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der Christian-Albrechts-Universität zu Kiel

INFLUENCE OF DIETARY FAT ON THE ORAL BIOAVAILABILTY OF THE FLAVONOL QUERCETIN

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Meiner Familie

Lesser, S. *Influence of Dietary Fat on the Oral Bioavailability of the Flavonol Quercetin. Doctoral dissertation, University of Kiel, 2006*

A multitude of potentially beneficial health effects are presently discussed to be exerted by flavonoids, a group of secondary plant metabolites. Knowledge about the bioavailability of these agents is a prerequisite for the estimation of their potential *in vivo* effects. The flavonol quercetin is one of the most predominant flavonoids with regard to quantity in the Western-style human diet and it is also one of the most studied. In plants and plant-derived food, quercetin dominates in its glycosilated form. Both the chemical composition of the sugar moieties and their position(s) influence the intestinal absorption of quercetin. In addition, the composition of the diet may exert an influence on the bioavailability of quercetin. The present study investigated the influence of the amount of dietary fat as well as the influence of the chemical composition of dietary fat (long-chain triacylglycerols, LCT, *vs.* medium-chain triacylglycerols, MCT) on the oral bioavailability of co-ingested quercetin in pigs.

The first study investigated the influence of different amounts of dietary fat in a test meal (enriched with lard; 3, 17, or 32% crude fat content) on the oral bioavailability of quercetin either applied as aglycone or as quercetin-3-*O*-glucoside (30 µmol/kg body weight). It was shown that an increase in the dietary fat content from 3 to 17% (wt/wt) significantly enhanced the bioavailability of quercetin with both sources of quercetin (aglycone and monoglucoside). No further enhancing effect on quercetin's bioavailability was observed with a diet containing 32% fat.

The second study was aimed to investigate the influence of the fatty acid pattern of dietary fat (LCT *vs.* MCT, 16% crude fat content) on the bioavailability of quercetin applied as aglycone. Intake of quercetin with the LCT as well as the MCT diet, compared with the low-fat diet, enhanced the bioavailability of quercetin by 12 and 38%, respectively, which was significant for the MCT diet only. The pharmacokinetic parameters of quercetin in plasma, however, were significantly influenced by the type of dietary fat. Maximal plasma levels of quercetin in the systemic circulation were reached significantly later with the MCT diet compared with the LCT or the low-fat diet. As no differential effect of the experimental diets on gastric emptying was observed in an additional experiment performed in rats, a delay in gastric emptying triggered by MCT diet might be largely excluded as a potential cause of the findings.

In summary, it is demonstrated that both the fat content and the fatty acid pattern of dietary fat influence the oral bioavailability of the plant polyphenol quercetin. With respect to the systemic availability of dietary flavonoids, diet composition has to be considered as an important factor.

Lesser, S. *Einfluss von Nahrungsfett auf die orale Bioverfügbarkeit des Flavonols Quercetin. Dissertation, Universität Kiel, 2006*

Für Flavonoide, eine Gruppe sekundärer Pflanzenmetabolite, werden eine Reihe potenziell gesundheitsfördernder Wirkungen diskutiert. Voraussetzung für die Einschätzung ihrer möglichen Wirkungen im menschlichen und tierischen Organismus ist eine ausreichend hohe Bioverfügbarkeit. Quercetin ist eines der quantitativ und qualitativ bedeutendsten Flavonole aus der Gruppe der Flavonoide. In Pflanzen und in pflanzlichen Lebensmitteln liegt Quercetin vorwiegend glykosidisch gebunden vor. Sowohl die chemische Zusammensetzung als auch die Position des/der verknüpften Zuckerreste(s) beeinflussen die intestinale Aufnahme von Quercetin. Zusätzlich scheint auch die Zusammensetzung der Mahlzeit einen Einfluss auf die systemische Verfügbarkeit von Quercetin auszuüben. In der vorliegenden Arbeit wurde sowohl der Einfluss des Fettgehaltes als auch der mögliche Einfluss der Fettsäurenkettenlänge (langkettige Triacylglycerine, LCT, *vs.* mittelkettige Triacylglycerine, MCT) in der Nahrung auf die orale Bioverfügbarkeit von zeitgleich eingenommenem Quercetin an Schweinen untersucht.

In der ersten Studie wurde der Einfluss unterschiedlicher Fettgehalte einer Testmahlzeit (angereichert mit Schmalz; 3, 17 bzw. 32% Rohfettgehalt) auf die orale Bioverfügbarkeit von Quercetin (Aglykon oder Quercetin-3-*O*-Glukosid; 30 µmol kg^{-1} Körpergewicht) untersucht. Dabei zeigte sich, dass eine Erhöhung des Nahrungsfettgehaltes von 3 auf 17% (wt/wt) die systemische Verfügbarkeit von Quercetin unabhängig von der applizierten chemischen Form (Aglykon oder Monoglukosid) signifikant steigert. Eine Erhöhung des Fettgehaltes der Testmahlzeit auf 32% führte zu keiner weiteren Steigerung der Bioverfügbarkeit von Quercetin.

Die zweite Studie untersuchte den Einfluss der Fettsäurenkettenlänge (LCT *vs.* MCT; 16% Nahrungsfettgehalt) auf die systemische Verfügbarkeit von Quercetin. Die Einnahme von Quercetin mit einer LCT- bzw. MCT-haltigen Testmahlzeit erhöhte die systemische Verfügbarkeit von Quercetin gegenüber der Einnahme ohne zusätzliches Fett (Standard Diät, 2% Fettgehalt) um 12 bzw. 38%, wobei der Effekt mit der LCT Diät nicht signifikant ausfiel. Die pharmakokinetischen Parameter von Quercetin im Plasma wurden signifikant durch die Art des Nahrungsfettes beeinflusst. Maximale Quercetin-Plasmaspiegel wurden signifikant später bei Einnahme mit MCT Diät im Vergleich zur LCT bzw. Standard Diät erreicht. Eine verzögerte Magenentleerung als Erklärung für diese Befunde wurde weitgehend ausgeschlossen, da in einer ergänzenden Studie an Ratten kein Unterschied in der Magenleerung nach Einnahme der verschiedenen Diäten beobachtet wurde.

Zusammenfassend konnte gezeigt werden, dass sowohl der Gehalt als auch die Fettsäurenkettenlänge von Nahrungsfett die orale Bioverfügbarkeit des pflanzlichen Polyphenols Quercetin beeinflussen. Somit hat die Zusammensetzung einer Mahlzeit einen signifikanten Einfluss auf die systemische Verfügbarkeit von Flavonoiden aus der Nahrung.

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ABBREVIATIONS

ABC	ATP-binding cassette
ATP	adenosine triphosphate
AUC	area under the curve
BBM	brush border membrane
BCRP2	breast cancer resistance protein 2
BSA	bovine serum albumin
BW	body weight
c_{480}, c_{720}	plasma concentration at 480 or 720 min after ingestion of test meal, respectively
CBG	cytosolic β-glycosidase
CCK	cholecystokinin
c_{max}	maximal plasma concentration
CO_2	carbon dioxide
COMT	catechol-*O*-methyl-transferase
CVD	cardiovascular diseases
DMSO	dimethyl sulfoxide
g	acceleration of gravity, 9.81 m s^{-2}
GIT	gastrointestinal tract
HSA	human serum albumin
i.v.	intravenous
LCT	long-chain triacylglycerols
LDL	low-density lipoproteins
LPH	lactase-phloridzin hydrolase
LSM	least-squares means
MCT	medium-chain triacylglycerols
MRP2	multidrug resistance associated protein 2
NEFA	non-esterified fatty acids
P	octanol/water partition coefficient
P	probability
p-gp	p-glycoprotein
Q3G	quercetin-3-*O*-glucoside
Q3,4'diG	quercetin-3-*O*, 4'-*O*-diglucoside
Q4'G	quercetin-4'-*O*-glucoside
SEM	standard error of the mean
SGLT1	sodium-dependent glucose co-transporter 1
t_{max}	time at maximal plasma concentration
v	volume
wt	weight

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GENERAL INTRODUCTION

Flavonoids are secondary plant metabolites possessing a polyphenol structure. In the 1930ies, flavonoids were thought to have vitamin properties, whereas they were considered as potential mutagens and carcinogens in the 1970ies. The attention focused on their anti-mutagenic and anti-carcinogenic activities in the 1980ies. In recent years, the antioxidant properties of flavonoids and their potential role in both, inhibition of low-density lipoprotein (LDL) oxidation and platelet aggregation, were reported (Hertog, 1996). Protective properties of flavonoids in conjunction with so-called 'free radical diseases', such as cardiovascular diseases (CVD), cancer, or cataract, are actually discussed and, in case of CVD, are supported by epidemiological studies. These findings have resulted in increased interest in the health-promoting aspects of flavonoids.

In order to evaluate their bioactivity *in vivo*, it is necessary to understand the factors influencing the absorption of flavonoids by the gastrointestinal tract and the nature of the conjugates and metabolites present in the circulation. This work is aimed to contribute to our knowledge on nutritional factors influencing oral bioavailability of flavonols, a bioactive and abundant subgroup of flavonoids, and to gain further insight into the mechanisms of flavonol absorption.

The first chapter of this thesis gives an overview on the present knowledge on flavonol bioavailability, with special emphasize on the flavonol quercetin. Quercetin is an abundant flavonoid in vegetal food, and due to its potent antioxidative properties it is also one of the most investigated polyphenols. In the second chapter, a study on the bioavailability of quercetin aglycone and quercetin-3-monoglucoside (isoquercitrin, Q3G) fed to pigs in test meals with different fat content is described. In a subsequent study, presented in chapter III, the influence of the fatty acid chain length of dietary triacylglycerols on flavonol bioavailability is examined. Chapter IV presents a general discussion of the results of both studies. In the appendix, preparation of the plasma samples and HPLC analysis are described in detail, as the methods are only briefly summarized in the two manuscripts. In addition, information on the fatty acid composition of lard used as experimental fat in the own studies is provided.

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CHAPTER ONE

Oral Bioavailability of the Flavonol Quercetin – A Literature Review.

I.1. Introduction

Flavonoids are secondary plant metabolites synthesized in virtually all higher plants. Together with lignans, stilbenes and phenolic acids they make up the highly diverse group of polyphenols (Scalbert & Williamson, 2000). Due to their wide distribution in the plant kingdom, they are present in many plant-derived foodstuffs. Primarily present in onions, apples, kale, broccoli and black tea they are part of the daily nutrition. Early interest in polyphenols was related to their "antinutritional" effects, i.e. decreasing absorption and digestibility of food through their ability to bind proteins and minerals. The astringency of many fruits and beverages is attributable to the precipitation of salivary proteins by plant polyphenols. Current interest is focussing on the beneficial health effects of dietary polyphenols. For hundreds of years, flavonoid rich plants are used in traditional medicine of many ethnics. Epidemiological studies have suggested associations between the consumption of polyphenol-rich food and beverages and the prevention of diseases (Rice-Evans *et al.*, 2000). Flavonoids are the most abundant polyphenols in our diets (Scalbert & Williamson, 2000). They are powerful antioxidants *in vitro*, but their overall functions *in vivo* have yet to be clarified, whether antioxidant, anti-inflammatory, enzyme inhibitor or inducer, or some other role (Rice-Evans *et al.*, 2000).

With the 'Western Diet', daily consumption of the main flavonoids quercetin, kaempferol, myricetin (flavonols), apigenin and luteolin (flavones) sums up to approximately 20-30 mg d^{-1} (Hertog *et al.*, 1993b). The flavonol quercetin is one of the most abundant flavonoids in plants and plant-derived food (Hertog *et al.*, 1992). In some countries and also via the internet, quercetin can already be purchased as an over-the-counter food supplement (Weldin *et al.*, 2003). Anyhow, it seems too early to suggest supplemental intake exceeding the amount consumed with an optimised plant-food based mixed diet. Bioavailability is a prerequisite for potential health effects, and yet there are still unsolved questions concerning flavonoids. Due to quite intense research on the gastrointestinal uptake and metabolism of flavonoids over the past decade, using humans, animal models and cell culture studies, a general working hypothesis has been established (chapter I.3.2.1.) (Scalbert & Williamson, 2000). Most recently, emphasis in research is drawn on factors affecting flavonoids bioavailability, such as alternative routes of uptake other than via portal vein blood (Murota & Terao, 2005), the food matrix (Wiczkowski *et al.*, 2003; Graefe *et al.*, 2001; de Vries *et al.*, 2001), or the influence of co-administered nutrients (Goldberg *et al.*, 2003; Azuma *et al.*, 2003).

I.2. Structure and sources of flavonoids

Flavonoids are widespread in the plant kingdom, with exception of algae and fungi (Bravo, 1998). In plants, this secondary metabolites exert controlling effects on the amount of phytohormons of growth and differentiation, play a vital role in catalysing transport of electrons in photosynthesis and have antioxidative, antifungal and antibacterial effects, thus contributing to the defence system of the plants (Harborne & Williams, 2000). This defence function might be supported by the many studies that indicate mutagenic responses of cell cultures to polyphenols like quercetin (Brusick, 1993). On the other hand, there is very little evidence to date to suggest that dietary polyphenols promote adverse metabolic reactions *in vivo* when consumed in nutritionally relevant and also much higher quantities. Polyphenols are partially responsible for the sensory and nutritional qualities of vegetal food, as astringency and bitterness of foodstuffs and beverages depends on their content of polyphenolic compounds. Several thousand of different flavonoids have been identified in plants, with large diversity in their structural features. Classification of flavonoids in different subclasses is based on variations in their carbon skeleton (Bravo, 1998). In Figure I.1, the six main subclasses are shown (Rice-Evans *et al.*, 1996).

