Sveriges lantbruksuniversitet
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Effect of dietary flavonoids on hepatic CYP2E1 and 3A4 activity

– Implication for food-drug interactions

Flavonoiders effekt på hepatisk CYP2E1 och 3A4 aktivitet

- Implikation för interaktioner mellan mat och läkemedel

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Abstract

Flavonoids are a group of phytochemicals ubiquitously present in vegetables, fruits, berries and herbs. It is suggested that they contribute to protection against several lifestyle diseases including cancer. Nevertheless, herbal extracts and foods rich in certain flavonoids have been shown to interact with the metabolism of drugs that may cause adverse effects. The interactions are often due to modulation of drug metabolising cytochrome P450 enzymes in the liver.

The present study examined inhibitory effects of the flavonoid quercetin on the cytochrome P450 isoform CYP2E1. Quercetin, myricetin and isorhamentin were flavonoids investigated for inhibitory effects on the isoform CYP3A4. The metabolising function of those specific cytochrome P450 isoforms is crucial for detoxification and/or bioactivation of foreign compounds. Catalytic activity was measured *in vitro* using human liver microsomes separated in male and female pools. Reversibility of enzyme inhibition was investigated and kinetic measurements were performed in order to determine inhibition mechanism. Based on results from animal experiments, the hypothesis was that the selected flavonoids inhibit enzyme activity and that the degree of inhibition differed between genders.

Quercetin inhibited CYP2E1 activity and quercetin and myricetin inhibited CYP3A4 activity as expected, but isorhamnetin had no inhibitory effects on CYP3A4. Inhibition was reversible in all enzyme pools, but the mechanism varied between competitive, uncompetitive and non-competitive inhibition. These observations are related to the flavonoid structure where the number and position of hydroxyl-groups determine the binding mechanism to the enzyme and thereby the level and mode of inhibition. Surprisingly, this study revealed no substantial gender disparities of flavonoid inhibition in human cytochrome P450 activity. Large variations between enzyme pools were observed within the same gender, suggesting inter-individual differences attributable to genetic and/or other lifestyle factors rather than gender.

This study provided gained insight in human cytochrome 450 regulation in presence of flavonoids and the results can contribute to research in drug development.

Keywords: Cytochrome P450, CYP450, CYP2E1, CYP3A4, Flavonoid, Quercetin, Myricetin, Isorhamnetin, food-drug interactions, FDI

Sammanfattning

Flavonoider är en grupp fytokemikalier som finns i de flesta grönsaker, frukt bär och örter. De har visat sig bidra till ett skydd mot flera livsstilssjukdomar, till exempel cancer. Örtextrakt och livsmedel med högt innehåll av vissa flavonoider har dock visat sig påverka nedbrytningen av läkemedel vilket kan orsaka oönskade biverkningar. Interaktionen involverar framförallt en kemisk modulering av läkemedelsnedbrytande, så kallade cytokrom P450-enzymer i levern.

Den här studien undersökte inhibering av flavonoiden quercetin på cytokromisoformen CYP2E1 samt flavonoiderna quercetin, myricetin och isorhamnetin på CYP3A4. Aktiviteten hos dessa cytokrom P450-isoformer utgör en vital funktion i nedbrytningen av främmande ämnen. Katalytisk aktivitet mättes *in vitro* i levermikrosomer från människor uppdelade i manliga och kvinnliga pooler. Inhiberingens reversibilitet undersöktes och kinetiska analyser utfördes för att ta reda på inhiberingsmekanismen.

Hypotesen, baserad på resultat från liknande djurstudier, var att de specifika flavonoiderna inhiberar enzymaktivitet och att inhiberingsgraden varierar mellan könen.

Resultaten visade att quercetin inhiberade CYP2E1 aktivitet och quercetin, myricetin inhiberade CYP3A4 aktivitet som förväntat, men isorhamnetin inhiberade inte CYP3A4. Inhiberingen var reversibel i alla enzympooler och typen av inhibering var antingen kompetitiv, okompetitiv eller icke-kompetitiv. Dessa observationer kan relateras till flavonoidernas kemiska struktur där antalet hydroxylgrupper och dess positioner avgör bindningen till enzymet och därav graden av inhibering. Den här studien visade dock inga väsentliga skillnader mellan könen i flavonoidernas förmåga att inhibera cytokrom P450-aktivitet i människor. Det var stor variation mellan enzympoolerna inom samma kön vilket tyder på interindividuella skillnader relaterade till genetiska eller andra livsstilsfaktorer snarare än kön.

Resultaten från studien kan bidra till ökad kunskap om cytokrom-P450-reglering i människa vilket kan stödja forskning inom läkemedelsutveckling.

Nyckelord: Cytokrom P450, CYP450, CYP2E1, CYP3A4, Flavonoid, Quercetin, Myricetin, Isorhamnetin, Läkemedelsinteraktioner, FDI

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Abbreviations

CYP450	Cytochrome P450
FDI	Food-drug interactions
ADE	Adverse drug event
NADPH	β-nicotinamide adenine dinucleotide phosphate
PNP	*p*-nitrophenol
BR	Benzyloxyresorufin
BFC	7-benzyloxy-4-trifluoromethylcoumarin
HFC	7-hydroxy-7-benzyloxy-triflouromethylcoumarine

1 Introduction

1.1 Background

Cytochrome P450 enzymes (CYP450s) are the main group of enzymes responsible for the metabolism and detoxification of foreign substances, commonly called xenobiotics. They are also involved in the biosynthesis and metabolism of endogenous compounds such as fatty acids and steroids. CYP450s are highly expressed in the liver, where they catalyse reactions that convert lipophilic compounds into more hydrophilic derivatives. Most drugs undergo this biotransformation in order to be catabolised and eliminated from the body (Gonzalez, 1988). However, other xenobiotic compounds such as dietary flavonoids can modulate the activity of CYP450 enzymes leading to an enhancement or inhibition of the catalytic activity. Consequently, the detoxification or functionality of drugs may be interrupted (Hodek et al., 2002).

In the present study, the flavonoids quercetin, myricetin and isorhamnetin were examined for their inhibitory effects on enzyme activity of two CYP450 isoforms. These flavonoids are naturally occurring in a wide range of vegetables in the human diet and known to interact with several CYP450 enzymes (Chen et al., 2009; Guo and Zheng, 2014; Lau and Chang, 2009). Emerging *in vitro* and *in vivo* evidence propose health effects of many flavonoids attributable to their anti-oxidative properties (C.f. Bodewes et al., 2011). Accordingly, supplements containing high concentrations of flavonoids are commonly consumed to prevent health issues. However, knowledge about the underlying mechanisms are limited. (Hollman, Cassidy et al. 2011). Moreover, when co-administrated with prescription drugs there is a considerable risk for adverse health effect since knowledge about potential food-drug interactions (FDI) is still scarce. Understanding the mechanisms behind FDI with CYP450 enzymes can improve the development of new medicines for animals and humans and minimize the risk for adverse effects of drugs (Wanwimolruk and Prachayasittikul, 2014).

Besides diet and other lifestyle factors, the expression and activity of CYP450 enzymes depend on genetic profile, age and gender (Zanger and Schwab, 2013). These inter-individual variations in enzyme activity make it difficult to predict individual drug response. The effect of dietary components on CYP450 activity may also vary between men and women. Gender related differences in the effect of phytochemicals on CYP450 activity has previously been reported in porcine microsomes (Ekstrand et al., 2015) but very few studies have investigated this phenomenon in humans. Therefore, this study on gender related differences in CYP activity in human microsomes provides new and important knowledge about CYP450 regulation in humans.

1.2 Objectives

The aim of the present study was to evaluate gender-related inhibitory effects of selected dietary flavonoids on hepatic CYP450 activity in human males and females *in vitro*. A secondary goal was to examine the mode of enzyme inhibition.

Specifically, quercetin was investigated for its inhibitory effect on CYP2E1 activity and quercetin, myricetin and isorhamnetin were investigated for their inhibitory effects on CYP3A4 activity. The choice of flavonoids and CYP450 isoforms was based on a recently published study which demonstrated that quercetin inhibited CYP2E1, and querctietin, myricetin and isorhamnetin inhibited CYP3A4 in a gender-related manner in pigs (Ekstrand et al., 2015).

Given that pigs and humans have a lot of physiological similarities, the hypothesis of this study was that in the presence of the selected flavonoids, human CYP2E1 and CYP3A4 activity would be inhibited in a gender dependent manner.

2 Literature review

With regards to the objectives of this study, the following paragraphs give an overview of the role of CYP450 enzymes in human metabolism with special emphasis on xenobiotic metabolism. Relevant information about mechanisms in CYP450 regulation is reviewed. Furthermore, the current knowledge status of food-drug interactions involving CYP2E1 and CYP3A4 and the selected flavonoids are presented.

2.1 The Cytochrome P450 system

Cytochrome P450 enzymes evolved in early organisms over 1.4 billion years ago as a system to cope with foreign substances. Environmental changes such as when vertebrates moved from the sea to land lead to increased exposure to new substances and contributed to the development of the CYP450 system and to a wide diversity of the enzymes (Gonzalez, 1988).

The research of CYP450 enzymes stretches back to the 1950's when Klingenberg, (1958) discovered a pigment with the maximum absorption of 450 nm when bound to carbon monoxide. A decade later it was further characterized as a heme protein that catalyses reactions in the cytochrome P450 monooxygenase system (Harding et al., 1964; Omura and Sato, 1962).

In animal species CYP450s are present at two different locations within the cell. Microsomal CYP450s are located in the endoplasmic reticulum. They are mainly involved in xenobiotic metabolism particularly in the liver. Mitochondrial CYP450s function in the biosynthesis of steroids also in extrahepatic tissues (Omura, 2010).

2.1.1 Reaction model

Most CYP450-mediated reactions involve insertion of a single oxygen atom derived from molecular oxygen into an organic molecule. Such reaction is classified as for example hydroxylation, sulfoxidation, epoxidation, *N*-dealkylation or *O*-dealkylation depending on the substrate to be converted. The general catalytic cycle starts when a substrate, typically hydrophobic, binds to the active site of the CYP450 enzyme forming an enzyme-substrate complex. The cycle continues with a series of reactions that require the enzyme cytochrome P450 *reductase* to generate a supply of electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH). The enzyme-substrate complex attracts dioxygen to the reduced heme group (Fe^{2+}) at the enzyme. In the end of the cycle, water and a hydroxylated, more hydrophilic product, dissociates from the enzyme complex (Ortiz de Montellano and Voss, 2005). A simplified paradigm of the overall reaction is presented in **Figure 1**. In reality the system is far more

complex and dynamic. It is thought that oxygenated intermediates can be released at any step in the cycle, potentially generating reactive oxygen species (ROS) and the reactions do not necessarily proceed in order (Krest et al., 2013).

