CH_3)OCOCH_2CH_2COOH$



PVP

Figure 2.7. *Structures of the common polymers PVP, HPMC, and HPMCAS are shown above. HPMC and HPMCAS share the same basic structure, but differ based on the constituents on each cellulose.*

separation between a drug and polymer [118]. Co-precipitation also involves the solubilization of drug and carrier polymer, however instead of spray drying, a non-solvent is added drop-wise to the drug and polymer solution. As more non-solvent is added, the drug and polymer co-precipitate out and form microparticles [119]. Hot melt extrusion is an older strategy to formulate ASDs, however it can still be used to deliver amorphous drugs into solution. The process involves creating a physical blend of drug and polymer, followed by an extrusion of that mixture

through perforated screens with circular holes (0.5-2.0 mm diameter) to form the ASD microparticles [120].

ASD provides an easier, more efficient strategy of increasing drug (or polyphenol) bioavailability than some other strategies previously mentioned. Advantages of ASDs include reduced particle size, improved wettability, particles with higher porosity [59, 61], drugs in amorphous state (vs. crystalline) [59, 60, 116], and the prevention of drug recrystallization [59, 61, 116]. Reducing the particle size of a drug is a key factor when trying to increase its apparent solubility. The smaller the particle, the higher chance it has to dissolve into solution. Wettability and porosity are related due to the fact they deal with a drug's interactions with dissolution medium. Wettability can be defined as a measurement of the contact angle formed between a surface and a liquid [121]. The smaller the contact angle (< 90˚), the more efficient wetting will be seen [121]. Higher porosity exposes more of a drug's surface area to the medium being used, allowing a higher rate of wetting, and overall improved dissolution. As discussed previously, the amorphous state of a drug offers many advantages as far as increased solution concentration and enhanced dissolution rates are concerned. Lastly, while not 100% effective, ASD typically prevents a dispersed drug from recrystallizing following release, which keeps the molecules amorphous (or in solution) and improves dissolution. Finally, from a practical standpoint, it may be advantageous to deliver quercetin via ASD rather than through the ingestion of compounds known to contain high amounts of quercetin. In foods, quercetin is mainly represented as glycosides, which may have overall negative effects on bioavailability, but with ASD there is the hypothesis that aglycone quercetin in an amorphous state will be delivered and eventually absorbed, increasing bioavailability of the native compound.

Along with the novelty and promise of improving oral bioavailability, there are some drawbacks to using solid dispersions. For example storage conditions, most notably moisture conditions, may have an effect on recrystallization of amorphous compounds [61, 122], resulting in lower solution concentrations and dissolution rates. In addition, solid dispersions prove to be troublesome when successful formulations are proposed for manufacturing scale-up [59]. That being said, the advantages seem to outweigh the disadvantages and there are even some proposed strategies to combat the disadvantages of ASD [61]. With more research, the use of ASD has potential to be a very effective strategy in improving hydrophobic drug apparent solubility, bioavailability, and bioactivity.

Prevention of Crystallinity

Once a drug is dispersed in ASD, the goal is to keep the material from recrystallizing, i.e. returning to its lowest energy state. Crystalline materials are subject to molecular aggregation in solution that can lead to the formation of nuclei and subsequent crystal growth continuation [123]. If nucleation and growth of crystals can be prevented *in vivo*, then supersaturation could occur and be maintained for a period of time sufficient for the amorphous drug to transit through the GI tract and be absorbed [124]. Supersaturation can be defined as an increase in solution concentration past a compound's thermodynamic solubility. As a result, the amorphous form of the compound may have significantly higher absorption rates than if it reaches the gut lumen in crystalline form. One study tested 34 polymers in combination with the poorly soluble drug ritonavir. HPMCAS had one of the highest ratios of crystal growth rate in the absence of polymer to growth rate when the polymer was present. This suggests that HPMCAS is effective when it comes to preventing drugs from recrystallizing after the amorphous state has been released. However, each polymer-drug interaction is unique and mechanistic understandings are limited so

it is difficult to translate the crystal growth inhibition property of HPMCAS across all drugs, and in our case, flavonols [124]. One proposed mechanism of preventing recrystallization suggests that the polymer acts as a physical barrier, preventing crystals from continually depositing themselves on one another and growing [125]. Physical barriers usually occur through hydrogen bonding, van de Waals forces, and electrostatic interactions between the polymer and drug upon dispersion. Those same forces are holding the entire dispersion together and play an integral role in the release of the drug in an aqueous environment.

Amorphous solid dispersion creates an environment where the molecules are considered physically unstable, that is, the molecule spends an extended period of time in a state that is not the most favorable [126]. Many studies have taken a look at how these interactions take place and the mechanisms involved. For example, one study showed that a crystalline salt precipitated into an amorphous state had significantly improved solubility-limited absorption. In fact, the precipitate was in amorphous form and the subsequent solution was supersaturated for more than 4 hours [126]. An extended timeframe of supersaturated drug solution *in vivo* has potential create greater trans-membrane and trans-cellular gradients, favoring passive diffusion, and also to saturate Phase II and III of xenobiotic metabolism, diminishing their ability to deter absorption, and increasing the amount of quercetin permeating through the enterocyte. So, while there would still be modification of the drug and efflux from enterocytes back into intestinal lumen, there would also be surplus concentrations of unmodified drug present in the cells due to supersaturation. These extra portions of drug have the potential to overcome Phase II metabolic transformation and Phase III efflux and could be absorbed into the bloodstream in its most biologically active form. Other researchers have proposed this concept as well [16].

Mechanism of Release

A key concept associated with the increase of a drug's solution concentration and bioavailability using ASD is its mechanism of release. The structure of polymer used plays an important role in this mechanism. This review will be focused on cellulose-derived polymers, which can typically be substituted at C-2, C-3, and C-6 (Fig. 2.8). When a polymer is substituted

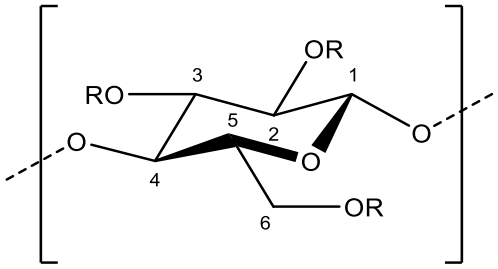


Figure 2.8. *The structure of cellulose is displayed with –OR groups representing the sites where functional groups may be attached for amorphous solid dispersion applications.*

it will have improved ability to interact with a drug and also water when in aqueous environments. For example, an R-group could contain a carboxylic acid group (-COOH), which will interact with both a drug and water through hydrogen bonding. The subsequent release of a drug in a matrix with a carboxylated cellulose is pH dependent. In the stomach, where the pH is below the pK_a, the carboxyl group is essentially 100% protonated. In this case, the polymer is neutral (no net charge) and does not repel itself and the drug molecule will be stable, physically held within the cellulose matrix. As the pH increases, i.e. as the matrix moves from stomach to intestinal environments, the carboxyl group becomes deprotonated (and net negatively charged) and subsequent electrostatic repulsion between multiple deprotonated carboxyl groups facilitates the swelling of the dispersion and release of amorphous drug. ASD is not exclusive to carboxyl substituents, however, and cellulose can be substituted with different constituents that will bind to drug molecules, each with a unique pK_a, which will affect the timing of release during

digestion. When using different functional groups, it is vital that the polymer does not completely disassociate with the drug when an aqueous environment is initially present, otherwise recrystallization is likely to occur. Drug hydrophobicity is high enough that immediate release of the entire amorphous load may result in only a very brief period of supersaturation due to rapid recrystallization. To maximize solution concentration, while still preventing recrystallization via delayed release of amorphous drug, amphiphilic polymers are suggested to be superior in maintaining supersaturated solutions because they possess both hydrophilic (enhanced release) and hydrophobic (prevention of recrystallization) surfaces and properties. These supersaturated solutions should be maintained long enough to see an increase in bioavailability when ASDs are tested *in vivo*.

Flavonols, quercetin included, are very crystalline in nature. This is a shared characteristic with some pharmaceuticals, which as described above, can be used in ASD to improve release during digestion. We hypothesize that formulating quercetin in ASD with a cellulose-derived polymer matrix will mimic the pharmaceutical industry technique and improve the bioavailability of the flavonol *in vivo*. With improved bioavailability, there is an expectation of improving quercetin-mediated preventative effects against biomarkers of diseases such as obesity and type-2 diabetes.

Evaluating ASD Effectiveness

Dissolution testing is commonly used when evaluating ASDs and their ability to improve dissolution, with the goal of creating and maintaining supersaturation *in vitro*. Dissolution involves the administration of a drug into a specified medium, followed by continuous agitation over a specific time period. These tests are used to provide researchers with a drug's dissolution profile, in other words, providing the rate of dissolved material into solution as well as the total

concentration of a particular drug in solution. Parameters of dissolution testing include AUC, C_{MAX}, and T_{MAX}, all of which have all been previously defined in *Pharmacokinetics of Quercetin*. Although these are blood measures, the same measures can be adapted for *in vitro* use. These tests can be performed under a number of conditions, but the most effective tests mimic physiological conditions. For example, dissolution can be performed to simulate gastric conditions (pH 1.5-2.8) or intestinal conditions (pH 5-7.6) [8]. Ideal dissolution profiles include high AUC measurements as well as increased C_{MAX} concentrations. Sometimes dissolution testing can be used as a predictor for a drug's bioequivalence, which can be defined as its *in vivo* dissolution performance [127]. This may not be true for all drugs and other compounds tested during dissolution, emphasizing the importance of true *in vivo* testing. A successfully performing drug *in vitro* would be expected to perform in the same fashion when tested in an animal or human model. Ideally, two identical doses, one in ASD and the other by itself, would have significantly different circulating concentrations *in vivo*, with the ASD providing the significantly higher of the two concentrations.

Newman *et al*. discuss factors such as drug particle size, dissolution media type and volume, apparatus and agitation rate and how all of these play roles in how an ASD will perform *in vitro*. Drug particle size can have a significant effect on the dissolution rate and overall profile of a drug. For example, ASDs containing nitrendipine, a drug used for hypertension and angina relief [128], were subjected to dissolution at numerous particle sizes (200 nm, 620 nm, 2.7 μm, 4.1 μm, and 20.2 μm) to assess how those sizes affected translated to bioavailability in rats. Results showed significantly different solubilization values (61.4%, 51.5%, 29.4%, 26.7%, and 24.7%, respectively), suggesting that smaller particle size is usually more advantageous for increasing the effectiveness of an ASD [8]. Dissolution medium is another determinant to how a drug will act

during dissolution. Newman *et al*. reviewed numerous studies and showed that most dissolutions were performed in 0.1 N HCl (21%), water (19%), and pH 6.8 solutions (17%) [8]. In their review they also assessed the United States Pharmacopeia (USP) guidelines for dissolution and suggested that experiments be performed under sink conditions, that is, at three times the volume of dissolution medium that is required to saturate a particular drug in that medium. However, in a survey conducted by the researchers, 86% of participants did not specify the conditions used (sink vs. nonsink) [8]. This is most likely due to the fact that sink conditions do not permit observation of supersaturation. In addition, the review mentioned that volume was dependent on the targeted GI location. For example, the jejunum has a pH of 6.9 and an average of 105 mL in volumetric capacity [129], and it would be advantageous for researchers to target these conditions with their media if the small intestine was of interest. That being said, other researchers have reported the pH of the jejunum to be as low as 4.4 during a fasted state. Nonetheless, depending on the target location for eventual delivery, the use of pH relevant media was strongly suggested.

According to USP, there are four official dissolution apparatuses to be used [130]. They include Apparatus 1 and 2 (basket apparatus and paddle apparatus, respectively), which are essentially the same vessel; the only difference being the agitation source (basket apparatus vs. paddle). Apparatus 3 uses glass reciprocating cylinders to introduce agitation and Apparatus 4 uses a flow-through cell device, maintaining a constant stream of dissolution medium throughout the device. Jacketed beakers and flasks can also be used for dissolution, with the addition of a plate-controlled stir bar maintaining constant rpm. Common agitation rates used in dissolution testing are 50 rpm (18%) and 75 rpm (10%) according to the review's analysis of numerous research experiments. It is suggested that agitation rates be related to mixing encountered in the body [8].

The review also points out how *in vitro* studies fail to mimic some relevant *in vivo* parameters an ASD would be facing. For example, GI fluids, bile salts, food matrices, and mucosal linings of the intestine can all have an effect the bioavailability, even after supersaturation is met. There have been some possible solutions in order to overcome some of these limitations. For example, one study examined the possibility of changing the dissolution medium that is typically used in order to replicate the environment of the small intestine. They proposed the additions of mono- and diglycerides and/or lipase to the medium in order to simulate the fats and oils present after a meal. Also suggested was using phosphate buffer instead of bicarbonate to avoid pH instability throughout the test and/or the addition of sodium taurocholate as a representative bile salt [7]. Overall, dissolution testing provides useful insight on how an ASD will perform *in vivo*, but there are still some obvious limitations [8]. It is imperative that ASDs be tested *in vivo* after successful *in vitro* dissolution results. The top performing and most promising ASDs should be the first candidates tested *in vivo*, seeing as they have the best chance at successfully increasing the bioavailability of certain drugs. Finally, when performing *in vivo* studies to assess ASD effectiveness, the most important factor is choosing which animal model to use, ideally using the animal that will most resemble the drug of interest bioavailability in humans. According to Newman *et al.*, dogs (41%) are a common model, followed by rats (24%), rabbits, and monkeys [8]. Recently, pigs have been considered as potential models, however, the ethical implications are still controversial [131].