The large structural diversity of flavonoids with presently more than 6000 different known forms (Harborne & Williams, 2000) is mainly due to different oxidation states of the heterocyclic ring, the pattern of hydroxylation, glycosilation, acylation with phenolic acids, and by the existence of stereoisomers, among other factors. Most flavonoids are usually found in plants bound to sugars as *O*-glycosides. Flavones may also occure as C-glycosides. The only exception to this rule are the flavanols, such as catechins and procyanidins, which are almost always present in the diet in the non-glycosilated form (Rice-Evans *et al.*, 1996). Predominantly, the bonds are beta-glycosidic and sugar moieties might be mono-, di- or oligosaccharides. The associated sugar moiety is very often glucose or rhamnose, but other sugars may also be involved (e.g. galactose, arabinose, xylose, glucuronic acid) (Manach *et al.*, 2004). The sugar molecules can bind to various positions in the parent flavonoid, although there is a preference for the 3-position (Hollman & Arts, 2000). About 150 naturally occurring glycosides of quercetin alone have been described (Williams & Harborne, 1994). Structures of selected flavonols are shown in Figure I.2. Free flavonoids, i.e. flavonoids without attached sugars, are named aglyca. Aglyca of flavonoids may be present in plant-derived food mainly as a result of storage and processing (Hollman & Arts, 2000).

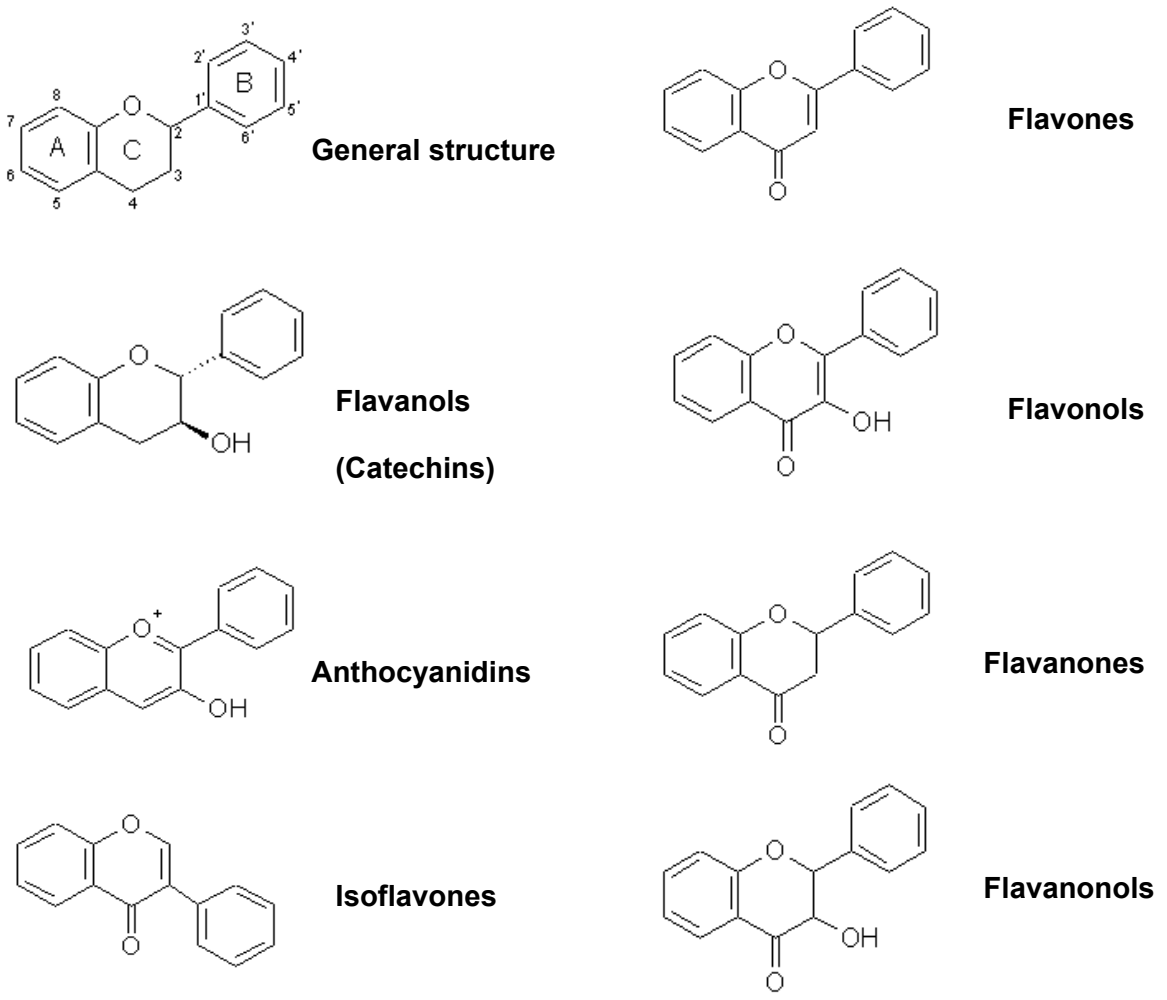
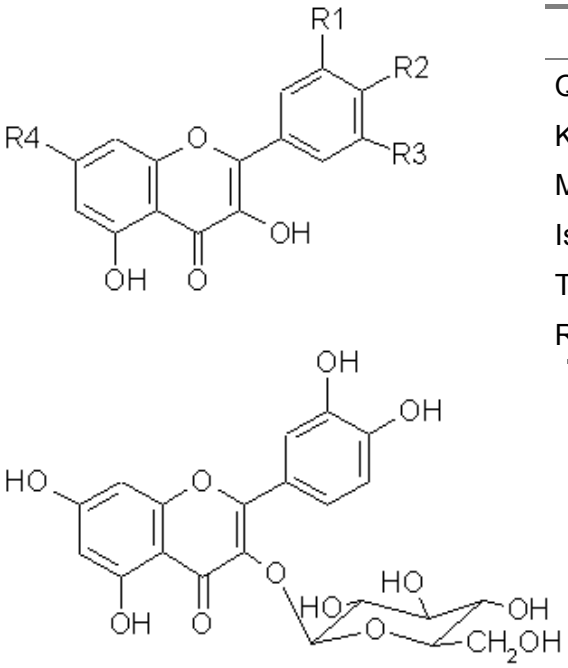


Figure I.1: Basic chemical structures of the main flavonoid subclasses

Synthesis of flavonoids by plants depends on light. Therefore, highest concentrations of flavonols and flavones occure in the epidermis of the sun-exposed parts of leafs and in the peel of fruits while only trace amounts are found below the soil surface (Hertog, 1996). An exception are onions which contain a large amount of quercetin glucosides (Price & Rhodes, 1997). Marked differences in concentrations of flavonoids exist between pieces of fruit in the same tree and even between different sides of a single piece of fruit, depending on exposure to sunlight (Price *et al.*, 1995). Traditionally used herbs and medicinal plants often have a high flavonoid content by nature, e.g. calendula flowers, ginkgo biloba leafs, elder flower, goldenrod, red clover or fennel seeds (Pietta *et al.*, 2003).

Flavonols, a sub-group of flavonoids, are present in human diets predominantly as quercetin and kaempferol. In vegetables, quercetin glycosides predominate, but glycosides of kaempferol, luteolin, and apigenin are also present (Herrmann, 1988). Quercetin levels in

vegetables were found to be generally below 10 mg kg^{-1}, except for onions, beans, broccoli and kale. Seasonal variations were found to be large in leafy vegetables such as lettuce and endive, which agrees with the light-dependency of flavonoid synthesis (Hertog *et al.*, 1992). Fruits contain almost exclusively quercetin glycosides (Herrmann, 1988). In most fruits the quercetin content averages 15 mg kg^{-1}, except for apples, apricot and black currants (37 mg kg^{-1}) (Hollman & Arts, 2000).



	R1	R2	R3	R4
Quercetin	OH	OH	H	OH
Kaempferol	H	OH	H	OH
Myricetin	OH	OH	OH	OH
Isorhamnetin	OCH_3	OH	H	OH
Tamarixetin	OH	OCH_3	H	OH
Rhamnetin	OH	OH	H	OCH_3

Isoquercitrin

(= Quercetin-3-*O*-Glucoside, Q3G)

Figure I.2: Chemical structure of selected flavonols

Apart from sun exposure, numerous additional factors may influence the flavonoid content of plants: variety, environmental factors like pathogen exposure, ripeness at the time of harvest, processing and storage (Asami *et al.*, 2003). As flavonoids are not evenly distributed in plant tissue, food fractionation during processing may result in a loss or enrichment. Simple peeling of fruits and vegetables can eliminate significant portions of polyphenols. In apples, for example, quercetin is located in the peel of certain cultivars with up to 1 g kg^{-1} fresh weight, while the peeled fruit contains no more quercetin glycosides at all (Burda *et al.*, 1990). Cooking may also have major effects. Onions and tomatoes, for example, lose ~75-80% of their initial quercetin content after boiling for 15 min, 65% after cooking in a microwave oven, and ~30% after frying (Crozier *et al.*, 1997). Mean levels of some flavonoids in selected plant food (raw material) are shown in Table I.1.

Table I.1: Flavonoids content in selected plant food[1]

Food source	Flavonoid	Content
Apples	Quercetin	20-36[2]
	(+)Catechin	0-17[3]
	(-)Epicatechin	2-101[3]
Apricot	Quercetin	25-26[2]
	(+)Catechin	26-57[3]
	(-)Epicatechin	67-171[3]
Bean, French	Quercetin	39[2]
	Kaempferol	<12[2]
Black currant	Quercetin	37[2]
	Kaempferol	1[2]
Broccoli	Quercetin	43[4]
	Kaempferol	94[4]
Endive	Quercetin	<1[2]
	Kaempferol	44-246[5], 46[2]
Grape, black	Quercetin	15[8]
	Myricetin	5[2]
Grape, white	Quercetin	12[8]
	Myricetin	5[2]
Kale	Quercetin	110[8]
	Kaempferol	211[8]
Onion	Quercetin	86-1694[7], 347[8]
Pear	Quercetin	6[8]
Plum	Quercetin	9[8]-15[2]
Salad varieties/ lettuce	Quercetin	0-157[5]
Strawberry	Quercetin	9[8]
	Kaempferol	12[8]
Tea infusion, black and green	Quercetin	10-25[9]
	Kaempferol	7-17[9]
	Myricetin	2-5[9]
	(-)Epicatechin	31-79[2]
Tomato	Quercetin	1-22[6], 8[8]
	Kaempferol	0-1[6]
Wine, red	Quercetin	4-16[9]
	Myricetin	7-9[9]
	(+)Catechin	0-208[2]
	(-)Epicatechin	15-88[2]

[1](Hollman & Arts, 2000), modified. Contents in mg kg^{-1} fresh weight or mg L^{-1} (only edible portions are considered), [2](Hollman & Arts, 2000), [3](Risch & Herrmann, 1988), [4](Price *et al.*, 1997), [5](DuPont *et al.*, 2000), [6](Stewart *et al.*, 2000), [7](Price & Rhodes, 1997), [8](Hertog *et al.*, 1992), [9](Hertog *et al.*, 1993a).

Because flavonoids are ubiquitously present in the plant kingdom, daily consumption of flavonoids with plant food can be assumed (Kühnau, 1976). The amount of daily intake depends on the specific diet of an individual and also varies with the flavonoid content in vegetal food and, therefore, is difficult to estimate. In addition, a lack of standardized analytical methods and the structural diversity of flavonoids have complicated estimation of their food content. In 1976, Kühnau *et al.* (1976) estimated intake of flavonoids in the USA to be as high as 1 g per day, which nowadays is regarded as overestimated (see below). Based on the determination of the content of five major flavonoids in food (Hertog *et al.*, 1992; Hertog *et al.*, 1993a), the association between intake of these flavonoids and disease occurrence in human subjects became calculable. Since March 2003, a database in which the flavonoid contents of 225 selected food and beverages are compiled from 97 bibliographic sources has been available on the US Departement of Agriculture webside (www.nal.usda.gov/fnic/foodcomp). Generally, databases are currently established (Watanabe *et al.*, 2004). This should allow more accurate estimations of dietary intake from dietary consumption monitoring. With regard to the fact that flavonoids are naturally present as glycosides in plants and the varying bioavailability of different flavonoid glycosides, however, compiling food composition tables with individual glycosides rather than the aglyca should be considered (Arts *et al.*, 2004).

In Western Europe, main intake of flavonoids with diet takes place in form of apples, onions, broccoli, berries, black and green tea, chocolate and red wine. Consumption of flavonols has been estimated to be in the range of 20-25 mg d^{-1} in Denmark, The Netherlands, and the USA. Quercetin intake was shown to vary from 3-34 mg d^{-1} (reported for 10^{th} and 90^{th} percentile, respectively) in between Dutch individuals, with a medium of 16 mg d^{-1} (Hertog *et al.*, 1993c). Mean consumption of flavonols and flavones in The Netherlands was 23 mg d^{-1} (n = 509), whereby quercetin accounted for the highest portion (~65%), as was similarily observed by Sampson *et al.* (2002) for US American health professionals (~75%)(n = 116 772). In another huge cohort study on 34 789 male American health professionals, the average total intake of flavonols and flavones was estimated to be 20.1 mg d^{-1}; the 3 primary flavonols ingested were quercetin (15.4 mg d^{-1}), kaempferol (3.6 mg d^{-1}), and myricetin (0.9 mg d^{-1}), composing more than 90% of the flavonoids ingested by the study cohort (Rimm *et al.*, 1996). In a Finnish cohort, median flavonoid intake was only 3.4 mg d^{-1}, but ranged from 0-41.4 mg d^{-1}. On average, about 95% of the total flavonoid intake was quercetin. The main sources of flavonoids were apples and onions (Knekt *et al.*, 1996). Using data from the Seven Countries study, Hertog *et al.* (1995) stated that in West Finland the only dietary flavonoid might be

quercetin. Taken together, quercetin intake reported in this study varied from 2.6 (West Finland) to 34.6 (Japan) mg d^{-1} with an average of ~18 mg d^{-1} throughout several regions of Europe, the USA and Japan. Arai *et al*. (2000) calculated an amount of 9.3 mg d^{-1} quercetin consumed by 115 Japanese women, with 16.7 mg d^{-1} as the sum of flavonols and flavones. Mean intake of flavonols in the German population was calculated using data from the National German Food Consumption Survey by Böhm *et al.* (1998). According to this analysis, daily per capita intake was 11.5 mg flavonols, mainly derived from fruits and vegetables, but also from black tea and red wine. In the Bavarian population, mean daily flavonol consumption was ~12 mg, with quercetin accounting for the largest portion of 10.3 mg (Linseisen *et al.*, 1997). Total flavonoid intake in Bavaria was 54 mg d^{-1}.