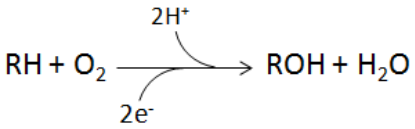


Figure 1. Cytochrome P450 catalysis. RH represents any substrate that binds to the enzyme's heme group (Fe^{3+}) at the active site. ROH represents the final hydroxylated and more hydrophilic product.

2.1.2 CYP450 isoforms

More than 15 000 CYP450 genes coding for varieties of the enzymes have been identified across all biological kingdoms. They are divided into gene families, subfamilies and finally individual genes based on their amino acid sequence profile. Humans possess only 57 active CYP450 genes (compared to 102 in mouse) grouped into 18 families and 44 subfamilies (Nelson et al., 2004). CYP450 family 1-3 are mainly involved in detoxification of exogenous compounds and the remaining CYP450 families regulate metabolism of endogenous compounds such as fatty acids and steroids (Ingelman-Sundberg and Sim, 2010).

The nomenclature of the enzymes is structured as follows: CYP450 followed by a number for family, a letter for subfamily and a number for the isoenzyme. For example, CYP2E1 and CYP3A4 investigated in this study represent different families. The expression of CYP450 enzymes varies widely between species, age, gender, homeostasis of the organism and is often tissue specific. Although expressed in almost all organs, human CYP450s are especially concentrated in the liver where CYP3A4 accounts for the largest fraction (30%), followed by CYP2C (20%), CYP1A2 (13%), CYP2E1 (7%), CYP2A6 (4%), CYP2D6 (1-5%) and CYP2A6 (<1%) (Shimada et al., 1994).

2.2 CYP450 in xenobiotic metabolism

Biotransformation of foreign compounds is an important function of CYP450 enzymes since a disruption of enzyme activity may produce toxicity that is detrimental for the health of the organism. The metabolic process comprises three steps: Phase I modification, Phase II conjugation and Phase III transport (Hoffmann et al., 2014). Microsomal CYP450 enzymes operate in the first phase by introducing polar groups to the foreign substance (Guengerich, 2001).

Most xenobiotics are metabolised by only a dozen human CYP450s belonging to CYP450 families 1-3. The most relevant enzymes involved in drug metabolism are six CYP450 isoforms that metabolise as much as 90 % of all prescribed drugs. The complex regulation of CYP450 activity makes it difficult to predict the fate of drugs and possible adverse drug events (ADE). Therefore, the drug industry is particularly interested in the research of CYP450 regulation (Lynch and Price, 2007; Zanger and Schwab, 2013).

The most common and well-studied mechanism underlying FDIs is inhibition of drug metabolising enzymes (Wienkers and Heath, 2005). In addition to inhibition of enzymes, drug

transporters are susceptible to inhibition and can thus influence drug metabolism (Arnaud et al., 2010). Furthermore, *increased* expression of enzymes or transporters may modulate xenobiotic metabolism, often via receptor-mediated gene transcription (Hewitt et al., 2007). The present study is exclusively devoted to direct enzyme inhibition.

2.2.1 Enzyme inhibition

Any substance, including many drugs and dietary components, that reduce the velocity of enzymatic reactions are called inhibitors. There are two distinct types of inhibition, namely reversible and irreversible inhibition as described by Mathews et al., (2012);

- *Reversible inhibition* is called so because of the weak binding between the inhibitor and the enzyme that easily dissociates and *reverses* the inhibition. The binding action depends on the molecular structure of the enzyme and the inhibitor that further distinguish different modes of reversible inhibition:
  - Competitive inhibitors compete with the substrate for binding to the active site of the enzyme. As a result, the proportion of bound substrate diminishes and the catalytic activity decreases. The inhibition can be avoided by increasing the substrate concentration.
  - Uncompetitive inhibitors bind *only* to the enzyme-substrate complex. Since the inhibitor and substrate bind to different sites they do not structurally resemble each other. Upon binding, a conformational change distorts the catalytic reaction that cannot be overcome by increased substrate concentration.
  - Mixed inhibitors bind either to free enzyme or to the enzyme-substrate complex also at a site distinct from the active site. A slight conformational change in the enzyme complex makes the substrate binding to the active site not optimal and hence, reduces the enzyme activity. Inhibition cannot be overcome by increased substrate concentration because the inhibitor and the substrate do not compete for the same site.
  - Non-competitive inhibition is a special case of mixed inhibition where the inhibitor has the same binding affinity to the free enzyme and the enzyme-substrate complex.

- *Irreversible inhibition,* also called mechanism-based inactivation, occurs when the inhibitor constructs strong, covalent bonds with the enzyme that are unlikely to break. It alters the structure of the enzyme so that the inhibition cannot be reversed.

In practice, reversibility is often studied *in vitro* by including a pre-incubation step prior to addition of the probe substrate. In case of irreversible inhibition, the degree of inhibition increases after inclusion of a pre-incubation step. In contrast, reversible inhibition is unaffected by pre-incubation due to the inhibitor's ability to rapidly dissociate from the enzyme (Mathews et al., 2012). The type of reversible inhibition can be further examined with help of a kinetics study explained in the next paragraph.

2.2.2 Michaelis Menten kinetics

In biochemistry, the Michaelis-Menten equation is the most applicable approach for describing enzymatic reactions **(Figure 2.)**. It is an essential tool when studying reversible CYP450 inhibition. The equation describes the reaction velocity as a function of substrate concentration that appears as a hyperbolic curve when viewed in a diagram **(Figure 3)**. V_{max} reflects the maximum reaction velocity in which the enzyme is saturated with substrate. The Michaelis constant (K_m) is the substrate concentration needed to achieve half of V_{max} and reflects the binding affinity of the substrate to the enzyme. A high K_m indicates low binding affinity and more substrate is required to achieve half of V_{max}.

$$V = \frac{V_{max}\,[S]}{K_m + \,[S]}$$

Figure 2. The Michaelis Menten equation

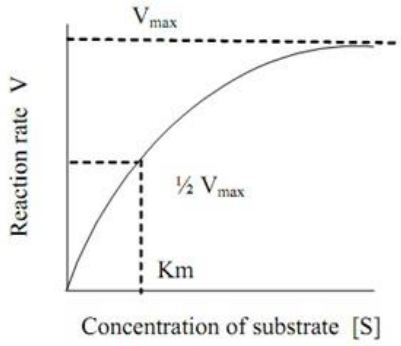


Figure 3. Michaelis Menten plot describing the reaction velocity, V, as a function of substrate concentration, yielding K_m and V_{max}. Berg JM, Tymoczko JL, Stryer L. Biochemistry. 5th edition. New York: W H Freeman; 2002. Section 8.4, The Michaelis-Menten Model Accounts for the Kinetic Properties of Many Enzymes. Available at: http://www.ncbi.nlm.nih.gov/books/NBK22430/ [2016-04-20]

In vitro studies of CYP450 inhibition use a range of combined substrate and inhibitor concentrations in order to investigate the mode of inhibition. The results viewed in a Michaelis-Menten plot **(Figure 3)** are interpreted as described by Berg et al., (2002):

- Competitive inhibition yields a high K_m but does not affect V_{max} since inhibition is overcome by higher substrate concentration.
- In uncompetitive inhibition, both K_m and V_{max} are reduced because the inhibitor increases the effective substrate binding but inhibit the catalytic process.
- In the case of mixed inhibition, K_m increases and V_{max} decreases because the binding of the inhibitor affect the substrate binding and vice versa.

- Non-competitive inhibition is characterized by an unchanged K_m value and decreased V_{max} due to the unaffected substrate binding and impaired enzyme functionality.

The half maximum inhibitory concentration (IC_{50}) and inhibition constant (K_i) are both terms that describe how effective the inhibitor is and are frequently used when evaluating the potency of drug candidates. More specifically, IC_{50} is the concentration of an inhibitor that causes 50 % inhibition of the CYP450 enzyme activity and K_i, also called the dissociation constant, is a kinetic measure that reflects the strength of interaction between the enzyme and the inhibitor. The K_i value for an inhibitor is interpreted analogous with the K_m for a substrate, i.e. a low K_i indicates a strong binding affinity of the inhibitor to the enzyme and a high K_i indicates weak binding capacity. Its formula depends on the mode of inhibition. In the case of non-competitive inhibition, the K_i value is equal to IC_{50}. During competitive and uncompetitive inhibition, K_i is considered to be half of IC_{50}. For mixed inhibition, the K_i value ranges from IC_{50} to $IC_{50}/2$ (Haupt et al., 2015). Whereas the IC_{50} value depends on enzyme-, inhibitor- and substrate concentrations as well as other experimental conditions, the K_i value is independent of the substrate concentration but depends on the enzyme and inhibitor (Cer et al., 2009).

2.3 Food-drug interactions involving CYP450

Many plant derived food components have the ability to interfere with CYP450 mediated drug metabolism that may cause FDI. The most recognised examples are studies of grape fruit juice and St. John's wort which have shown clinically significant interactions with the metabolism of prescribed drugs. Other *in vitro* and *in vivo* studies have indicated interactions between extracts of fruit, berries and herbs with several CYP450 enzymes (Wanwimolruk and Prachayasittikul, 2014). For example, extracts from blueberry, ginkgo biloba and garlic, including their bioactive components, have been studied for their potential role in food-drug interactions. The interaction often leads to a change in bioavailability of the drug, sometimes beneficial for the patient, in other cases it causes adverse or even dangerously toxic effects (Wanwimolruk and Prachayasittikul, 2014). The grade of interaction depends on the characteristics of the drug and active food constituents, the involved CYP450 enzymes, dosage, and health status of the patient. Supplements containing high levels of interacting components are available at the market and claimed to be beneficial for health. From a clinical perspective, the fact that supplements are not uncommonly taken concomitantly with drugs is of special concern (Wanwimolruk and Prachayasittikul, 2014).