Bioactivity of Quercetin

Obesity and Diabetes

Diabetes affects 9.3% of the United States population, or a total of 29.1 million people. In addition, 27.8% of those 29.1 million people with diabetes are undiagnosed and unaware of their

health status [92]. A larger percentage of the population is pre-diabetic, and this is where research is focused on prevention methods. Obesity is also a major issue in the United States. Obesity can be defined as the disproportional relationship between energy expended and energy intake, leading to the uncontrolled growth of fat cells [21]. The Journal of American Medicine reports that more than one-third of adults and 17% of youth in the U.S. are obese [93]. Stemming from the fact that obesity and diabetes affect so many people, research towards preventing and ameliorating these diseases is abundant. A review of research relating quercetin to prevention of obesity and diabetes is presented. Animal doses were converted to human equivalent doses (HED) based on the findings in Reagan-Shaw *et al* [132].

Chronic intake of quercetin has the potential to prevent excessive adipocyte growth and a reduction in weight gain from individuals who consume high fat diets. Rivera *et al*. demonstrated this in obese Zucker rats, dosing them with 2 or 10 mg/kg over the course of 10 weeks. Both doses improved dyslipidemia, however only the higher of the two doses significantly reduced body weight gain in the rats [133]. A daily intake of 10 mg/kg (HED = 1.62 mg/kg), is higher than what is seen in the typical human diet, so it would be interesting to see if a lower dose would show the same effects in the rats. In another study, after three weeks of quercetin supplementation in C57BL/6J mice, an increase of energy expenditure was seen, however after eight weeks those same effects were not detected. The researchers suggested that the mice had metabolically adapted to the quercetin supplementation which diminished the early on energy expenditure and overall nutrient partitioning. However in the same study, a significant reduction in markers of inflammation in the mice was seen after eight weeks. This illustrates the complex mechanisms involved in metabolism of flavonols, warranting further research into the subject.

One recent study has suggested that quercetin could be used as a supplement for diabetic women during pregnancy to reduce blood glucose levels [134]. In addition, quercetin has also been shown to play a crucial role in the potentiation of glucose-induced insulin secretion [135], as well as protecting β-cell function, and reducing oxidative stress [135-137]. Jeong *et al*. have investigated the effects of quercetin on diabetes mellitus in 4 week old C57BL/KsJ-db/db mice. Their research concluded that quercetin at 72 mg/kg per day and 140 mg/kg per day (HED = 11.7 mg/kg and 23.2 mg/kg, respectively) could be effective in improving hyperglycemia, dyslipidemia, and antioxidant status in type 2 diabetic mice [138]. In humans this would only be achievable with diet supplementation of quercetin. Shetty *et al*. have shown that with a quercetin-incorporated diet at 1 g/kg body weight (HED = 162 mg/kg), diabetic status in rats with streptozotocin-induced diabetes could be ameliorated in about 25% of their study's rats fed with quercetin [139]. At that concentration, a reduction in diabetes should be expected, however, consuming that much quercetin is unrealistic in translation to a human study. Another study showed that quercetin treatment in diabetes-induced rats prevented the development of early diabetes tissue injury by decreasing oxidative stress and blocking mediators of tissue damage [140]. Intriguingly, natural sources are also being explored to provide protection against obesity and diabetes. Okra extract and its major flavonoids, isoquercetin and quercetin 3-O-gentiobioside, have been shown to reduce blood glucose and serum insulin level and improved glucose tolerance in high-fat diet-induced obese mice. In addition, a reduction in total cholesterol levels in okra extract treated obese mice was noticed [141].

While these are only a few examples of the research being completed, it is easy to see the wide range of hypotheses being tested. Moving forward, it is key to work from what has been proven successful and develop what are truly novel, viable ideas.

Other Bioactivity

Obesity and diabetes are only two of many areas of research being tested for prevention by quercetin. Quercetin has been involved in numerous treatment, diet-incorporation studies that deal with other health adversities and diseases. A brief review of some other studies outside of obesity and diabetes prevention is presented. One common area of research is with quercetin and its potential to treat and prevent cancer. One study found that quercetin was effective in preventing prostate cancer in male rats in which cancer was chemically-induced [142]. Another study examined honey, a product rich in flavonols like quercetin, chrysin, kaempferol, and naringenin. It has been speculated that flavonoids in honey may be able to inhibit P-glycoprotein (P-gp), an efflux pump for pharmaceutical drugs and other xenobiotics, and reverse multidrug resistance that can develop in cancer cells. This phenomenon could be a new, potentially huge cost savings technique to alleviate cancer cell proliferation with less negative effects than normal treatment methods [143]. Chemical modification of flavonols have been shown to improve upon their chemotherapeutic effects in comparison to their naturally occurring forms [144]. In addition, diet supplementation of quercetin-enriched foods suggests increased bioavailability (> 10 μM) and a positive correlation with not only cancer treatment, but also decreased risk of cardiovascular disease [91]. Besides cancer, quercetin can modulate acute vasodilation in normotensive, normocholesterolemic human subjects after the administration of oral quercetin. Interestingly, the hypothesized mechanism of the vasodilator effects was a result from the deconjugation of a quercetin metabolite, quercetin-3-O-glucuronide [145].

Quercetin possesses other bioactivities, both *in vitro* and *in vivo*, such as diminishing the negative effects of vascular smooth muscle disorders [146], lowering blood pressure [147, 148], providing an alternative to synthetic fungicides [149], decreasing hepatic lipogenesis [150], and

reduced risk of coronary heart disease [151]. These are just a few of many proposed bioactivities of the flavonol. Other research, though, has provided a few shortcomings of quercetin supplementation. Failure to observe lower plasma triglycerides and total, LDL, VLDL, and HDL cholesterol in hypertensive patients has been reported, as well as no effect on fasting blood glucose levels [147]. Intake of quercetin and other flavonols was measured in elderly women and related to the risk of total and site-specific cancers and no correlation was significant between flavonol consumption and cancer risk reduction [152].

Chapter 3: Novel Cellulose-Based Amorphous Solid Dispersions Enhance Quercetin Solution Concentrations *In Vitro*

Introduction

Quercetin (Fig. 3.1) is a dietary flavonol (a subclass of flavonoids) present at high levels in foods including apples, onions, and broccoli [36, 153]. Quercetin intake has been associated with many potential health benefits, including reduced risk of cardiovascular disease [35, 91], cancer [142, 143, 154], and diabetes and obesity [31, 134, 136].

Poor quercetin oral bioavailability severely limits its potential to benefit health. This low bioavailability is largely due to its crystallinity, and hence poor solubility (ranging from 2.15-7.7 μg/mL at 25°C [64, 65]) in the aqueous milieu of gut lumen, as well as extensive metabolism and subsequent luminal efflux by gut epithelial cells (Phase-II and Phase-III xenobiotic metabolism,

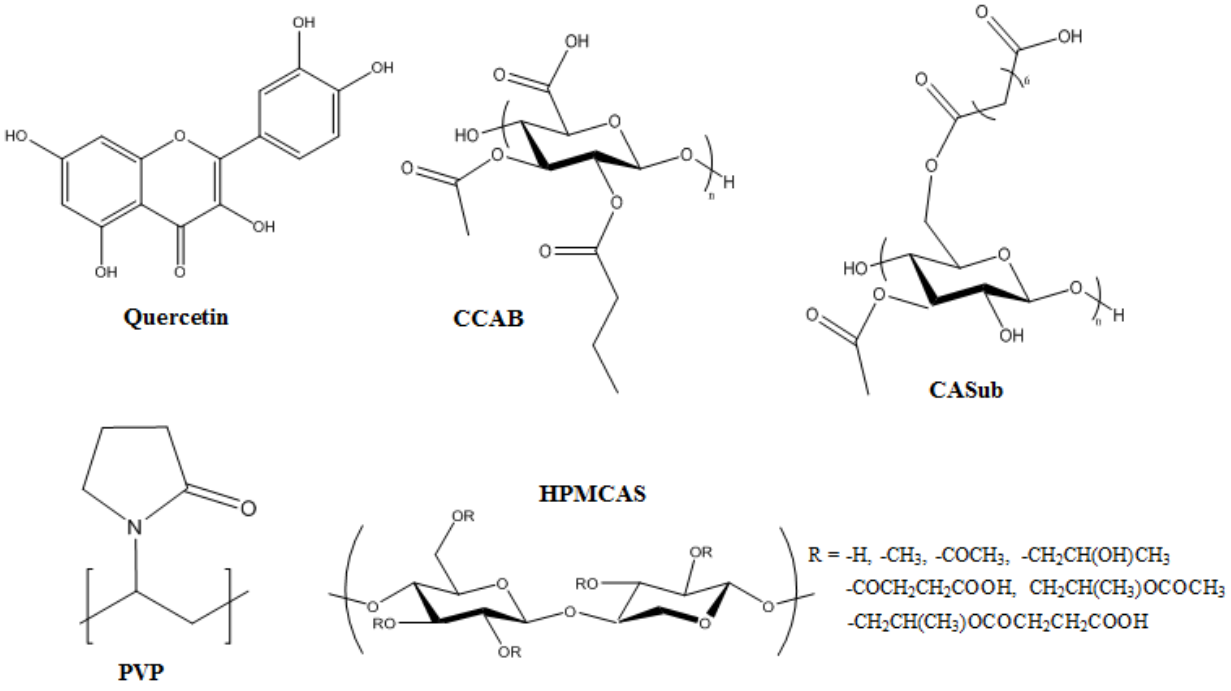


Figure 3.1. *Chemical structures of quercetin, CCAB, CASub, PVP, and HPMCAS. The cellulosic structures are not meant to convey regioselective substitution; depictions of substituent location are merely for convenience and clarity of depiction.*

respectively). Improved quercetin solubility may increase bioavailability by increasing the amount available for absorption, and by saturating Phase-II and Phase-III metabolic enzymes; both effects are likely to result in increased net flux into circulation.

Many techniques have been employed to improve quercetin oral bioavailability, such as protein- or cellulose-based nanoparticles [155-157], encapsulation [158], nanoemulsifying drug delivery systems [159], and ASD [6, 124, 160]. ASD preparation with polymer dispersants is an attractive way to stabilize the high energy, amorphous drug in a glassy polymeric matrix. ASD not only provides supersaturated drug solutions, but also enhances permeation by increasing the drug concentration gradient across the enterocyte. Polymer selection is key for ASD performance because the dispersion must be miscible, with strong polymer-drug interactions (e.g. hydrogen bonding) for stability against crystallization [59, 160]. Amphiphilic polymers possessing carboxylic acid functionality perform well in ASD due to strong polymer-drug interactions; their pH responsiveness is also valuable. At gastric pH, the protonated form protects the drug and minimizes release, while deprotonation at near-neutral intestinal pH swells the polymer and triggers drug release [6, 160]. Cellulose derivatives are popular ASD polymers due to their generally benign nature and high T_g values. CASub and cellulose acetate adipate propionate (CAAdP) were synthesized in the Edgar lab and show high promise for ASD [6, 161].

ASD has been only lightly explored for quercetin dissolution enhancement. Lauro *et al.* achieved slight dissolution enhancement using ASDs prepared with cross-linked sodium carboxymethylcellulose and sodium starch glycolate [65]. Similarly, Lauro *et al.* used spray dried dispersions with cellulose acetate trimellitate and cellulose acetate phthalate to improve quercetin release at pH 6.8 [162]. Recently, several polymers were evaluated for their ability to improve quercetin dissolution *in vitro* [6]. HPMCAS afforded ASDs containing up to 50%

quercetin content; optimal dissolution was obtained from 10% Q ASDs, optimally in polymer blends containing 10% of the water-soluble PVP [163-165]. Employing PVP in blends with other cellulosic polymers may generally enhance drug release, while retaining the excellent stabilization from the cellulosic polymer [166].

The objective of this study was to assess the performance of the novel polymer CASub for making ASDs with quercetin and creating supersaturated quercetin solutions at physiological pH, vs. crystalline quercetin as negative control and HPMCAS/quercetin ASD as positive control. We hypothesized that 1) CASub would provide enhanced solution concentration and preferable dissolution kinetics compared to HPMCAS, and 2) that blending CASub with PVP would further enhance quercetin dissolution during simulated fasting at intestinal pH (6.8).

