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Sammanfattning

Blåbär (*Vaccinium myrtillus*) har ett högt innehåll av fenoler (flavonoider och fenolsyror). Vissa av dessa fenoler har visats metaboliseras i levern av cytokrom P450, ett enzymatiskt system vilket även är har en huvudsaklig roll gällande metaboliseringen av läkemedel. Det har rapporterats att ett flertal flavonoler (en flavonoid subgrupp), så som quercetin, myricetin, isorhamnetin och rutin har interagerat med CYP450, t.ex. via inhibering. Inhibering av CYP450 kan antingen öka eller minska effekten av läkemedel. Därav bör interaktioner mellan blåbär och metabolisering av läkemedel närmare studeras. Skillnader i metabolism mellan män och kvinnor bör också studeras i.o.m. att CYP450 gener uttrycks olika hos män och kvinnor. Mikrosomer från levern är användbara i *in vitro*-modeller, vilka ofta tillämpas i ett tidigt skede av läkemedelsutvecklingsprocessen, då för att identifiera potentiella interaktioner mellan läkemedel och livsmedel samt mellan läkemedel och läkemedel. På grund av likheterna mellan gris och människa med avseende på CYP450 i lever samt på grund av den stora tillgängligheten av grislever via slakterier, har mikrosomer från gris blivit en attraktiv model i biokemiska studier av könsrelaterade skillnader i livsmedels-läkemedelsinteraktioner.

Målet för denna studie var att utvärdera om och hur blåbär och fenoliska förningar i blåbär inhiberar CYP450 isoform 1A och 3A. Fem flavonoler från blåbär valdes utefter deras potential att interagera med CYP450. I dagsläget, har inga studier andra studier påträffats avseende blåbär och de valda komponenternas inhiberande effekt på CY1A och CYP3A. Fyra fenolsyror som finns i blåbär valdes också ut för att studeras p.g.a. deras antioxidativa kapaciteter (som i mycket liknar flavonolernas). Välkända och specifika probe substrat användes tillsammans med mikrosomer från svin (entire male and female pigs). Kinetiska mätningar utfördes för att utreda inhiberingsmekanism. Potentiella skillnader mellan könen utvärderades.

CYP3A aktivitet inhiberades av myricetin; icke-kompetitivt hos hanar (Ki=112.7) och inte alls hos honor, av isorhamnetin; kompetitivt hos hanar (Ki=71.2) och icke-kompetitivt hos honor (Ki=93.7). CYP1A aktivitet inhiberades av myricetin; kompetitivt hos hanar (Ki=2.5) och hos honor (Ki=4.6), av isorhamnetin; kompetitivt hos hanar (Ki=99.9) och hos honor (Ki=10.8), av quercetin; kompetitvt hos hanar (Ki=0.2) och irreversibelt hos honor (IC_{50}=1.3).

Resultaten ifrån denna studie indikerar att aktiviteten av CYP450 1A och 3A inhiberas av vissa komponenter från blåbär och att detta är en effekt som kan ha könsrelaterade skillnader.

Nyckelord: Cytokrom P450, CYP1A, CYP3A, Blåbär (Vaccinium myrtillus), Läkemedelsinteraktioner, Myricetin, Isorhamnetin, Quercetin

Abstract

Bilberry fruit (*Vaccinium myrtillus*) is a rich source of bioactive compounds, including flavonols and phenolic acids. At least some of these compounds were shown to be metabolized in the liver by cytochrome P450 (CYP450) enzyme system, which is also important in the metabolism of pharmaceuticals. Several flavonols (a flavonoid subgroup) such as quercetin, myricetin, isorhamnetin and rutin have been reported to integrate with CYP450, i.e. by inhibition. Thus, a possibility of bilberry interaction with pharmaceutical metabolism should be carefully studied. Gender-related differences of such interactions should also be considered given that CYP450 genes in male and female are differentially expressed. Hepatic microsomes are useful in vitro model commonly used at early stages of drug development to identify potential food-drug or drug-drug interactions. Pig is increasingly used as an animal model for human biomedical studies. The comparability of hepatic CYP450 between pigs and humans as well as easy availability of porcine livers from abattoirs make porcine microsomes an attractive model to study gender-related differences in food-drug interactions.

The aim of this study was to evaluate the inhibitory effect of selected phenolic compounds from bilberry fruit on the activities of CYP1A and CYP3A in porcine hepatic microsomes. Five flavonols and four phenolic acids were selected for the study. This selection was based on two criteria, abundance in bilberry and potential interaction with CYP450. To estimate CYP1A and CYP3A activities, the probe substrates 7-ethoxyresorutin and 7-benzyloxy-4-triflouromethylcoumarin (BFC) were used, respectively.

This study demonstrated that CYP3A activity was non-competitively inhibited by myricetin in male pigs only (Ki=112.7) and-, competitively by isorhamnetin in both male (Ki=71.2) and female pigs (Ki=93.7). CYP1A activity was competitively inhibited by myricetin in male (Ki=2.5) and female pigs (Ki=4.6) and by isorhamnetin in male (Ki=99.9) and in female pigs (Ki=10.8). Inhibition mode of CYP1A by quercetin differed between genders being competitive in male (Ki=0.2) and irreversible in female pigs (IC_{50}=1.3).

In conclusion, this study suggests that the activity of hepatic CYP450 1A and 3A are inhibited by the flavonols myricetin, isorhamnetin and querctin. Moreover, this study provides first evidence of gender-related differences in this inhibition.

Keywords: *Cytochrome P450, CYP1A, CYP3A, Bilberry (Vaccinium myrtillus), Food-drug interactions, Myricetin, Isorhamnetin, Quercetin*

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Abbreviations

BBJ	Blueberry juice
BFC	7-Benzyloxy-4-Triflouromethylcoumarin
BROD	Benzyloxyresorufin O-Debenzylase
BUS	Buspiron
CYP1A	Cytochrome P450 1A
CYP3A	Cytochrome P450 3A
CYP450	Cytochrome P450
DDI	Drug-Drug Interactions
EROD	7-Ethoxyresorufin *O*-Deethylase
ES	Enzyme-substrate
FDI	Food-Drug Interactions
HFC	7-Hydroxy-4-Triflouromethylcoumarin
IBS	Irritable bowel syndrome
MROD	Methoxyresorufin O-Demethylation
NIP	Nifedipine
PHEN	Phenacetin
TST	Testosterone 6β-hydroxylation
ZAT	Zatosetron

1 Introduction

1.1 Background

Bilberry fruit (*Vaccinium myrtillus*) is traditionally used both as a food and as a folk medicine (Sjörs, 1989). As a food, bilberry fruit is eaten fresh, dried or processed into jams, pie fillings, liqueurs, wine and may serve as a preservative (Small & Catling, 1999, 170- 176). Due to its high phenolic content, bilberry extract is extensively used as an ingredient in supplements and natural medicines (Yamamoto *et al.*, 2013). Bilberry extract contains several bioactive flavonoids (including flavonols) and phenolic acids, with different bioactivities that may have implications for health, such as antioxidative effects (Kahkonen *et al.*, 2001). A few flavonoids exert cancer chemopreventive effects and are thus used in cancer chemotherapy (Yang *et al.*, 2001; Shih *et al.*, 2000).

In folk medicine, bilberry was used to treat inflammations in mouth and trout, mild diarrhea, to improve night vision and to lessen bleeding before surgery (Small & Catling, 1999, 172-73). Beliefs of bilberry as a medical crop is partly supported by *in vitro* findings (Biedermann *et al.*, 2013; Master *et al.*, 2012; Triebel *et al.*, 2012; Basu *et al.*, 2010; Karlsen *et al.*, 2010). However, scientific evidence for that is limited (Canter & Ernst, 2004; Conquer *et al.*, 1998).

Potential health benefits of bilberry might be compromised by the presence of compounds which can interfere with drug metabolism and detoxification, leading to so called food-drug interactions, FDI (Thummel & Wilkinson, 1998). FDI may occur during intestinal absorption, hepatic metabolism and excretion (Guengerich & Shimada, 1998). Partly, FDI occur through induction or inhibition of cytochrome P450 (CYP450), which is responsible for the biotransformation of a majority of drugs (Guengerich & Shimada, 1998). For example, components from grapefruit (Ho *et al.*, 2001), St. Johnś wort (Obach, 2000)and blueberry juice (Hanley *et al.*, 2013) inhibit the activity of CYP450 and, depending on substrate (drug), may either increase or decrease its effect (Moon *et al.*, 2006). It is believed that quercetin, myricetin, rutin (Ho *et al.*, 2001) and isorhamnetin interact with CYP450 (Kimura *et al.*, 2010). The nature of these interactions is not yet well documented and very little in known how gender affects FDI (Dresser *et al.*, 2000). It is well known that CYP450 genes are differentially expressed in males and females (Rasmussen *et al.*, 2011) and, CYP450 activity is also gender related (Skaanild & Friis, 1999)

Liver microsomes contain the full range of CYP450 and thus, are a useful model for *in vitro* studies of CYP450 activity (Bambal & Clarke, 2010,70). Human microsomes have been extensively studied but, due to a limited supply and high costs,

animal models are being frequently used (Skaanild, 2006). The porcine (pig/mini-pig) CYP450 enzyme system and drug metabolism is similar to human, suggesting that porcine microsomes can be an attractive model to study food-drug interactions (Skaanild, 2006). Data from *in vitro* studies are often used in early stages of drug development, to screen a compound against potential FDI and DDI and, provide information for designing possible future clinical studies (Ito *et al.*, 2004).