2.4 Inhibition by dietary flavonoids

Flavonoids constitute a large group of plant pigments present in vegetables, fruits, berries and herbs that are consumed by most people on a daily basis. They have been extensively studied for their bioactive properties *in vitro* and *in vivo*. The anti-oxidative properties of flavonoids explain the apparent anti-carcinogenic activities where CYP450 enzymes is highly involved in the mechanism of action (Kale et al., 2008). Flavonoids can interact with CYP450 enzymes by modulating (inhibition or induction) biosynthesis of the enzymes or the catalytic activity. It may occur at a transcriptional or gene translational level as well as direct modulation of

CYP450 activity (Hodek et al., 2002). Flavonoids possessing a hydroxyl group generally inhibit CYP450 enzymes whereas flavonoids lacking hydroxyl groups may induce CYP450-dependent activities (Buening et al., 1981).

In nature, flavonoids occur mostly as glycosides where one of the hydroxyl groups is replaced by a sugar molecule such as glucose, rhamnose or rutinose. Sugar attached flavonoids have increased water solubility and are considered non-absorbable in the gut. Nevertheless, colon bacteria hydrolyses them into more lipophilic free flavonoids (aglycones) that are able to pass the intestinal border by simple diffusion (Hodek et al., 2002). In general, the flavonoid content in dietary plants is more concentrated in the seeds and outer part of the plant. Growing conditions and thermal processing as well as the extraction method are factors that influence the flavonoid contents measured in food (Harris et al., 2015; Mitchell et al., 2007; Wach et al., 2007). Hollman and Katan (1999) reported an average daily flavonoid intake of 23mg/day in the Netherlands where quercetin and myricetin were included in the observation, but stressed that it varies widely depending on the diet.

Quercetin, myricetin and isorhamnetin **(Figure 4)** are all members of the flavonol subgroup of flavonoids. Major food sources include onions, apples, kale, broccoli, apples, cherries, berries tea and red wine (Moon et al., 2006). Flavonols and other groups of flavonoids are bioactive constituents in many herbal extracts that are known to modulate CYP450 activity. Gingko Biloba for instance is used in alternative medicine to treat cerebrovascular diseases and memory impairment where quercetin, myricetin and isorhamnetin among other flavonoids are considered to be the responsible mediators (Cho and Yoon, 2015; Lau and Chang, 2009). A three-ring molecule with hydroxyl (OH-) groups attached make up the backbone of flavonols. Their similar structure means that they share some similarities in properties. The number of hydroxyl groups seems to correlate to the degree of inhibition. Myricetin and quercetin have showed stronger inhibition than flavonoids with less hydroxyl groups, and myricetin having one more hydroxyl group than quercetin was the more potent *in vitro* inhibitor of CYP2E1 and CYP3A4 activity (Ho et al., 2001; Li et al., 1994).

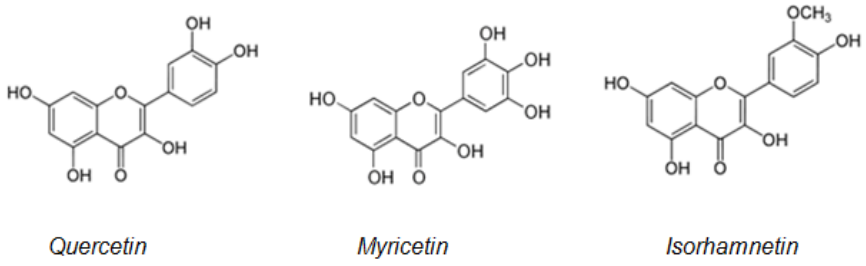


Figure 4. Chemical structure of the flavonoid aglycones of quercetin, myricetin and isorhamnetin.
Structures were retrieved from Mitchell et al., (2007) (Quercetin), Ong and Khoo, (1997) (Myricetin) and Wikipedia (2009). *Isorhamnetin*. https://upload.wikimedia.org/wikipedia/commons/5/54/3-methylquercetin.svg [2016-04-20] (Isorhamnetin)

2.4.1 Quercetin

Quercetin is the most abundant flavonoid in the human diet and hence also the most studied. It is distributed in a wide range of vegetables and consumed daily by most people worldwide. A review from the U.S. Department of Agriculture lists raw capers as the richest food source of quercetin (234mg/100g of the edible portion), followed by lovage (170mg/100g) (Bhagwat et al., 2014). In the western world, apples, onions and red wine account for the largest share of quercetin consumption although they contain much less quantities per 100g (Rodgers and Grant, 1998). Absorption of quercetin in the intestines is accompanied by methylation, sulfatination and glucuronidation, yielding many metabolites that are further metabolised in liver and kindeys. Twenty-one quercetin related compounds have been found in human urine (Kelly, 2011).

Quercetin inhibit several CYP isoforms including CYP1A2 and CYP2E1 *in vitro* and CYP3A4 both *in vitro* and *in vivo* (Moon et al., 2006). A number of clinical studies indicate positive health effects of quercetin, for example blood pressure lowering effects. In most other areas such as cancer prevention the research is still limited to *in vitro* experiments (Kelly, 2011).

2.4.2 Myricetin

Various species of berries contain relatively high contents of myricetin as well as fennel and parsley leaves although carob is the richest dietary source (Bhagwat et al., 2014). Myricetin is structurally similar to quercetin and therefore have similar functions. Generally, it is present in the same vegetables, but to a much lesser extent than quercetin (Bhagwat et al., 2014; Ong and Khoo, 1997).

Research on myricetin is often devoted towards its protective role in metabolic and cancer diseases whereas studies on specific CYP450 inhibition by myricetin are less common. However, Ekstrand et al., (2015) demonstrated that myricetin competitively inhibit CYP1A activity and non-competitively inhibit CYP3A in porcine liver microsomes. Studies of rat liver microsomes have found myricetin to be an inhibitor of CYP3A, CYP2C9 (Choi et al., 2010) and a stronger inhibitor of CYP1A2 than quercetin (Li et al., 1994). Furthermore, it exhibited mixed-type inhibition of CYP2A6 in human liver microsomes (Tiong et al., 2010).

2.4.3 Isorhamnetin

Isorhamnetin is a metabolite of quercetin and also occurs naturally in vegetables. Most vegetables included in the report from U.S. Department of Agriculture contain only traces or no isorhamnetin. Nevertheless, parsley, kale and sea buckthorn contain amounts comparable to quercetin (Bhagwat et al., 2014). In consort with quercetin and myricetin, isorhamnetin has demonstrated cytoprotective and antioxidative capacity along with anti-adiposigenesis and anti-inflammatory effects (Si et al., 2012).

Isorhamnetin has been found to inhibit hepatic porcine CYP1A and CYP3A activity in a competitive manner (Ekstrand et al., 2015). Furthermore, hepatic CYP2B6 activity was inhibited *in vitro* by isorhamnetin aglycone in contrast to the glyosidic counterpart which did not exhibit inhibition (Lau and Chang, 2009).

A compilation of CYP450 enzymes inhibited by quercetin, myricetin and isorhamnetin respectively is presented in **Table 1**.

Table 1. Overview of previously investigated inhibitory effects on CYP450 activity by quercetin, myricetin and isorhamnetin

CYP450 isoform	Flavonoid	Model	Effect	Reference
CYP1A1	Quercetin	Recombinant	Inhibition	Schwarz et al., 2011
	Myricetin	Recombinant	Inhibition	
	Isorhamnetin	Recombinant	Inhibition	Takemura et al., 2010
CYP1A2	Quercetin	HLM	Non-competitive inhibition	Rastogi and Jana, 2014
	Quercetin	Recombinant	Inhibition	Takemura et al., 2010
	Isorhamnetin	Recombinant	Inhibition	
CYP1B1	Quercetin	Recombinant	Inhibition	Takemura et al., 2010
	Isorhamnetin	Recombinant	Inhibition	
CYP2A6	Quercetin	HLM	Competitive inhibition	Tiong et al., 2010
	Myricetin	HLM	Mixed type inhibition	
CYP2B6	Quercetin	HLM	Inhibition	Lau and Chang, 2009
	Isorhamnetin	HLM	Inhibition	
CYP2C8	Quercetin	Recombinant	Irreversible inhibition	Pang et al., 2012
CYP2C9	Quercetin	HLM	Competitive inhibition	Rastogi and Jana, 2014
CYP2C19	Quercetin	HLM	Competitive inhibition	
CYP2D6	Quercetin	HLM	Competitive inhibition	
	Quercetin	LM male rats	Inhibition	Vijayakumar et al., 2015
CYP2E1	Quercetin	Male porcine LM	Competitive inhibition	Ekstrand et al., 2015
CYP3A	Myricetin	Male porcine LM	Non-competitive inhibition	
	Isorhamnetin	Male & female porcine LM	Competitive inhibition	
CYP3A4	Quercetin	HLM and HIM	Inhibition	Fasinu et al., 2013
	Myricetin	Recombinant	Inhibition	Choi et al., 2010

HLM = human liver microsomes, LM = liver microsomes, HIM = human intestinal microsomes

2.5 Cytochrome 2E1

The isoform CYP2E1 is expressed in various organs and tissues and is one of the major hepatic CYP450 enzymes in humans. It metabolises relatively small hydrophobic endogenous and exogenous compounds including several steroids as well as drugs such as chlorozoxazone and paracetamol. Chlorozoxazone is frequently used as a probe substrate since CYP2E1 exhibits high specificity for the compound. The present study used another specific substrate, *P*-Nitrophenol (PNP). It rapidly metabolises to nitrocatechol and is therefore suitable for *in vitro* assays (Cederbaum, 2014).

Research on CYP2E1 regulation is mostly focused on its association with various cancers since the enzyme is an efficient activator of chemical carcinogenesis (Zanger and Schwab, 2013). Induction of CYP2E1 is linked to several types of cancer caused by the generation of reactive oxygen species (ROS) that cause cell damage. The enzyme is inducible by many of its own substrates and several hormones at a transcriptional, translational and posttranslational level (Gonzalez, 2007). Ethanol is an example of an inducing CYP2E1 substrate that is a causative player in alcoholic and non-alcoholic liver disease caused by ROS production (Aubert et al., 2011). Moreover, CYP2E1 expression is elevated during several other physiological conditions including diabetes, obesity, fasting and smoking (Caro and Cederbaum, 2004; Girre et al., 1994).