Experimental

Materials

Quercetin (≥ 95% by HPLC), epicatechin (EC) (≥ 90% by HPLC), and KCl (solid, anhydrous, ≥ 99%) were purchased from Sigma-Aldrich (St. Louis, MO). Cellulose acetate propionate (CAP-504-0.2; degree of substitution (DS) (acetate) = 0.04, DS (propionate) = 2.09; M_n = 15,000); CCAB; DS (butyrate) = 1.62, DS (acetate) = 0.06, DS (carboxylic acid) = 0.28); Mw = 252,000) and cellulose acetate (CA 320S, DS (acetate) = 1.82) M_n = 50,000) were from Eastman Chemical Company (Kingsport, Tennessee). HPMCAS (wt %: methoxyl 20-24%, hydroxypropyl 5-9%, acetyl 5-9%, succinoyl 14-18%; Mw = 18,000) was from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Chemical structures of ASD polymers used (HPMCAS, PVP, CCAB, and CASub) are provided in Fig 3.1. Acetonitrile (ACN, HPLC-grade), methylene chloride (HPLC-grade), tetrahydrofuran (THF), reagent ethanol, sodium phosphate monobasic, and sodium hydroxide (NaOH) were purchased from Fisher Scientific (Waltham, MA) and used

as received. HCl (12.1M) was obtained from Macron Chemicals (Center Valley, PA). Suberic acid, adipic acid, methyl ethyl ketone (MEK), *p*-toluenesulfonic acid (PTSA), triethylamine (Et_3N), and oxalyl chloride were purchased from ACROS Organics (Thermo Fisher, Waltham, MA). 1,3-Dimethyl-2-imidazolidinone (DMI) was purchased from ACROS Organics and dried over 4 Å molecular sieves. Water was purified by reverse osmosis and ion exchange using a Barnstead RO pure ST (Barnstead/Thermolyne, Dubuque, IA, USA) purification system. LCMS grade ACN, water and formic acid were obtained from VWR (Radnor, PA).

Synthesis of CASub

CASub was synthesized as previously reported [161]. Preparation of monobenzyl suberate: suberic acid (87 g, 0.5 mol), benzyl alcohol (81 g, 0.75 mol), PTSA (0.95 g, 5 mmol), and toluene (200 mL) were stirred in a flask equipped with Dean-Stark trap and heated at reflux for 3 h until the theoretical amount of water was collected. The resulting mixture was cooled to room temperature, water (200 mL) was added, and the pH adjusted to 9 with 6M NaOH. The aqueous layer was separated by centrifuge, mixed with ethyl ether (150 mL), and the pH adjusted to 2 with 6M HCl. The ether layer was separated and concentrated under reduced pressure to afford a colorless oil. ^{1}H NMR ($CDCl_3$): δ 1.33 (m, 4H), 1.68 (m, 4H), 2.36 (m, 4H), 5.09 (s, 2H), and 7.32 (m, 5H).

Synthesis of monobenzyl suberoyl chloride: Monobenzyl suberate (30 g, 113 mmol), DMF (3 drops), and 200 mL dichloromethane were cooled in a round bottomed flask to 0°C. Oxalyl chloride (25.4 g, 200 mmol) was added drop by drop under vigorous magnetic stirring, then stirred 2 h at room temperature till gas formation ceased. Solvent was removed under reduced pressure, 10 mL toluene was added, and then it was concentrated again under reduced pressure. The product was a yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.34 (m, 4H), 1.66 (m, 4H), 2.36 (t,

2H), 2.86 (t, 2H), 5.12 (s, 2H), 7.35 (m, 5H).

Benzyl CASub synthesis: CA 320S (1.00 g, 4.19 mmol) was dissolved in DMI (20 mL), Et_3N (1.95 mL, 13.83 mmol, 3.3 eq) was added all at once, then monobenzyl suberoyl chloride (3.7 g, 12.57 mmol, 3 eq) was added. After 20 hours at 90°C under nitrogen, the reaction mixture was cooled, then added to ethanol (250 mL) dropwise to precipitate the product, which was isolated by vacuum filtration, then washed with 200 mL water and re-dissolved with 25 mL THF and re-precipitated in 200 mL hexane. The product was characterized by ^{1}H NMR. δ 1.3 ($COCH_2CH_2CH_2CH_2CH_2CH_2CO$ of suberate), 1.6 ($COCH_2CH_2CH_2CH_2CH_2CH_2CO$ of suberate), 2.10–2.46 ($COCH_2CH_2CH_2CH_2CH_2CH_2CO$ of suberate and $COCH_3$ of acetate), 3.00–5.20 (cellulose backbone), 5.10 (s, $CH_2C_6H_5$), 7.35 (CH_2C_6H5).

Hydrogenolysis of benzyl CASub: Benzyl CASub (1 g) was dissolved in 100 mL THF, then $Pd(OH)_2/C$ (500 mg) was added. The mixture was stirred, after removal of the air by vacuum, at a high speed under H_2 (balloon) for 24 h at room temperature. Products were isolated by filtering through Celite, then the same protocol was repeated one more time and, the solvent was removed under reduced pressure, and the product precipitated in ethanol (100 mL). ^{1}H NMR CASub (DMSO): δ 1.2 ($COCH_2CH_2CH_2CH_2CH_2CH_2CO$ of suberate), 1.4-1.6 ($COCH_2CH_2CH_2CH_2CH_2CH_2CO$ of suberate), 2.10–2.46 ($COCH_2CH_2CH_2CH_2CH_2CH_2CO$ of suberate, and $COCH_3$ of acetate), 3.00-5.20 (cellulose backbone).

Preparation of ASDs via spray drying

CCAB (0.9 g) was dissolved in 120 mL THF at room temperature for 15 h. Q (0.1 g) was dissolved in 20 mL acetone separately and stirred for 20 min. THF (20 mL) was added, then this quercetin/acetone/THF solution was added to the polymer/THF solution dropwise. All CCAB/quercetin solutions were prepared similarly. ASDs were prepared by spray drying the

polymer/quercetin solutions using a nitrogen-blanketed spay dryer (Buchi B-290). Instrument parameters were as follows: inlet temperature 90°C, outlet temperature 75°C, aspirator rate 80%, 40% pump rate, compressed nitrogen height 30 mm and nozzle cleaner 2. HPMCAS (1.8 g) was dissolved in 15 mL THF and 15 mL acetone, stirred overnight, then quercetin (0.2 g) was added to the solution and stirred for 15 min before spray drying. A similar procedure was followed to prepare CASub spray dried particles, except that acetone was used as solvent. PVP (0.4 g or 0.2 g) was dissolved in 10 mL ethanol and CCAB (1.4 g)/quercetin (0.2 g) was dissolved in 80 mL acetone. A similar protocol was followed for PVP/CASub blends with ethanol/THF. Our convention for naming treatments is to list the % polymer(s), with the remainder being quercetin. For example, 10% Q/90% CCAB is referred to as 90 CCAB in the text, figures and tables. ASDs prepared were: 90 CCAB, 75 CCAB, 50 CCAB, 10 PVP:80 CCAB, 20 PVP:70 CCAB, 90 HPMCAS, 90 CASub, 10 PVP:80 CASub, 20 PVP:70 CASub.

ASD Characterization: XRD

X-ray powder diffraction patterns were measured with a Bruker D8 Discover X-ray Defractometer (Billerica, MA) with a Lynxeye detector and a KFL CU 2K X-ray source. Samples were run with a 1 mm slit window between a scan range of 10˚ to 50˚ 2θ.

ASD Characterization: DSC

DSC analyses were performed on a Trios TA Instrument (New Castle, DE) with dry samples (5 mg) loaded into Tzero^{TM} aluminum pans. Each sample was equilibrated at 20˚C and then heated to 200˚C at 20˚C/min. Then samples were quench-cooled to −50˚C and reheated to 200˚C at 20˚C/min. T_g values were recorded as the step-change inflection point from second heat scans.

Determination of crystalline and amorphous quercetin solubility

Crystalline solubility: An excess of crystalline quercetin was added to 15 mL pH 6.8 buffer solution, or pH 2.5 solution. Solutions were equilibrated at 37 ˚C/48 h with constant agitation, protecting them from light.

Amorphous solubility: Supersaturated quercetin solutions were prepared by adding a specific amount of quercetin stock solution (4 mg/mL in MeOH) to 15 mL buffer at 37 ˚C. Potassium phosphate buffer (100 mM, pH 6.8) with 100 µg/mL PVP (Kollidon® 12 PF); or acidified distilled water (pH 2.5) with 100 µg/mL PVP was used. The polymer was added to inhibit drug crystallization during the experiment and accurately determine the "amorphous solubility" of quercetin. Total quercetin concentration of was 80 µg/ml.

Crystalline and supersaturated solutions were centrifuged at 35,000 rpm (274,356 *x* g) for 30 minutes to separate the precipitated drug phase using an Optima L-100 XP ultracentrifuge equipped with Swinging-Bucket Rotor SW 41 Ti (Beckman Coulter, Inc., Brea, CA). Following centrifugation, the supernatant was collected, diluted (1:1) with methanol, and the final concentrations were measured by HPLC using an Agilent HPLC 1260 Infinity system (Agilent Technologies, CA, USA) with an Agilent Eclipse plus C18, 4.6 x 250 mm, 5 µm analytical column (Agilent technologies, CA, USA). The mobile phase was 40 % acetonitrile: 60 % DI water acidified with phosphoric acid at pH 2.5. The experimental conditions were: 1.3 mL/ min flow rate, 370 nm wavelength and 20 µL injection volume, and controlling the column temperature at 35 ˚C. The retention time was 3.6 minutes.

UPLC verification of quercetin content in ASDs

Incorporation of quercetin into the ASD was quantified by extraction and UPLC-MS/MS. Each batch of ASD particles was evaluated for quercetin content (wt %, n = 4). Quercetin-

containing ASDs were dissolved in ethanol (~0.26 mg/mL), and 50 μL of this solution was combined with 50 μL internal standard solution [epicatechin (EC), 0.8 mg/mL in ethanol] and 50 μL 0.1% formic acid in 80% water/20% 80:20 ACN/THF. Solid quercetin [i.e. 100% quercetin, ≥ 95% purity] was used as control and analyzed similarly. See *UPLC-MS/MS* below for full UPLC methodological details.

In vitro dissolution

In vitro dissolution was performed under non-sink conditions to evaluate quercetin dissolution concentrations and kinetics achieved via ASD delivery, compared to crystalline quercetin alone, under conditions similar to some normal human gastrointestinal conditions. Fasted gastric pH was simulated using pH 1.2 buffer (500 mL of 0.2 M KCl was combined with 850 mL of 0.2 M HCl, and then diluted to 2 L with MilliQ water). Small intestinal pH was simulated using pH 6.8 buffer (6.8 g/L sodium phosphate monobasic in MilliQ Water, adjusted with 0.2 M NaOH to pH 6.8). Dissolution was performed as described previously with modifications [160]. Jacketed flasks (250 mL, 37°C) were employed. Dissolution medium consisted of 100 mL gastric or small intestinal buffer. All treatments contained a fixed queretin amount (7 mg) to ensure supersaturation if all dissolved, and were continuously stirred with a magnetic stir bar at 400 rpm for 2 h (gastric) or 8 h (intestinal). Sample aliquots (1 mL) were taken at 30, 60, 90, 120 min (gastric) or 30, 60, 90, 120, 180, 240, 300, 360, 420, and 480 min (small intestinal) and each aliquot was replaced with 1 mL buffer to maintain constant volume. Aliquots were centrifuged (10 min, 37°C, 47,000 x *g*) on a Beckman Coulter Avanti JE high-speed centrifuge (Sunnyvale, CA). Following centrifugation, the supernatant (i.e. soluble fraction) was collected, diluted (1:1) with ethanol, and stored at −80°C for further analysis.

UPLC-MS/MS

Internal standard solution (50 µL, 0.8 mg/mL EC in ethanol), 50 µL diluted dissolution supernatant, and 900 µL of a solution of 0.1% formic acid in 80% water/20% 80:20 ACN/THF were added to Waters UPLC vials (Milford, MA) and mixed. Analyses were performed on a Waters Acquity H-class UPLC separation model (milford, MA) equipped with a Waters Acquity UPLC BEH C18 column (2.1 mm x 50 mm, 1.7 µm particle size). Column and sample temperatures were maintained at 40˚C and room temperature (24 ± 1˚C), respectively. The binary mobile phase consisted of 0.1% (v/v) aqueous formic acid (phase A) and 0.1% formic acid in 80% ACN/20% THF (phase B) (all solvents LC-MS grade except THF). System flow rate was 0.6 mL/min. A linear elution gradient was employed as follows: 80% A at 0 min, 10% A at 2.20 min, 100% A at 2.25 min. An injection volume of 2 µL was employed. A second injection of 100 µL DMSO was used in between sample injections to remove any carryover quercetin, with isocratic mobile phase (100% B for 2 min), and subsequent reconditioning for 2.25 min to initial gradient conditions. Electrospray (ESI)-MS/MS analysis was performed in negative mode on a Waters Acquity TQD (triple quadrupole) mass spectrometer equipped with a Z-spray electrospray interface as described in Goodrich & Neilson with modifications [167]. Capillary voltage was −1.5 kV, cone voltage 56 V for quercetin and 34 V for EC, source temperature 150˚C, and desolvation temperature 500˚C. Desolvation and cone gasses were N_2 at flow rates of 1,000 and 50 L/hr, respectively. Detection was performed by multi-reaction monitoring (MRM) of parent pseudomolecular ($[M-H^-]$) ion to daughter (fragment) ion transitions during collision-induced dissociation (CID, Ar gas: 0.25 mL/min). The MRM transitions for quercetin and EC were 300.77 m/z → 150.88 m/z and 288.79 m/z → 245.02 m/z, respectively, with collision

energies of 20 eV for quercetin and 10 eV for EC. Quantification was based on an internal standard curve prepared using varying levels of quercetin with the fixed internal standard, EC.