In the present study, bilberry fruit (*Vaccnium myrtillus*), five flavonols (quercetin, rutin, myricetin isorhamnetin) and three phenolic acids (p-coumaric acid, caffeic acid and gallic acid) found in bilberry fruit were investigated for their potential to inhibit CYP450 activity *in vitro*. Both reversible and irreversible inhibitions were examined. The present study also evaluated the possibility of gender-related differences in CYP450 activity.

1.2 Objective

The overall objective of this study was to evaluate the inhibitory potential of flavonols and phenolic acids from bilberry fruit on porcine CYP450 activity.

Specific aims of the study were:

(1) To investigate *in vitro* effect of rutin, myricetin, quercetin, isorhamnetin, p-coumaric acid, gallic acid and caffeic acid on CYP1A and CYP3A activities in porcine liver microsomes.
(2) To investigate gender-related differences in response of CYP1A and CYP3A to these phenolic compounds.

2 Literature review

2.1 Content of phenolic compounds bilberry fruit (*Vaccinium myrtillus*)

The content of phenolic compounds in bilberry fruit (*V. myrtillus*) is mainly represented by flavonoids and phenolic acids (Häkkinen *et al.,* 1999).

Flavonoids are characterized by antioxidative-, (Kähkönen *et al.,* 2001; Kähkönen & Heinonen, 2003), enzyme inhibition-, chelating- and reduction activities (Di Carlo *et al.,* 1999). Many flavonoids are well distributed in most plant foods, where they are present in glycosylated forms (Mikulik-Petkovesk, 2012). In the human diet, berries have been suggested to be the most concentrated source of glycosylated flavonols (Mikulik-Petkovesk, 2012). Flavonols are one of several flavonoid subgroups which, in bilberry fruit are represented e.g. by quercetin and myricetin and their glycosylates, isorhamnetin (Mikulik-Petkovesk, 2012) which is the 3´-methylated form of quercetin (Breinholt *et al.,* 2002) and, rutin, which is a quercetin glycosylate (Moze *et al.,* 2011).

Phenolic acids exert similar effects to flavonoids, (Hakkinen *et al.,* 1999; Yang *et al.,* 2001) and are represented in bilberry fruit e.g. by *p*-coumaric acid and caffeic acid, which are derivates of hydroxycinnamic acid and gallic acid, a derivate of hydroxbenzoic acid (Häkkinen *et al.,* 1999). The content and concentration of phenolic compounds in bilberry fruit vary, depending on several factors, e.g. geographic location (Åkerström *et al.,* 2010) and season (Moze *et al.,* 2011).

Recent findings suggested that flavonoids such as flavonols may be responsible for several health beneficial effects such as, antiallgeric (quercetin), antiatherogenic (quercetin), anticancer (quercetin, rutin), antidiabetic (quercetin), antidiarrhoeal (quercetin, myricetin), antihepatotoxic (quercetin), anti-inflammatory (quercetin, myricetin), antipsasmotic (quercetin) and antiulcer activity (quercetin, rutin) (Di Carlo *et al.,* 1999). Flavonoids are generally accepted as health beneficial and thus, individual flavonoids as well as flavonoid rich bilberry extract have become attractive ingredients in dietary supplements, functional foods and herbal medicine, which are easily available, e.g. in supermarkets (Yamamoto *et al.,* 2013; Conquer *et al.,* 1998; Kalogeromitros *et al.,* 2008). In U.S many of these products are accompanied with some kind of medical claim (Kalogeromitros *et al.,* 2008). The regulations and demands of clinical studies are not as rigorous as for other conventional drugs (Dresser *et al.,* 2000).

2.2 Food-drug interaction (FDI)

The increased consumption of supplements, herbal medicines and functional foods has evoked awareness of food/herb-drug interactions (Hansen & Nilsen, 2008). Food-drug interactions (FDI) occur when components from the food by some means interact with drug metabolism and modify both drug bioavailability and drug effect (Guengerich & Shimada, 1998). FDI may take place during intestinal absorption, hepatic metabolism and excretion (Guengerich & Shimada, 1998). The present study focused on hepatic metabolism, as a site of drug biotransformation. Some commonly used herbs and fruits can cause serious alterations in human health if consumed simultaneously with certain drugs (Conney, 2003). Grapefruit (Ewards *et al.,* 1996; Ho e*t al.,* 2001; Girennavar *et al.,* 2005), St. John´s wort (Obach *et al.,* 2000; Hansen & Nilsen, 2008(Hansen & Nilsen, 2008), ginkgo biloba, (von Moltke *et al.,* 2004), chicory root (Rasmussen *et al.,* 2012) and blueberry juice (Hanley *et al.,* 2012) are foods that have recently been shown to interact with drug metabolism. The active components and the mechanisms are though not yet fully understood (Thummel & Wilkonson, 1998).

2.2.1 Biotransformation of drugs in the liver

Hepatic biotransformation of drugs (activation/deactivation) determines the bioavailability and thus the effect of the drug (Guengerish & Shimada, 1998). In the liver, cytochrome P450 (CYP450) is responsible for a majority of drug biotransformations (Guengerish & Shimada, 1998). Phenacetin (Bourrié *et al.,* 1996), nifedipine (Anzenbacher *et al.,* 1997), zatosetron (Ring *et al.,* 1994) and testosterone (Anzenbacher *et al.,* 1997) are a few examples of drugs that are biotransformed in the human liver. Usually, biotransformation is divided into two phases, of which phase 1 includes CYP450 oxidation, resulting in increased solubility (polarity) of the drug and provision of sites for conjugation with polar units e.g. glucuronate or sulfate (Thummel & Wilkonson, 1989). The following phase II metabolism (conjugation) is then carried out by other enzymes to further increase the solubility of the xenobiotic and/or endogenous metabolites and facilitate their excretion from the body (Preusse & Skaanild, 2011, 148).

2.3 Cytochrome P450

Cytochrome P450 (CYP450) is a superfamily of hemoprotein containing mixed function monooxygenases, which are responsible for phase I biotransformation of drugs and other xenobiotic compounds such as, environmental pollutants and, endogenous substances such as steroids and fatty acids (Conney, 2003). In human, approximately 55 isoforms of CYP450 are known, of which only some (belonging to CYP450 family 1, 2 and 3) are relevant for drug metabolism (Thummel & Wilkinson, 1998).

2.3.1 CYP450 isoform 1A

Cytochrome P450 subfamily 1A (CYP1A) in human, consists of two major isoforms, 1A1 and 1A2 (Venkatakrishnan *et al.,* 2001). CYP1A1 is not significantly expressed in the human liver, but was found in the intestine and lungs (Venkatakrishnan *et al.,* 2001). CYP1A2 is the major CYP1 gene family product, present in hepatic microsomes (Moon *et al.,* 2005), representing approximately 13% of the total amount of CYP450 in the human liver (Bambal & Clarke, 2010, 60).

CYP1Aś are involved in biotransformation (activation and deactivation) of many procarcinogens such as aromatic heterocyclic amines and mycotoxins (Macé *et al.,* 1997), CYP1A´s are popular targets in research on cancer therapy and prevention (Conney, 2003). A high CYP1A1 expression in human is associated with increased risk of lung cancer in smokers, probably due to an increased activation of procarcinogens from the cigarette smoke (Venkatakrishnan *et al.,* 2001), i.e. polycyclic aromatic hydrocarbons (PAH´s) such as benso(a)pyrene (Schwartz *et al.,* 2005). Both CYP1A isoforms are involved in the metabolism of the estrogen 17β-estradiol (E_2) (Takemura *et al.,* 2010).

CYP1A2 metabolizes caffeine, theophylline, imipramine, acetaminophen, carcinogenic aromatic and heterocyclic amines (Bambal & Clarke, 2010, 66) and, is often subject of studies on cancer chemoprevention (Shih *et al.,* 2000; Conney, 2003). Cancer chemopreventive agents, e.g. quercetin are found in many plant foods (Shih *et al.,* 2000). It has been pointed out that components which acts as chemopreventive agents in one experimental setting, may have the opposite (hazardous) effect in another setting , thus the cancer chemopreventive regimen should also be tailored to the individuals where the pathways of carcinogenesis is well known (Conney, 2003).

2.3.2 CYP450 family 3A

The cytochrome P450 subfamily 3A (CYP3A) in human consists of three isoforms, namely CYP3A3, 3A4 and 3A5 (Thummel & Wilkinson, 1998). CYP3A4 is the quantitatively most important in human, representing approximately 30% of the total amount of CYP450 in human liver (Thummel & Wilkonson, 1998). CYP3A3 shares over 98% cDNA homology with CYP3A4 and it is not yet established whether 3A3 is an own gene product or an allelic variation of 3A4 and, the two isoforms are thus collectively named CYP3A4 (Thummel & Wilkinson, 1998). CYP3A5 is only found in 10-30% of the hepatic samples (in levels of 10-30% of CYP3A4 levels) and, is structurally distinct from CYP3A4 (Thummel & Wilkinson, 1998).

Approximately 50% the oxidative metabolism of drugs in the liver is mediated by CYP3A4 (Bambal & Clarke, 2010, 60), representing increased risk for drug interactions (Zuber *et al.,* 2002). CYP3A may also chemically activate or deactivate carcinogens in the liver (Dresser, 2000).