Numerous phytochemicals are considered anti-carcinogenic attributable to their ability to inhibit CYP2E1 (Barcelo et al., 1996; Wargovich, 2006). Furthermore, CYP2E1 inhibitors such as the irreversible inhibitor disulfiram are utilized in clinical practises against alcohol abuse (Pelkonen et al., 2008). The antioxidant disulfiram, diethyldithio-carbamate and the active constituent in garlic, diallylsulfide, are known irreversible inhibitors of CYP2E1 (Cho and Yoon, 2015). The proclaimed health beneficial ingredient in red wine, resveratrol, is a non-competitive CYP2E1 inhibitor (Piver et al., 2001). Additional phytochemicals present in various food products and herbs have indicated inhibitory effects including licochalcone from liquorice (He et al., 2015), isoflavones from soy (Fan et al., 2013), black tea catechins (Catterall et al., 2003), black pepper (Kang et al., 1994) and ginseng (Kim et al., 1997). Most studies were conducted in the liver or intestine of mice and rats and the results can therefore not apply to humans. Zuber et al., (2002) pointed out that although active components from milk thistle inhibited CYP2E1 in human liver microsomes, the plasma concentrations of those compounds following dietary uptake do not reach the concentration required for inhibition. Therefore, CYP2E1 inhibition by milk thistle in the diet is unlikely.

2.6 Cytochrome 3A4

CYP3A4 is the most abundant CYP450 enzyme in the liver and the main metabolising enzyme of approximately 50% of all prescribed drugs (Pelkonen et al., 2008). The small intestine also hosts significant CYP3A4 content where it substantially contributes to phase I metabolism of xenobiotics (von Richter et al., 2004). It is involved in bioactivation of procarcinogens such as aflatoxin (Gonzalez and Gelboin, 1994). Furthermore, several clinical studies have reported FDI involving irreversible inhibition of CYP3A4 activity. Orally administrated drugs were insufficiently degraded and accumulating in its active form in the blood circulation, sometimes causing unfavorable toxicity (Zhou et al., 2007; Chen et al., 2012).

The diverse functions of CYP3A4 can be attributed to its chemical structure. It exhibits a broad substrate preference due to its large and flexible active site that allows large or multiple

small molecules to bind. Substrates include several clinical drugs such as immunosuppressants, antibiotics and statins along with endogenous steroids such as testosterone, progesterone and bile acid (Zanger and Schwab, 2013). A recommended probe substrate for CYP3A4 inhibition assays is 7-benzyloxy-4-trifluoromethylcoumarin (BFC) which was used in this study. Although BFC is metabolised by several CYP450 isoforms, dealkylation to hydroxy-4-trifluoromethylcoumarin (HFC) by CYP3A4 is especially sensitive to inhibition (Renwick et al., 2000; Stresser et al., 2000).

Consistent with low substrate selectivity, CYP3A4 is susceptible to inhibition by a broad variety of substances. Known inhibitors are structurally divergent, ranging from antifungal agents, antimicrobial and antihypertensives to herbal and food constituents (Pelkonen et al., 2008). Grapefruit juice is a well recognised CYP3A4 inhibitor and responsible for several clinically relevant FDI proved in clinical studies (Ameer and Weintraub, 1997). The juice is rich in flavonoids that individually also have inhibitory effects, for example naringin (Veronese et al., 2003). Resveratrol is a bioactive component of red wine, this flavonoid has been shown to interact with drug metabolism in human volunteers (Chow et al., 2010).

Research of CYP3A4 inhibition has revealed a few contradictory findings. The anti-depressive herb St. John's wort for instance inhibited CYP3A4 *in vitro* (Obach, 2000) but prolonged exposure increased CYP3A4 expression (Lau et al., 2011). A possible explanation proposed by Xie and Kim (2005) is that multiple inhibitors and inducers in the herb influence the enzyme expression in a feedback fashion. Gurley et al., (2005) showed that St. John's wort strongly induced CYP3A4 expression and to a less extent CYP2E1 in elderly subjects and discouraged concomitant ingestion of herbal supplements with prescription drugs to avoid herb-drug interactions.

Quercetin is one of the major phytochemicals in St. John's wort and has also showed contradictory effects on CYP3A4 activity. It inhibited felodipine metabolism in human liver microsomes (Fasinu et al., 2013) but *in vivo* studies of rats have showed conflicting results where both inhibition (Choi et al., 2011) and induction have been detected. The glucoronidated metabolites of quercetin following oral administration was thought to cause the enzyme activation (Yu et al., 2011).

Similar to CYP2E1 inhibition, CYP3A4 was inhibited by licochalcone, black pepper and irreversibly by resveratrol (Piver et al., 2001). Tiliroside is a flavonoid present in several berry species such as raspberries and is a strong CYP3A4 inhibitor (Sun et al., 2010). Likewise, Gingko Biloba inhibited CYP3A4 activity through inhibition of BFC metabolism in human microsomes (Gaudineau et al., 2004).

There are many more inhibitors of CYP2E1 and CYP3A4 metabolism than the above mentioned examples. This literature review presented the most relevant and investigated inhibitors deriving from dietary sources.

2.7 Gender related differences in CYP450 activity

Since CYP450 genes are located on autosomal chromosomes, gender differences in gene expression are not expected. Observed variations in expression and activity can therefore be attributed to endogenous hormonal influences (Meibohm et al., 2002). However, Penaloza et al., (2014) asserted that inborn differences between male and female CYP450 expression do exist through different methylation patterns in the embryo that persist in primary cells. They

found certain methylation patterns associated with higher expression of CYP1A1, CYP2E1 and CYP7B1 in tissues and cell cultures of female mice compared to male.

Human studies on gender differences in CYP450 regulation is often studied by quantifying the metabolism of probe substrates for specific CYP450 isoforms. Epidemiological studies with clinical drugs have reported 24-40 % higher CYP3A activity in women (Kahan et al., 1986; Krecic-Shepard et al., 2000; Lew et al., 1993; Meibohm et al., 2002). However, other studies suggested that the higher CYP3A metabolism is due to lower expression of the drug transporter P-glycoprotein in women (Kinirons et al., 1999; Lan et al., 2000; Meibohm et al., 2002). Furthermore, *in vitro* studies could not detect gender differences in CYP3A activity in human liver microsomes (Schmucker et al., 1990; Shimada et al., 1994).

Several studies have reported higher activity of CYP1A2 in men compared to women, (Bartoli et al., 1996), (Bock et al., 1994; Ou-Yang et al., 2000; Relling et al., 1992). Men also expressed higher CYP2E1 activity indicated by faster oral clearance of the probe substrate chlorzoxazone than women. However, the difference was less pronounced after normalisation for body weight, indicating bodyweight-related disparities rather than gender-related (Kim and O'Shea, 1995). A number of human studies have reported significantly higher CYP2D6 activity in women than in men, these findings are of clinical importance since the isoform is an exclusive metaboliser of several therapeutic agents (Hägg et al., 2001; Labbe et al., 2000; Tamminga et al., 1999).

Despite its clinical relevance, gender related differences in CYP450 regulation only recently attracted researcher's attention. The influence of dietary components such as flavonoids on CYP450 regulation may be gender-dependent but is not well studied. Ekstrand et al., (2015) found that myricetin, isorhamnetin and quercetin affected porcine CYP1A, CYP3A and CYP2E1 activity and the mode of inhibition in a gender-dependent manner. Thus, studying this phenomenon in humans is a unique addition to the research of CYP450 regulation.

3 Material and method

An *in vitro* experiment was set up to analyse gender related differences in degree of enzyme inhibition and the type of inhibition.

CYP2E1activity was measured using the probe substrate PNP and CYP3A4 activity was measured using BFC as probe substrate. Well established HPLC methods were used to quantify 4-nitrocatechol, product of PNP metabolism, and 7-hydroxy-4-trifluoromethylcoumarin (HFC), product of BFC metabolism. The procedure consisted of mixing and incubating inhibitor (quercetin, myricetin, isohamnetin), enzyme, substrate, electron donor (NADPH) and buffer in test tubes followed by measuring product formation.

Substrate specificity and enzyme inhibition in the presence of an inhibitor were examined by using human recombinant enzymes expressed in *Saccharomyces cerevisiae*. The same enzyme was used to test if the catalytic activity followed a Michaelis-Menten curve.

The following step was to carry out the same experiment in female and male human liver microsomes. Two female and two male pools were analysed, each pool containing samples from approximately 20 individuals. If inhibition occurred, enzyme reversibility was tested through inclusion of a pre-incubation step. In case of reversible inhibition, multiple substrate concentrations were investigated to achieve the maximum velocity (V_{max}) of the enzyme activity, the Michaelis-Menten constant (K_m) and the inhibition constant (K_i) in order to determine the mode and strength of inhibition. Inhibition was defined as a 50 % or more decrease in catalytic activity in at least one sample within enzyme pools. The decrease was in comparison to the control which was set to have 100 % catalytic activity.

An overview of the experimental design is shown in **Figure 5.**

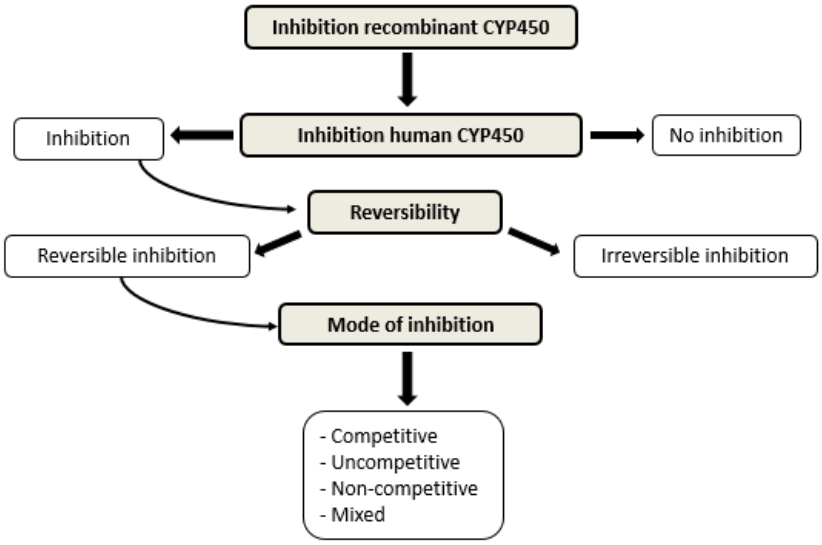


Figure 5. Overview of the experimental design.