Data Analysis and Statistics

Dissolution results are reported as soluble quercetin vs. time. Dissolution kinetics were determined from this data. Pseudo-pharmacokinetic parameters (area under the concentration-time curve: AUC; maximal observed solution concentration: C_{MAX}; time at which maximal solution concentration was observed: T_{MAX}) were computed from quercetin concentration/time data using standard plugins for Microsoft Excel (Redmond, WA). Prism v. 6.0d (GraphPad, la Jolla, CA) was used to perform statistical comparisons. Dixon's Q-test ($\alpha = 0.05$) was utilized to identify and exclude any outliers as necessary. Significant differences in dissolution parameters between treatments were determined using one-way ANOVA with Tukey's HSD *post hoc* test performed on treatment means. Significance was defined as $P < 0.05$.

Results and Discussion

Three promising carboxylated cellulose derivatives were selected for ASD preparation in order to increase quercetin apparent solubility (Fig. 3.1). CCAB is a new commercial carboxylated cellulose ester [168], and HPMCAS is a cellulose ether-ester that is in commercial use as an efficient ASD polymer [6]. CASub was recently designed by the Edgar and Taylor groups as a promising ASD polymer and crystal growth inhibitor [161]. The above-mentioned polymers may not only stabilize amorphous quercetin in the solid state and prevent recrystallization after release, but also provide targeted pH-controlled release to the small intestine where quercetin absorption occurs. We also investigated blends of CASub and CCAB with PVP to promote enhanced quercetin dissolution, since quercetin and thus its ASDs with polymers like CASub and CCAB (δ = 22.66 and 24.44, respectively) are rather hydrophobic. ASD drug concentration

influences both the practicality of the method and ASD performance, so to explore this influence, quercetin concentration in the ASD was varied (10%, 25% and 50% quercetin (w/w)), using readily available commercial CCAB (90 CCAB, 75 CCAB, 50 CCAB) as the test system. To compare effectiveness of novel polymers and polymer blends, dissolution profiles were compared against commercial polymers HPMCAS and CCAB.

Solid State Characterization of quercetin-loaded ASDs

Morphology was examined by SEM (Fig. 3.2). All ASD particles exhibited a smooth surface, indicating amorphous dispersion, except for 50% quercetin loaded CCAB that appeared to contain quercetin crystals. Particles have corrugated morphology, crushed, indented and collapsed, typical of polysaccharide-based spray-dried ASDs.

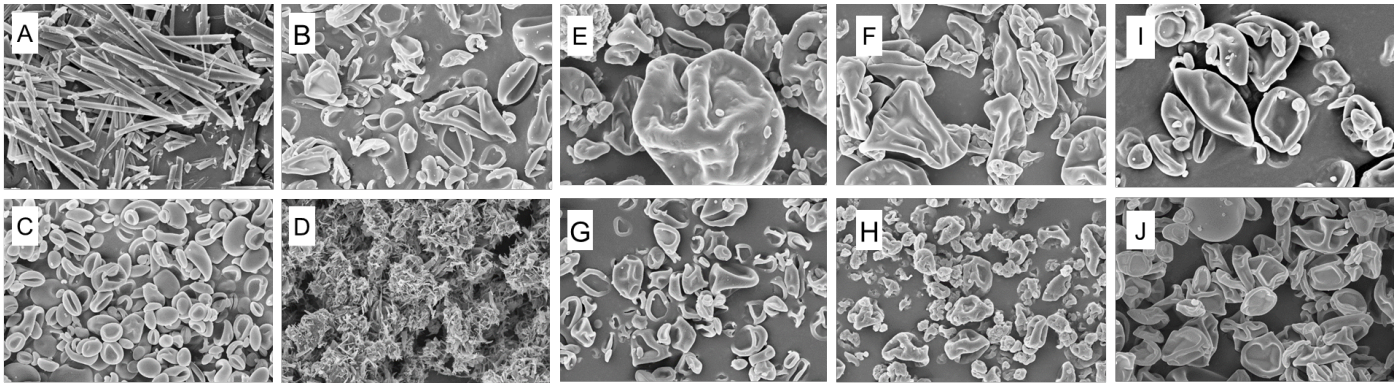


Figure 3.2. *SEM images (mag. 10X) for crystalline quercetin (A), 90 CCAB (B), 75 CCAB (C), 50 CCAB (D), 10 PVP:80 CASub (E), 20 PVP:70 CASub (F), 10 PVP:80 CCAB (G), 20 PVP:70 CCAB (H), 90 HPMCAS (I), and 90 CASub (J) are shown to illustrate particle size range (1-3 μm) and morphology.*

XRD was used to determine whether dispersions were amorphous. All XRD spectra showed only amorphous haloes (Fig. 3.3), except for that of crystalline quercetin and 50% quercetin in CCAB (Fig. 3.3A). XRD data strongly support the amorphous nature of these dispersions except for 50 CCAB, which was therefore excluded from further testing.

DSC was used to further examine dispersion morphology of quercetin and polymers; data from CASub and CCAB is presented in Fig. 3.4. Although crystalline quercetin melts at 326 °C,

DSC scans were kept ≤ 185°C, due to concerns about potential crosslinking of these polymers (containing both OH and CO_2H groups) above that temperature [169, 170]. For ASDs a glass transition (T_g) temperature lower than that of the pure polymer is expected. Polymer T_g values are 175°C (PVP), 144°C (CASub) and 134°C (CCAB). ASDs (10% quercetin) all had lower T_g values than the corresponding pure polymer, indicating that quercetin acted as a plasticizer. Since quercetin melts higher than the decomposition temperatures of several of our polymers, absence of quercetin T_m and T_c in the ASDs could not be confirmed. DSC T_g values along with the XRD data were sufficient to confirm the amorphous character of quercetin in these dispersions.

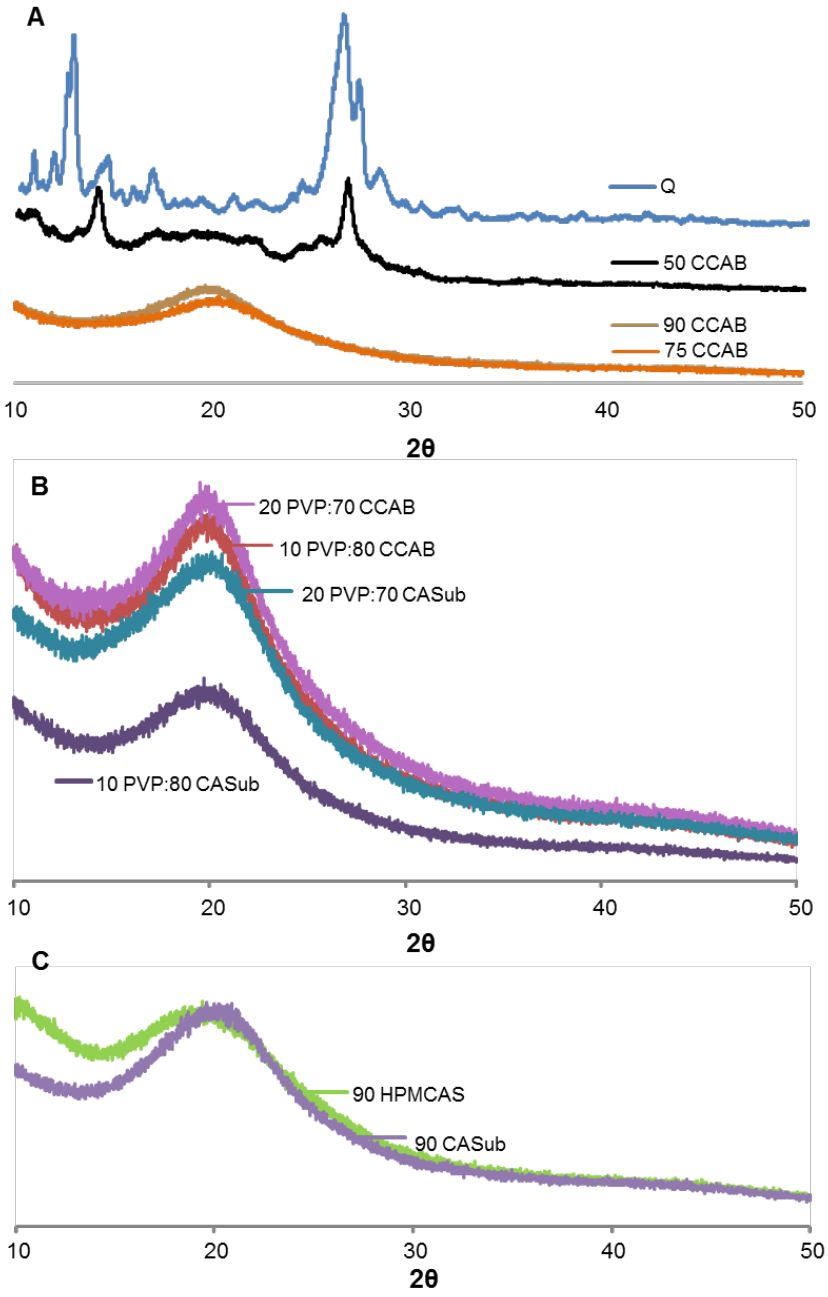


Figure 3.3. *XRD spectra of quercetin, 50 CCAB, 75 CCAB, and 90 CCAB (A), PVP blends with both CCAB and CASub (B), 90 HPMCAS (C), and 90 CASub (C).*

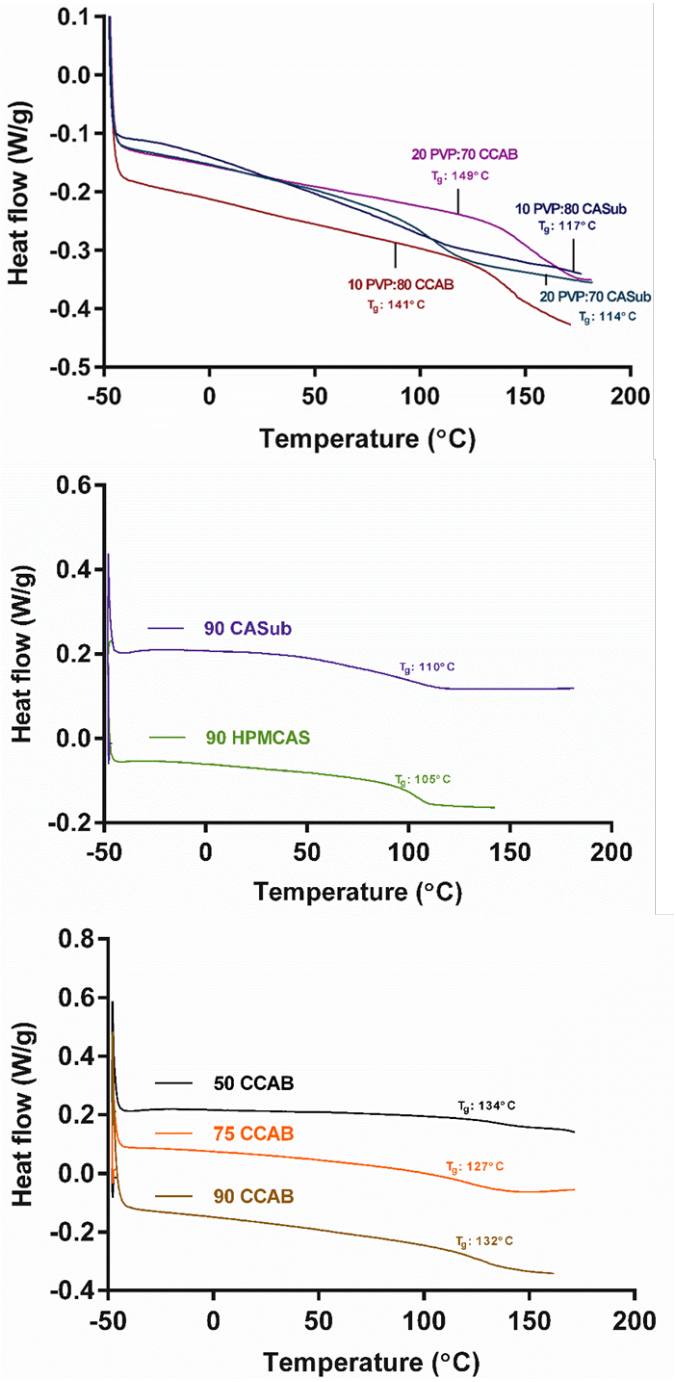


Figure 3.4. *DSC second heating curves of quercetin-loaded ASDs.*

UPLC determination of ASD quercetin loading

In order to characterize ASD formulations and standardize quercetin levels (7 mg/flask) for *in vitro* dissolution experiments, quercetin purity was measured, and quercetin-containing ASDs were analyzed, by UPLC-MS/MS (Table 3.1). ASD quercetin contents conformed closely to targeted levels, though greater variability was seen at higher quercetin contents.