2.4 Microsomal *in vitro* studies of CYP450

2.4.1 Model organisms for *in vitro* studies of CYP450 activity

One of the first CYP450 being isolated and characterized were CYP1A2 in rat, (Zuber *et al.,* 2002). Later it was found that CYP1A2 is conserved throughout species, including human (Zuber *et al.,* 2002). However, there are many substrates, which are metabolized in humans and rats by different isoforms, which makes rat a less suitable model for human studies (Zuber *et al.,* 2002).

Pig is a good model for human CYP1A (Zuber *et al.,* 2002) and CYP3A (Zuber *et al.,* 2002; Soucek *et al.,* 2001; Skaanild & Friis, 1999; Monshouer *et al.,* 1998; Anzenbacher *et al.,* 1997) and, are often used in preclinical pharmacological and toxicological studies, because of its many similarities (both physiological and metabolic) with human (Suenderhauf & Parrott, 2013).

Compared to human liver microsomes, where CYP1A1 isoform is not expressed, conflicting data have been published on the presence of CYP1A1 in the pig liver (Achour *et al.,* 2011). In many studies, CYP1A1 mRNA was detected in the liver of

Meishan and Landrace pig (Kojima & Degawa, 2013; Messina *et al.,* 2011), although other reported only the presence of CYP1A2 (Achour *et al.,* 2011). Activity of 7-ethoxyresorufin-O-deethylase (EROD) is commonly used as a probe reaction to estimate CYP1A activity. Both the expression of CYP1A and EROD activity differed between male and female pigs (Skaanild & Friis, 1999). In female pigs, CYP1A activity was higher in female compared to male (Rasmussen *et al.,* 2012).

In pigs, the isoforms CYP3A29 and CYP3A39, representing 14% of total CYP450 content, are homologous to human CYP3A4/5 (Anchour *et al.,* 2011). In porcine thus, the amount of hepatic CYP3A is less than in human (**Table 1**).

Table 1. Percentage of the most important drug metabolizing enzymes present in human liver relative to their homologues in porcine liver microsomes and amount of drug metabolism in human liver.

	Most relevant drug metabolizing CYP450 isoforms									
	3A	**1A2**	**2A6**	**2B6**	**2C8**	**2C9**	**2C19**	**2D6**	**2E1**	**3A5**
Total, %										
Human:	**34[1]**	**13[1]**	5[1]	3[1]	6[1]	17[1]	0.3[1]	2[1]	15[1]	2[1]
Porcine:	**14[2]**		34[2]			16[2]		26[2]		
Drug metabolism (%)										
Human:	**50[1]**	**Less[1]**			15[1]		25[1]	Less[1]		

[1] Bambal & Clarke, 2010, 60, [2] Achour *et al.,* 2011

2.4.2 CYP450 regulation

The mechanism of CYP450 regulation is well studied in human (Venkatakrishnan *et al.,* 2001); however, less data on porcine CYP450 is available (Preusse & Skaanild, 2012) . Regulation of CYP450 expression depends on various factors and their combination, including the presence of substrate, genetic background, hormonal status, age, gender etc. (Tsyrlov *et al.,* 1994; Hodek *et al.,* 2002; Rasmussen *et al.,* 2011; Zamaratskaia *et al.,* 2005; Brunius *et al.,* 2012; Zamaratskaia *et al.,* 2011 Zamaratskaia *et al.,* 2012). The mechanisms of this regulation involve orphan nuclear receptors, the major transcriptional factor of CYP450 expression regulation (Wang & LeCluyse, 2003). Some CYP450 are regulated on a post-transcriptional level (Brunius *et al.,* 2012). Additionally, CYP450 activity can be directly regulated

by the presence of inhibitors, for example bioactive compounds in the diet (Hodek *et al.*, 2007).

Both breed- and gender-related differences in CYP450 activity have been demonstrated in porcine microsomes (Kojima & Degawa, 2013; Rasmussen *et al.*, 2012; Rasmussen *et al.*, 2011; Skaanild & Friis, 1999). Even though the total amount of CYP450 did not differ between male and female pigs, the expression and catalytic rate of CYP1A-mediated EROD and CYP3A-mediated testosterone 6β-hydroxylation differed between male and female pigs (Skaanild & Friis, 1999). The activity of CYP1A2 was observed to be higher in female compared to male pigs (Skaanild & Friis, 1999). The same study also demonstrated that the gene expression and protein concentration of CYP1A2 were higher in female than in male pigs (Skaanild & Friis, 1999).

2.4.3 CYP450 catalytic activity

Most CYP450 isoforms in liver microsomes metabolize a variety of substrates and thus participates in different catalytic activities (Guengerich, 1997). The catalytic activities of individual CYP450 isoforms are commonly assessed using probe (or marker) substrate(s) (Venkatakrishnan *et al.*, 2001). A probe substrate is a molecule which is metabolized by one specific isoform and thus, metabolism of a probe substrate can be used to estimate activity of a specific isoform (Garcia-Serna & Mestres, 2011). Catalytic activities of individual CYP450 enzymes depends on various factors and vary between individuals (Bourrié *et al.*, 1996), between species (Zamaratskaia *et al.*, 2012) and between genders within the same species (Rasmussen *et al.*, 2011).

CYP1A

In human, CYP1A activity is usually estimated as a rate of ethoxyresorufin *O*-deethylation (EROD) (Anzenbacher *et al.*, 1997; Guengerich, 1997). EROD activity was detected in the microsomes from minipig, although obtained values were somewhat lower than in the microsomes from human (Anzenbacher *et al.*, 1997). EROD activity was also detected in conventional pigs, however, it was suggested that EROD might not accurately reflect CYP1A1 activity, as it is probably metabolized by two isoforms (Zamaratskaia *et al.*, 2011; Zamaratskaia & Zlabek, 2009). Porcine CYP1A activity may also be estimated as a rate of methoxyresorufin *O*-demethylation (MROD) (Zamaratskaia & Zlabek, 2009). Phenacetin is metabolized to paracetamol (a mild analgestic) in humans and is a typical probe substrate for human CYP1A (Bourrié *et al.*, 1996). The catalytic activities of CYP1A-mediated EROD,

MROD and phenacetin O-deethylation in human and porcine, is illustrated in **Table 2**.

Table 2. The catalytic activities of CYP1A in human (H) and porcine (P) microsomes are estimated using different common substrates. Results are presented in units used in the original references.

Microsomal CYP1A			
Substrate	**Reaction**	**Specie**	**Catalytic activity**
Ethoxyresorufin	O- deethylation	H	190 pmol/min/nmol [1]
	O- deethylation	P	27.3 pmol/min/nmol [1]
	O- deethylation	H	851 pmol/min/mg [2]
	O-deethylation	H	70 pmol/min/mg [4]
	O-deethylation	H	300 pmol/min/mg [4]
Mehthoxyresorufin	O-demethylation	P	0.8 pmol/min/mg [3]
Phenacetin	O-deethylation	H	6800 pmol/min/mg [4]

[1]Anzenbacher *et al.*, 1997, [2]Zlabek & Zamaratzkaia, 2012, [3]Zamaratskaia & Zlabek, 2009, [4]Bourrié *et al.*, 1996

CYP3A

BFC (7-benzyloxy-4-trifluoromethylcoumarin) O-debenzylation to HFC (7-hydroxy-4-triflouromethylcoumarin) is mediated by CYP3A in both human (Hansen & Nilsen, 2008) and porcine microsomes (Tomankova *et al.*, 2012; Zamaratskaia *et al.*, 2012). In pigs, BFC is used as a probe substrate for CYP3A activity (Zamaratskaia *et al*., 2012; Tomankova *et al.*, 2012). However, it is still not known if CYP3A29 and CYP3A39 are the main isoforms mediating BFC metabolism. In pigs, CYP3A differed between Landrace and Duroc pigs (Zamaratskaia *et al.*, 2012). Zatosetron (Ring *et al.*, 1994) and nifedipine (Anzenbacher *et al.*, 1997) are both metabolized by CYP3A in human. The catalytic rate at which nifedipine is metabolized in human is almost identical to minipig (Anzenbacher *et al.*, 1997). Testosterone 6β-hydroxylation is catalyzed in human and porcine microsomes by CYP3A in a similar manner, suggesting that testosterone can be used as a probe substrate to estimate both human and porcine CYP3A activity (Anzenbacher *et al.*, 1997). Catalytic rates of CYP3A-mediated BFC, nifedipine, zatosetrone and testosterone metabolism in human and porcine, is presented in **Table 3**.

Table 3. Common substrates to estimate microsomal CYP3A catalytic activities are estimated using common substrates, such as BFC (7-benzyloxy-4-trifluoromethylcouarin), nifedipine, zatosetron and testosterone, in human (H) and porcine (P) microsomes. Results are presented in units used in the original references.

P-Lan= porcine landrace, **P-Dur**= porcine Duroc=, **P-cross**= porcine cross-breed, **Pmini**= porcine minipig.