3.1 Chemicals

Quercetin dehydrate 98%, 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-hydroxy-7-benzyloxy-triflouromethylcoumarine (HFC), 7-benzyloxyresorufin (BR), reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma-Aldrich (Steinheim, Germany). Isorhamnetin and myricetin were purchased from Fluka (Buchs, Switzerland). HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Quercetin, isorhamnetin and myricetin were dissolved in methanol, BFC in dimethyl sulfoxide and HFC and NADPH in 0.05 M potassium phosphate buffer (pH 7.4). All stock solutions were stored at −80 °C, and working solutions at −20 °C.

3.2 Microsomes

CYP2E1 and CYP3A4 human recombinants, expressed in *Saccharomyces cerevisiae* and microsomes from liver, pooled separately from human female and male were purchased from Sigma-Aldrich (Steinheim, Germany). Additionally, human liver microsomes pooled from 20 female individual donors and 20 male individual donors were purchased from Life technologies Europe BV (Netherlands). Protein concentration in all human liver microsomes was 20 mg/ml.

3.3 Analysis of CYP2E1

Hydroxylation of PNP to *p*-nitrocatechol was measured to determine the catalytic activity of CYP2E1 (Tassaneeyakul et al., 1993). Incubations contained 10 pmol of human recombinant cDNA-expressed CYP2E1 or 0.2 mg of human liver microsomal protein, 0.2 mM of PNP and 1.0 mM NADPH in incubation buffer (0.1 M potassium phosphate, pH 6.8) and 2µl of 16, 32 or 128 µl inhibitor or methanol (control). For the kinetic measurements, samples with 16 µl

inhibitor were not prepared. The final incubation volume was 0.25 ml. Incubation was performed in a water bath at 37 °C, for 30 min. The reaction was terminated with 0.02 ml of 40% trichloroacetic acid. The tubes were vortexed and centrifuged at 10000g for 5 min. Supernatants were transferred to HPLC vials and concentrations of p-nitrocatechol were measured using the HPLC system consisting of a pumping system (L-7100), autosampler (AS 7200), fluorescence detector (L-7485), UV detector (L-7400) set at 345 nm, and D-7000 HPLC Manager software (Merck, Hitachi, Tokyo, Japan). Hypersil ODS column (5 μm, 60 × 4.6 mm, Hewlett–Packard, Palo Alto, CA, USA) equipped with a guard column was used. P-nitrocatechol was eluted using an isocratic mobile phase consisting of 0.1% trifluoroacetic acid in 25% acetonotrile at a flow rate of 1.0 ml/min. PNP hydroxylation was also investigated in human recombinant CYP3A4 according to the same procedure.

3.4 Analysis of CYP3A4

Metabolism of BFC to HFC was measured to determine the activity of CYP3A (Renwick et al., 2000). Incubations contained 5 pmol of human recombinant cDNA-expressed CYP3A4 or 0.05 mg of human liver microsomal protein, 25 μM of BFC and 1.0 mM NADPH in incubation buffer (50 mM potassium phosphate, pH 7.4) and 2μl of 16, 32 or 128 μl inhibitor or methanol (control). For the kinetic measurements, samples with 16 μl inhibitor were not prepared. The final incubation volume was 0.5 ml. Incubation was performed in a water bath at 37 °C, for 10 min. The reaction was terminated with 0.5 ml of ice cold methanol. The tubes were vortexed and centrifuged at 10000g for 5 min. Supernatants were transferred to HPLC vials and concentrations of HFC were measured using the same HPLC system as for CYP2E1, except for the detector. HFC was eluted using an isocratic mobile phase of 50 mM phosphate buffer:methanol:acetonitrile (52:45:3, v/v) at a flow rate of 1.0 ml/min and detected with a fluorescence detector (410 and 538 nm for excitation and emission wavelengths). In addition to O-dealkylation by BFC, O-dealkylation of the substrate benzyloxyresorufin (BR) was investigated in human recombinant CYP3A4.

3.5 Data analysis

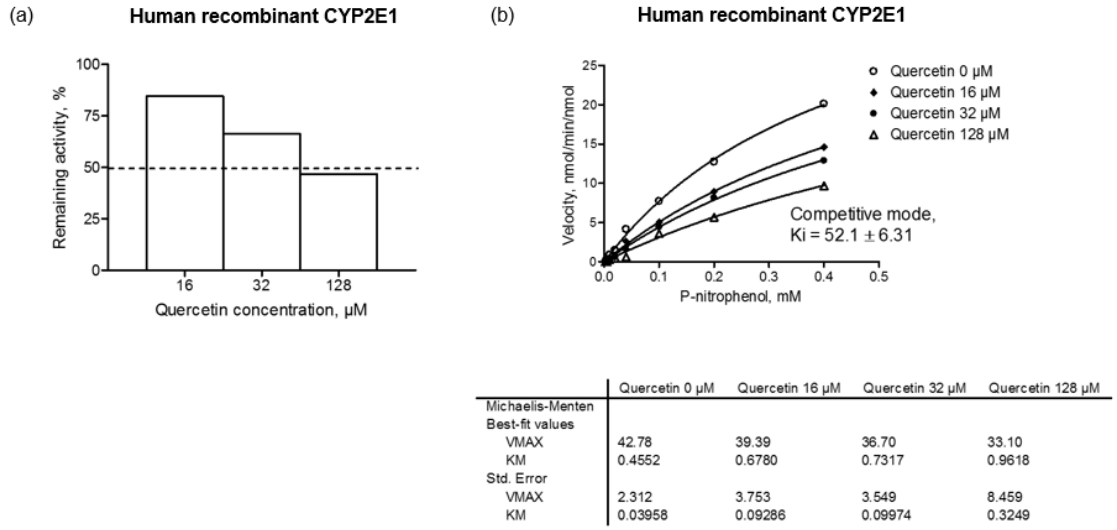
Enzyme kinetic and inhibition parameters were estimated using GraphPad Prism 4.0 (GraphPad software Inc., CA, USA). Michaelis constant (K_m), maximal velocity (V_{max}) and dissociation constant for inhibitor binding (K_i) values were obtained from Michaelis–Menten non-linear regression equation.

4 Results

4.1 CYP 2E1 activity

Recombinant CYP2E1 activity

PNP was hydroxylated to 4-nitrocatechol by recombinant CYP2E1. The catalytic activity decreased with increasing concentration of quercetin compared to the control **(Figure 6a)**. The mode of inhibition was competitive as indicated by a higher K_m-value in the presence of inhibitor (**Figure 6b**).



	Quercetin 0 µM	Quercetin 16 µM	Quercetin 32 µM	Quercetin 128 µM
Michaelis-Menten Best-fit values				
VMAX	42.78	39.39	36.70	33.10
KM	0.4552	0.6780	0.7317	0.9618
Std. Error				
VMAX	2.312	3.753	3.549	8.459
KM	0.03958	0.09286	0.09974	0.3249

Figure 6. *In vitro* inhibition of PNP hydroxylation by quercetin in CYP2E1 human recombinant, expressed in *Saccharomyces cerevisiae*. Remaining enzyme activity in percentage in the presence of 16, 32 and 128 µM of quercetin (a), saturation curve with reaction velocity in different substrate concentrations and K_m and V_{max} values in presence of different quercetin concentrations (b).

Hepatic CYP2E1 activity in human microsomes

The inhibitory effects of quercetin in human microsomes differed between the two microsome pools within the same gender. At a substrate concentration of 200μM, the enzyme activity in micrsosomes from pool 1 was not affected by quercetin, irrespective of female or male samples, and with or without pre-incubation (**Figure 7a**). When a lower substrate concentration was used (20μM PNP), inhibition was observed in male but not female microsomes (**Figures 7a and 7b**). In microsome pool 2, enzyme inhibition was observed in both female and male samples with 20 μM PNP concentration and in the female microsomes with higher substrate concentration. Including a pre-incubation step in the procedure did not increase the level of inhibition (**Figures 7c and 7**). Combining the results from the two microsome pools indicate enzyme inhibition in both genders using the lower substrate concentration of 20μM PNP and no pre-incubation. Pre-incubation did not further increase the degree of inhibition (**Figure 7e and 7f**).

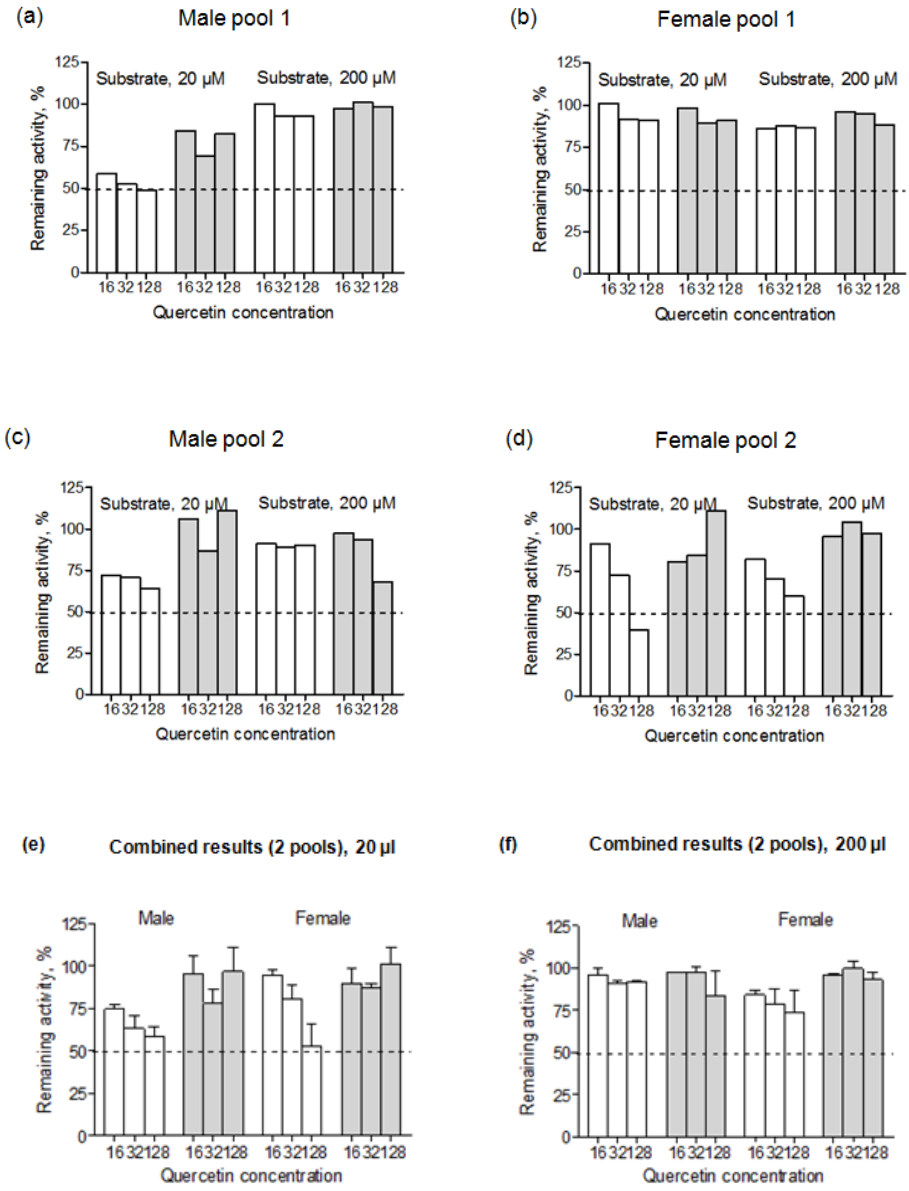
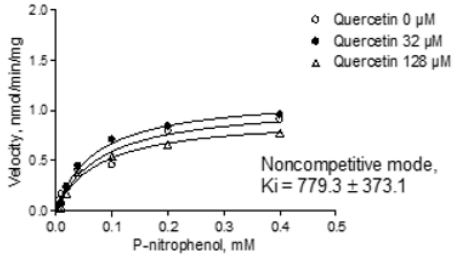