Table 3.1 Actual (quercetin) content of prepared ASD formulations

ASD Formulation[a]	Quercetin (wt %)[b]
Quercetin	91.9 ± 4.63[c]
90 CCAB	10.4 ± 1.68
75 CCAB	28.2 ± 4.29
50 CCAB	45.4 ± 4.95
10 PVP:80 CCAB	9.44 ± 0.128
20 PVP:70 CCAB	9.13 ± 0.258
90 HPMCAS	12.5 ± 0.279[d]
90 CASub[e]	12.0 ± 0.585[d]
10 PVP:80 CASub[e]	10.6 ± 0.718[d]
20 PVP:70 CASub[e]	9.92 ± 0.287[d]

[a]Values in this column refer to formulation targets (wt %); convention for naming treatments is to list the % polymer(s), with the remainder being quercetin
[b]Data shown are mean ± SEM (n = 4, unless otherwise specified)
[c]Supplier specification indicates quercetin purity ≥ 95% (wt %)
[d]n = 3 due to limited quantities of ASDs available
[e] DS(0.9), M_w = 20,000-25,000 g/mol

Determination of crystalline and amorphous quercetin solubility

We attempted to confirm the crystalline solubility and measure quercetin amorphous solubility. Amorphous forms of compounds have a maximum apparent solubility, which represents the maximum amount of free drug achievable in solution, termed amorphous solubility [171]. The experimental amorphous solubility can be measured by creating supersaturated solutions by a solvent-shift method, and then measuring the concentration of drug in the supernatant. However, for compounds that are fast crystallizers (like quercetin), it is necessary to add a small amount of polymer (in this case PVP) to stabilize the supersaturated solution, inhibiting crystallization, and permitting accurate measurement of amorphous solubility. It was

impossible to measure quercetin amorphous solubility in the absence of polymer, because the drug crystallized upon contact with the aqueous solution. We measured quercetin crystalline solubility at low (2.5) pH, where quercetin is un-ionized, and at pH 6.8 where it is partially ionized. Quercetin amorphous solubility was also measured at both pH values. Crystalline solubility values were determined in the absence and presence of PVP, while amorphous solubility was determined only in the presence of PVP. Amorphous solubility was significantly higher than its crystalline counterpart at both pH values. Quercetin crystalline solubility was similar to literature reports, and amorphous solubility appears to be at least 31 mg/mL at small intestine pH.

Table 3.2. Crystalline and amorphous solubility of quercetin in varying dissolution medium.

Medium	Crystalline solubility (µg/ml)	Amorphous solubility (µg/ml)
Acidified water (pH 2.5)[a]	0.64 ± 0.11	N/A[b]
Acidified water (pH 2.5)[a] + 100 µg/ml PVP	1.03 ± 0.20	23.48 ± 0.06
100 mM potassium phosphate buffer (pH 6.8)	1.03 ± 0.08	N/A[b]
100 mM potassium phosphate buffer (pH 6.8) + 100 µg/ml PVP	N/A[b]	31.29 ± 1.80

[a] Acidified with phosphoric acid
[b] Measurement not performed in absence of polymer due to fast Q crystallization upon contact with the solution
[c] Measurement not performed

Impact of quercetin content upon release

ASDs containing 10 and 25 wt% quercetin in CCAB were evaluated with regard to quercetin release. As presented in Fig. 3.5A, both 10% and 25% quercetin-loaded CCAB ASDs (90 CCAB, 25 CCAB) effectively protected quercetin from release at acidic pH; indeed, solution concentrations were significantly lower than that from crystalline quercetin alone. At the neutral pH that mimics the small intestine, release of quercetin from the 10 % quercetin dispersion (90

CCAB, Fig. 3.5B) reached much higher solution concentrations than from the 25% quercetin ASD (75 CCAB) or from crystalline quercetin alone. This is predictable given the quite hydrophobic nature of quercetin (more quercetin in the ASD makes it more hydrophobic, slowing drug release), and is consistent with our results with CAAdP and other polymers [6]. As a result we chose to compare subsequent ASD formulations with different polymers using 10% quercetin in each ASD.

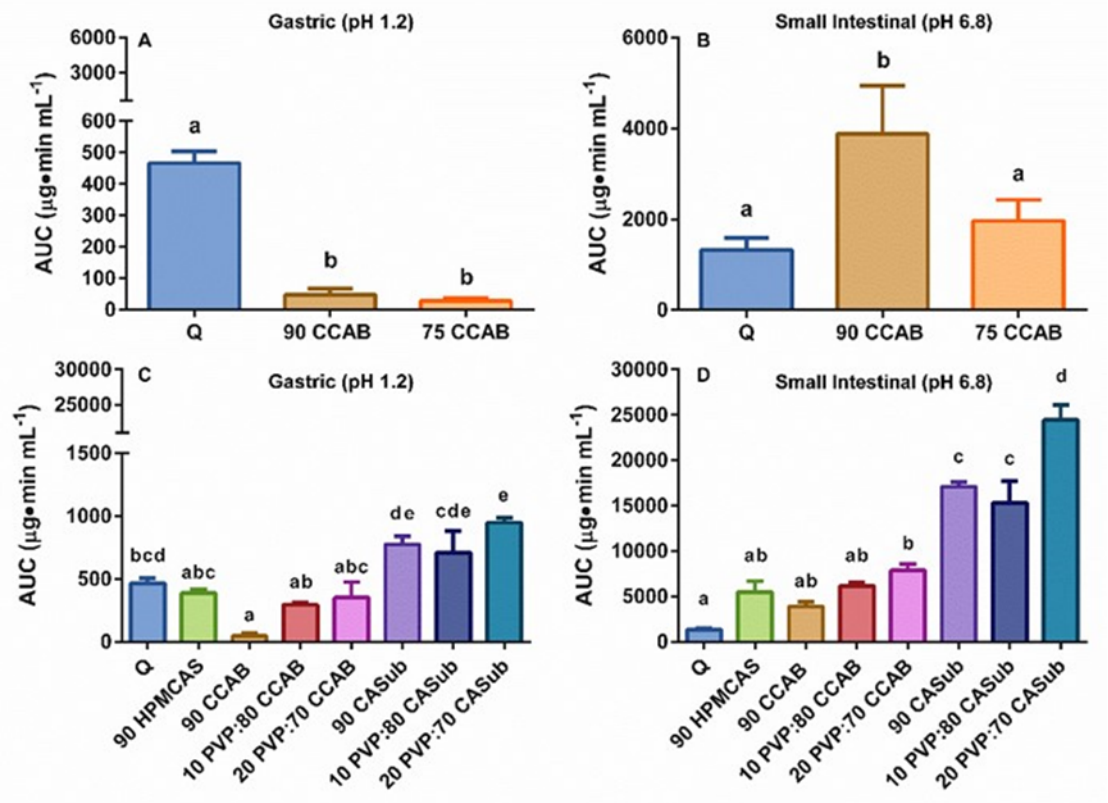


Figure 3.5. *Dissolution area under the curve (AUC) values for all treatments. Quercetin compared to 90 CCAB and 75:25 CCAB is given for both gastric pH (A) and intestinal pH (B). Quercetin compared to all treatments that employed 10% quercetin loads are shown for both gastric pH (C) and intestinal pH (D). Different letters above each bar represent statistically significant differences in AUC between treatments ($p<0.05$) by 1-way ANOVA with Tukey's post hoc test.*

Simulated gastric conditions (pH = 1.2)

Crystalline quercetin and all quercetin ASDs had extremely low dissolution in the gastric environment (Fig. 3.5C, Table 3.3). This was expected due to the inherent properties of crystalline quercetin, as well as ASDs made with carboxylated cellulose derivatives (protonated,

thus neutral/poorly soluble at acidic pH). The most quercetin released at low pH was from 10% quercetin dispersed in 20% PVP:70% CASub, reaching only a mean C_{MAX} of 13.4 μg/mL and AUC of 972 μg·min/mL. All other polymers were relatively successful in preventing quercetin release at gastric pH. Even the AUC from 20%PVP:70% CASub at pH 1.2 was quite low compared to observed AUC values for any treatment at pH 6.8.

Table 3.3. Pseudo-pharmacokinetic parameters of quercetin at gastric pH (1.2).

ASD Formulation	AUC[a] (μg min/mL)	C_{MAX}[a] (μg/mL)	T_{MAX}[a] (min)
Quercetin	164 ± 76.0	4.61 ± 1.76	30
90 CCAB	47.1 ± 20.6	1.21 ± 0.493	120
75 CCAB	27.7 ± 8.68	0.582 ± 0.225	90
50 CCAB	181 ± 35.7	2.92 ± 0.462	30, 60[b]
10 PVP:80 CCAB	295 ± 20.1	4.33 ± 0.206	120
20 PVP:70 CCAB	350 ± 124	5.27 ± 1.92	120
90 HPMCAS[c]	387 ± 30.3	5.55 ± 0.833	90
90 CASub[c]	774 ± 64.4	11.5 ± 1.78	30
10 PVP:80 CASub[c]	707 ± 172	12.3 ± 0.298	90
20 PVP:70 CASub[c]	972 ± 58.0	13.4 ± 0.707	90

[a]Data are mean ± SEM AUC, average C_{MAX} and T_{MAX} (n = 4 except where indicated)
[b]C_{MAX} occurred twice at 2 separate time points
[c]n = 3

Simulated small intestinal conditions (pH = 6.8)

Dissolution experiments were performed at pH 6.8 to mimic the small intestine, and under non-sink conditions where ≥ 31-fold supersaturation would be achieved if all drug dissolved, in order to permit observation and quantification of the supersaturation expected from ASDs. AUC measurements for all formulations are presented in Fig 3.5D**;** AUC, C_{MAX}, and T_{MAX} values are summarized in Table 3.4. All ASDs examined provided some degree of supersaturation vs. quercetin alone. ASDs prepared with only HPMCAS or CCAB provided only modest improvements in quercetin solution concentrations; this is particularly interesting for HPMCAS, which has some solubility in water (ca. 23 mg/mL) [6]. In contrast, CCAB blended with PVP, and CASub either by itself or blended with PVP all give very significantly supersaturated quercetin solutions. Optimum quercetin dissolution was observed with CASub

and PVP:CASub blends. Overall, 10% quercetin dispersed in 20 PVP:70 CASub provided the most significant enhancement ($p < 0.05$) in quercetin apparent solution concentration, with an 18-fold increase in AUC compared to crystalline quercetin (~ 24,400 vs. 1,330 μg·min/mL, respectively). This ASD was also able to produce the highest maximum solution concentration (C_{MAX} = 78.3 μg/mL) over the course of 8 hours at pH 6.8. This average C_{MAX} value is 12.7-fold higher than the average C_{MAX} value attained by crystalline quercetin (6.16 μg/mL). The degrees of enhancement of quercetin solution concentrations achieved are comparable to those achieved from different amorphous matrices by others [172] and in our previous work [6].

Table 3.4. Pseudo-pharmacokinetic parameters of quercetin at intestinal pH (6.8).

ASD Formulation	AUC[a] (μg min/mL)	C_{MAX}[a] (μg/mL)	T_{MAX}[a] (min)
Quercetin	1330 ± 133	6.16 ± 0.141	120
90 CCAB	3880 ± 529	18.4 ± 2.15	60
75 CCAB	1940 ± 254	7.16 ± 1.49	60
50 CCAB	2550 ± 137	8.21 ± 0.760	N/A[b]
10 PVP:80 CCAB	6210 ± 382	20.0 ± 1.33	480
20 PVP:70 CCAB	7850 ± 733	27.4 ± 3.03	420
90 HPMCAS[c]	5480 ± 1210	64.1 ± 19.7	30
90 CASub[c]	17100 ± 485	48.7 ± 1.74	N/A[b]
10 PVP:80 CASub[c]	15300 ± 2434	50.4 ± 4.58	90
20 PVP:70 CASub[c]	24400 ± 1640	78.3 ± 7.12	N/A[b]

[a]Data are mean ± SEM AUC, average C_{MAX} and T_{MAX} (n = 4 except where indicated)
[b]T_{MAX} values listed as "N/A" had 4 separate times where C_{MAX} occurred
[c]n = 3

Blending with the miscible and hydrophilic PVP enhanced quercetin release from both CCAB and CASub as anticipated. The concern was whether the lower concentration of the effective crystallization inhibitor (CCAB (Marks., Nichols, Edgar, unpublished) or CASub [173]) would lead to loss of quercetin solution concentration due to crystallization. This does not appear to have been the case, and the blending approach was effective at synergistically combining PVP release properties with CASub/CCAB crystallization inhibition properties. Our data suggest that

the combination of a hydrophobic polymer (CCAB and CASub) with one that is hydrophilic (PVP) appears to gives the ASD the ability to enhance its release profile and then prevent quercetin recrystallization once in solution. These combinations provide both relative protection from gastric conditions and excellent release and stabilization upon reaching small intestinal pH.