Microsomal CYP3A			
Substrate	***Reaction***	***Species***	***Catalytic activity***
BFC	O-debenzylation	P-cross	35.1 pmol/min/mg product[1]
	O-debenzylation	P-Lan	334 pmol/min/mg[2]
	O-debenzylation	P-Dur	275 pmol/min/mg[2]
Nifedipine	Oxidation	H	2205 pmol/product/min/nmol[3]
	Oxidation	Pmini	2193 pmol/product/min/nmol[3]
Zatosetron	N-oxidation	H	1790 pmol product/min/mg protein[4]
Testosterone	6β-hydroxylation	H	1340 pmol/product/min/nmol[3]
	6β-hydroxylation	P	1330 pmol/product/min/nmol[3]

[1]Tomankova *et al.*, 2012, [2]Zamaratskaia *et al.*, 2012, [3]Anzenbacher *et al.*, 1997, [4] Ring *et al.*, 1994

2.5 Inhibition of CYP450

There are several mechanisms of inhibition and, some inhibitors may demonstrate more than one type of inhibition (Thummel & Wilkinson, 1998). Generally, *in vitro* studies, of CYP450 are carried out using a probe substrate to measure and quantify the inhibition over a range of substrate and inhibitor concentrations (Thummel & Wilkonson, 1998). Most CYP450 follow Michaelis-Menten kinetics, showing a hyperbolic curve when the initial velocity, V_0 is studied as a function of product formation (Berg *et al.*, 2007, 216-228; Thummel & Wilkinson, 1998). To investigate the reversibility of inhibition, a pre-incubation of microsomes with inhibitor before addition of a probe substrate is usually used (Thummell & Wilkinson, 1998). An increase of inhibition degree after inclusion of a pre-incubation step, suggests irreversibility, meaning that the enzyme-substrate complex does not dissociate, whereas the degree of reversible inhibition is not affected by pre-incubation and then, the enzyme and substrate complex dissociates rapidly (Thummel & Wilkinson, 1998).

Due to a huge number of reactions catalyzed by CYP1A and CYP3A, inhibition of these isoforms by dietary components may result in alterations in essential physiological processes (Bushra *et al.,* 2011). This underlines the importance of dietary components and hepatic metabolism (Bushra *et al.,* 2011).

2.5.1 Irreversible inhibition

Irreversible inhibition is also called mechanism based or suicide inactivation, and causes permanent reduction in enzyme activity (Thummel & Wilkinson, 1998). The concentration of the inhibitor, needed to decrease the activity of the enzyme by 50% (IC50) is used as a characteristic measure, enabling comparisons between different inhibitors (Thummel & Wilkinson, 1998).

Examples of irreversible inhibitors found in food is e.g. the grapefruit constituent 6´,7´-dihydroxybergamottin (furanocoumarin) which markedly inhibits metabolism of CYP3A substrates, both *in vitro* and *in vivo* (Edwards *et al.,* 1996; Thummel & Wilkinson, 1998). Grapefruit juice itself which is a well-known irreversible inhibitor of CYP3A activity (Seden *et al.* 2010; Hanley *et al.,* 2012). Several steroids also inhibited (inactivated) CYP3A activity in human, irreversibly (Thummel & Wilkinson, 1998) and in pig (Rasmussen *et al.,* 2011; Zamaratskaia *et al.,* 2012).

2.5.2 Reversible inhibition

If the degree of inhibition is unaffected by inclusion of a pre-incubation step, then the inhibition is considered to be reversible and the mode (mechanism) of inhibition may be further determined (competitive-, uncompetitive-, noncompetitive or mixed) using several substrate concentrations (Thummel & Wilkinson, 1998).

Competitive inhibitors have a higher affinity to the enzyme than to the enzyme-substrate complex and, as more substrate is added, the inhibition will be overcome, why these inhibitors will be recognized by increased KM (substrate concentration that yield a half-maximal velocity) and unchanged Vmax (maximum velocity) (Berg *et al.,* 2007, 216-228).

Non-competitive inhibitors can bind to the enzyme simultaneously as the enzyme as the substrate, but at different sites, and are being recognized by a decreased Vmax and unchanged KM (Berg *et al.,* 2007, 216-228).

Uncompetitive inhibitors have higher affinity to the enzyme-substrate complex than to the enzyme (Berg *et al.,* 2007, 216-228). Some typical chemical inhibitors of CYP1A and CYP3A are listed in **Table 4**.

The inhibition constant is also called dissociation constant (Ki) and is the binding affinity of the reversible inhibitor, indicating the potency of the inhibitor (Berg *et*

al., 2007, 226). The most potent reversible inhibitors have Ki-values under 1µM (Thummel & Wilkinson, 1998). Examples of potent reversible inhibitors include azole antifungal agents (e.g. ketoconazole) and HIV protease inhibitors (Thummel & Wilkinson, 1998). Reversible inhibition by blueberry juice (BBJ) was recently demonstrated, which inhibited CYP3A triazolam- (sedative-hypnotics) and buspirone (anti-anxiety medicine) metabolism in human liver microsomes *in vitro* (Hanley *et al.*, 2012). Pre-incubation did not substantially enhance the degree of inhibition, indicating a reversible mode of inhibition (Hanley *et al.*, 2012). The same blueberry juice, in a human intervention, changed the metabolic pattern of major drug metabolizing enzymes (CYP450) in the liver (Hanley *et al*., 2012). The active components in the blueberry juice were never examined (Hanley *et al.*, 2012).

The concentration of the inhibitor [I] relative to its inhibition constant *in vitro* Ki, that is [I]/Ki ratio is used to estimate the degree of inhibition (Thummel & Wilkins, 1998; Ito *et al.*, 2002). Ki values above 75-100µM is usually not physiologically relevant inhibitors (Thummel & Wilkinson, 1998).

Table 4. Chemical *in vitro* inhibitors of CYP1A and 3A in human (H), porcine (P) and mice (M). --- indicates no inhibition.

aNF =a-napthoflavone, **FUR** =furafylline**, KET** =ketoconazole, **7βest** =7β-estradiol, **TAO** =triacetyloleandomycin, **DBM** =6´,7´-dihydroxybergamottin, **PHE** =phenacetin, **EROD** =7-ethoxyresorufin O-deethylation, **BROD** =7-benzyloxresorufin, **BFC** =7-benzyloxy-4-trifluorometylcoumarin, **MID** =midazolam 1´-hydroxylation, **NIF**= nifedipine oxidation, **TST =**testosterone 6β-hydroxylation

CYP450	***Inhibitor***	***Reaction***	***Specie /Sex***	***IC50 (µM)***	***Ki (µM)***	***Mechanism***
1A	**aNF**	PHE[5]	H		0.013[5]	Competitive[5]
	FUR	PHE[1]	H	4,7[1]		competitive[1]
	KET	PHE[1]	H	~3[1]		competitive[1]
		EROD[3]	P	---	---	---[3]
		BROD[3]	P/male		10.4[3]	non-competitive[3]
		BROD[3]	P/female		14.2[3]	non-competitive[3]
3A		BFC[4]	P			non-competitive[4]
		BFC[8]	H	0.1[8]		
		MID[6]	H		14.9[6]	non-competitive[6]
	7βest	BFC[4]	P			irreversible[4]
	TAO	NIF[7]	H			inhibition [7]
		NIF[7]	P			inhibition[7]
	DBM	TST[2]	M	25[2]		Irreversible[2]

[1]Bourrié *et al.*, 1996, [2]Edwards *et al.*, 1996, [3]Zlabek & Zamaratzkaia, 2012, [4]Zamaratskaia *et al.*, 2012, [5]von Moltke *et al.*, 1996, [6]Gibbs *et al.*, 1999, [7]Anzenbacher *et al.*, 1997

2.6 CYP450 inhibition by flavonoids

In general, flavonoids are *inter alia* characterized as potential enzyme inhibitors and, due to their wide distribution in plant food their role as potential CYP450 inhibitors makes them important for evaluating FDI (Di Carlo *et al.,* 1999). Several reports have demonstrated that a variety of flavonoids may interact with CYP450-mediated drug metabolism, by inhibiting CYP450 activity in the liver of humans and animals (Tsyrlov *et al.*, 1994; Obach *et al.,* 2000; von Moltke *et al.,* 2012; Chaudhary & Willett, 2006; Kimura *et al.,* 2010; Ring *et al.,* 1994; Ho *et al.,* 2001; Obach *et al.,* 2000; von Moltke *et al.,* 2012).

The bioactivity of several flavonols might include interactions with CYP450 (Bushra *et al.,* 2011). Thus, a simultaneous intake of drugs and flavonols may result in food-drug interactions such as inhibition (Breinholt *et al.,* 2002).

The structure of flavonols is a crucial factor for determining its effect on CYP450 activity (Ho & Saville, 2001). The strength of inhibition of CYP450 by flavonols differs depending on number and location of hydroxyl groups of the flavonol skeleton (Breinholt *et al.,* 2002). In liver microsomes, flavonols, such as kaempferol is 3´-hydroxylated and tamarixetin is 4´-demethylated to quercetin (Breinholt *et al.,* 2002). This metabolism was strongly inhibited by the CYP1A inhibitor (and drug) fluvoxamine (kaempferol: IC50=1.2µM and tamarixetin: IC50=1.4µM), suggesting that flavonoids and drugs are metabolized by the same CYP450 enzymes (Breinholt *et al.,* 2002).

The location as well as the number of hydroxyl groups on the flavonoid skeleton may play a crucial role for the CYP450 inhibition potency of specific flavonols (Ho *et al.,* 2001).