Figure 7. *In vitro* inhibition by quercetin in human hepatic male and female microsomes containing CYP2E1. PNP hydroxylation of male (a) and female (b) human microsomes from pool 1and male (c) and female (d) human micrsosomes from pool 2 in two different substrate concentrations. Combined results from pool 1 and pool 2 with 20µM substrate concentration (e) and 200µM (f). White bars indicate no pre-incubation and grey bars indicate 15 minutes pre-incubation.

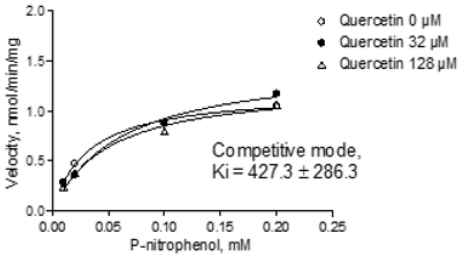
A non-competitive mode of inhibition was observed in male microsomes from pool 1(**Figure 8a**) and competitive in the microsomes from pool 2 (**Figure 8b**). For the female samples, no inhibition was detected in pool 1 (**Figure 8c**) and competitive inhibition was seen in microsome pool 2 (**Figure 8d**).

(a) **Male pool 1**



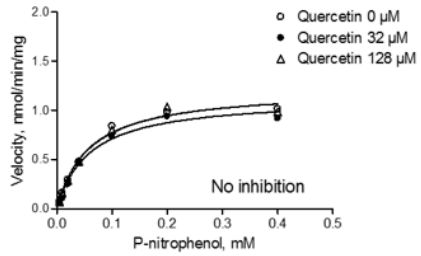
	Quercetin 0 µM	Quercetin 32 µM	Quercetin 128 µM
Michaelis-Menten Best-fit values			
VMAX	1.067	1.146	0.9306
KM	0.08175	0.07138	0.07771
Std. Error			
VMAX	0.1193	0.06335	0.07898
KM	0.02570	0.01153	0.01883

(b) **Male pool 2**



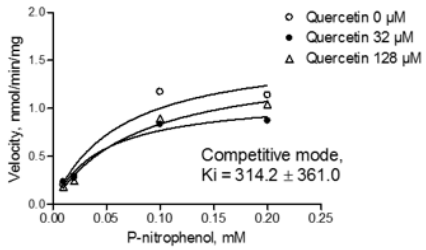
	Quercetin 0 µM	Quercetin 32 µM	Quercetin 128 µM
Michaelis-Menten Best-fit values			
VMAX	1.201	1.496	1.278
KM	0.03226	0.06230	0.05004
Std. Error			
VMAX	0.03043	0.1560	0.09995
KM	0.002720	0.01791	0.01160

(c) **Female pool 1**



	Quercetin 0 µM	Quercetin 32 µM	Quercetin 128 µM
Michaelis-Menten Best-fit values			
VMAX	1.218	1.132	1.221
KM	0.05751	0.05928	0.05837
Std. Error			
VMAX	0.05916	0.07306	0.07548
KM	0.008642	0.01174	0.01112

(d) **Female pool 2**



	Quercetin 0 µM	Quercetin 8 µM	Quercetin 128 µM
Michaelis-Menten Best-fit values			
VMAX	1.624	1.121	1.493
KM	0.06208	0.04573	0.07844
Std. Error			
VMAX	0.3861	0.1372	0.1881
KM	0.04076	0.01704	0.02502

Figure 8. Saturation curve of PNP hydroxylation in human hepatic microsomes containing CYP2E1. (a) male microsomes from pool 1, (b) male microsomes from Pool 2, (c), female microsomes from pool 1 and (d) female microsomes from pool 2.

PNP hydroxylation by recombinant CYP3A4

Formation of nitrocatechol from PNP by human recombinant CYP3A4 was not detected in the HPLC analysis and therefore no area under the curve could be obtained.

4.2 CYP 3A4 activity

Recombinant CYP3A4 activity

O-dealkylation of BR was observed in human recombinant CYP3A4, although the catalytic activity was not inhibited in the presence of quercetin, myricetin and isorhamnetin compared to the control **(Figure 9a)**. The other probe substrate BFC demonstrated O-dealkylation to HFC and inhibition in the presence of quercetin and myricetin, but not isorhamnetin. Quercetin was the strongest inhibitor **(Figure 9b)**.

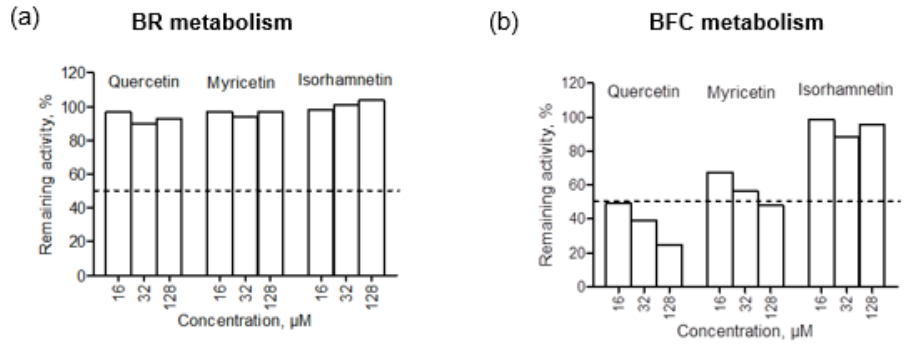
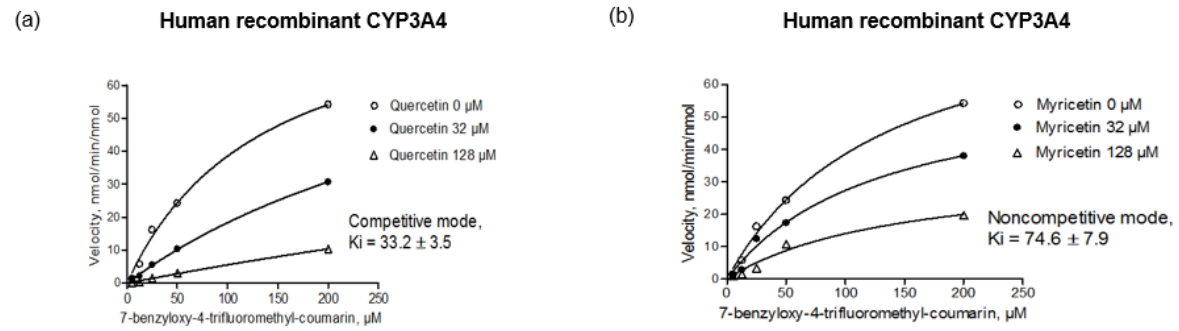


Figure 9. *In vitro* inhibition of O-dealkylation of BR (a) and BFC (b) by quercetin, myricetin and isorhamnetin in human recombinant CYP3A4, expressed in *Saccharomyces cerevisiae*.

Quercetin inhibited the catalytic activity in human recombinant CYP3A4 competitively (**Figure 10a**) and myricetin in a non-competitive manner (**Figure 10b**).



	Quercetin 0 µM	Quercetin 32 µM	Quercetin 128 µM
Michaelis-Menten Best-fit values			
VMAX	91.17	93.81	79.48
KM	136.6	410.8	1328
Std. Error			
VMAX	9.538	9.873	77.29
KM	27.79	61.09	1470

	Myricetin 0 µM	Myricetin 32 µM	Myricetin 128 µM
Michaelis-Menten Best-fit values			
VMAX	91.17	62.61	34.48
KM	136.6	129.3	146.6
Std. Error			
VMAX	9.538	9.547	9.042
KM	27.79	38.95	73.28

Figure 10. Saturation curve of BFC O-dealkylation by quercetin (a) and myricetin (b) in human recombinant CYP3A4 expressed in *Saccharomyces cerevisiae*.

Hepatic CYP3A4 activity in human microsomes

Both female and male human microsomes exhibited decreased catalytic activity in the presence of quercetin (**Figure 11a**) and myricetin (**Figure 11b**) but not isorhamnetin (**Figure 11c**). Inhibition was more pronounced in the presence of quercetin than myricetin. There was no difference between genders and no effect of pre-incubation, indicating reversible inhibition.