Thus, quercetin constitutes at least a significant percentage of total daily dietary flavonoid intake. Additionally, it is one of the most intensively studied polyphenolic compound in human diet due to its particular biological activities (Scalbert & Williamson, 2000). In comparison to the amount of dietary intake of some well established vitamins, e.g. vitamin C (~115 mg d^{-1}), vitamin E (~14 mg α-tocopherol equivalents d^{-1}), and pro-vitamin A (β-carotene, ~2.8 mg d^{-1}) (Karg, 2004), the level of daily flavonoid intake is considerably high.

I.3. Bioavailability of flavonols

Biological effects of any substance depend on its bioavailability. After giving a brief introduction into bioavailability and pharmacokinetic parameters in general, this chapter focuses primarily on those aspects concerning the bioavailability of flavonols, with special emphasis on quercetin, with regard to the subject of this thesis.

I.3.1. GENERAL ASPECTS OF BIOAVAILABILITY

Bioavailability of a substance is determined by its absorption, disposition, metabolism, and excretion (ADME). Drug metabolism occurs already during *first pass* (in the gut mucosa and liver) and thereafter by *second pass* (organ/tissue specific) metabolism. In a nutritional sense, apart from the amount of a dietary substance and its effective metabolites reaching the systemic circulation (and thereby other body tissues), the critical concentration required for biological activity as well as its biological effect(s) are also comprised in the definition of bioavailability. *In vitro* or cell culture experiments can only address partial aspects of bioavailability, such as cellular mechanisms of absorption, biological effects on certain cell types, etc. Thus, estimation of true bioavailability can only be done from experiments using intact individuals.

Bioavailability of a compound is usually determined by its concentration-time profile in the systemic circulation. One has to keep in mind, however, that blood sampling does not yield information on organ- or tissue-specific distribution, accumulation, and metabolism.

Pharmacokinetic parameters of a substance are derived from the plasma concentration-time curve. The area under the plasma concentration-time curve (AUC) is generally used as a measure of bioavailability (Figure I.3). Other parameters of interest with regard to bioavailability of a substance are the maximal plasma concentration (c_{max}), time until c_{max} is reached (t_{max}), the velocity of absorption (k_a, $t_{a1/2}$), elimination kinetics (k_{el}, $t_{1/2}$), and the timeframe of presence of effective concentrations in the body. Accuracy of the plasma concentration-time curve and of calculated parameters, of course, strongly depends on the frequency of sampling.

For calculation of the absolute oral bioavailability (BV_{abs}) of a substance, AUC_{po} (after oral intake) is related to the AUC_{iv} (after intravenous injection, normalized to the same dose), with $BV_{abs} = (AUC_{po}/AUC_{iv}) \times 100\%$ (Chen *et al.*, 2005). In contrast, relative oral bioavailability

is calculated from orally given doses. The effect of an experimental variant is thereby evaluated by comparing the AUC_{po} measured in the experimental setting to the AUC_{po} measured in a control setting of the experiment, e.g. a different oral formulation.

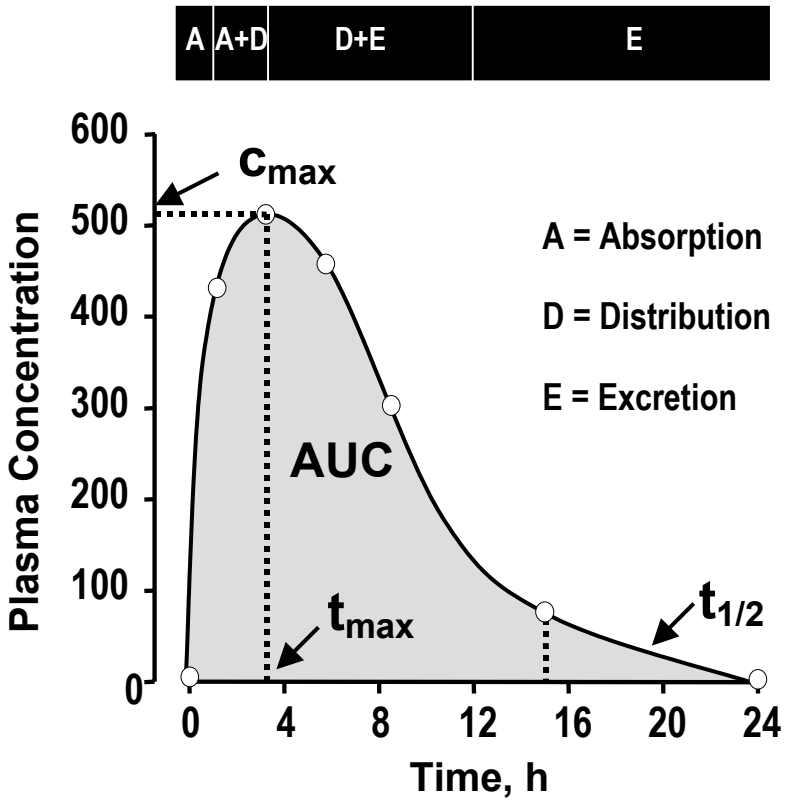


Figure I.3: Model of a plasma concentration-time curve after administration of a single oral dose

AUC, area under the curve; c_{max}, maximum plasma concentration; t_{max}, time at maximum plasma concentration; $t_{1/2}$, terminal elimination half-life.

I.3.2. BIOAVAILABILITY OF QUERCETIN

I.3.2.1. Absolute systemic availability

Data on disappearance from the gut (apparent absorption) are often the only quantitative data available from human studies. In a study by Hollman *et al.* (1995), half of the quercetin glycosides from onions disappeared in ileostomized but otherwise healthy volunteers (n = 9) until the end of the small intestine and were considered to be absorbed. Furthermore, disappearance of 24% quercetin aglycone and 17% rutin (quercetin-3-*O*-glucorhamnoside) have been measured in the same study. Walle *et al.* (2000) calculated an even higher systemic availability of quercetin from quercetin monoglucosides (within the range of 65-81%) in ileostomized volunteers (n = 4). It has to be kept in mind, however, that compared to the normal situation, an increased number of micro-organisms might have been present at the end of the small intestine in ileostomy patients (Graefe *et al.*, 1999). In a human intestinal perfusion study (proximal jejunum), ~60% of quercetin-3-*O*, 4'-*O*-diglucoside (Q3,4'diG) disappeared from an onion and broccoli containing extract (Petri *et al.*, 2003).

In an isolated rat small intestine perfusion model, quercetin absorption was 38% in the jejunum and 19% in the ileum during 90 min of perfusion at a rate of 8 mL min^{-1} (Spencer & Rice-Evans, 2003). In this study, however, mucosal and biliary elimination was not considered. Crespy *et al.* (2003) found bioavailability of quercetin of only 9% in a *in situ* perfusion study on rats, which was the lowest of all flavonoids examined (9-49%). The low systemic availability of quercetin was due to a high secretion of conjugated quercetin and quercetin derivatives by the liver and intestinal mucosa. First pass elimination was highest for quercetin (52% mucosal re-secretion plus 6% biliary secretion) among the flavonoids investigated. Absorption of at least 20% of labelled quercetin from the digestive tract was deduced from recovery of radioactivity in the bile and urine of rats in an early study (Ueno *et al.*, 1983). Recently, however, Graf *et al.* (2005) found only 6% of an oral quercetin dose (4.9 mg kg^{-1} BW) to be absorbed into the systemic circulation of rats, using radioactively labelled quercetin-4'-*O*-glucoside (Q4'G). After applying a dose of 10 mg kg^{-1} BW to rats, Chen *et al.* (2005) even observed a total bioavailability of 48% for quercetin. In pigs, after intake of 50 mg quercetin kg^{-1} BW, Ader *et al.* (2000) determined a 17% total bioavailability of the aglycone in comparison to i.v. application.

Thus, data on systemic availability of quercetin vary substantially between studies, the methods applied, and the species investigated.

I.3.2.2. Mechanisms of intestinal absorption

During the last decade, knowledge about the mechanisms involved in the intestinal absorption of flavonols has consistently improved, although several questions still remain open. The small intestine is one of the major sites for absorption of dietary flavonols. Thus, quercetin monoglucosides are absorbed from the small intestine, as can be deduced from the fast appearance (within 30 min) of quercetin derivatives in blood of men and animals after oral intake (Cermak *et al.*, 2003; Olthof *et al.*, 2000; Hollman *et al.*, 1997). Quercetin from more complex glycosides, like rutin, however, is absorbed from the distal small and the large intestine (Morand *et al.*, 2000b). Quercetin itself is a rather lipophilic molecule, which may passively diffuse through cell membranes (Walgren *et al.*, 2000a; Movileanu *et al.*, 2000; Walgren *et al.*, 1998; Saija *et al.*, 1995). In rats, absorption of quercetin may even occur from the stomach (Crespy *et al.*, 2002). In contrast, quercetin glycosides, the predominant form in food (chapter I.2.), are relatively polar by nature, and this strongly limits their passive transcellular diffusion.

Enzymatic hydrolysis of quercetin monoglucosides is assumed to take place either in the gut lumen (Walle *et al.*, 2000) or, in some cases, intracellularly after uptake into enterocytes. β-Glycosidic bonds are resistant to hydrolysis by pancreatic enzymes (Arts *et al.*, 2004). An intracellular β-glucosidase (CBG, EC 3.2.1.21), however, with a rather broad substrate specificity, purified from jejunal cells of rats and both human small intestinal and liver cells, can cleave at least some flavonol monoglucosides, e.g. Q4'G, but only weakly affects glucosides linked in the 3-position, like quercetin-3-*O*-glucoside (Q3G) (Day *et al.*, 1998; Ioku *et al.*, 1998). Intracellular β-glucosidase has also been found in pig jejunal mucosa (McMahon *et al.*, 1997) and liver (Lambert *et al.*, 1999).

Intracellular cleavage of flavonol monoglucosides requires their mucosal uptake. For quercetin monoglucosides, experimental evidence for uptake into enterocytes via the intestinal sodium-dependent glucose co-transporter (SGLT1) has been presented (Wolffram *et al.*, 2002; Ader *et al.*, 2001; Walgren *et al.*, 2000b; Gee *et al.*, 1998). Investigations with everted rat jejunal sacs indicated that SGLT1 is capable of transporting quercetin monoglucosides (Gee *et al.*, 2000; Gee *et al.*, 1998). This finding was further substantiated by studies using human Caco-2 cells (an immortalized human colon cancer cell line), Chinese hamster ovary cells (G6D3 cells) stably transfected with SGLT1 (Walgren *et al.*, 2000b), and brush-border membrane vesicles from pig jejunum (Cermak *et al.*, 2004).

Cleavage of 3-*O*-linked monoglucosides requires presence of additional β-hydrolases in the gut. The brush border membrane (BBM) enzyme lactase-phloridzin hydrolase (LPH, EC 3.2.1.62) is another mammalian enzyme active against β-glycosidic bonds, cleaving both Q3G and Q4'G, but not rutin (Day *et al.*, 2003; Sesink *et al.*, 2003; Németh *et al.*, 2003; Day *et al.*, 2000b). LPH is located in the BBM of mucosal cells of the small intestine with the active site facing the intestinal lumen. The flavonol aglycone, liberated adjacent to the BBM by LPH, is assumed to passively diffuse across the BBM (Day *et al.*, 2000b). Stemming from shedded epithelial cells, CBG and LPH may also be present in the gut lumen (Walle *et al.*, 2000), but Petri *et al.* (2003) have shown that mucosal enterocytes, rather than luminal contents, are responsible for the majority (79-100%) of β-glycosidase activity in human jejunum.

In some studies evidence has been presented for a minor hydrolysis of dietary flavonol glycosides with large interindividual variability in the human oral cavity by both bacteria and shedded epithelia cells (Walle *et al.*, 2005; Parisis & Pritchard, 1983).

Sugar moieties containing rhamnose, for example as present in rutin, seem to sterically hinder cleavage of the β-glucosidic bond by endogenous enzymes and, thus, are generally not absorbed from the small intestine. Microbial α-rhamnosidase and β-glucosidase are needed to release the aglycone from such more complex glycosides (Day & Williamson, 2003; Erlund *et al.*, 2000; Crespy *et al.*, 1999) with subsequent absorbtion of quercetin from the distal small and the large intestine. Microbial degradation of liberated quercetin, however, may concurrently limit its bioavailability (chapter I.3.3.4.) (Cermak *et al.*, 2003; Morand *et al.*, 2000b).

Figure I.4 shows a model summarizing potential ways of the intestinal handling of polyphenols, which is unrestrictedly valid for flavonols (Scalbert & Williamson, 2000).

I.3.2.3. Metabolism, distribution, and elimination

Conjugation with glucuronic acid and sulfates are so-called phase II detoxification reactions in the metabolism of xenobiotics, rendering a substance more hydrophilic and, in turn, accelerating and increasing its renal and/or biliary excretion. Similarly, methylation is an inactivating reaction by blocking hydroxyl groups. Sulfation is a major pathway of metabolism at low concentrations for many xenobiotics, although this pathway can become saturated. Flavonols are to a great extend metabolised already during intestinal absorption within the intestinal mucosa and, subsequently, in the liver (*first pass*). This metabolisation

mainly includes *O*-methylation of hydroxyl groups at the catechol B-ring (catechol-*O*-methyltransferase, COMT, EC 2.1.1.6), glucuronidation (uridine-5'-diphosphate glucuronosyl-transferase, UGT, EC 2.4.1.17), and sulfation (sulfotransferase, EC 2.8.2.1) (Petri *et al.*, 2003; Donovan *et al.*, 2001).