2.6.1 CYP1A inhibitors

Quercetin was shown to inhibit CYP1A activities estimated as EROD and MROD activity in human microsomes (Tsyrlov *et al.,* 1994). In the same human microsomes, the potency of quercetin to inhibit MROD (IC50=0.4µM) was high compared to EROD (IC50=4.1µM) (Tsyrlov *et al.,* 1994). In mice microsomes, the same inhibition pattern was observed, indicating that the potencies of quercetin to inhibit EROD and MROD are comparable between human and mice microsomal CYP1A (Tsyrlov *et al.,* 1994). As a component of St. John´s wort, quercetin inhibited EROD activity in supersomal human recombinant CYP1A with Ki =0.2µM and IC50= 6.0µM (Chaudhary & Willett, 2006). Kinetic studies, using phenacetin as a substrate, demonstrated that PHE was non-competitively inhibited (Ki =3.3µM, IC50=

<10µM) by 10 µM quercetin, in recombinant, microsomal CYP1A (Obach, 2000). As a component found in Ginkgo biloba, quercetin was studied in hepatic microsomes, donated from two humans and, phenacetin to paracetamol was inhibited, with IC50=3.0µM (Moltke *et al.* (2012). Summary of CYP1A inhibitors are presented in **Table 5**.

Table 5. CYP1A inhibition by flavonols. Summary of studies presenting *in vitro* inhibition of human c-DNA derived CYP1A (H/cDNA) activity, in human microsomal CYP1A (H/mcs), in human recombinant CYP1A (H/r-mcs) and mice (M) by flavonols.

EROD =7-ethoxyresorufin *O*- deethylation, **MROD**= methoxyresorufin O-deethylation, **PHE**= phenacetin *O*-deethylation, **inhib**= inhibition, **n.i.**= indicates no inhibition, a.u.= arbitrary units

CYP1A						
Inhibitor		***Organism/ enzyme source***	***Sub-strate***	***Kinetic***		
Flavonol	***Conc.***			***IC_{50} (µM)***	***K_i (µM)***	***Mode of inhibition***
Quercetin	0-25µM[1]	H/c-DNA[1]	EROD[1]	41.1[1]		inhib[1]
	0-25µM[1]	M/c-DNA[1]	EROD[1]	20.3[1]		inhib[1]
	0-25µM[1]	H/c-DNA[1]	MROD[1]	0.4[1]		inhib[1]
	0-25µM[1]	M/c-DNA[1]	MROD[1]	29.1[1]		inhib[1]
	a.u.	H/r-mcs[2]	PHE[2]	<10[2]	3.3[2]	non-competitive[2]
	<10µg/ml[3]	H/mcs[3]	PHE[3]	3.0[3]		inhib[3]
	0.1-10µM[4]	H/r-mcs[4]	EROD[4]	6.0[4]	0.25[4]	inhib[4]
Myricetin	0.1-10µM[4]	H/r-mcs[4]	EROD[4]	34[4]	1.4[4]	inhib[4]
Rutin	0.1-10µM[4]	H/r-mcs[4]	EROD[4]	N.I.[4]	N.I.[4]	n.i.[4]

[1] Tsyrlov *et al.*, 1994, [2]Obach *et al.*, 2000, [3] von Moltke *et al.*, 2012, [4]Chaudhary & Willett, 2006

Myricetin inhibited EROD activity in supersomal recombinant human CYP1A, with Ki=1.4µM and IC50=34µM (Chaudhary & Willett, 2006).

The potency of isorhamnetin to inhibit CYP1A2 was strongly reduced by steric collisions in its way, suggesting that isorhamnetin not fit well into the hydrophobic pocket (active site) of CYP1A2 (Takemura *et al.*, 2010). For strong CYP1 inhibition to occur flavonoids require a 2-3 double bonds on the C-ring (Takemura *et al.*, 2010).

Rutin (10µM) did not inhibit CYP1A1 in human microsomes (Chaudhary & Willett, 2006).

2.6.2 CYP3A inhibitors

Quercetin inhibited CYP3A-mediated triazolam hydroxylation (Moltke *et al.,* 2010), zatosetron N-oxidation (Ring *et al.,* 2004) and quinine 3-hydroxylation (Ho *et al.,* 2001) in human microsomes. Quercetin from grapefruit, inhibited 55-60% (30-45% activity remaining) of CYP3A-mediated quinine 3-hydroxylation, in two human donors, with IC50 values of 82µM and 41µM (Ho *et al.,* 2001). The potency of quercetin to inhibit CYP3A-mediated triazolam hydroxylation (IC50=4.1µM) (von Moltke *et al.,* 2010) was higher than its potency to inhibit CYP3A-mediated zatosetron oxidation (Ki=13.6µM) (Ring *et al.,* 2004). The inhibition mode of CYP3A-mediated zatosetron oxidation, by quercetin was uncompetitive (Ring *et al.,* 2004). Quercetin inhibited human recombinant CYP3A testosterone 6β-hydroxylation, (IC50= 22.1µM) (Kimura *et al.,* 2010) more potently than CYP3A phenacetin O-deethylation, IC50=82µM (Obach *et al.,* 2000).

Myricetin (aglycon) inhibited CYP3A-mediated triazolam hydroxylation, with an IC50 value of 6.4 µM (von Moltke *et al.,* 2012). When different concentrations (10-200 µM) of myricetin was studied, 78-94% of CYP3A quinine 3-hydroxylation (22-6% remaining) was inhibited, which was more than by quercetin but, the IC50 value could not be calculated (Ho & Saville, 2001). Kinetic studies of CYP3A-mediated testosterone hydroxylation demonstrated approximately 70% irreversible inhibition (~30% activity remaining) by myricetin, with a Ki value of 44.5µM (Kimura *et al.,* 2010).

Isorhamnetin inhibited testosterone hydroxylation (≥80% inhibition and ≥20% remaining) more than myricetin and less than quercetin (Kimura *et al.,* 2010)

CYP3A-mediated quinine 3-hydroxylation was not inhibited by rutin at low and medium concentrations but, a modest inhibition was observed at high concentrations (Ho & Saville, 2010) and, no significant inhibition of CYP3A-mediated testosterone hydroxylation was observed (Kimura *et al.,* 2010). A summary of CYP3A inhibitors is presented in **Table 6**.

The potency of flavonols to inhibit CYP3A quinine 3-hydroxylation increased by increased number of hydroxyl substituents on the flavonol seleton (Ho *et al.,* 2001). Myricetin, with 6 OH-groups was found to be the most potent inhibitor of CYP3A quinine 3-hydroxylation, while quercetin with 5 hydroxyl groups was the second most potent and, rutin with 4 hydroxyl groups was found to have least potency (Ho *et al.,* 2001). Presence of hydroxyl groups at the R4 position on the B ring (as quercetin have), specifically enhanced the inhibition potency (Ho *et al.,* 2001).

It should be emphasized that the concentrations of flavonoids tested in *in vitro* studies were likely higher than typical bioavailable concentrations in humans. Thus, the results should be interpreted with caution.

Table 6. CYP3A inhibition by flavonols. Summary of studies presenting *in vitro* inhibition of human microsomal CYP3A (H/mcs) and in human recombinant CYP3A (H/r-mcs) by flavonols.

ZAT =zatosetron N-oxidation, **QUI** =quinine 3-hydroxylation, **TRI** =triazolamhydroxylation, **TST**= testosterone 6β-hydroxylase, **n.d.** =not detected

CYP3A						
Inhibitor		**Organism/ enzyme source**	**Sub-strate**	**Kinetic**		
Flavonol	**Conc.**			**Ki µM**	**IC_{50} µM**	**Inhibition (mode)**
Quercetin	12.5-100µM[2]	H/mcs[2]	ZAT[2]	13.6[2]		Uncompetitive[2]
	10-200µM[3]	H/mcs[3]	QUI[3]		41[3]	Inhib.[3]
	10-200µM[3]	H/mcs[3]	QUI[3]		82[3]	Inhib.[3]
	9.9µM[5]	H/mcs[5]	TRI[5]		4.1[5]	Inhib.[5]
	100µM[1]	H/r-mcs[1]	TST[1]		22.1[1]	Inhib.[1]
Myricetin	10-200µM[3]	H/mcs[3]	QUI[3]		n.d.	Inhib.[3]
	9.9µM[5]	H/mcs[5]	TRI[5]		6.4[5]	Inhib.[5]
	100µM[1]	H/r-mcs[1]	TST[1]	44.5[1]		Inhib.[1]
Isorhamnetin	100µM[1]	H/r-mcs[1]	TST[1]	16.6[1]		Inhib.[1]
Rutin	100µM[1]	H/r-mcs[1]	TST[1]	-	-	No inhib.[1]
	10-200µM[3]	H/mcs[3]	QUI[3]	-	-	No-modest inhib.[3]

[1] Kimura *et al.*, 2010, [2] Ring *et al.*, 1994, [3]Ho *et al.*, 2001, [4], [5] von Moltke *et al.*, 2012

3 Material & Method

3.1 Chemicals

Nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 7-benzyloxy-4-triflouromethylcoumarin (BFC; CYP3A substrate), 7-hydroxy-4-triflouromethylcoumarin (HFC; product of BFC metabolism), 7-ethoxyresorufin (CYP1A substrate), resorufin (product of 7-ethoxyresorufin metabolism), rutin, myricetin, quercitin, isohamnetin, p-coumaric acid, gallic acid and caffeic acid (inhibitors) were obtained from Sigma-Aldrich (Steinheim, Germany). Methanol and acetonitrile of HPLC grade were obtained from Merk (Darmstadt, Germany).