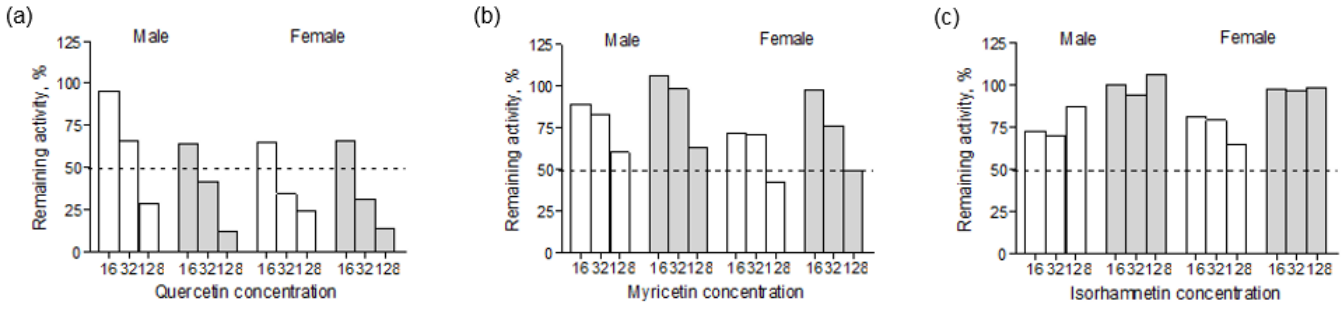
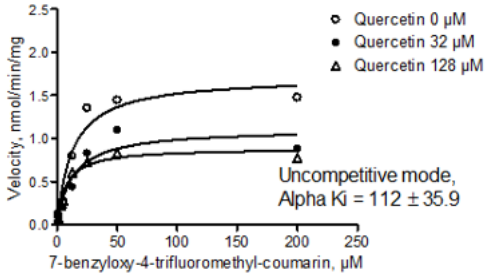


Figure 11. *In vitro* inhibition of O-dealkylation of BFC by quercetin (a), myricetin (b) and isorhamnetin (c) in human hepatic male and female microsomes containing CYP3A4. White bars indicate no pre-incubation and grey bars indicate 15 minutes pre-incubation.

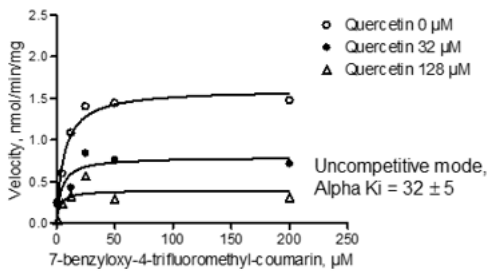
Quercetin acted as an uncompetitive inhibitor on human microsomal CYP3A4 activity in both genders and microsome pools (**Figure 12 a-d**). Myricetin exhibited a consistent non-competitive mode of inhibition between genders and microsome pools (**Figure 13 a-d**).

(a) **Male pool 1**



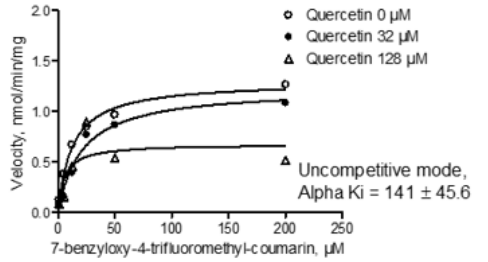
	Quercetin 0 µM	Quercetin 32 µM	Quercetin 128 µM
Michaelis-Menten Best-fit values			
VMAX	1.712	1.110	0.8869
KM	12.83	13.06	7.346
Std. Error			
VMAX	0.1852	0.1760	0.06215
KM	4.837	7.190	2.126

(b) **Male pool 2**



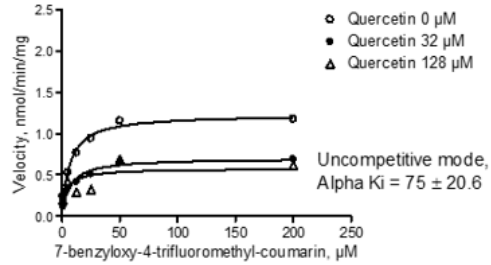
	Quercetin 0 µM	Quercetin 32 µM	Quercetin 128 µM
Michaelis-Menten Best-fit values			
VMAX	1.609	0.7905	0.3956
KM	6.464	4.251	3.041
Std. Error			
VMAX	0.08142	0.1176	0.08989
KM	1.406	3.941	3.795

(c) **Female pool 1**



	Quercetin 0 µM	Quercetin 32 µM	Quercetin 128 µM
Michaelis-Menten Best-fit values			
VMAX	1.294	1.229	0.6760
KM	12.72	21.28	5.736
Std. Error			
VMAX	0.04858	0.09809	0.1724
KM	1.669	5.152	6.575

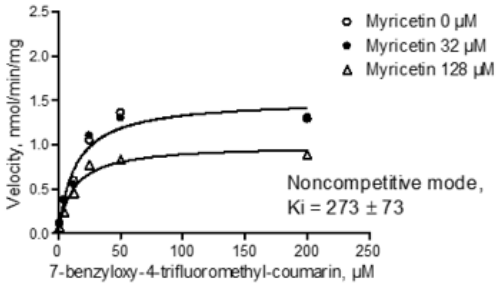
(d) **Female pool 2**



	Quercetin 0 µM	Quercetin 32 µM	Quercetin 128 µM
Michaelis-Menten Best-fit values			
VMAX	1.229	0.7020	0.5745
KM	6.581	6.837	3.969
Std. Error			
VMAX	0.05029	0.03584	0.1150
KM	1.150	1.474	3.965

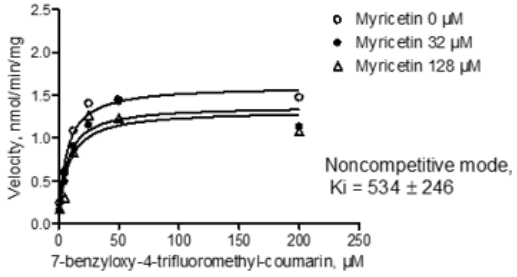
Figure 12. Saturation curve of BFC O-dealkylation in human hepatic male (a-b) and female (c-d) microsomes from two microsome pools, in the presence of quercetin.

(a) **Male pool 1**



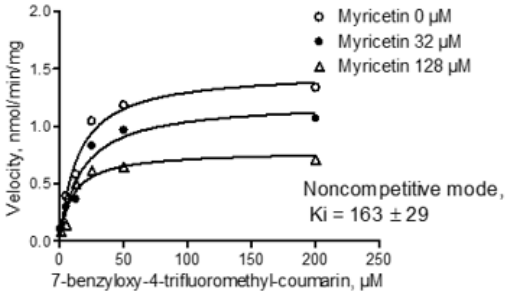
	Myricetin 0 µM	Myricetin 32 µM	Myricetin 128 µM
Michaelis-Menten Best-fit values			
VMAX	1.506	1.509	0.9965
KM	13.55	13.90	12.07
Std. Error			
VMAX	0.1402	0.1467	0.07377
KM	4.330	4.604	3.172

(b) **Male pool 2**



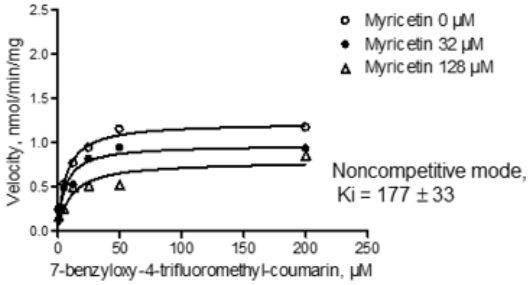
	Myricetin 0 µM	Myricetin 32 µM	Myricetin 128 µM
Michaelis-Menten Best-fit values			
VMAX	1.609	1.376	1.321
KM	6.464	6.718	7.696
Std. Error			
VMAX	0.08142	0.1385	0.1831
KM	1.406	2.872	4.340

(c) **Female pool 1**



	Myricetin 0 µM	Myricetin 32 µM	Myricetin 128 µM
Michaelis-Menten Best-fit values			
VMAX	1.475	1.211	0.7815
KM	14.12	16.75	10.03
Std. Error			
VMAX	0.08923	0.1213	0.07515
KM	2.896	5.427	3.616

(d) **Female pool 2**



	Myricetin 0 µM	Myricetin 32 µM	Myricetin 128 µM
Michaelis-Menten Best-fit values			
VMAX	1.229	0.9763	0.7894
KM	6.581	5.877	10.46
Std. Error			
VMAX	0.05029	0.07999	0.1043
KM	1.150	2.133	5.118

Figure 13. Saturation curve of BFC O-dealkylation in human hepatic male (a-b) and female (c-d) microsomes from two microsome pools, in the presence of myricetin.

4.3 Summary of results

The results from the kinetic measurements are summarised in **Table 2.**

Table 2. Results from inhibition assays of recombinant, male and female CYP2E1 and CYP3A4 from two pools respectively in the presence of flavonoids

Enzyme	Inhibitor	Enzyme pool	Sex	Mode of inhibition	K_i, μM
CYP2E1	Quercetin	Rec	-	Competitive	52.1 ± 6.31
		1	M	Non-competitive	779.3 ± 373.1
		2	M	Competitive	427 ± 286.3
		1	F	No inhibition	
		2	F	Competitive	314.2 ± 361.0
CYP3A4	Quercetin	Rec	-	Competitive	33.2 ± 3.5
		1	M	Uncompetitive	112 ± 35.9
		2	M	Uncompetitive	32 ± 5
		1	F	Uncompetitive	141 ± 45.6
		2	F	Uncompetitive	75 ± 20.6
CYP3A4	Myricetin	Rec	-	Non-competitive	74.6 ± 7.9
		1	M	Non-competitive	273 ± 73
		2	M	Non-competitive	534 ± 246
		1	F	Non-competitive	163 ± 29
		2	F	Non-competitive	177 ± 33
CYP3A4	Isorhamnetin	Rec	-	No inhibition	-
		1	M	No inhibition	-
		1	F	No inhibition	-

Rec = recombinant, M = male, F = female

5 Discussion

Modulation of CYP450 activity has a large impact on xenobiotic metabolism. Extensive research in the field of CYP450 regulation has successfully contributed to better predictions of food-drug interactions (FDI) although many challenges still remain. Knowledge about gender differences in drug metabolism is for instance limited. Flavonoids in our diet are appreciated for their positive health effects but may influence drug efficacy by modulating drug metabolising enzymes. The modulation may be gender-dependent, an aspect that should be taken into account when health care professionals inform a patient about medications and when establishing drug dose recommendations. The results of this study can contribute to understanding the implication of dietary flavonoids in drug interactions.

Gender differences in inhibition effects by quercetin on CYP2E1 activity has been identified in pigs *in vitro* (Ekstrand et al., 2015). Pig is a widely used animal model in human CYP450 studies because the metabolism and disease development in pigs are comparable to in humans. Therefore, similar gender differences were expected in this study. That study considered a 50 % or more decrease in catalytic activity compared to the control might be of physiological relevance. Accordingly, decrease in catalytic activity was regarded as inhibition if the activity was reduced by at least 50 % in at least one sample within the enzyme pool.