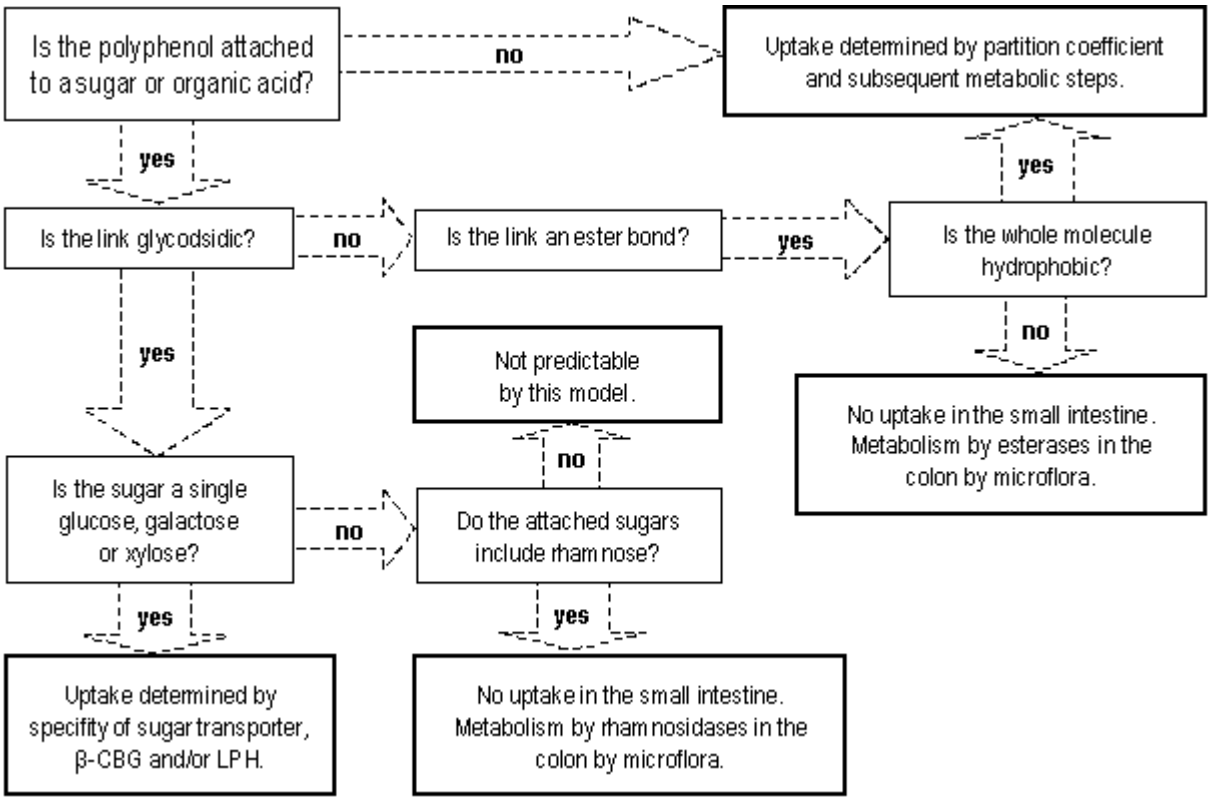


Figure I.4: Model for prediction of the absorption of polyphenols in humans based on evidence from *in vivo* and *in vitro* studies (Scalbert & Williamson, 2000)

In blood and urine from humans, at least 23 different metabolites of quercetin have been described including mixed sulfates, glucuronides, methylated derivatives and also glucosides (Mullen *et al.*, 2004). In rat plasma, more than 18 different flavonol metabolites with varying degrees of glucuronidation, methylation and/or sulfation were detected after intake of radioactively labelled Q4'G (Graf *et al.*, 2005; Mullen *et al.*, 2002). Intestinal conjugation

mechanisms are highly efficient, but statements on activities of the different enzymes involved vary. In a rat small intestine perfusion model, Spencer & Rice-Evans (2003) observed an almost complete (98%) glucuronidation of quercetin. In an *in situ* perfusion study with quercetin (15 nmol/min) in rats (bile was diverted), Crespy *et al*. (1999) observed 64% as glucuronidated derivatives and methoxylated forms of quercetin, 36% were present as sulfated forms, whereas no mixed glucurono-sulfoconjugates were detected in the intestinal effluent. Morand *et al.* (1998) found that in rats, after an adaption period of 3 weeks to a 0.2% quercetin-containing diet, the main circulating metabolites were sulfo-glucuronides (91.5%) of quercetin and isorhamnetin, whereas only 8.5% where pure quercetin glucuronides.

Highest activity of UGT for conjugation of quercetin within the intestinal tract of rats has been reported for the duodenum (~150 pmol mg protein^{-1} min^{-1}) in comparison to jejunum (~80 pmol mg protein^{-1} min^{-1}) and stomach (~60 pmol mg protein^{-1} min^{-1}) (Murota & Terao, 2005). No value for liver UGT conjugation activity on quercetin was reported in this study. An earlier study in rats, however, revealed that UGT activity on (-)epicatechin is higher in the mucosa of small and large intestine than in the liver, while highest activity of sulfotransferase occurred in liver (Piskula & Terao, 1998a). UGT in microsomes of rat liver were less active on quercetin than those of rat intestine (Morand *et al.*, 1998). In humans, UGT activity on quercetin in the liver has been observed (Day *et al.*, 2000a), but mRNA of additional UGT1A8 and UGT1A10, capable of flavonoid glucuronidation, has been isolated from jejunum, ileum, and colon but were not found in liver (Cheng *et al.*, 1999). Furthermore, mRNA of UGT1A1 was found to be induced immediately after intake of Q3,4'diG in isolated human enterocytes (Petri *et al.*, 2003). In general, after oral intake, the early occurrence of conjugated metabolites in plasma observed in different species points towards intensive *first pass* metabolism by the gut mucosa (Erlund *et al.*, 2000; Ader *et al.*, 2000; Morand *et al.*, 1998). In portal vein blood of pigs only conjugated quercetin was found, supporting the view that the small intestine is the major site of conjugation (Cermak *et al.*, 2003).

Although sulfation of quercetin may occur in intestinal cells, the liver was found to be mainly responsible for the formation of sulfo-conjugates of quercetin. Different recombinant human sulfotransferases show a high sulfating potential against flavonoids (Pai *et al.*, 2001). It is unclear, however, to what extend those enzymes are present in the small intestine. Studies on rats indicate that the origin of most circulating sulfated flavonoids is the liver (Donovan *et al.*, 2001; Piskula & Terao, 1998a), but sulfation has also been observed in the gut mucosa (Crespy *et al.*, 1999). Differences between species with respect to tissue distribution and

activity of sulfotransferases are likely, as sulfation of xenobiotics in pigs does not seem to take place (Caldwell, 1980).

O-Methylation of quercetin by COMT has been observed both in gut and liver of rats (Donovan *et al.*, 2001; Piskula & Terao, 1998a). Also in humans and in pigs, methylated metabolites of quercetin have been found after oral application (Hubbard *et al.*, 2003; Cermak *et al.*, 2003; Ader *et al.*, 2000; Manach *et al.*, 1998). Main sites of quercetin *O*-methylation are the 3'- and 4'-hydroxyl groups, yielding isorhamnetin or tamarixetin, respectively. Differences in the pattern of monomethylated flavonol metabolites have been observed between different species (see chapter IV).

In summary, flavonols undergo ample postabsorptive conjugation reactions within the gut mucosa and liver, explaining the absence of free aglyca in plasma in any species investigated after oral intake of either free quercetin or quercetin glycosides (Cermak *et al.*, 2003; Day *et al.*, 2001; Manach *et al.*, 1999).

Because quercetin conjugates can be retrieved from the gut lumen of bile-diverted animals after perfusion of the intestine with quercetin containing solutions, direct mucosal re-secretion of conjugates back into the lumen can be postulated (Crespy *et al.*, 2003; Crespy *et al.*, 1999). Apical efflux was indeed identified to be mediated by several transport proteins of the BBM, belonging to the family of so-called ATP-binding cassette (ABC) transporters such as the multidrug resistance associated protein 2 (MRP2), p-glycoprotein (p-gp) and breast cancer restistance protein 1 (BCRP1) (see chapter I.3.3.3.) (Ofer *et al.*, 2005; Sesink *et al.*, 2005; Wang *et al.*, 2005).

Flavonols that leave the enterocytes during intestinal absorption at the basal pole, seem to generally enter into blood capillaries and are transported via the portal vein towards the liver (Cermak *et al.*, 2003). Murota & Terao (2005) recently reported that quercetin and metabolites were additionally found in the lymph of rats (c_{max} = 3.1 µM, t_{max} = 30 min) after administration of 30 µmol quercetin kg^{-1} BW. In this study, quercetin was solubilised in propylene glycol and after intragastric application, a NaCl solution containing glucose was continuously applied.

A major portion of flavonols are excreted into bile already during the first passage through the liver. In rats, ~6% of a perfused amount of quercetin (~14 nmol/min) was found to take this route (Crespy *et al.*, 2003). These findings are supported by other bioavailability studies on

rats, where also significant amounts of quercetin have been found in bile (Arts *et al.*, 2004; Crespy *et al.*, 2002).

Ueno *et al.* (1983) reported that more than 30% of quercetin was detected as CO_2 72 h after intraperitoneal injection of 315 mg kg^{-1} BW, whereas in bile duct-cannulated rats (secretion into the gut lumen via bile is blocked) no exhalation of labelled CO_2 was detected. Because degradation of quercetin to CO_2 is due to bacterial metabolism, this finding exemplifies that biliary excretion of quercetin is a major elimination pathway. The amount of flavonoids excreted via bile, however, strongly depends on the nature of the flavonoid. Thus, only 1% of a perfused dose of catechin (flavanol), was found to be secreted into bile in rats, whereas genistein (isoflavone) was extensively secreted (32%) (Crespy *et al.*, 2003). Flavonols and their conjugates excreted into the bile or by mucosal mechanisms may partially be re-absorbed, but eventually reach the large intestine, where they can be hydrolysed by microbial enzymes and either are re-absorbed or further degraded (chapter I.3.3.4.).

Elimination half-life of quercetin after oral intake was observed to be 11-28 h in humans (Manach *et al.*, 2005; Graefe *et al.*, 2001; Hollman *et al.*, 1997) in comparison to 3.8 h in pigs (Ader *et al.*, 2000), and more than 8 h in rats as derived from (Manach *et al.*, 1997). Walle *et al.* (2001) even found the half-life of radioactively labelled quercetin metabolites (not necessarily intact flavonols) in human plasma ranging from 20-72 h. The long half-life would favour accumulation in plasma after repeated intake.

In blood, protein binding of quercetin is more than 98% (Gugler *et al.*, 1975), with albumin being the most abundant carrier protein in human blood (~40 mg L^{-1}). In serum, albumin major regions for binding are located in subdomains IIA and IIIA, providing hydrophobic pockets (He & Carter, 1992). Flavonoids in general display moderate binding affinities for albumin, with flavonols and flavones being most tightly bound (Dufour & Dangles, 2005). Glycosilation and sulfation, however, lower the affinity of flavonoids to albumin by one order of magnitude depending on the conjugation site (Dufour & Dangles, 2005). The slow systemic elimination of quercetin in humans might be at least partially explained by its particularly high binding affinity to human serum albumin (Dufour & Dangles, 2005; Zsila *et al.*, 2003; Manach *et al.*, 1995). Significant differences with respect to affinity and binding site were observed for the highly homologous human (HSA) and bovine (BSA) serum albumin, and may therefore contribute to different plasma half-lifes in different species (Dufour & Dangles, 2005).

In humans, elimination of flavonols from the systemic circulation into urine seems to account for only a minor portion. Renal excretion of conjugated quercetin after intake of quercetin glucosides (50 mg aglycone equivalents) accounts for ~2.5% (0.3-6.4%) of the ingested dose, as deduced from 14 bioavailability studies (Manach *et al.*, 2005). Excreted quercetin and kaempferol in human urine are mainly in the form of glucuronides (Wang *et al.*, 2003). Urinary recovery of other flavonoids, like catechins or isoflavones, is higher than that of quercetin in humans (Scalbert & Williamson, 2000). In rats, after i.v. application of quercetin, Q3G and rutin, 2.4, 6.7, and 9.2% of the applied dose were retrieved from urine (Choudhury *et al.*, 1999). After oral application of a high dose of ^{14}C-quercetin (630 mg kg^{-1} BW) to bile-duct cannulated rats, ~9% of the radioactivity was excreted via urine (Ueno *et al.*, 1983).

Tissue distribution of circulating flavonols has not been thoroughly investigated until now. Thus, the ability of flavonols to accumulate within target tissues is so far mainly a matter of speculation as data are scarce, even from animal studies. In a pilot study, de Boer *et al*. (2005) have studied the tissue distribution of quercetin in rats (n = 6 per group) and, in a preliminary experiment, also in pigs (n = 2). After supplementation of rats with ~50 or 500 mg quercetin kg^{-1} BW d^{-1} for 11 weeks, the highest flavonol concentration was present in lungs (4.0 and 15.3 nmol g^{-1} tissue) and the lowest in brain, white adipose tissue, and spleen. In the pigs, supplemented for 3 d with 500 mg quercetin kg^{-1} BW d^{-1}, the highest concentration was found in liver (5.9 nmol g^{-1}) and kidney (2.5 nmol g^{-1}), but no samples from lung tissue were taken in this species. Accumulation of flavonols in plasma and heart was lower in pigs than in rats, whereas distributions in liver, brain and spleen did not differ. It should be emphasized, however, that organ concentrations clearly remain below the plasma concentration of quercetin and quercetin derivatives.