3.2 Study design

The flowchart in **Figure 1** illustrates the design of the experimental process. First, bilberry compounds (one concentration per compound) were evaluated for their ability to inhibit CYP3A and CYP1A activity. If the activities were inhibited by at least 21%, the reversibility of the inhibition was evaluated by the including of a preincubation step. Reversible inhibitors were further analyzed for the inhibition mode using multiply substrate concentrations. The details of analysis, substrate and inhibitor concentrations are described in sections 3.3.

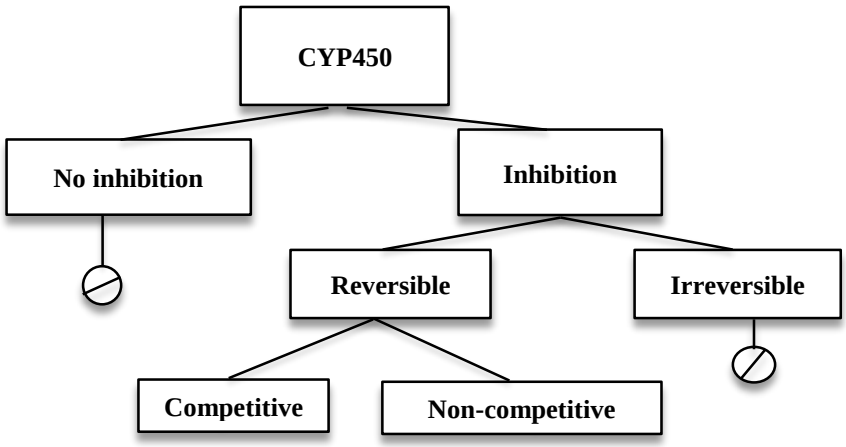

Figure 1. The flow illustrates the design of the experimental process.

3.3 Biochemical analysis

3.3.1 Bilberry extract preparation

The powder of dried bilberry fruits (0.5 mg) was extracted by overnight incubation, at 35°C in 50% methanol and 1-2 M HCl (Häkkinen *et al*. 1998). The mixture then was evaporated to dryness, re-dissolved in 1.5 ml of 100% methanol and filtered before use. Extract was further diluted with 100% methanol to obtain 6 working solutions in a 2000, 200, 20, 8 and 2 dilution of undiluted extract. Working solutions were stored at -80°C. A number of working solution indicates dilution factor from the undiluted extract (undiluted bilberry extract -1, extract diluted 2000 times -2000).

3.3.2 Microsomal preparation

The calcium aggregation method was used for microsomal preparation. Briefly, 2 g liver tissue was homogenized in Tris-EDTA buffer (50 mM Tris-HCl, 150mM Potassium chloride, 2 mM EDTA, pH 7.4). The obtained homogenate was diluted with Tris-EDTA buffer containing 8 mM $CaCl_2$, incubated for 4 minutes and centrifuged at 25.000 x g for 30 minutes at 4° C (Rasmussen *et al*., 2011). All steps were carried out on ice. The microsomal protein concentration was measured with a commercially available kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer´s instructions. Microsomes were diluted to a final protein concentration of 10 mg/ml and stored at -80° C until used.

3.3.3 Cytochrome P450 enzymatic assays

CYP1A activity. A stock solution in resorufin was prepared in 100% methanol. Standard curves were constructed using resorufin in a concentration range from 0.05 to 5 pmol/ ml in the mixture of 50 mM potassium phosphate buffer, pH 7.4:methanol (1:1) in duplicate. CYP1A activity was assessed by incubating 0.20 mg/ml of microsomal proteins with NADPH at a concentration of 0.5 mM and the substrate, 7-ethoxyreorufin at a concentration of 2 µM. After 5 minutes incubation in a waterbath at 37° C, the reaction was terminated by 500 µl of ice-cold methanol, followed by centrifugation at 7500 x g for 10 minutes. The supernatants were analyzed with HPLC. HPLC conditions used were: isocratic mobile phase: 20 mM phosphate

buffer pH 6.8:methanol:acetonitrile (52:45:3), flowrate: 1.0 ml/minute, fluorescence detector of 560 and 586 nm (excitation and emission), injection volume: 10µl and run time 3 minutes. CYP1A activity is reported as picomoles of resorufin formed per minute per milligram of microsomal protein.

CYP3A activity. A stock solution HFC was prepared in 100 % methanol. Known amounts of HFC in a range of 2 to 2000 pmol/ml were added to the mixture of 50 mM potassium phosphate buffer, pH 7.4:methanol (1:1) in duplicate. CYP3A activity was assessed by incubating 0.25 mg/ml of microsomal proteins with NADPH at a concentration of 0.5 mM and the substrate, BFC at a concentration of 100µM. After 10 minutes of incubation in a water bath at 37° C, the reaction was terminated by 500 µl of ice-cold methanol. The resulting mixtures were centrifuged at 7500 x g for 10 minutes and the supernatants were analyzed with HPLC. HPLC conditions used were: isocratic mobile phase: 20 mM phosphate buffer pH 6.8: methanol: acetonitrile (52:45:3), flowrate: 0.6 mL/min, fluorescence detection at 410 and 538 nm (excitation and emission), injection volume: 10 µl and run time 8 min. CYP3A activity is reported as picomoles of HFC formed per minute per milligram of microsomal protein.

In both assays, a Hypersil ODS column (3µm, 60 x 4.6 mm Hewlett-Packard) equipped with a guard column was used.

Inhibition studies were performed using two pools of microsomes from three male pigs in each and, two pools of microsomes from two female pigs in each.

An overview of the assay conditions is presented in **Table 7**.

Table 7. Assay conditions for CYP1A and CYP3A.

Substrate	Isoform	Microsomal protein amount	Incubation	Substrate conc.	NADPH
Ethoxyresorufin	1A	0.20 mg/ml	5 min	2 µM	0.5 mM
BFC	3A	0.25 mg/ml	10 min	100µM	0.5 mM

3.3.4 Bilberry extract

Various concentrations of methanol bilberry extract were incubated with microsomes, NADPH and a specific probe substrate (either BFC for CYP3A or 7-ethoxyresorufin for CYP1A). Serial dilutions were prepared by addition of methanol to

yield six concentrations of bilberry extract and, the extract concentrations were reported in arbitrary units indicating the dilution of a stock extract (0.5 mg of bilberry powder in 1 ml of methanol). The incubation was allowed to proceed as above described in enzymatic assays.

3.3.5 Individual compounds from bilberry extract

Inhibition of CYP3A and CYP1A activities by following compounds typically found in bilberries: rutin, myricetin, quercetin, isorhamnetin, p-coumaric acid, gallic acid and caffeic acid. First, bilberry compounds (one concentration per compound) were screened for their ability to inhibit CYP3A and CYP1A activity *in vitro*. Solutions were added to eppendorf tubes in the following order and quantity: inhibitor (16 µM in final incubation volume), freshly pre-mixed incubation buffer, substrate and NADPH; the reaction was initiated by addition of microsomal protein. The final concentration of methanol was 0.1% in the incubation volumes. The same amount of methanol was added to the control incubations. Percentage of inhibition calculations were based on differences between the control incubations (without inhibitor, 100%) and the incubations in the presence of inhibitor. A control activity without addition of inhibitor was assessed within each assay. The activity was regarded as inhibited if it differed from the control activity by at least 21%.

3.3.6 Enzyme inhibition kinetics

The compounds, showing ability to inhibit either CYP3A or CYP1A, were further tested using 3 concentrations (16, 32 and 128 µM). The incubations were performed with and without inclusion of pre-incubation step (5 min before addition of substrate) in order to assess reversibility of inhibition. If inhibition degree increased after inclusion of a pre-incubation step, the inhibition was considered as irreversible and 50% inhibition of enzyme activity (IC_{50} value) was determined. If inhibition degree was not affected by inclusion of pre-incubation step, the inhibition was considered as reversible and mode of inhibition was further determined by kinetic analysis using several substrate concentrations. CYP3A activity was determined at BFC concentrations from 0.1 to 150 µM and CYP1A activity was determined at 7-ethoxyresorufin concentrations from 0.1 to 4 µM.

The incubations were processed as described in enzymatic assays section. All measurements were performed in duplicate.

3.4 Statistical analysis

The inhibition mode (competitive and non-competitive) and inhibition constants (Ki) were determined with GraphPad Prism version 4.0 for Windows, GraphPad Software (San Diego, California, USA). The choice of the best-fit enzyme model was based on F-test.

Enzyme activities were considered as altered when inhibition degree exceeded 20%. No further statistical analysis (p-values) were performed to avoid false positive results due to low intra-assay variations.

4 Results

4.1 CYP450 activity in the presence of bilberry

Inhibition by bilberry extract on CYP450 activity

The undiluted bilberry extract (**Figure 2**) markedly modified the activity of the CYP450 isoform 1A in male (remaining activity varied from 61.2 to 64.9%) but not in female pigs. The dilution of the extract eliminated this inhibition. The activity of CYP3A increased in male and decreased in female pigs in the presence of bilberry extract.

Reversibility of inhibition

Out of the selected compounds (rutin, myricetin, quercetin, isorhamnetin, p-coumaric acid, gallic acid and caffeic acid, only myricetin, quercetin and isorhamnetin showed potential to inhibit CYP450 activity by at least 21% (remaining CYP450 activity below 79%)(**Figure 3**).