Table 3.5. Hildebrand solubility parameters of polymers used to prepare quercetin-loaded ASDs.

Polymer	Solubility parameter, δ ($MPa^{1/2}$)
PVP	28.39
CCAB	24.44
CASub (DS 0.9)	22.66
HPMCAS	22.42

Polymer solubility parameters are important, albeit imperfect, predictors of whether the polymer has the right hydrophobic/hydrophilic balance (they do not have the ability to discriminate between ionized and un-ionized carboxyl, which for this purpose is an important flaw), and are presented in Table 3.5. The dissolution curves obtained with different ASDs are shown in Fig. 3.6 (pH 6.8) and Fig. 3.7 (pH 1.2). Higher solubility parameters indicate greater polymer hydrophilicity, therefore the polymers arranged by decreasing hydrophobicity are PVP < CCAB < CASub < HPMCAS. The correlation between quercetin release and polymer solubility parameter is rather weak; this has been observed also in other polymer-drug systems (Mosquera-Giraldo, Meng, Edgar, Slipchenko, unpublished). Within more confined data sets, solubility parameters can have predictive value; thus addition of the quite hydrophilic PVP enhances release from the more hydrophobic CCAB and CASub matrices in predictable fashion. Based on solubility parameters alone, for example, the low quercetin solution concentration obtained from the 10% quercetin ASD in CCAB ASD was unexpected; a maximum quercetin solution concentration of only 18.4 μg/mL (Fig. 3.6B) was attained. Lower concentration with CCAB than with CASub was surprising since CCAB has higher calculated solubility parameter

than CASub. CASub was especially effective at enhancing quercetin solution concentration, reaching a maximum of 48.7 μg/mL within the first hour of dissolution (Fig. 3.6B). This is fully consistent with the known excellence of CASub as a crystallization inhibitor [173]. When blended with 10% PVP, release and thus solution concentration did not improve noticeably (50.4 μg/mL), but with 20% PVP in the ASD (20 PVP:70 CASub), solution concentration improved markedly to 78.3 μg/mL (Fig. 3.6D) HPMCAS, the most hydrophobic polymer in the set of polymers investigated, provided interesting quercetin ASD behavior. Release from the HPMCAS ASD was very rapid, reaching an average solution concentration of 64.1 μg/mL (Fig. 3.6B) within 30 min. This solution was clear, but quickly became cloudy, and measured solution concentration quickly dropped off, indicating that HPMCAS was ineffective at preventing quercetin crystallization from supersaturated solution.

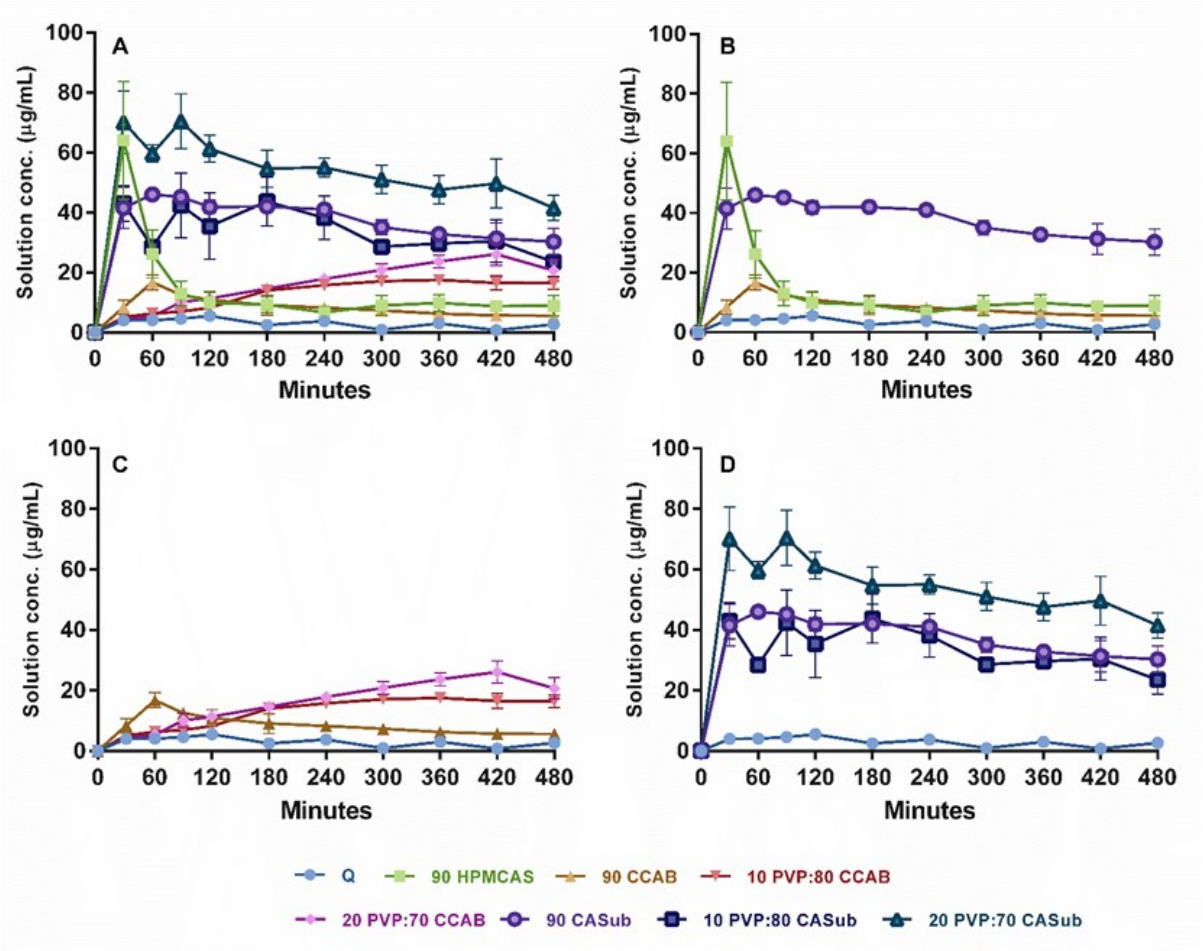


Figure 3.6. *Average solution concentrations of quercetin (mean ± SEM, n = 3-4) plotted over time at pH 6.8 for all treatments (A), 10% quercetin-loaded ASDs only shown for comparison of dissolution properties for each polymer utilized (B), the impact of PVP blending with CCAB (C) and CASub (D). All graphs contain crystalline quercetin as a control for comparison.*

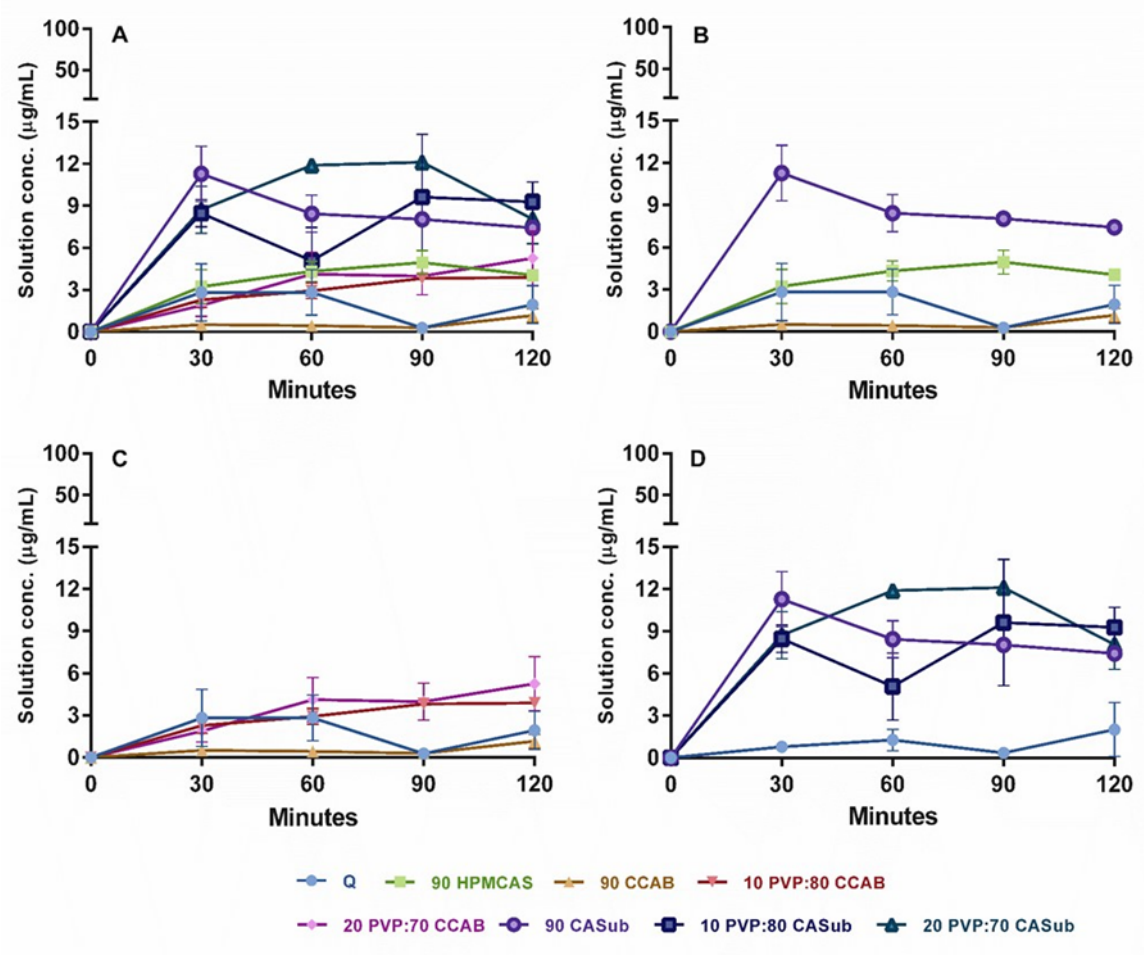


Figure 3.7. *Average solution concentrations of quercetin (mean ± SEM, n = 3-4) plotted over time at pH 1.2 for all treatments (A), 10% quercetin-loaded ASDs only shown for comparison of dissolution properties for each polymer utilized (B), the impact of PVP blending with CCAB (C) and CASub (D). All graphs contain crystalline quercetin as a control for comparison.*

Conclusions

Amorphous solid dispersions at 10 wt% quercetin were prepared using the cellulose esters CCAB, HPMCAS, and CASub. quercetin was also amorphous at 25% in CCAB, but was not fully amorphous at 50% quercetin in CCAB. All ASDs of quercetin in these carboxyl-containing cellulose esters protect effectively against quercetin release at fasting gastric pH, giving substantially lower quercetin concentrations than from quercetin without polymer. In contrast, all three cellulose esters provide supersaturated solutions of quercetin at small intestinal

pH, at which pH most of the carboxylic acids are ionized. The degree of supersaturation from CCAB-only dispersions was slight, due in part to ineffective drug release, while release from HPMCAS ASD was rapid, achieving substantial supersaturation. However HPMCAS proved to be a poor inhibitor of quercetin crystallization from supersaturated solutions, resulting in rapid de-supersaturation. CASub on the other hand provided both substantial quercetin release at pH 6.8, and stable, high supersaturation. Furthermore, incorporation of the more hydrophilic and water-soluble 10% PVP into ASD blends was effective, enhancing release from CCAB at 10% PVP, and significantly enhancing release and supersaturation from CASub ASDs at 20% PVP. Thus CASub and its blends with PVP are highly effective polymers for enhancing quercetin solution concentration *in vitro*, and provide a promising opportunity for increasing quercetin bioaccessibility and bioavailability *in vivo*.

Overall these results confirm our hypotheses, and significantly illuminate structure-property relationships of ASD polymers. We can term polymer properties like sufficiently high T_g (50°C or more above the highest likely ambient temperature) and solubility parameters within an effective range (hydrophobic enough to interact with hydrophobic drugs, hydrophilic enough to release them) as *necessary* but clearly not *sufficient* polymer properties for effectiveness in ASD. This work further confirms the value of the pH-responsive carboxylic acid functional group in providing neutral pH release as well as desirable specific polymer-drug interactions, but this parameter alone is also *insufficient* to fully predict success or failure. This work also provides a further example of the value of polymer blends for achieving performance levels (in this case both release and crystallization inhibition) that would be difficult to achieve by ASD of drug, in this case quercetin, with a single polymer. More detailed study of expanded sets of polymers is necessary to further sort out the structural features required for effective ASD.