It is assumed that cleavage of glucuronides in target tissues might occur (de Boer *et al.*, 2005; O'Leary *et al.*, 2003; O'Leary *et al.*, 2001). Shimoi *et al.* (2001) suggested that inflammation enhances the release of aglyca from glucuronides because β-glucuronidase activity is elevated in this situation. Thus, despite the excessive postabsorptive metabolisation of flavonols described above, the presence of aglyca in tissues might be conceivable.

I.3.3. FACTORS INFLUENCING INTESTINAL ABSORPTION OF QUERCETIN

Numerous factors may influence the amount of intestinally absorbed quercetin, including the type and linkage position of sugar moieties, the vehicle of application, the degree of solubilisation, food matrix effects, and food composition (Graefe *et al.*, 2001; Erlund *et al.*, 2000; Hollman *et al.*, 1999; Hollman *et al.*, 1997). In addition, individual factors such as enzyme activities, including polymorphisms, and composition of the intestinal microflora are of relevance. These influencing factors are reviewed in this section.

I.3.3.1. Physico-chemical aspects

Chemical form

In this context, the pattern of glycosilation is considered as chemical form. In general, quercetin monoglucosides, such as Q3G, are better bioavailable than the aglycone and more complex glycosides. This was observed in pigs (Cermak *et al.*, 2003; Ader *et al.*, 2000), humans (Olthof *et al.*, 2000; Hollman & Katan, 1997; Hollman *et al.*, 1995), and rats (Gee *et al.*, 2000; Morand *et al.*, 2000a; Morand *et al.*, 2000b). The higher bioavailability of quercetin from monoglucosides is essentially due to the involvement of LPH, which promotes uptake of aglyca deriving from monoglucosides (see chapters I.3.2.1. and I.3.3.2.). Furthermore, SGLT1 may facilitate uptake of quercetin monoglucosides, such as Q3G and Q4'G, across the BBM into enterocytes (chapter I.3.2.1.). No aglyca or glycosides other than monoglucosides, however, are transported by SGLT1 (Wolffram *et al.*, 2002; Ader *et al.*, 2001; Gee *et al.*, 1998). More complex flavonol glycosides need microbial cleavage before liberation of the aglyca and intestinal uptake (chapter I.3.2.1.).

Solubility

The octanol/water partition coefficient K_{part} (= P), expressed as log P, is a measure for the lipophilicity of a substance and provides information on the efficacy of passive transfer of a substance across membranes (Crespy *et al.*, 2003). In general, a positive log P value indicates a lipophilic substance and a negative value a hydrophilic substance; log P = 0 indicates an equal partition between both phases. According to Brown *et al.* (1998), log P of quercetin is 0.08, suggesting an approximately equal distribution between aqueous and lipophilic compartments. The coefficients of kaempferol (1.84) and luteolin (1.35) indicate a better solubility in the hydrophobic environment, which can be explained by fewer hydroxyl groups

of these compounds compared to quercetin. Rothwell *et al.* (2005) reported log *P* values for quercetin and kaempferol, respectively, of 1.82 and 3.11. Log *P* values for quercetin and kaempferol of 2.74 and 2.69 were calculated by Sergedienė *et al.* (1999), and of 2.15 and 2.94 by Crespy *et al.* (2003). Discrepancies of log *P* values are most likely attributable to different calculation/measurement methods applied.

In accordance with the more lipophilic nature of quercetin aglycone, Azuma *et al.* (2002) observed, that co-administration of lipids and emulsifiers enhanced the absorption of the aglycone. They found an accelerated and enhanced uptake of quercetin in rats when quercetin aglycone was administered in water supplemented with 3% (wt/v) emulsifiers (sucrose fatty acid ester, polyglycerol fatty acid ester, or sodium taurocholat) and either 10% (wt/wt) lecithin or 20% (wt/wt) soybean oil. However, administration with pure lecithin or soybean oil in water without additional emulsifiers did not significantly enhance uptake of quercetin. In this context, bile constituents, such as sodium taurocholat and phospholipids are considered to play an important role in flavonol absorption (Azuma *et al.*, 2002; Piskula & Terao, 1998b).

According to Piskula & Terao (1998b), the solubility of quercetin at a dose of 5 mg mL^{-1} decreases in a 25% propylene glycol/water (v/v) mixture by almost 3 orders of magnitude and in pure water by 5 orders of magnitude as compared to solvation in pure propylene glycol, which is in good agreement with results from Azuma *et al.* (2002). However, no direct correlation between the extent of absorption and quercetin´s solubility could be established (Piskula, 2000).

Organic solvents, such as DMSO, propylene glycol, or solutions containing more than 30% ethanol, are frequently used vehicles for administration of quercetin in rat studies (Azuma *et al.*, 2002; da Silva *et al.*, 1998; Ueno *et al.*, 1983), leading to a higher flavonol absorption under experimental conditions (Piskula & Terao, 1998b). Flavanols have also been administered complexed with phospholipids to achieve a higher bioavailability (Pietta *et al.*, 1998).

I.3.3.2. Lactase-phloridzin hydrolase (LPH)

LPH (EC 3.2.1.62) is a mammalian β-glycosidase present in the BBM of the small intestine (chapter I.3.2.1.). The enzyme is mentioned here again because of its pivotal role in the rapid *in vivo* uptake of quercetin from quercetin monoglucosides (Day *et al.*, 2000b). It has become

increasingly clear during the last years that LPH is a major determinant for the intestinal handling of quercetin monoglucosides (Day *et al.*, 2003; Sesink *et al.*, 2003). For example, in a study on *in situ* perfused rats (jejunum and ileum) after selective inhibition of LPH, a 67% reduction of Q3G hydrolysis and, concomitantly, a 75% reduction in quercetin plasma levels as compared to rats without LPH inhibition was demonstrated (Sesink *et al.*, 2003).

LPH is traditionally regarded to be a β-galactosidase necessary for digestion of the disaccharide lactose. In *in vitro* studies with purified LPH from lamb it was confirmed that several quercetin glucosides are substrates for this enzyme (Day *et al.*, 2000b). Somewhat surprising, it was consistently shown that LPH exerts a low activity against quercetin-3-*O*-galactoside (Arts *et al.*, 2004; Németh *et al.*, 2003). Thus, it must be assumed that the spatial configuration of the hydroxyl group in the C4-position of the sugar, marking the difference between glucose and galactose, is a major determinant for hydrolysis by LPH (Arts *et al.*, 2004). According to Day *et al.* (2000b) the flavonols Q3G and Q4'G are substrates for the lactase domain and not for the phloridzin hydrolase domaine of LPH, as may have been expected due to the flavonoid structure of phloridzin.

LPH levels vary widely, with 75% of the worlds population showing lactose maldigestion caused by physiologically low levels of LPH in adulthood. This may have implications for flavonol absorption (Day & Williamson, 2003), although a correlation of quercetin bioavailability and LPH activity has not been established yet.

I.3.3.3. Intestinal efflux transporters

Conjugated flavonol metabolites and flavonol monoglucosides (Q4'G) were found to be partly re-secreted into the gut lumen by enterocytes (Walgren *et al.*, 2000a; Crespy *et al.*, 1999). Apical efflux is mediated by members of the ABC transport protein family present at the BBM, such as the multidrug resistance associated protein MRP2, p-glycoprotein (p-gp) and breast cancer resistance protein BCRP1 (Ofer *et al.*, 2005; Sesink *et al.*, 2005; Wang *et al.*, 2005). These export carriers play a central role in the intestinal defence of organisms against toxic compounds.

In humans, evidence for mucosal re-secretion was presented for the first time in ileostomy volunteers (Walle *et al.*, 2000). Subsequently, efflux transporters have been studied in different model systems. In rats, luminal efflux of quercetin conjugates out of enterocytes up

to 52% of an applied dose was observed, much higher than their biliary secretion (6%) (Crespy *et al.*, 2003; Crespy *et al.*, 1999).

The spectrum of substrates of MRP2 comprises mainly organic anions, like glutathione and glucuronic acid conjugates of lipophilic compounds (Borst & Elferink, 2002). During studies in Caco-2 cells and Chinese hamster ovary cells (G6D3 cells) stably transfected with SGLT1, net uptake of quercetin monoglucosides was only observed after inhibition of MRP2 (Walgren *et al.*, 2000a; Walgren *et al.*, 2000b). In these studies, transport of Q4'G by MRP2 was reported for the first time. Tissue distribution of MRP2 is restricted to apical membranes of polarized cells and the transporter is mainly expressed in liver, intestine and kidney (van Zanden *et al.*, 2005; Walgren *et al.*, 2000a).

P-glycoprotein is a 170 kDa plasma glycoprotein encoded by human MDR1 gene (Zhou *et al.*, 2004). It is expressed constitutively in a number of tissues, and is found at high levels on the apical surface of epithelial cells in the liver (bile canaliculi), kidney (proximal tubulus), pancreas (pancreatic ductal cells), small intestine and colon (columnar mucosal cells), and adrenals. Moreover, p-gp is present at the blood-brain barrier, limiting penetration of a number of drugs into the brain (Zhou *et al.*, 2004). Similar to MRP2, p-gp is involved in the development of drug resistance. The described effects of flavonoids, including quercetin, on p-gp function are in part contradictory (Ofer *et al.*, 2005; Wang *et al.*, 2005).

BCRP1, a transport protein initially discovered in breast cancer cells, has a relatively broad tissue distribution and functions similar to MRP2. Expression of BCRP1 in human jejunum is comparable with MRP2 (Dietrich *et al.*, 2003). It was recently shown that quercetin aglycone and its glucuronide conjugates are substrates for BCRP1 (Sesink *et al.*, 2005).

I.3.3.4. Intestinal microflora

In humans colonic contents contains ~10^{10}-10^{12} bacteria g^{-1} (Day & Williamson, 2003). This microflora disposes of a range of enzymes that differ from endogenous enzymes present in the gut. The microbes may cleave sulfates, glucuronides and glycosides. Thus, for example, rutin and quercetin-3-rhamnoside require microbial enzymes for their deglycosilation. They are hydrolysed by strains of colonic *Bacteroides distasonis*, *B. uniformis*, and *B. ovatus*, expressing α-rhamnosidase and β-glucosidase (Bokkenheuser *et al.*, 1987). Free aglyca may diffuse passively through the gut wall (chapter I.3.2.1.) or are further degraded (see below).

In humans, the strictly anaerobic bacterium *Eubacterium ramulus* occurring at numbers of approximately 10^8 g^{-1} dry faeces has been identified as one major quercetin degrading micro-organism, capable of growing on Q3G as the sole carbon and energy source (Braune *et al.*, 2001). *Eubacterium ramulus* can degrade a number of flavonoids, including Q3G, quercetin and kaempferol, to phenolic acids, being capable of cleaving the flavan ring system in the presence of glucose (Blaut *et al.*, 2003). Phenolic metabolites of flavonoids from microbial degradation are mainly derivatives of phenylpropionic, phenylacetic and benzoic acids with different hydroxylation and methylation patterns (Gonthier *et al.*, 2003). In the case of flavonols, relevant degradation products are 3,4-dihydroxybenzoic, 3,4-dihydroxyphenyl-acetic, and 3-methoxy-4-hydroxybenzoic acid (Aura *et al.*, 2002; Pietta *et al.*, 1997; Merfort *et al.*, 1996). Simple phenolic acids may account for 30-60% of the ingested flavonols (Pietta, 2000). After absorption from the colon, they may contribute to changes in total antioxidant capacity of plasma, as some of them still possess a catechol structure (Scalbert & Williamson, 2000).

Carbon dioxide (CO_2), detected in exhaled air and stemming from microbial degradation of quercetin, has been identified to be another major metabolite. In three healthy volunteers, 23-81% of orally (100 mg) and intravenously (2.5 mg) applied ^{14}C labelled quercetin appeared as CO_2 (Walle *et al.*, 2001). In intact rats, Ueno *et al.* (1983) found more than 30% of a high dose ^{14}C-quercetin (630 mg kg^{-1} BW) to be decomposed to CO_2 within 24 h after oral administration by gavage.

Although not of direct relevance for flavonols, it is worthy to mention, that microbial metabolism may also yield metabolites with an enhanced biological activity compared to the parent compound. The most prominent example is the isoflavone daidzein, which is converted to equol by the gut flora in ~30-40% of the human population. There is emerging evidence, that equol producers demonstrate stronger effects of some biomarkers, such as bone mineral density, after isoflavone consumption, compared to non-producers, due to the high estrogenic activity of equol as compared to its parent substance daidzein (Setchell *et al.*, 2002).

Enterohepatic recirculation

Conjugated flavonols re-enter the small intestine via bile or directly by mucosal re-secretion (chapter I.3.2.2.). The secreted conjugates are unlikely to passively diffuse across the intestinal wall due to a negative charge at physiological pH (Williamson *et al.*, 2000). In the distal ileum and in the colon, however, the conjugates may be subjected to bacterial enzymes.

Liberated aglyca may again enter the blood and contribute to plasma levels. This enterohepatic recycling could prolong the presence of flavonols within the body. Evidence for an enterohepatic recycling of flavonols is mainly based on the occurrence of secondary peaks in plasma concentration-time curves at timepoints well after t_{max} following oral (Crespy *et al.*, 1999; Manach *et al.*, 1996) or i.v. administration (Ader *et al.*, 2000).

A recent study on the enterohepatic recirculation of quercetin used surgically 'linked' rats. The bile from a quercetin-injected (10 mg kg^{-1} BW) donor rat was cannulated into the duodenum of the 'linked' recipient rat (Chen *et al.*, 2005). In this study, a major impact of enterohepatic recirculation on the bioavailability of quercetin was excluded, because quercetin was detected in the portal vein of the recipient rat only at trace amounts 6 h after dosing of quercetin-containing bile. After intra-gastric administration of bile containing quercetin metabolites (obtained from the donor rat) to a conscious rat, again only trace amounts of quercetin were recovered in portal vein blood during an 8 h-experiment. The lack of enteric re-absorption of quercetin from biliary quercetin conjugates might be explained by rapid degradation of quercetin in the lower intestinal tract. Evidence for rapid metabolism of quercetin within the large intestine derives from recent observations on the decomposition of quercetin, isorhamnetin and rutin (50 or 100 µmol l^{-1}) using a colon-simulation technique (COSITEC) with pig cecal contents (Cermak *et al.*, 2005). Further support for a massive degradation of the ring structure of quercetin also derives from the above mentioned fact, that up to 80% of an oral quercetin dose was exhaled as CO_2 in healthy volunteers (Walle *et al.*, 2001).