Myricetin and isorhmnetin reversibly inhibited CYP1A- and CYP3A-activity (degree of inhibition was not affected by inclusion of pre-incubation step) in the microsomes from both male and female pigs (**Figure 4**). Inhibition of CYP1A by quercetin exhibited gender-specific pattern being reversible in the microsomes from male, and irreversible in the microsomes from female pigs.

Inhibition mode and Ki values

CYP1A activity was competitively inhibited by myricetin and isorhamnetin in both male and female pigs. Both inhibitors expressed higher potential to alter CYP1A activity in female pigs as indicated by lower Ki values. Quercetin competitively inhibited CYP1A activity in male pigs, while in female pigs quercetin acted as an irreversible inhibitor, with an IC50 value of 1.3 μM (**Figure 5)**.

Myricetin non-competitively inhibited CYP3A activity in the microsomes from male but not female pigs. Isorhamnetin competitively inhibited CYP3A in both male and female microsomes, although Ki value was somewhat lower in male compared to female pigs (71.2 vs. 93.7 μM, respectively) (**Figure 6)**.

The results from kinetic measurements are summarized in **Table 8**.

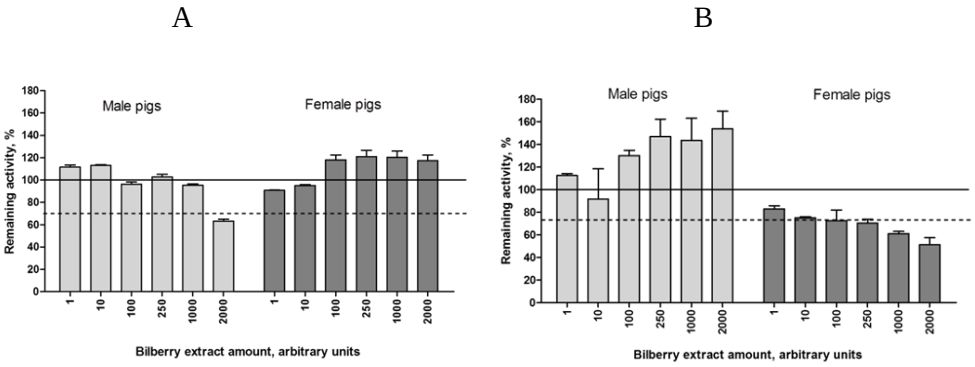

Figure 2. Inhibition by bilberry extract, diluted (arbitrary units), with the most dilute. A) CYP1A activity and B) CYP3A activity, in the presence of bilberry extract.

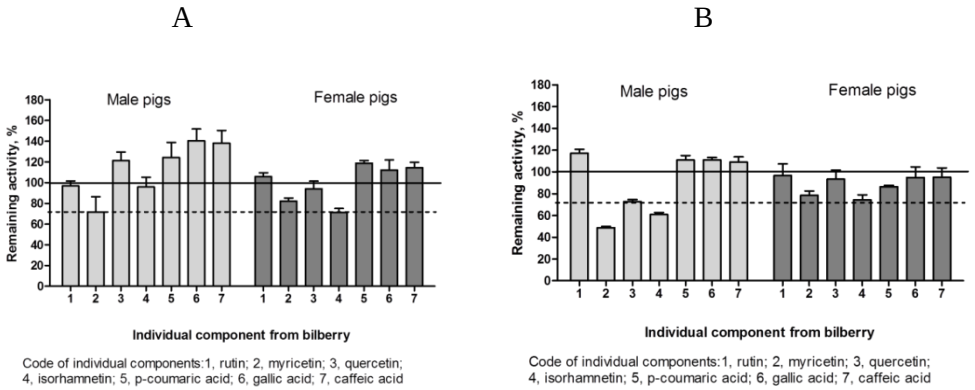

Figure 3. Inhibition by individual bilberry components: A) CYP1A activity and, B) CYP3A activity and, only myricetin, quercetin and isorhamnetin showed potential to inhibit CYP450 activity by at least 21%.

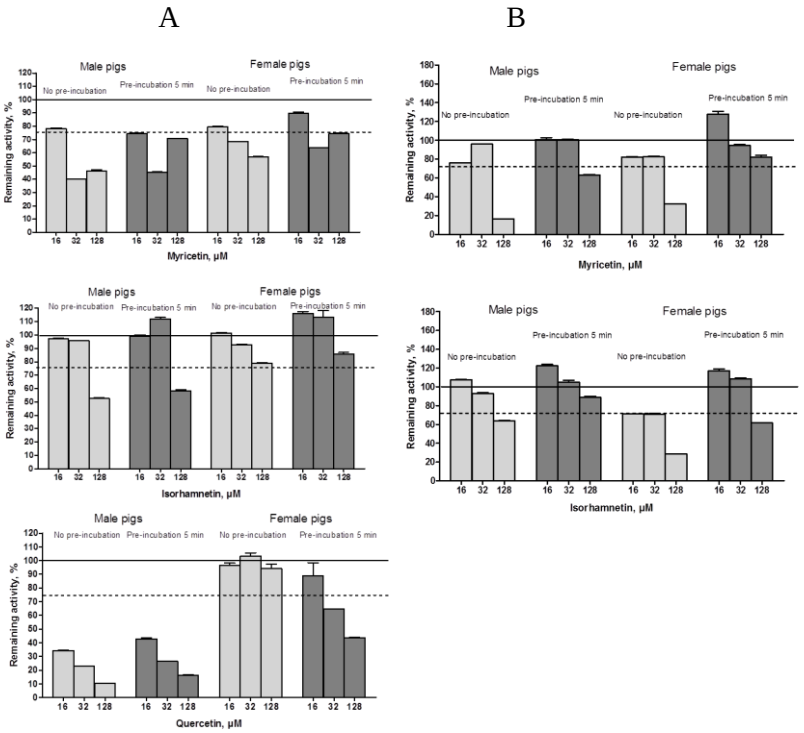

Figure 4. Reversibility, A) CYP1A in the presence of myricetin, isorhamnetin and quercetin and B) CYP3A in the presence of myricetin and isorhamnetin

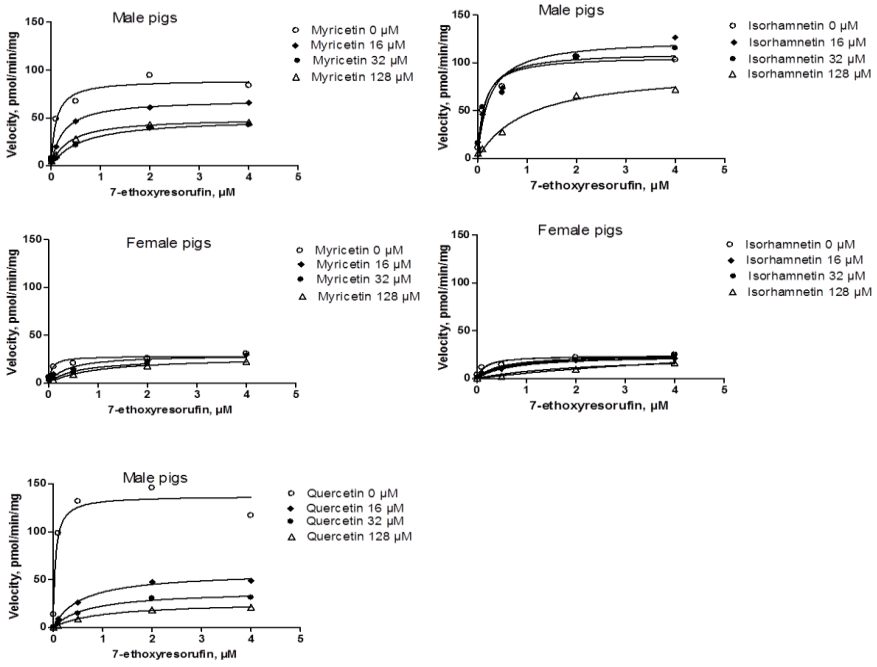

Figure 5. CYP1A saturation curve, EROD.

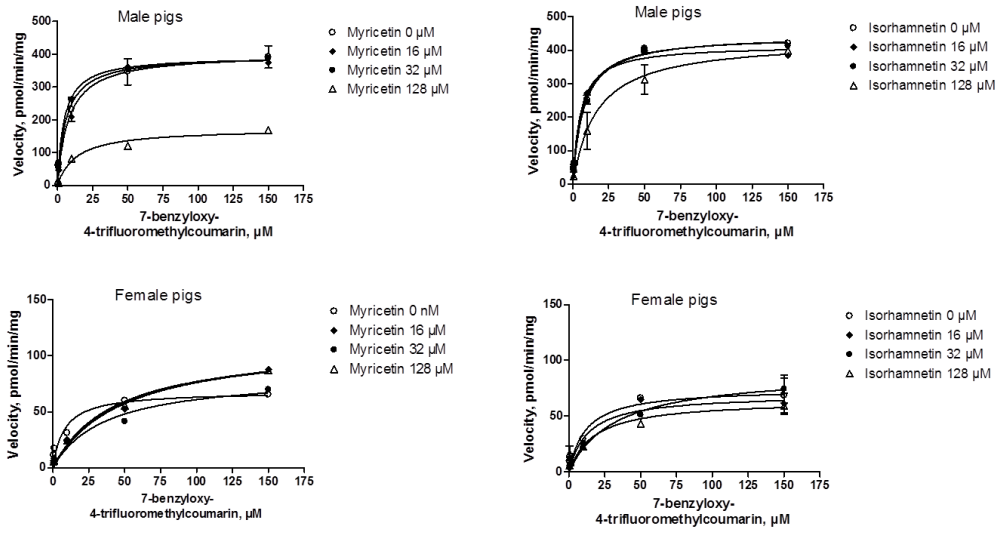

Figure 6. CYP3A (BFC) saturation curve.