Because quercetin clearly inhibited recombinant CYP2E1, it was expected to have inhibitory effects on CYP2E1 activity in human microsomes. Surprisingly, no inhibition was observed at the substrate concentration of 200 µM. A theoretical explanation is that the microsomes contained other enzymes that metabolise PNP. That is however unlikely because PNP is a known selective CYP2E1 substrate (Cederbaum, 2014). Nevertheless, to test this hypothesis, PNP hydroxylation was investigated in human recombinant CYP3A4. Formation of P-nitrocatechol was not detected, confirming that CYP3A4 is not involved in PNP metabolism. Another explanation for the absence of inhibitory effects by quercetin on human microsomal CYP2E1 in this experimental set might be too high concentration of substrate used (200 µM). Indeed, quercetin inhibited recombinant CYP2E1 in a competitive manner, and this type of inhibition can be overcome by using high substrate concentrations. To verify this hypothesis, an experiment with lower substrate concentration (20 µM) was set up. In this case, inhibition was observed in the microsomes from male but not from female donors. This result is consistent with a similar inhibition study on pigs (Ekstrand et al., 2015). On the contrary, in microsome pool 2 the degree of inhibition was stronger in female than in male samples. The combined results including two microsome pools suggest inhibition in both genders but the

level of inhibition cannot be associated with gender due to the large variations between pool 1 and pool 2.

The competitive inhibition observed in male and female samples from pool 2 was in agreement with the theory that PNP has the ability to overpower the inhibitory effect of quercetin. However, because the K_i values were much higher than the highest inhibitor concentration used, the accuracy of inhibition mode determination is questionable. Increasing the inhibitor concentration was not considered since the results would be out of physiologically relevant levels. Most probably, quercetin is a weak human CYP2E1 inhibitor and clinically important *in vivo* effects are unlikely. The results from pool 1 further supports this idea, where K_i was the highest of all in male and no inhibition was detected in female.

CYP3A4 metabolises a broad spectrum of compounds and accounts for a large portion of xenobiotic metabolism, thus investigating effects of a number of dietary flavonoids is relevant. Gender differences in CYP3A activity have been observed in humans and specifically involving myricetin and isorhamnetin in pigs (Ekstrand et al., 2015), but prior to this study it has not been investigated in humans.

Two probe substrates, BR and BFC, were used to investigate O-dealkylation in recombinant CYP3A4. Because none of the flavonoids inhibited BR metabolism, it was not further investigated. Quercetin and myricetin inhibited BFC metabolism in human recombinant CYP3A4 as expected. Isorhamnetin had no inhibitory effects despite its similar structure to quercetin. It confirms the fact that the number and position of hydroxyl groups in the flavonol structure determines the inhibitory capacity.

Similarly to CYP2E1 inhibition, quercetin inhibited human recombinant CYP3A4 competitively. Myricetin demonstrated non-competitive inhibition, indicating binding to a different site of the enzyme compared to quercetin. The results imply that the substrate can overcome inhibition by quercetin but not by myricetin. For *in vivo* relevance, the non-competitive inhibitor myricetin would be a more potent inhibitor than the competitive inhibitor quercetin given that the drug (substrate) concentration is high enough. However, in this study quercetin was a competitive inhibitor only in recombinant CYP3A4 but not in human microsomes which resemble *in vivo* conditions better than recombinant enzyme.

Both quercetin and myricetin acted as reversible inhibitors in the present study, an irreversible mechanism would have a larger impact *in vivo* because the inhibition is stronger. Isorhamnetin had no inhibitory effect. Interestingly, isorhamnetin inhibited CYP3A BFC metabolism in male and female hepatic porcine microsomes (Ekstrand et al. 2015). A species difference in CYP3A4 regulation might explain the absence of inhibition by isorhamnetin in the present study. Another possibility is that the other member of the CYP3A family, CYP3A5, influenced BFC metabolism in the pig study and/or in this study. The exact isoform that is inhibited by isorhamnetin remains to be investigated.

According to the literature, flavonoids possessing many hydroxyl groups are more potent CYP450 inhibitors *in vitro* (Ho et al., 2001; Pang et al., 2012). In this case, quercetin was a stronger inhibitor than myricetin of both recombinant and microsomal CYP3A4 enzymes despite one less hydroxyl group. The result suggests that other factors influence differences in inhibition potency. Possibly, flavonoids interact differently depending on the choice of substrate and the specific CYP450 isoform. Inhibition by quercetin should be of greater importance *in vivo* considering it is more prevalent in food than myricetin.

The K_i values reveal some fluctuations in degree of inhibition between microsome pools. Inhibition by quercetin may seem to be stronger in male CYP3A4 whereas inhibition by myricetin was more pronounced in females indicated by lower K_i values in those samples. However, the variation was larger between the pools than between genders. Therefore, it cannot be concluded that quercetin and myricetin inhibit human CYP3A4 activity in a gender-dependent manner.

Taken together, this study confirmed that quercetin inhibits human CYP2E1 activity and quercetin and myricetin inhibit human CYP3A4 activity *in vitro*. In contrast to pigs, there was no clear pattern of gender related differences in humans. Genetic or lifestyle factors together with weak inhibition effects probably explain the observed variations in enzyme activity between the samples. Studies on gender-related inhibition mechanisms should therefore include enzyme pools from several origins. It is also possible that gender differences exist but cannot be detected in the present study due to the other factors influencing enzyme activity. In studies using animal models, individual characteristics and environmental and dietary factors (breed and feed) are strictly controlled. Such control cannot be achieved in human studies. Nevertheless, it may explain why gender differences were observed in pigs but not in humans. This aspect should be taken into account when applying results from animal studies to human CYP450 regulation.

FDI involving quercetin and myricetin are plausible considering CYP3A4 metabolises most drugs. However, the K_i values in the present study were in general higher than the maximum plasma concentration of bioactive compounds, only quercetin inhibited CYP3A4 at levels relevant *in vivo*. It should also be noted that *in vitro* models studying one mechanism should be extrapolated *in vivo* with caution. CYP450 activities are regulated at several levels in the body and influenced by both genetic and environmental factors. There may be overlaps in bioactivation and detoxification, CYP3A4 can for instance both activate and detoxicate aflatoxin (Buening et al., 1981). This complexity is also noticeable when the effects of flavonoids show contradictory results in short term versus long term exposure. Whether the investigated flavonoids have the ability to cause clinically relevant FDI must be investigated *in vivo*. *In vitro* experiments including the present study are useful as an initial approach to predict FDI.

It can be argued that use of relatively high concentrations of inhibitor (above the concentration found in circulation) might be regarded as a study limitation. However, Ki values (in contrast to IC50) are not dependent on experimental conditions, and provide relevant information on potency of the inhibitor independently on the concentration used in the study. Indeed, *in vitro* studies aimed to investigate inhibition mechanism commonly use inhibitor concentration higher than physiologically relevant to provoke the effect and elucidate the mechanism behind it. The second study limitation is a low number of microsomal pools included in the study. Only 2 pools from male and 2 from female donors were used. Even each of these pools consisted of microsomes from 20 or more individuals, taken into consideration between-individual variations, it is difficult to make generalizations about two large subpopulations (human females and males, each comprising about 5 billion individuals). Nevertheless, such studies, while preliminary and explorative, are of value and might give new insight into interpretation of existing data and suggest new avenues for future research.

6 Conclusions

This study suggests that (1) quercetin reversibly inhibits *in vitro* activity of human hepatic CYP2E1 and (2) quercetin and myricetin but not isorhamnetin reversibly inhibit *in vitro* activity of human hepatic CYP3A4. Inter-individual differences in enzyme activity in presence of those flavonoids are most likely due to other factors than gender.

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8. Appendix: Popular summary

What you eat may influence how your body deals with drugs

- and even depend on your gender!

The scientific evidence are clear, piling up your plate with vegetables every day is good for you and it prevent diseases. Why are the greens so healthy? Part of the answer could be: Flavonoids. These molecules are not essential nutrients but found in nearly all vegetables. They help keeping your cells healthy by for example using their antioxidative power to scavenge evil free radicals. What is less desirable about flavonoids is that they can interact with drug metabolising enzymes, so called cytochrome P450 enzymes. A well known example is grapefruit juice which contains flavonoids that have been shown to inhibit cytochrome P450 enzyme activity. An inhibited enzyme is unable to break down drugs or other foreign compounds properly. The consequence may be that the drug loses its effect or in the worst case, causes adverse effects.

I have studied this so called food-drug interaction a bit deeper and found some unexpected results. I investigated inhibition of two cytochrome P450 isoforms: CYP2E1 and CYP3A4. They are both highly involved in the breakdown of foreign compounds in the liver. In pigs, it was previously shown that CYP2E1 and CYP3A4 activity were inhibited by flavonoids differently in males compared to females. Since pigs are commonly used as an animal model for studying human CYP450 regulation, I was eager to investigate if similar gender-related differences exist also in humans.

The experiment was executed *in vitro* (in test tubes) where liver enzymes deriving from male and female donors were mixed with a flavonoid. Three flavonoids were investigated for their inhibitory effects: quercetin, myricetin and isorhamnetin. Quercetin is one of the most common flavonoids in our diet, myricetin and isorhamnetin are less present but contribute to the overall flavonoid intake. Apples, onions, tea and red wine account for a large portion of our dietary sources of flavonoids.

To my surprise, no gender differences in the degree of enzyme inhibition by flavonoids were seen. Instead the inhibition differed greatly between donor pools within the same gender, suggesting that currently unknown factor beyond sex affect the inhibition. Thus, drug metabolism is regulated differently in humans compared to in pigs and there must be other factors responsible for the inter-individual differences in enzyme activity, for instance genetic profile, age or health status of the donors.

Inhibition of CYP2E1 and CYP3A4 was observed in both genders in the presence of quercetin and myricetin but not isorhamnetin. A big question: will the quercetin and myricetin you eat impede your ability to break down drugs? According to the results from my study, probably not, because the inhibition was weak and you would need to eat unrealistic amounts of quercetin and myricetin. On the other hand, food contains lots of other flavonoids that may also inhibit drug metabolising enzymes. If you are on medication and take additional herbal supplements the flavonoid intake will be considerably higher and thereby a food-drug interaction may occur. However, food-drug interactions depend on several other factors, therefore a conclusion regarding the effect of quercetin and myricetin in living persons cannot be drawn from the results of my research. But it gives a hint of potential food-drug interactions that should be further investigated in clinical studies.