The results of this study, particularly with CASub, warrant *in vivo* investigation of quercetin-loaded ASDs as method for increasing quercetin bioavailability upon oral administration. They predict that significant supersaturation should be achievable *in vivo*; it will be of great interest to see whether this results in higher permeation *in vivo*, and in saturation of metabolic enzymes, thereby providing enhanced bioavailability, particularly of the unmetabolized native quercetin. If successful, such an ASD approach should enable animal and human *in vivo* bioavailability enhancement studies, and provide predictable absorbed doses that will enable informative dose-response studies, thus leading to exploration of whether the potential health benefits of quercetin can be realized in humans.

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Chapter 4: Research Limitations

With this research we have assessed the performance of quercetin in ASD with cellulosic polymers at two physiologically relevant pH levels, 1.2 (gastric) and 6.8 (small intestinal). Our emphasis was on increasing solution concentrations of quercetin at pH 6.8, since the small intestine is the singular most absorptive region in the GI tract. We were simulating the environment a quercetin-loaded ASD would be found in solely on pH and ion concentration. There are obvious limitations to this strategy. First, the pH in the small intestine flucuates based on the location (duodenem, jejunum, ileum) and fed vs. fasted state of an individual. The pH can range from 4.9 to 8.0 based on these factors [7]. Using pH 6.8 we were able to target the mid-jejunum [174], however, other regions of the small intestine could have been focused on which would have changed the pH of our buffer. In addition, there are several components within the lumen of the small intestine that can affect the dissolution of quercetin. Our research model was simulating a fasted state, with no added digestive enzymes or other food components, focusing on dissolving quercetin in a specific pH buffer. In the future, it may be beneficial to investigate how the dissolution of quercetin fairs when digestion is also simulated, i.e. fed state simulation. With this in place, luminal components that may have a signficant effect on quercetin dissolution can be studied. Components include bile salts, mucosal lining of the intestine, bicarbonate concentration, and dietary components such as lipids. As one example, research has shown that dietary fat (lipid) can improve quercetin bioavailability through the formation of micelles, allowing easier diffusion through the small intestine [55, 175]. It would be interesting to see the effects of a variable such as dietary fat on the *in vitro* dissolution of quercetin.

Chapter 5: Cellulose Acetate Suberate Amorphous Solid Dispersions Effects on Quercetin Oral Bioavailability in Wistar-Furth Rats

Introduction

Quercetin (Fig. 5.1) is a naturally occurring flavonol, one of the most common classes of flavonoids found in foods [11], and has many potential health benefits upon consumption [14, 91, 137, 143]. Its potential bioactivity against diseases such as cancer, cardiovascular disease, diabetes, and obesity has drawn significant research interest.

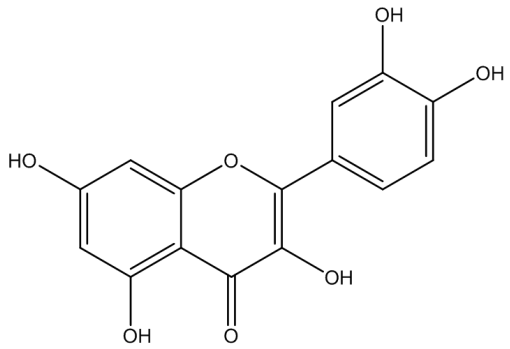

Figure 5.1. *The chemical structure of Q.*

The problem that arises with achieving the proposed benefits of quercetin is that its oral bioavailability is poor, which significantly reduces its potential bioactivities. For example, it has been shown that after quercetin administration, as low as 5.3% of a dose can be present in systemic circulation unmodified [72]. The same study, however, did show that total bioavailability of quercetin and its Phase II metabolites was 59.1% of the original dose, suggesting extensive metabolism (i.e. modification). In rat models, quercetin bioavailability has been shown to be highly variable, ranging from 0.19-188 μmol·h/L (Table 5.1). One of the main reasons behind quercetin possessing such low bioavailability is its structure, and resulting properties in solution. First, quercetin is especially crystalline, meaning that the energy needed to break the intramolecular bonds it creates within in its natural crystalline matrix is high enough that water

will not effectively dissolve it. Second, quercetin is hydrophobic, so its association with water molecules is minimal. The aqueous solubility of quercetin has been reported as 2.15-7.70 μg/mL at 25°C [64, 65]. Solubility is crucial for quercetin release into the aqueous milieu of the gut lumen and transport across the unstirred water layer onto the apical surface of gut epithelial cells (i.e. bioaccessibility). Low solubility leads to minimal portions of a consumed dose of quercetin available for absorption by the epithelial cells, in other words, low bioaccessibility. Additionally, solubility is critical for maximizing the trans-epithelial concentration gradient used to drive epithelial permeation.

Table 5.1. Quercetin bioavailability data from *in vivo* rat studies.

Dose[a]	AUC[b]	C_{MAX}[c]	Ref.
10	0.19	0.695	[72]
10	7.5	N/A	[74]
50	48.435	4.9×10^{-3}	[75]
100	80.3	9.5×10^{-3}	
50	187.85	19.536	[76]
100	0.414	0.9437	[77]

[a]Dose in mg/kg, [b]AUC in μmol·h/L, [c]C_{MAX} in μmol/L

Xenobiotic metabolism is another hurdle that quercetin is forced to overcome in order to be bioavailable. Major barriers within metabolism that quercetin must overcome include the enzyme-rich small intestine and primary detoxification organs such as the liver and kidneys. During xenobiotic metabolism, quercetin does not undergo Phase I, but is highly involved in Phase II/III where metabolic enzymes and transporters work to inactive quercetin through modification (Phase II) [2] and efflux back into the lumen of the GI tract for excretion (Phase III) [5]. Even circulating quercetin is still at risk of undergoing metabolic transformation through detoxification and excretion by the liver [90]. Finding a strategy to overcome poor bioavailability

of quercetin caused by poor solubility and extensive metabolism is critical for delivering the health benefits of quercetin at the population level.

Solubility plays such a crucial role in total bioavailability that an abundance of research has investigated strategies to increase solubility of flavanols. Techniques that have been used include liposomes [111], cyclodextrins [176], solid lipid nanoparticles [177], and amorphous solid dispersion (ASD) [124, 160]. Overall, ASD is a novel approach at increasing the bioavailability of flavonols, and is usually used with poorly water-soluble drugs in the pharmaceutical industry [59, 110, 178]. It is a strategy that utilizes polymers to disrupt crystallinity by trapping hydrophobic materials in amorphous state, and, using pH-dependent release, increase apparent solution concentration. Although there has been some *in vitro* evidence of enhanced dissolution of quercetin through ASD [162, 179], improving the *in vivo* bioavailability of quercetin via ASD has yet to be truly investigated.

Previously, we found that 10% quercetin-loaded ASDs provide optimal *in vitro* dissolution profiles for quercetin. Furthermore, we were able to successfully incorporate amorphous quercetin (10% w/w) into ASD with cellulose acetate suberate (CASub) (Fig. 5.2)

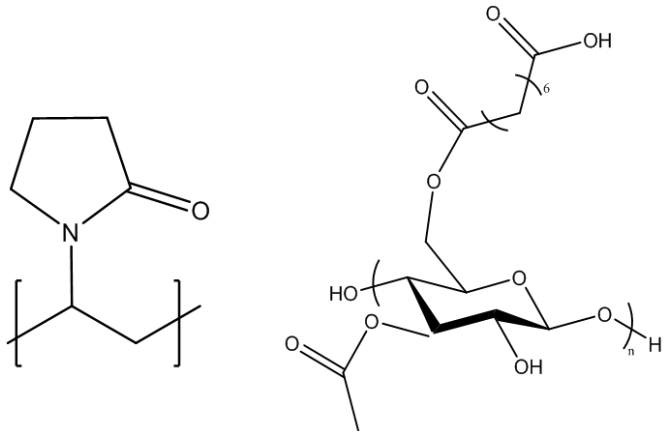

Figure 5.2. *The chemical structures of PVP (left) and CASub (right). Regioselectivity of CASub is random, substituents placed on the cellulose backbone for convenience.*

and significantly improve upon its dissolution (18-fold increase in *in vitro* AUC at pH 6.8). The addition of the water-soluble polymer polyvinylpyrrolidone (PVP) (Fig. 5.2) facilitated enhanced release and improved upon the dissolution of quercetin (Gilley et al. manuscript submitted). The objective of this study was therefore to evaluate the effectiveness of the novel polymer, CASub, used in ASD with quercetin in order to increase the bioavailability of quercetin *in vivo*. In addition, we are interested to see if bioavailability can be enhanced when PVP is blended with CASub in ASD. This is the first study of its kind utilizing CASub in ASD with quercetin. we hypothesized that delivering quercetin in ASD will 1) significantly enhance the total bioavailability of quercetin and 2) increase the ratio of unmodified quercetin to Phase-II metabolites of quercetin in systemic circulation, compared to crystalline quercetin.

Experimental

Materials

Quercetin (≥ 95% by HPLC) and epicatechin (EC) (≥ 90% by HPLC) were purchased from Sigma-Aldrich (St. Louis, MO). Cellulose acetate (CA 320S, DS (acetate) = 1.82) M_n = 50,000) was from Eastman Chemical Company (Kingsport, Tennessee). Polvinylpyrrolidone (PVP) (M_n = 58,000) was purchased from ACROS Organics (New Jersey, USA). Acetonitrile (ACN, HPLC-grade), methylene chloride (HPLC-grade), tetrahydrofuran (THF), betadine solution and reagent ethanol were purchased from Fisher Scientific (Waltham, MA). Formic acid (99.9%) was obtained from Macron Chemicals (Center Valley, PA). Suberic acid, adipic acid, methyl ethyl ketone (MEK), *p*-toluenesulfonic acid (PTSA), triethylamine (Et_3N), and oxalyl chloride were purchased from ACROS Organics (Thermo Fisher, Waltham, MA). 1,3-Dimethyl-2-imidazolidinone (DMI) was purchased from ACROS Organics and dried over 4 Å molecular sieves. Water was purified by reverse osmosis and ion exchange using a Barnstead RO pure ST

(Barnstead/Thermolyne, Dubuque, IA, USA) purification system. Sterile saline solution (0.9% Sodium chloride) was purchased from Teknova (Hollister, CA). Catheter lock solution (500 IU heparin/mL final solution in glycerol) was purchased from SAI Infusion Technologies (Lake Villa, IL). Thicken Up® Instant Food & Drink Thickening Powder was purchased from Nestlé HealthCare Nutrition, Inc. (Florham Park, NJ).

Synthesis of CASub

CASub was synthesized as previously described in Chapter 3.

Preparation of ASDs via spray drying

CASub (1.8 g) was dissolved in 30 mL acetone, stirred overnight, then quercetin (0.2 g) was added to the solution and stirred for 15 min before spray drying (final ratio: 10% quercetin, 90% CASub). PVP (0.4 g) was dissolved in 10 mL ethanol and CASub (1.4 g)/quercetin (0.2 g) was dissolved in 80 mL THF (final ratio: 10% quercetin, 20% PVP, 70% CASub). ASDs were prepared by spray drying the polymer/quercetin solutions using a nitrogen-blanketed spay dryer (Buchi B-290). Instrument parameters were as follows: inlet temperature 90°C, outlet temperature 75°C, aspirator rate 80%, 40% pump rate, compressed nitrogen height 30 mm and nozzle cleaner 2. The convention for naming treatments is to list the % polymer(s), with the remainder being quercetin (both treatments contain 10% quercetin). For example, 10% quercetin/90% CASub is referred to as 90 CASub in the text, figures and tables.

ASD characterization

ASDs were characterized as reported in Chapter 3.

UPLC verification of quercetin content in ASDs

Incorporation of quercetin into ASDs was quantified by extraction and UPLC-MS/MS as previously described (Gilley et al. manuscript submitted). Each ASD was evaluated for quercetin

content (wt %, n = 3). ASDs were dissolved in ethanol (~0.26 mg/mL), and 50 µL of this solution was combined with 50 µL internal standard solution [epicatechin (EC), 0.8 mg/mL in ethanol) and 900 µL 0.1% formic acid in 80% water/20% 80:20 acetonitrile/tetrahydrofuran. Solid quercetin [i.e. 100% quercetin w/w, ≥ 95% purity) was used as control and analyzed similarly.

In vivo bioavailability assessment

Inbred Male Wistar-Furth rats (N = 30, age = 8 wks, avg. weight upon receipt = 243 mg) with exteriorized jugular catheters and pin ports were obtained from Envigo Research Models and Service (Indianapolis, IN). Catheter patency was checked and maintained every 4 d per Envigo protocols. Rats were weighed upon receipt, at the start of administration of the quercetin-supplemented diet (Day 0) and immediately before treatment administration (Day 10). The animals were housed 1 rat per cage on a 12-hour light/dark cycle (70˚F, 40% relative humidity). A basal diet (TD.150827, Envigo) supplemented with 0.1% quercetin was provided for 7 d in order to acclimate rats to habitual quercetin exposure so that pharmacokinetic data would be closely representative of typical dietary exposure, followed by a 2 d quercetin-free (basal diet alone) washout period. Food and water were provided *ad libitum*. During the 7 d quercetin-supplemented period, rats (n = 30) consumed an average of 23.0 g ± 0.00196 g feed/day, providing 73.4 mg quercetin /kg BW. Rats were then randomized to 3 treatments (n = 10) and administered one of three treatments: crystalline quercetin, and one of two quercetin-loaded ASDs: 10% (w/w) quercetin in cellulose acetate suberate (CASub), or an ASD containing 10% (w/w) quercetin in 20% (w/w) polyvinylpyrrolidone (PVP) blended with 70% cellulose acetate suberate (CASub). The total quercetin dose was 50 mg/kg BW for all treatments.