Tabel 8. Mechanism of inhibition of specific CYP450 isoforms by myricetin, isorhamnetin and quercetin in porcine liver microsomes.

CYP	**Individual phenolic compound**	**Sex** (pigs)	**K_i** (µM)	**IC_{50}** (µM)	**Mode of inhibition**
1A	Quercetin	M	0.2		Competitive
		F		1.3	Irreversible
	Myricetin	M	2.5		Competitive
		F	4.6		Competitive
	Isorhamnetin	M	99.9		Competitive
		F	10.8		Competitive
3A	Myricetin	M	112.7		Non-competitive
		F			No inhibition
	Isorhamnetin	M	71.2		Competitive
		F	93.7		Competitive

M=male, F=female

5 Discussion

CYP450 is involved in the metabolism of various endogenous and xenobiotic compounds; thus, inhibition of individual isoforms by dietary components may have undesirable and even harmful effects on essential physiological processes. Additionally, such inhibition might alter metabolism of drugs. Thus, the effect of food components on CYP450 might be carefully studied.

Previous studies demonstrated that several dietary phenolic compounds, such as quercetin, myricetin and isorhamnetin have potencies to inhibit activities of some CYP450 isoforms in human, rat and mice at concentrations in the range 8-200μM.

The concentrations of the inhibitors in the present study (16 μM or 8 μM in the incubation volume of 500 μl) corresponded to the concentration of quercetin measured in the liver of pig fed a single quercetin dose (25 mg/kg) (Bieger *et al.*, 2008). In the kinetic analysis, higher inhibitor concentrations were also included (32 and 128 μM) to increase the accuracy in Ki estimation.

The present study was designed to elucidate potential inhibition of CYP1A and CYP3A by several phenolic compounds, in porcine hepatic microsomes. Hepatic microsomes from male and female pigs were used in the study. It was recently suggested that interactions between CYP450 and xenobiotic compounds may differ between male and female (Rasmussen *et al.*, 2011). Moreover, *in vitro* CYP3E activities were inhibited by 17βoestradiol in the microsomes from male but not from female pigs (Zamaratskaia *et al.*, 2007; Rasmussen *et al.*, 2011).

The present results provide further evidence on gender-related regulation of CYP450. First, bilberry extract affected CYP1A and CYP3A activities different in male and female pigs. CYP1A activity was lower in the presence of bilberry extract only in male and, CYP3A only in female pigs. Further analysis using individual component confirmed gender-related differences.

Quercetin was a potent competitive inhibitor of CYP1A in male pigs and, irreversible inhibitor in female pigs. The gender-related pattern of CYP1A inhibition by quercetin might explain overall inhibition of male CYP1A by bilberry extract. Human CYP1A measured as EROD activity was also inhibited by quercetin (Tsyrlov *et al.*, 1994) even though the inhibition was more potent in porcine than in human.

Isorhamnetin and myricetin inhibited CYP1A in the microsomes of both male and female pigs. Present findings are in line with those on recombinant CYP1B1 and CYP1A1 (Chaudhary & Willet, 2006), in which myricetin and quercetin were

identified as inhibitors of EROD. Chaudhary & Willet (2006) did not, however, evaluate the mode of inhibition.

Quercetin is a well-known inhibitor of human CYP3A. The CYP3A-mediated biotransformation of many drugs, i.e. zatosetron (Ring *et al.*, 1994), quinidine (Ho *et al.*, 2001), triazolam and testosterone (Kimura *et al.*, 2010; von Moltke *et al.*, 2004; Obach, 2000) were inhibited by quercetin in humans. The known *in vivo* interaction between grapefruit juice and the drug nifedipine, in humans is believed to be at least partly due to the presence of quercetin in grapefruit juice (Ho *et al.*, 2001). Interestingly, CYP3A-mediated BFC metabolism was not inhibited by quercetin in pig. This implies specie-related differences in food/feed-drug interactions. More studies are needed to investigate the effect of quercetin on porcine CYP3A activity. In such studies, several substrates for CYP3A should be included.

The potency of myricetin to inhibit CYP3A activity in male pigs (no inhibition in female pigs) was weak, compared to its potency to inhibit CYP1A EROD. Other metabolic reactions that are inhibited by myricetin, includes human CYP3A-mediated metabolism of the drugs triazolam (Moltke *et al.*, 2012), quinidine (Ho e*t al.*, 2001) and testosterone (Kimura *et al.*, 2010).

In human microsomes, rutin inhibited CYP3A-mediated metabolism of steroids such as testosterone as well as the drugs quinidine (Ring *et al.* 1994). In this study, no inhibition by rutin was observed.

Phenolic acids, used in the present study did not modify either CYP1A or CYP3A activities. Previous findings suggested that some phenolic acids are metabolized by CYP450 (Martignoni *et al.*, 2006). In this case, the competitive inhibition of CYP450 substrate metabolism would be expected. However, no inhibition was observed. More studies are needed to identify the role of phenolic acids in the regulation of CYP450 activity.

The risks for berry component-drug interactions are not yet well documented. Further research is needed to assess the degree and mode of possible interactions. In such research, both in vivo and in vitro studies need to be included. Gender-related differences in CYP450 activity observed in the present and previous studies (Kojima & Degawa, 2013; Rasmussen *et al.*, 2012; Rasmussen *et al.*, 2011; Skaanild & Friis, 1999) should be taken into account when designing future studies on interactions between food/feed components and CYP450 activity.

The results from the present study contribute to further understanding on the interactions between the dietary phenolic components, quercetin, myricetin and isorhamnetin and, CYP450-mediated metabolism. Further investigations are needed to determine physiological significance of this inhibition and its relevance for food-drug interactions in humans.

6 Conclusions

The study suggests that individual components in bilberry fruits have an inhibitory effect on CYP1A and CYP3A activity. The mode and degree of this inhibition differed between male and female. Further research is needed to elucidate exact mechanism and physiological significance of the interaction between phenolic compounds and CYP450-mediated hepatic metabolism.

7 References

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9 Appendix: Popular summary

Are bilberry fruit and its phenolic constituent´s capable of changing drug metabolism and, thus the effect of drugs?

An increased awareness about food-drug interactions may reduce unwanted side-effects and severe complications such as those arising from a simultaneous intake of drugs with food/supplements. In this study, bilberry fruit and phenolic components from bilberry fruit was investigated for their potential to interact with the major drug metabolizing enzyme system, cytochrome P450 (CYP450). Interactions between CYP450 activity (activation/deactivation of drugs) and certain food or food components may result in an increased (or decreased) bioavailability of the drug and thus, an increased (or decreased) drug effect.

The most known example of FDI is that of grapefruit and the CYP450 isoform 3A (CYP3A), which is the quantitatively most important CYP450. Grapefruit interact with almost all drugs and may increase bioavailability of the drug and, thus the effect of the drug as much as five-fold. Active components and mechanisms of FDI are not yet fully understood. However, individual dietary flavonoids, which are most known for their many, health beneficial effects, have demonstrated potency to inhibit CYP450 and, should thus also be seen as a threat.

Pig is a good and commonly used animal model for human CYP450 activity and, drug metabolism. In this study, hepatic microsomes from pig, was used to study the activity of CYP1A and CYP3A in the presence of bilberry extract and its phenolic constituents (quercetin, myricetin, isorhamnetin, rutin, p-coumaric acid, caffeic acid and gallic acid). Previous studies have showed that gender-related differences in CYP450 activity occur in porcine, and in human, even though these results are still questioned. In the present experiment, hepatic microsomes from both male and female pigs were used.

In this study, further evidence on gender-related regulation of CYP450 was demonstrated. First, the activity of CYP1A decreased markedly in male but not in female in the presence of bilberry extract, while the activity of CYP3A increased in male and decreased in female.

Secondly, individual flavonols (quercetin, myricetin and isorhamnetin) confirmed the gender-related differences. Quercetin, the most potent of the inhibitors investigated, inhibited CYP1A in male pigs competitively and irreversibly in female

pigs. The gender-related pattern of CYP1A inhibition by quercetin might explain overall inhibition of male CYP1A by bilberry extract. Previous studies, on human CYP1A activity show a lower degree of inhibition by quercetin. Isorhamnetin and myricetin inhibited CYP1A in both male and female pigs. These findings are in line with those on human CYP1A1 (Chaudhary & Willet, 2006).

Even though quercetin is a well-known CYP3A inhibitor in human, CYP3A-mediated BFC metabolism was not inhibited by quercetin in pig. This implies specie-related differences in food/feed-drug interactions, in line with previous findings. Myricetin was a weak inhibitor of CYP3A activity in male pigs (no inhibition in female pigs) compared to its potency to inhibit EROD by CYP1A. No inhibition was observed for rutin or any of the phenolic acids which was investigated in pigs in this study even though there are published data on such interactions in previous literature.

For future studies on interactions between food/feed components and CYP450 activity, this report recommends to take gender-specific patterns into account. To determine physiological significance and relevance for FDI further investigations are needed.