I.3.3.5. Food matrix and food composition

Different studies investigated the oral bioavailability of quercetin after application of either the pure aglycone or glycosides dissolved in various organic solvents (Olthof *et al.*, 2000), in water (Erlund *et al.*, 2000), in an encapsulated form (Hollman *et al.*, 1995), mixed among meals (Goldberg *et al.*, 2003; Cermak *et al.*, 2003), or as a natural ingredient of food (Wiczkowski *et al.*, 2003; Young *et al.*, 1999).

The presence of food within the gastrointestinal tract (GIT) is known to markedly alter the oral absorption of xenobiotic compounds by exerting significant effects on motility, transit profiles, pH, blood/lymph flow, secretion, absorption, and intestinal solubilization capacity

(Charman *et al.*, 1997). In addition, effects depend, among others, on the dose and chemical form of the flavonol, as well as on size and composition of the meal.

In addition to the presence or absence of food, the applied source of the flavonol seems to exert an influence. Within plant material, flavonoids are embedded into the plant matrix, e.g. in onion scales or leafy vegetables. During digestion, this matrix is mechanically and enzymatically disrupted and the flavonol glycosides are liberated. Some difference in bioavailability has been observed when flavonols were administered as pure substances in contrast to administration within native food. Much higher plasma concentrations (up to 5 µM) were achieved when quercetin glucosides were administered to fasted volunteers solubilized in a water-alcohol mixture (Olthof *et al.*, 2000) than when an equivalent quantity was ingested with food such as onions, apples, or a complex meal (0.3-0.75 nM) (Manach *et al.*, 1998; Hollman *et al.*, 1997). Hollman *et al.* (1997) pointed out, that differences between onions and, e.g., apples in cell wall structure, location of glycosides within cells and/or their binding to cell constituents may affect liberation of quercetin from these foodstuffs in the GIT. Graefe *et al.* (2001) conducted a four-way crossover study in 12 healthy human volunteers to determine the influence of the food matrix on the absorption of quercetin. Each subject received an onion supplement or Q4'G (both equivalent to 100 mg quercetin), as well as rutin and buckwheat tea (both equivalent to 200 mg quercetin). There was no significant difference in the bioavailability and pharmacokinetic parameters between the onion supplement or Q4'G. Peak concentrations were reached 4.3 ± 1.8 h after administration of buckwheat tea and 7.0 ± 2.9 h after ingestion of rutin. To a minor extend, the plant matrix influences both the rate and extend of absorption in the case of buckwheat tea administration compared with the isolated compound.

In addition to the food matrix, the composition of a meal may affect the bioavailability of flavonoids. In this context, influences of alcohol and of the macronutrients fat, protein and also carbohydrates have been proposed.

Ethanol from beverages such as wine may aid in extracting the flavonoids from the chyme and act as a permeation enhancer. The findings of Goldberg *et al.* (2003) supported this theory, as they observed higher quercetin plasma levels in humans when the same amount of quercetin (422 nmol kg^{-1} BW) was administered dissolved in white wine with 11.5% alcohol content (c_{max} = 127 µg quercetin L^{-1} serum), in comparison to administration of quercetin dissolved in white grape juice (c_{max} = 63 µg L^{-1}) or vegetable juice (c_{max} = 47 µg L^{-1}). Azuma *et al.* (2002) also found an enhancing effect of ethanol on the bioavailability of quercetin (150

μmol kg^{-1} BW) in rats, but significant effects of ethanol were only observed at rather high doses of alcohol (at least 30%, v/v). In contrast to these results, a study by de Vries *et al.* (2001) on quercetin bioavailability (14-16 mg) from native food reported that the alcoholic beverage red wine in comparison to a portion of onions or a glass of tea is a poor source of flavonols, especially of quercetin, in humans.

Tea is another major source of flavonoids. It was speculated that the addition of milk to a tea infusion may inhibit absorption of tea flavonoids due to binding of polyphenols to proteins (Serafini *et al.*, 1996). It was convincingly demonstrated, however, that addition of milk does not affect the absorption of flavonoids from tea in man (Hollman *et al.*, 2001; Het Hof *et al.*, 1998). In another study on the bioavailability of polyphenols, the authors observed a decrease in (-)epicatechin (flavanol) from dark chocolate consumed with 200 mL milk or when the same amount of (-)epicatechin was ingested with milk chocolate, in comparison to dark chocolate. They suggested that the reduced bioavailability could be due to interactions between chocolate flavonoids and milk proteins, thus reducing the absorption of catechins (Serafini *et al.*, 2003). Association of quercetin with protein deriving from food, especially soy and whey protein, in chyme has been proofed *in vitro* (Rawel *et al.*, 2002; Kroll *et al.*, 2002). For quercetin, primarily the hydroxyl groups on the ring B and C can react with lysine and tryptophane in proteins by forming covalent bonds (Rawel *et al.*, 2002; Kroll *et al.*, 2002).

Some studies indicate that the presence of lipids and emulsifying agents may improve the absorption of specific flavonoids. For example, catechins from green tea (Pietta *et al.*, 1998), oligomeric proanthocyanidins from grape seeds, and silibinin from milk thistle are absorbed to a higher extent when administered as phospholipid complexes rather than in the free form (Pietta, 2000). Azuma *et al.* (2002) suggested, that incorporation of quercetin into lipid micelles was an important factor for a higher absorption of quercetin. The effect of co-ingested lipids and emulsifiers on the accumulation of quercetin metabolites in blood plasma after short-term ingestion of onions by rats were investigated (Azuma *et al.*, 2003). More than 4.6% (wt/wt) of soybean oil in the diets significantly enhanced the accumulation of quercetin metabolites in the plasma. Co-administration of fish oil or beef tallow showed effects comparable to that of soybean oil at concentrations of 9.5% (wt/wt), and lecithin was more effective than the other three lipids. The addition of emulsifiers, sodium caseinate and sucrose fatty acid ester, to the drinking water while administering soybean oil containing diet was able to again enhance bioavailability of quercetin significantly. These results indicate that co-

ingested lipids and emulsifiers could enhance the bioavailability of quercetin glucosides in onion.

No effect of varying amounts of cornstarch on quercetin bioavailability was observed in preliminary trials (Azuma *et al.*, 2003). In contrast, Schramm *et al.* (2003) observed that co-administration of sugar and bread test meals increased the bioavailability of cacao flavonols. In this study no effects on the pharmacokinetics of quercetin by protein rich test meals were observed and the co-administration of butter only non-significantly enhanced the systemic availability of quercetin.

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CHAPTER TWO

Bioavailability of Quercetin in Pigs is Influenced by the Dietary Fat Content[1,2]

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ABSTRACT

The flavonol quercetin is one of the most prevalent flavonoids found in edible plants. In this study, the influence of dietary fat on oral bioavailability of quercetin was investigated. Quercetin (30 μmol/kg body weight) was administered either as the lipophilic aglycone or as the more hydrophilic quercetin-3-*O*-glucoside in test meals differing in fat content (3, 17 or 32 g fat/100g diet) to growing pigs. Blood samples were drawn repeatedly over a 24-h period and analyzed by HPLC. The main metabolite found in plasma was always conjugated quercetin. Quercetin bioavailability from each diet was always higher from the glucoside than from the aglycone. Irrespective of the chemical form applied, the bioavailability of quercetin was higher in the 17% fat diet compared with the 3% fat diet ($P < 0.05$). No further effect on bioavailability was observed when the flavonols were administered with diets containing 32% fat. The elimination of quercetin was significantly delayed after its application with fat-enriched diets ($P < 0.05$). Thus, in addition to the chemical form of the flavonol, the fat content of the diet influences oral bioavailability of quercetin.

Key words: flavonoids, bioavailability, quercetin, fat, pigs

INTRODUCTION

The flavonol quercetin is one of the most prevalent flavonoids found in edible plants. In plants and subsequently in plant-derived foods, quercetin is present mainly as glycosides (1,2). It has been shown repeatedly that the sugar moiety is a major determinant governing the intestinal absorption of quercetin (3-6). In addition to the chemical form of the flavonol, the applied vehicle or the composition of the diet seems to have a substantial effect on quercetin bioavailability (7-10). In a previous study by our group, the bioavailability of quercetin from quercetin-3-*O*-glucoside (Q3G)[4] was significantly enhanced when Q3G was administered to pigs in a meal rich in fat and low in carbohydrates, compared with a standard carbohydrate-rich pig diet (3). One reason for this finding could have been the difference in fat concentration in the experimental diets because lipids in combination with emulsifiers were

[4]Abbreviations used: AUC, area under the curve; BBM, brush border membrane; BW, body weight; c_{480}, c_{720}, plasma concentration at 480 or 720 min after ingestion of test meal, respectively; c_{max}, maximal plasma concentration; LPH, lactase-phlorizin hydrolase; LSM, least-squares means; Q3G, quercetin-3-*O*-glucoside; SGLT1, sodium-dependent glucose transporter; t_{max}, time at maximal plasma concentration.

shown to enhance the absorption of orally administered quercetin in rats (8). Therefore, in this study we investigated the effect of the dietary fat content on the oral bioavailability of quercetin in pigs. Quercetin was administered either as the rather lipophilic aglycone or as the more water-soluble Q3G in diets with differing fat contents.

MATERIALS AND METHODS

Animals, diets and experimental procedure

Cross-bred growing male castrated pigs ($n = 7$) with a body weight (BW) of 30-35 kg were purchased from a local farmer. The pigs were surgically equipped with permanent catheters (Cook Deutschland GmbH) placed in the jugular vein. They were restrictively fed (80% of voluntary feed intake) a commercial pig diet composed of barley, wheat and defatted soybean meal (Plambeck Kraftfutter). The composition of this diet (designated as 3% fat diet) is shown in Table II.1. Vitamins and minerals were supplemented according to the recommendations of the German Society of Nutritional Physiology (11). Water was freely available by nipple drinkers.

Each pig received in consecutive experiments either quercetin aglycone or Q3G (30 μmol/kg BW) mixed into a test meal directly before administration. The test meals consisted either of the regular pig diet (3% fat diet) or of an isoenergetic amount of the same diet enriched with either 15 or 30 g lard/100 g (wt/wt) (designated as the 17 and 32% fat diets, respectively) (Table II.1). Lard was obtained from Fischermanns GmbH. Blood samples (8 mL each) were collected over a period of 24 h. After each experiment, there was a wash-out period of 24 h.

Processing of plasma samples and HPLC analysis

Blood samples were drawn into heparinized containers, immediately centrifuged (1500 x *g*, 10 min, 4°C) and stored at –70°C until analysis by HPLC as described previously (3,12,13). All samples were treated enzymatically with β-glucuronidase/sulfatase before the extraction of flavonol compounds.

All flavonols were obtained from Carl Roth GmbH. β-Glucuronidase/sulfatase type H-2 (crude enzyme extract from *Helix pomatia*) was purchased from Sigma-Aldrich AG.

Table II.1: Composition of the diets[1]

	% fat in the diet		
Component	**3**	**17**	**32**
		g/kg diet	
Dry matter	869.2	888.8	914.2
Crude fat	32.9	174.1	318.2
Crude protein	161.8	142.4	115.6
Ash-free NDF[2]	120.5	109.6	86.6
NFC[3]	500.1	419.0	353.2
Starch	399.1	347.3	292.0
		MJ/kg diet	
Gross energy	16.2	19.6	22.7

[1]Isoenergetic amounts of each diet were fed: 200.0 g of the 3% fat diet, 162.0 g of the 17% fat diet, and 136.2 g of the 32% fat diet.

[2]NDF, neutral detergent fiber.

[3]NFC, nonfiber carbohydrates.

Statistical analysis

The area under the plasma concentration-time curve (AUC) was determined according to the linear trapezoidal rule. For each pig and treatment, total bioavailability (AUC_{total}) was calculated by adding up the AUC values of quercetin and its metabolites with an intact flavonol structure (isorhamnetin and tamarixetin). AUC data, maximal plasma concentrations (c_{max}), those at 480 (c_{480}) and 720 min (c_{720}) after ingestion of the test meal, and time at maximal plasma concentration (t_{max}) values were analyzed with the MIXED model procedure from SAS (Version 8.2, SAS Institute) based on the model: $Y_{i,j,k} = \mu + D_i + F_j + (D \times F)_{i,j} + A_k + e_{i,j,k}$ where μ = mean, D_i = diet (i = 3, 17, or 32% fat diet), F_j = administered flavonol (j = quercetin aglycone or Q3G), (D x F) = diet x administered flavonol interaction, A_k = animal, and $e_{i,j,k}$ = residual error. The individual pig was treated as random factor. AUC data, c_{max}, c_{480}, c_{720} and t_{max} values are presented as least-squares means (LSM) ± SEM. A P-value < 0.05 was considered significant.

RESULTS

Irrespective of the administered flavonol or the diet fed, the main metabolite in plasma after β-glucuronidase/sulfatase treatment of the samples was always quercetin (78.8 ± 0.8%, $n = 38$). In addition to quercetin, the monomethylated derivatives isorhamnetin (3'-*O*-methyl quercetin, 11.0 ± 0.5%) and tamarixetin (4'-*O*-methyl quercetin, 10.3 ± 0.4%) were found. No differences in the relative abundance of these metabolites were observed after intake of the aglycone or of the glucoside.