Each treatment was dispersed in thickened water (22.4 mg/mL) so as to not dissolve the treatment (ensuring crystalline quercetin and/or stable, intact ASD was delivered), but to entrap each treatment within the thickened water for delivery. The treatments were administered via intragastric gavage. Gavage needles were purchased from Kent Scientific (Torrington, CT). The concentration was 12.5 mg quercetin/6 mL thickened water for all three treatment suspensions, with actual concentrations adjusted based on the body weight of each individual rat. The quercetin only suspension was made up of 12.5 mg quercetin, and the other 2 treatments were 125 mg total dispersion (12.5 mg quercetin + 112.5 mg cellulose derivative (CASub or PVP:CASub). Blood (~ 200 µL) was drawn through the exteriorized catheter at t = 0 (immediately prior to gavage), 0.5, 1, 2, 4, 6, 8, 12, 18, and 24 hr post-gavage to assess the bioavailability of quercetin. Blood was collected in serum tubes, clotted and centrifuged for 10 min at 4˚C and 1,500 x g to separate serum. Serum was extracted and stored at -80˚C in tubes containing 25 µL dried aqueous ascorbic acid (1% w/v).

Sample preparation

From there, quercetin and its metabolites were extracted from serum as described in Neilson *et al*. with minor modifications [180]. Solid phase extraction (SPE) was performed with Waters Oasis HLB (30 mg, 1 cc) SPE cartridges (Milford, MA). Cartridges were conditioned with 1 mL methanol (MeOH) and 1 mL H_2O. 50 µL serum sample was loaded on the cartridge, followed by washing with 2 mL of 1.5 M aqueous formic acid (v/v) and 2 mL of 5% aqueous MeOH (v/v). quercetin and its metabolites were then eluted with 2 mL of acidified MeOH (0.1% formic acid, v/v). Extracts were then dried under vacuum, resolubilized in 25 µL of starting mobile phase conditions (80%: 0.1% (v/v) aqueous formic acid (phase A) and 20%: 0.1% formic

acid in 80% ACN/20% THF (phase B)), vortexed for 30 s, and stored for analysis by UPLC-MS/MS.

Remaining Work

The finals steps of this research have yet to be completed. The serum samples have been prepared and are ready for UPLC analysis. From there the data will be collected, and quercetin concentrations will be calculated for each time point. Based on the results of those calculations, pharmacokinetic parameters will be determined. Most notably, AUC curves will be generated for each animal's total quercetin serum concentration. Other parameters will include C_{MAX}, T_{MAX}, and $t_{1/2}$. After this has been completed, overall significant differences will be determined using a one-way ANOVA with Tukey's HSD post-hoc test perform comparisons between treatments if necessary.

Chapter 6: Summary

We were able to achieve successful incorporation of amorphous quercetin into ASD with several cellulosic polymers including 6-carboxycellulose acetate butyrate (CCAB), hydroxypropylmethylcellulose acetate succinate (HPMCAS) and cellulose acetate suberate (CASub). ASD formulations were formed via spray drying. Formulations were stable and the amorphous status of quercetin in the dispersions was confirmed by SEM, XRD, DSC, and FT-IR. Only one formulation (50 CCAB) was unable to stabilize amorphous quercetin and it was removed from the experiment as a result. Through dissolution studies at physiologically relevant pH levels, ASDs were successful at protecting quercetin from potential deterioration and recrystallization at gastric pH (1.2), while providing substantial pH-dependent release at intestinal pH (6.8), resulting in significantly enhanced dissolution compared to crystalline quercetin alone. This is one of the major benefits of using amphiphilic cellulosic polymers in ASD with hydrophobic materials such as quercetin. Preliminary dissolution testing provided us with the optimal quercetin load to use in ASD (10%), and that was employed throughout all subsequent treatments. CASub was superior at increasing the apparent solubility, and total AUC, of quercetin ($p < 0.05$) compared to quercetin alone and most other treatments. The addition of 20% PVP (% w/w) in a blend with 70% CASub (% w/w) proved to be the best at improving quercetin dissolution. Quercetin AUC was increased 18-fold when incorporated into 20 PVP:70 CASub, a statistically and physiologically significant increase in apparent solubility at physiological relevant pH (6.8, small intestine). This study was the first to use the novel polymer created in the Edgar lab, CASub, in ASD with quercetin.

Moving forward, if these *in vitro* results can be translated to *in vivo* research, then we hypothesize a significant increase in the bioavailability of quercetin, and subsequent

improvement in its bioactivity. If quercetin concentrations in the GI lumen can increase 18-fold, this strongly suggests that our hypothesis will be confirmed. There is the chance, however, that because our *in vitro* work was solely pH based, with no added digestive enzymes or other luminal components, the bioavailability of quercetin may not significantly increase in the fashion we have predicted. With more *in vitro* dissolution studies using physiological releveant media we will be better able to translate our predictions to *in vivo* work. From the results of our research, ASD appears to be one of the best strategies to substantially increase luminal solution concentrations of quercetin, and CASub has the potential to emerge as a leading candidate for cellulosic polymers to use to do so. Our research has provided foundation for future animal research investigating quercetin-loaded ASDs potential ability to increase bioavailability upon consumption. In addition, it can be investigated whether or not the addition of PVP into ASD blends can further improve bioavailability of quercetin and other polyphenols.

To accomplish the proposed future research, we have utilized an animal (rat) pharmacokinetic study (Chapter 5). There are no other pharmacokinetic studies that utilize CASub in ASD with quercetin, so this is truly novel. We have used oral gavage of quercetin or quercetin in an ASD to give us the ability to deliver equivalent doses of quercetin to all animals, truly testing the effectiveness of ASD *in vivo*. Results of the bioavailability assessment will be obtained in the near future. Ultimately, we would like to test this in a clinical pharmacokinetic study. Until we can see significant improvements in [human] bioavailability of quercetin, we cannot predict increased quercetin bioactivity against obesity, type II diabetes, or other chronic metabolic disorders. If bioavailability of quercetin improvement can be observed *in vivo*, both in animals and humans, the next step would be to test the outcomes of those increased bioavailabilities on the bioactivity of quercetin. These types of study would need to be several

weeks to months long in order to investigate the true outcomes of chronic consumption of quercetin. Long term, the goal of this research is to develop a reliable strategy to increase the bioavailability, and subsequent bioavactivity, of dietary polyphenols. This strategy would be able to help those that suffer from diabetes and obesity, as well as other diseases that quercetin, its metabolites, and other polyphenols have been shown to address.

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Appendix A: Attributions

CASub Synthesis: Hale Cigdem Arca and Brittany L.B. Nichols.

Spray-drying ASDs: Hale Cigdem Arca and Brittany L.B. Nichols.

DSC and FT-IR analyses to confirm successful incorporation of amorphous quercetin: Hale Cigdem Arca and Brittany L.B. Nichols.

SEM imaging of ASDs: Steven McCartney (Nanoscale Characerization and Fabrication Laboratory, Virginia Tech).

XRD analyses: Ann Norris (Sustainable Biomaterials, Virginia Tech).

Crystalline and amorphous quercetin solubility experiments: Dr. Lynne Taylor and Laura Mosquera-Giraldo.

Appendix B: Methods

UPLC-MS/MS

Analyses were performed on a Waters Acquity H-class UPLC separation model (Milford, MA) equipped with a Waters Acquity UPLC BEH C18 column (2.1 mm x 50 mm, 1.7 µm particle size). Column and sample temperatures were maintained at 40˚C and room temperature (25 ± 1˚C), respectively. The binary mobile phase consisted of 0.1% (v/v) aqueous formic acid (phase A) and 0.1% formic acid in 80% ACN/20% THF (phase B) (all solvents LC-MS grade except THF). System flow rate was 0.6 mL/min . A linear elution gradient was employed as follows: 80% A at 0 min, 10% A at 2.20 min, 100% A at 2.25 min. An injection volume of 2 µL was employed. A second injection of 100 µL DMSO was used in between sample injections to remove any carryover quercetin, with isocratic mobile phase (100% B for 2 min), and subsequent reconditioning for 2.25 min to initial gradient conditions. Electrospray (ESI)-MS/MS analysis was performed in negative mode on a Waters Acquity TQD (triple quadrupole) mass spectrometer equipped with a Z-spray electrospray interface. Capillary voltage was −1.5 kV, cone voltage 56 V for quercetin and 34 V for EC, source temperature 150˚C, and desolvation temperature 500˚C. Desolvation and cone gasses were N_2 at flow rates of 1,000 and 50 L/hr, respectively. Detection was performed by multi-reaction monitoring (MRM) of parent psuedomolecular ($[M-H^-]$) ion to daughter (fragment) ion transitions during collision-induced dissociation (CID, Ar gas: 0.25 mL/min). The MRM transitions for quercetin and EC were 300.77 m/z → 150.88 m/z and 288.79 m/z → 245.02 m/z, respectively, with collision energies of 20.0 eV for quercetin and 10 eV for EC. Quantification was based on an internal standard curve prepared using varying levels of quercetin with the fixed internal standard, EC.

UPLC Verification of Quercetin Content in ASDs

Sample preparation

1. Weigh approximately 13 mg ASD ($n = 4$) and dissolve in a 50 mL volumetric flask with reagent alcohol (ethanol).
2. Sonicate each flask for 1 min to ensure all solids are dissolved.
3. Combine 50 µl dissolve ASD in EtOH, 50 µl epicatechin (internal std.), and 990 µl 0.1% formic acid in 80% water/20% 0.1% formic acid in 80:20 ACN:THF.

Dissolution

Buffer preparation (1 L)

1. Weigh 6.805 g sodium phosphate monobasic in a 250 ml volumetric flask and add to volume with MilliQ water. Stir until all particles have been dissolved. Add that mixture to a 1 L volumetric flask.
2. Add 112mL 0.2M NaOH to the 1 L volumetric flask containing the solution from #1 above.
3. Fill the 1 L volumetric flask containing solutions from #1 and #2 with MilliQ water up to volume.

4. Adjust pH to 6.8 using 0.2M NaOH solution. Use pH meter, stir with metal spatula or magnetic stir bar. Add 0.2M NaOH *slowly*, stir for 20 seconds and wait for the pH reading to be "STABLE". Continue adding NaOH solution until pH of 6.8 is met.

Dissolution protocol

1. Clean all dissolution glassware before starting. Soap & water, then a little acetone, then rinse with water.
2. Attach tubes from water heater/circulator to dissolution flasks (bottom of flask → inlet flow & top → outlet flow).
3. Place stir bar into each dissolution flask and fill with 100 mL phosphate buffer.
4. Turn on heated water circulator at 37˚C and stirring plate to 400 rpm. On the stir plate, the setting for 400 rpm is about halfway between 4 and 5. [After ~10 minutes, check temp. of water to ensure 37˚C, circulator typically will have to be set at 34.5˚C.]
5. Weight out ASD microparticles and deposit into their respective, labeled flasks.
6. Stir for 8 hours, only stopping for ~1 min immediately prior to sampling (1 mL aliquots).
    a. Samples taken at 30, 60, 90, 120, 180, 240, 300, 360, 420, and 480 min.
    b. Aliquots are put into labeled 1.5 mL centrifuge tubes.
    c. Replace all aliquots with 1 mL of buffer solution to keep volume constant.
7. Insert rounded ends, white, centrifuge inserts in opposite slots of rotor JA-20. Each centrifuge cycle should have the same # of samples (usually 2) in each insert so the weight is equal while spinning.
8. High speed centrifuge parameters
    a. Rotor: 20
    b. RPM: 47,000 x g Time:
    c. 10 min
    d. Temp: 37˚C
    e. Accel = MAX
    f. Decel = Slow.
9. Supernatant collected (0.5 mL) and diluted with EtOH (0.5 mL) into a new, labeled tube. Samples stored at -80˚C.

Sample preparation

1. Remove samples from freezer to thaw. Be sure to have one centrifuge tube of epicatechin thawed. Typically takes up to 30 min to have all samples completely thawed.
    a. After about 20 min, vortex each sample for ~30 seconds to help speed up the thawing process.
2. While waiting for the samples to thaw, start labeling UPLC vials.
3. Add 50 µl epicatechin solution, 50 µl dissolution sample, and 990 µl 0.1% formic acid in 80% water/20% 80:20 ACN:THF to each UPLC vial. Add them in that order. Cap the vials, shake a few times to mix, and they are ready for UPLC analysis.
    a. Before taking 50 ul of each dissolution sample, individually vortex for 15-30 seconds, then add the sample to the UPLC vial.