After the intake of quercetin aglycone with the 3% fat diet, the mean peak plasma concentration (c_{max}) was reached ~100 min (t_{max}) after intake (Table II.2). When the flavonol was administered together with the 17 or 32% fat diets, plasma concentrations of quercetin rose more sharply (Figure II.1) and reached their peak levels significantly earlier (Table II.2). Thereafter, quercetin levels with the 3% fat diet decreased continuously until they were below the detection limit after 24 h (Figure II.1). The elimination of quercetin was clearly delayed after its application with the fat-enriched diets; 480 min after intake (c_{480}) with the 32% fat diet, the quercetin plasma concentration (0.054 ± 0.011 µmol/L, $n = 7$) was higher than the value obtained with the 3% fat diet (0.015 ± 0.011 µmol/L, $n = 7$, $P < 0.05$). However, the respective plasma concentration obtained with the 17% fat diet (0.050 ± 0.013 µmol/L, $n = 6$) did not differ from 3% fat diet value ($P = 0.07$) or 32% fat diet value ($P = 0.81$). The values determined at 720 min after intake (c_{720}) with the 17 and 32% fat diets (0.073 ± 0.016 µmol/L and 0.070 ± 0.015 µmol/L, respectively) were both significantly higher than the corresponding value attained after intake with the 3% fat diet (0.015 ± 0.015 µmol/L, $P < 0.05$) (Figure II.1). The fat content of the diet had a significant effect on total bioavailability (AUC_{total}) of quercetin from the aglycone-containing diets (Table II.2). Compared with the 3% fat diet, bioavailability was significantly increased with the fat-enriched diets, with no significant difference between the 17 and 32% fat diets.

After intake of Q3G with the 3% fat diet, the mean peak plasma concentration of quercetin was reached after ~70 min (Table II.2). When the flavonol glucoside was administered together with fat-enriched diets, the plasma concentrations of quercetin reached their peak levels earlier, which was significant only for the 32% fat diet. However, the c_{max} value of plasma quercetin was lowest after intake with 32% fat (Table II.2). Similar to what was observed with the diets containing the aglycone, the elimination of quercetin was delayed with the fat-enriched diets (inset Figure II.1). Plasma concentrations after 480 min differed

significantly among all 3 diets (0.053 ± 0.020 µmol/L in the 3% fat diet, 0.123 ± 0.020 µmol/L in the 17% fat diet, $P < 0.05$ vs. 3% fat diet, and 0.174 ± 0.020 µmol/L in the 32% fat diet, $P < 0.05$ vs. 3% and vs. 17% fat diet, $n = 6$). The c_{720} values from both the 17 and 32% fat diets (0.071 ± 0.015 µmol/L and 0.090 ± 0.020 µmol/L, respectively) were also significantly higher than the plasma concentration after intake with the 3% fat diet (0.026 ± 0.015 µmol/L, $P < 0.05$). Hence, the fat content of the diet had a significant effect on AUC_{total} from Q3G (Table II.2).

Table II.2: Relative bioavailability and pharmacokinetic parameters of quercetin in pigs after intake of quercetin aglycone or quercetin-3-*O*-glucoside in test meals differing in their fat content[1]

Diet	c_{max}[2]	t_{max}[3]	AUC_{total}[4]	Relative bioavailability[5]
Quercetin aglycone				
% fat	*µmol/L*	*min*	*min x µmol/L*	*%*
3	0.518 ± 0.056 [a]	102.9 ± 8.0 [a]	117.3 ± 18.5 [b]	100
17	0.583 ± 0.060 [a]	70.0 ± 8.6 [b]	184.5 ± 19.8 [a]	157
32	0.563 ± 0.056 [a]	51.4 ± 8.0 [b]	176.0 ± 18.5 [a]	150
Quercetin-3-*O*-glucoside				
% fat	*µmol/L*	*min*	*min x µmol/L*	*%*
3	0.906 ± 0.089 [a]	70.0 ± 7.9 [a]	205.5 ± 19.8 [b]	100
17	0.895 ± 0.089 [a]	50.0 ± 7.9 [a,b]	270.9 ± 19.8 [a]	132
32	0.642 ± 0.089 [b]	45.0 ± 7.9 [b]	249.7 ± 19.8 [a]	122

[1]For composition of the diets, see Table II.1; values are LSM ± SEM, $n = 6$; for quercetin aglycone in the 3 and 32% fat diets, $n = 7$. Means in a column not sharing a superscript letter within the quercetin aglycone group or within the quercetin-3-*O*-glucoside group differ, $P < 0.05$.

[2]c_{max}, maximum plasma concentration of quercetin.

[3]t_{max}, time between administration of test meal and the appearance of c_{max}.

[4]AUC_{total}, area under the plasma concentation-time curve from 0 to 24 h for the sum of quercetin and its metabolites (isorhamnetin and tamarixetin).

[5]Relative bioavailability within the quercetin aglycone group or within the quercetin-3-*O*-glucoside group.

In the case of Q3G intake, the significantly increased AUC_{total} values obtained with the fat-enriched diets were due solely to the delayed elimination of quercetin (inset Figure II.1).

The amount of quercetin and methylated quercetin metabolites in the systemic circulation was also significantly influenced by the chemical form of quercetin administered. With quercetin aglycone, the mean total bioavailability from any of the diets was always significantly lower than from the respective diets containing the quercetin glucoside ($P < 0.05$) (Table II.2).

The fat content of the diet and the chemical form of the flavonol, however, did not have a significant interaction.

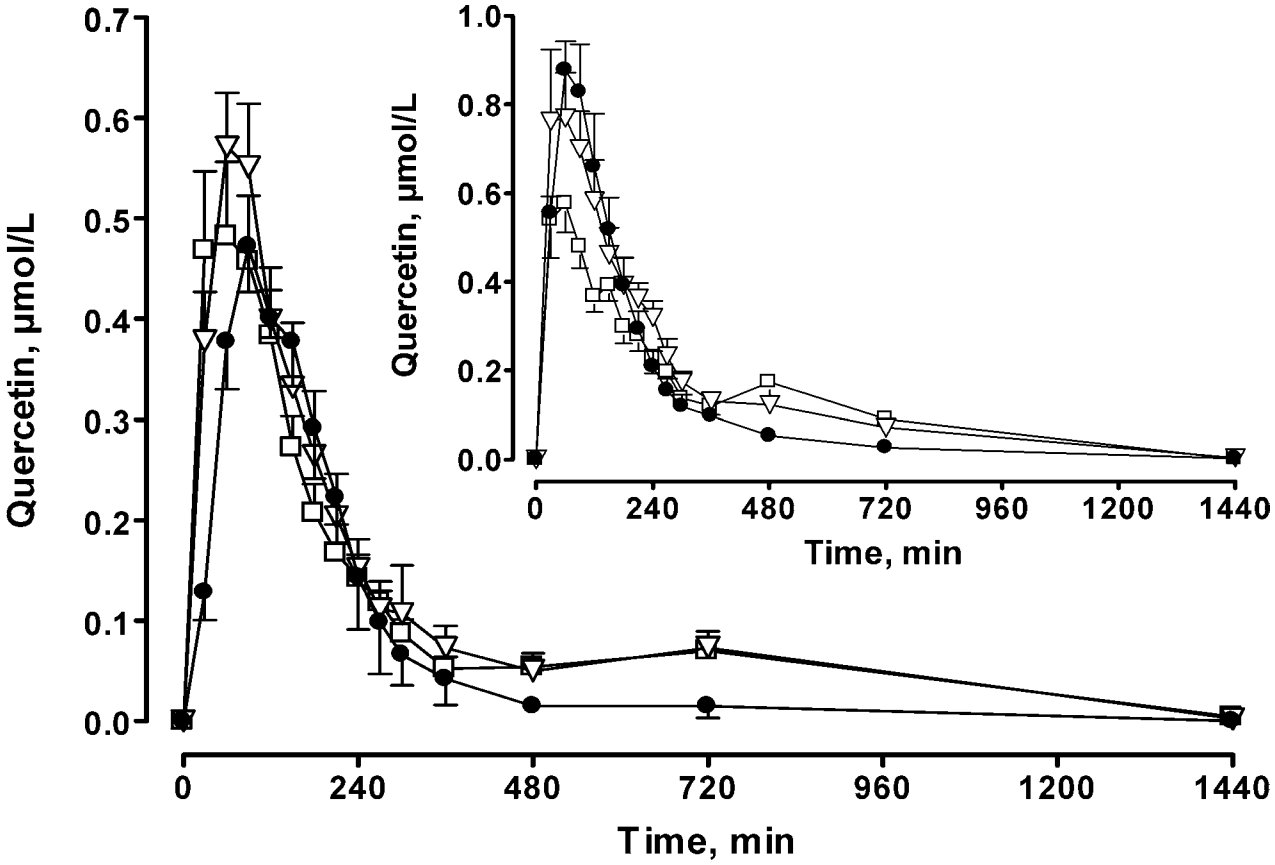

Figure II.1: Plasma concentration-time curves of quercetin after oral administration of quercetin aglycone or of quercetin-3-*O*-glucoside (inset) to pigs (30 µmol/kg BW each) in test meals that differed in their fat content

Filled circles represent values for 3% fat diet, open triangles for the 17% fat diet, and open squares for the 32% fat diet (w/w). Data are means ± SEM (*n* = 6; for quercetin aglycone in the 3 and 32% fat diets, *n* = 7).

DISCUSSION

In a recent experiment, total oral bioavailability of quercetin in pigs was increased by 140% when Q3G was administered with meat compared with a standard pig diet (3). One possible explanation for this finding could have been the higher fat concentration of meat. We therefore conducted the present study to investigate the influence of the dietary fat content on the oral bioavailability of quercetin from both quercetin aglycone and Q3G.

In pig plasma, quercetin and its metabolites with a flavonol structure are present in substantial amounts only as conjugates (3,12). Therefore, we treated all plasma samples with β-glucuronidase/sulfatase to release the flavonol aglycones.

In all experiments, quercetin was found in plasma within 30 min after ingestion. This suggests that absorption had already occured in the upper small intestine, irrespective of the diet and of the flavonol administered.

The increase in the dietary fat content from 3 to 17% crude fat enhanced the total bioavailability of quercetin (quercetin and its metabolites, isorhamnetin and tamarixetin) from the quercetin aglycone containing diet by ~50%. This effect was already maximal because a further increase in dietary fat had no additional effect. It is generally believed that the rather lipophilic quercetin aglycone diffuses passively through the brush border membrane (BBM) of enterocytes (14,15). Azuma *et al.* (8) observed that coadministration of lipids (soybean oil or lecithin) and emulsifiers such as the bile constituent taurocholate was able to enhance and accelerate the intestinal absorption of quercetin in rats. In another rat study, the absorption of the flavanol catechin was enhanced when green tea catechins were administered as a phospholipid complex rather than as free catechins (16). Thus, it is likely that in the presence of dietary fat, quercetin was partly incorporated into mixed bile salt micelles in the lumen of the duodenum. This could have promoted its solubility and transport through the unstirred water layer and passive diffusion through the BBM.

Interestingly, an enhancing effect of dietary fat on total bioavailability of quercetin was also found after administration of Q3G-containing diets. At first glance, this was an unexpected observation, because the more hydrophilic glucoside of quercetin should not directly interact with the absorption of lipids. In agreement with this finding, Azuma *et al.* (17) recently observed an enhancement of quercetin bioavailability from quercetin glucosides in onions by administration with at least 4.6% fat. In the present study, the effect of dietary fat on quercetin bioavailability from Q3G was due exclusively to a delayed elimination of quercetin from

plasma. In contrast, the addition of fat to the quercetin aglycone-containing diets also enhanced the absorption of the flavonol. Several studies pointed to the involvement of β-glycosidases such as the BBM enzyme, lactase-phlorizin hydrolase (LPH, EC 3.2.1.62), in the intestinal absorption of Q3G (18-20). According to those studies, Q3G is hydrolyzed by LPH in the small intestine and the liberated aglycone diffuses passively across the BBM into the enterocyte. Because the liberation of quercetin from Q3G by LPH occurs adjacent to the BBM, enhanced solubility and diffusion through the unstirred water layer as promoted by the formation of micelles cannot be expected in this case. However, an enhancing effect of dietary fat on the reabsorption of quercetin during enterohepatic circulation can be expected after the administration of both Q3G and quercetin aglycone. Stimulation of biliary secretion by dietary fat intensifies excretion of quercetin glucuronides and/or sulfates with the bile (21). After microbial deconjugation of the excreted conjugates in the lower small and large intestine, quercetin could be partially re-absorbed (22). This could explain the higher plasma concentrations of quercetin several hours after ingestion with the fat-enriched diets.

The earlier appearance of maximal quercetin plasma concentrations after ingestion with fat could be explained by a partial incorporation of the quercetin conjugates formed in the enterocyte into chylomicrons with consecutive export into the peripheral blood via lymph. Thus, part of the quercetin would bypass the liver and, consequently, increase the plasma concentration. However, because no data are available concerning the incorporation of quercetin conjugates into lipoproteins during absorption, this remains speculative at present.

In our previous study, administration of Q3G together with meat enhanced total quercetin bioavailability by 140% (3), whereas the addition of fat in the present study enhanced bioavailability of Q3G by only ~30%. This suggests that other dietary factors in addition to fat influence the bioavailability of quercetin. It is conceivable that the lack of carbohydrates in the meat could have favored carrier-mediated uptake of Q3G by the intestinal sodium-dependent glucose transporter (SGLT1) (3). Unspecific binding to food components such as soy protein could have limited the absorption of quercetin from the test meal used compared with the meat meal (23). In this regard, it is important to note that the absolute amount of protein in the meat meal was only ~10% of that in the standard meal used (3).

In the present study, all test meals were isoenergetic to avoid differences in the rate of stomach emptying, which in turn would influence absorption kinetics. Hence, the test meals differed inevitably in protein (32, 23, and 16 g crude protein) and carbohydrate content (100, 68, and 48 g nonfiber carbohydrates) in the 3, 17, and 32% fat test meals, respectively. These

differences in protein and carbohydrate content were rather small, however, compared with the relative differences in fat content (7, 28, and 43 g crude fat in the 3, 17, and 32% fat test meals, respectively). Thus, in our opinion it is most likely that the differences in the fat content were responsible for the observed effects on quercetin bioavailability.

Regardless of these latter considerations, Q3G was always more bioavailable than quercetin aglycone with each of the different diets. This observation agrees with our previous study using pigs (3), and is also known from studies on humans (7) and rats (24). Most authors explain the higher bioavailability of Q3G compared with the aglycone by the occurrence of higher local quercetin concentrations adjacent to the BBM due to the Q3G-hydrolyzing activity of LPH (see above). In addition, transport of Q3G by SGLT1 could also contribute to the higher bioavailability of quercetin from Q3G compared with quercetin aglycone. Several studies showed an interaction of Q3G with SGLT1 (5,25,26). A recent study demonstrated transport of a quercetin glucoside by SGLT1 (27). In experiments with pig small intestine BBM vesicles, we also found evidence for transport of Q3G by SGLT1 (28).

In summary, we showed that the dietary fat content influences the bioavailability of quercetin. Bioavailability from both quercetin aglycone and quercetin-3-*O*-glucoside is enhanced in a diet enriched with fat compared with a low-fat diet. This could be explained by an improved solubility and an accelerated absorption of the lipophilic quercetin aglycone via lipid micelles and a prolonged enterohepatic circulation.

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CHAPTER THREE

The fatty acid pattern of dietary fat influences the oral bioavailabiliy of the flavonol quercetin in pigs

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ABSTRACT

In a previous study, we have shown that the dietary fat content influences the bioavailability of the flavonol quercetin. In this study, the influence of the fatty acid pattern of dietary fats on oral bioavailability of quercetin was investigated. Quercetin (30 µmol/kg body weight) was administered to growing pigs (n = 6) in test meals consisting either of 200 g of a standard pig diet (2% crude fat) or of the same diet supplemented with 15 g fat/100 g diet using either medium-chain (MCT) or long-chain fatty acid triacylglycerols (LCT). Blood samples were drawn repeatedly over a period of 24 hours and analysed by HPLC. In addition, the influence of the different diets on gastric emptying was investigated in rats. In pigs, the bioavailability of quercetin was measured by quantifying its plasma metabolites with an intact flavonol structure. Bioavailability was enhanced by 38% ($P<0{\cdot}05$) and 12% ($P>0{\cdot}05$) after intake with the MCT and LCT diets, respectively, compared to standard diet. Maximum plasma concentrations of quercetin were reached significantly later with the MCT diet in comparison to the LCT or standard diet ($P<0{\cdot}05$). No differences in dry matter of gastric content were observed 60 min after intake of the experimental diets in rats.

Thus, administration of quercetin together with a diet containing MCT fat enhances the bioavailability of the flavonol. Interestingly, absorption of quercetin was significantly delayed with this particular diet. However, this was probably not due to a slower gastric emptying of the MCT diet.

Key words: Quercetin: Bioavailability: Fat: Pig

INTRODUCTION

Flavonoids are polyphenolic secondary metabolites that occur ubiquitously in higher plants. Their abundance in the human diet has raised an increasing interest in their bioavailability and biological properties. Flavonoids exert several biological activities, which are mainly related to their ability to influence various enzymes and/or to their antioxidant properties (Middleton *et al.* 2000). Detailed assessment of the bioavailability of putatively beneficial polyphenolic food constituents is vital for the evaluation of health effects in man and animals (Manach *et al.* 2005). The flavonol quercetin is one of the major representatives of flavonoids in many edible plants (Herrmann 1988; Hertog *et al.* 1992) and also one of the most studied.

In a previous study of our group conducted in pigs, oral bioavailability of quercetin aglycone from a test meal was enhanced by 57% by the addition of 15% fat (wt/wt) to a low-fat standard pig diet (Lesser *et al.* 2004). We speculated whether the enhancement of quercetin bioavailability was due to a better solubility of the relatively lipophilic quercetin aglycone in the intestinal tract in the presence of fat (Azuma *et al.* 2002). In this case, an increased absorption from fat containing diets should be largely independent of the type of dietary fat. Another possible explanation could be the generation of chylomicrons in the intestinal mucosa. Dietary fat mainly comprises triacylglycerols with long-chain fatty acids (chain length $>C_{12}$). After luminal hydrolysis, mucosal uptake and resynthesis of triacylglycerols, long-chain fatty acid triacylglycerols (LCT[4]) are incorporated into chylomicrons and are consecutively transported via lymph. In contrast, medium-chain fatty acid triacylglycerols (MCT, chain length C_8-C_{12}) are hydrolysed and predominantly exported from the enterocytes as NEFA into the mesenterical blood; they reach the liver via the portal vein (Bloom *et al.* 1951; Hashim *et al.* 1964; for review: Harkins & Sarett 1968). Transport together with LCT within chylomicrons via the lymphatic vessels (Murota & Terao 2005) could enhance quercetin bioavailability due to bypassing of the liver, and, thereby, reduce biliary elimination during the first liver passage (*first pass* effect). If this was true, mainly fats containing LCT should increase the bioavailability of flavonols but not fats comprised of MCT due to the different handling of LCT and MCT during intestinal absorption (Harkins & Sarett 1968). In order to differentiate between these two possibilities, i.e. better solubility *vs.* bypassing of the liver, we fed quercetin (30 µmol/kg BW) to pigs with test meals consisting of a low-fat pig diet (standard diet), or of the same diet enriched with either 15 g MCT or LCT fat/100 g diet, respectively. Although quercetin is present mainly in glycosidic form in the diet, hydrolysis of quercetin glycosides by the brush border membrane enzyme lactase-phloridzin hydrolase or by cytosolic β-glycosidase is a prerequisite for its absorption in the small intestine (Manach & Donovan 2004). Thus, further biotransformation of the released aglycone and export of the formed quercetin metabolites into the blood or intestinal lymph should be independent of the quercetin source. The aglycone is more lipophilic than its glucosides and, thus, better soluble in the presence of dietary fat. For these reasons, we chose to use quercetin aglycone in our study instead of quercetin glycosides.

[4]**Abbreviations**: AUC, area under the curve; BW, body weight; CCK, cholecystokinin, c_{max}, maximum plasma concentration; LCT, long-chain fatty acid triacylglycerols; MCT, medium-chain fatty acid triacylglycerols; t_{max}, time at maximum plasma concentration.

MATERIALS AND METHODS

Animals and diets

Cross-bred growing male castrated pigs (n = 6) with a body weight (BW) of 30-35 kg were purchased from a local farmer. The pigs were surgically equipped with permanent catheters (Cook Deutschland GmbH, Mönchengladbach, D) placed in the left jugular vein. The pigs were restrictively fed (80% of voluntary feed intake) with a commercial pig diet composed mainly of wheat, barley, and defatted soybean meal (Plambeck Kraftfutter, Brügge, D). The composition of this diet (designated as standard diet) is shown in Table III.1.

Table III.1: Composition of diets*

	Diets		
Components	**Standard**	**LCT**	**MCT**
	g/kg diet		
Dry matter	857·2	882·7	881·4
Crude fat	16·7	159·2	162·8§
Crude protein	172·3	140·5	144·8
Ash-free NDF	147·3	176·6	124·6
NFC	468·6	360·6	407·6
Starch	426·1	365·2	369·6
	MJ/kg diet		
Gross energy	15·6	19·0	19·1

LCT, long-chain fatty acid triacylglycerols; MCT, medium-chain fatty acid triacylglycerols; NDF, neutral deterent fiber; NFC, nonfiber carbohydrates.

*Isoenergetic amounts of each diet were fed: 200 g of the standard diet, and 162 g of LCT and MCT diets, each.

§Comprised of 90% MCT and 10% LCT.

Test meals were isoenergetic and consisted of the standard diet or of the same diet enriched with either 15 g lard or MCT oil/100 g diet (wt/wt; designated as LCT or MCT diet, respectively) (Table III.1). The fatty acids of the MCT oil consisted solely of octanoic and decanoic acid with a mean chain length of $C_{8\cdot8}$. The lard was composed of LCT with a mean chain length of $C_{17\cdot3}$, as determined by saponification number. Predominant fatty acids in lard are C18:1 (43 g/100 g), C16:0 (24 g/100 g), C18:0 (14 g/100 g) and C18:2 (9 g/100 g), with no fatty acids of less than 14 carbon atoms (Belitz & Grosch 1992). Commercial lard was obtained from Fischermanns GmbH (Duisburg, D), and the MCT oil was a kind gift from Unilever (Vlaardingen, NL). Vitamins and minerals where supplemented according to the recommendations of the German Society of Nutritional Physiology (Ausschuss für Bedarfsnormen der Gesellschaft für Ernährungsphysiologie 1987). Water was supplied for *ad libitum* intake by nipple drinkers.

Additionally, 18 male *CD* rats (Charles River, Sulzfeld, D) with a mean BW of approximately 250 g were used for an experiment aimed to investigate gastric emptying after consumption of the specific diets. The rats were housed individually and kept on a 12 h light/dark cycle. They were accustomed to eating the standard pig diet for one week prior to the experiment.

The experiments were approved by the Animal Welfare Officer of the University of Kiel and by the relevant legal authorities (Ministry of Agriculture and Environment of Schleswig-Holstein).

Experimental procedure – bioavailability in pigs

Each pig received a dose of 30 µmol quercetin/kg BW mixed into the test meal directly before feeding. Thereby, each of the three different quercetin containing diets was administered in consecutive experiments to each pig. Test meals were isoenergetic. Fifteen blood samples (8 mL each) were collected from each pig over a period of 24 h after intake of each test meal. After every experiment, there was a wash-out period of 24 h.

Experimental procedure – gastric emptying in rats

Two days prior to the experiment, the rats were allocated to three groups (n = 6 per group) and trained to eat meals of 5 g of the respective test diets (standard, LCT, or MCT) within 15 min at beginning and end of the dark period. The BW did not differ between the groups (253·4 (SEM 5·0), 256·3 (SEM 6·3), and 254·5 (SEM 4·5) g for the standard, LCT, and MCT

group, respectively). After fasting for 11 h (water allowed), the rats were fed the respective test meals containing 0·5 mmol quercetin/100 g diet (equimolar to the concentration in the standard diet pig test meal). The rats were anaesthetized with CO_2 1 h after intake of the meals and immediately decapitated. Blood was collected, the abdominal cavity was opened, the stomach was proximally and distally ligated and removed. Stomach content was collected by rinsing the stomach with 2 mL of pure water. The gastric content was dried (106 °C) until weight constancy. Dry matter was related to the dry matter of the respective diet ingested.

Processing of plasma samples and HPLC analysis

Blood samples from pigs and rats were drawn into heparinized containers and immediately centrifuged (1500 x g, 10 min, 4 °C). Plasma was stored at –70 °C until analysis by HPLC as described previously (Hollman *et al.* 1996; Ader *et al.* 2000). By the sensitive HPLC postcolumn chelation method applied, only flavonols possessing an intact ring structure with a free 3-OH group are detected (here: quercetin, isorhamnetin, and tamarixetin). Therefore, all plasma samples were treated enzymatically with β-glucuronidase/sulfatase (type H-2, crude enzyme extract from *Helix pomatia*, Sigma-Aldrich AG, Taufkirchen, D) before the extraction of flavonol compounds. All flavonols were obtained from Carl Roth GmbH (Karlsruhe, D).

Statistical analyses

Data are presented as mean values ± SEM. The area under the plasma concentration-time curve (AUC) was determined according to the linear trapezoidal rule. For each pig and treatment, total bioavailability (AUC_{total}) was calculated by adding up the AUC values of quercetin and its metabolites with an intact flavonol structure (isorhamnetin and tamarixetin). AUC data, maximum plasma concentration (c_{max}), and time at maximum plasma concentration (t_{max}) were analysed using repeated measures one-way ANOVA. The amount of dry matter from the gastric content and plasma flavonol concentrations of the rats were analysed using one-way ANOVA. Tukeys' multiple comparison was applied as post-test (Graphpad Prism 4, Graphpad Software Inc., San Diego, CA, USA). A P-value $<0{\cdot}05$ was considered significant.

RESULTS

Bioavailability in pigs

After administration of quercetin with the test meals to pigs, metabolites with an intact flavonol structure appeared in plasma within 30 min. Plasma levels of flavonols decreased below detection limit (7 nmol/L) within 24 h. In accordance with previous studies (Cermak *et al.* 2003; Lesser *et al.* 2004), the main metabolite after β-glucuronidase/sulfatase treatment of the pig plasma samples was quercetin, while isorhamnetin (3'-*O*-methyl quercetin) and tamarixetin (4'-*O*-methyl quercetin) were found in a proportion of approximately 10% each, irrespective of the dietary treatment.

After intake of quercetin with the standard diet, the mean peak plasma concentration (c_{max}) was reached 95 min (t_{max}) after intake (Table III.2). Thereafter, quercetin levels with the standard diet decreased continuously until they were below detection limit after 24 h (Figure III.1).

Table III.2: Pharmacokinetic parameters and relative bioavailability of quercetin in pigs after intake of quercetin in test meals differing in their fat content and/or fatty acid pattern*

Diet§	c_{max}		t_{max}		AUC_{total}		Relative bioavailability
	µmol/L		*min*		*min x µmol/L*		*%*
	Mean	SEM	Mean	SEM	Mean	SEM	
Standard	0·323	0·038	95·0[a]	18·0	86·6[b]	11·3	100
LCT	0·334	0·034	65·0[a]	5·0	96·7[b]	8·5	112
MCT	0·288	0·030	170·0[b]	20·0	119·7[a]	8·7	138

LCT, long-chain fatty acid triacylglycerols; MCT, medium-chain fatty acid triacylglycerols; c_{max}, maximum plasma concentration of quercetin; t_{max}, time between administration of test meal and the appearance of maximum plasma concentration of quercetin; AUC_{total}, area under the plasma concentration-time curve from 0 to 24 h for the sum of quercetin and its metabolites isorhamnetin and tamarixetin.

*Values are means of six pigs for each dietary treatment.

[a,b]Mean values within a column with unlike superscript letters were significantly different (P<0·05).

§For composition of diets, see Table III.1.

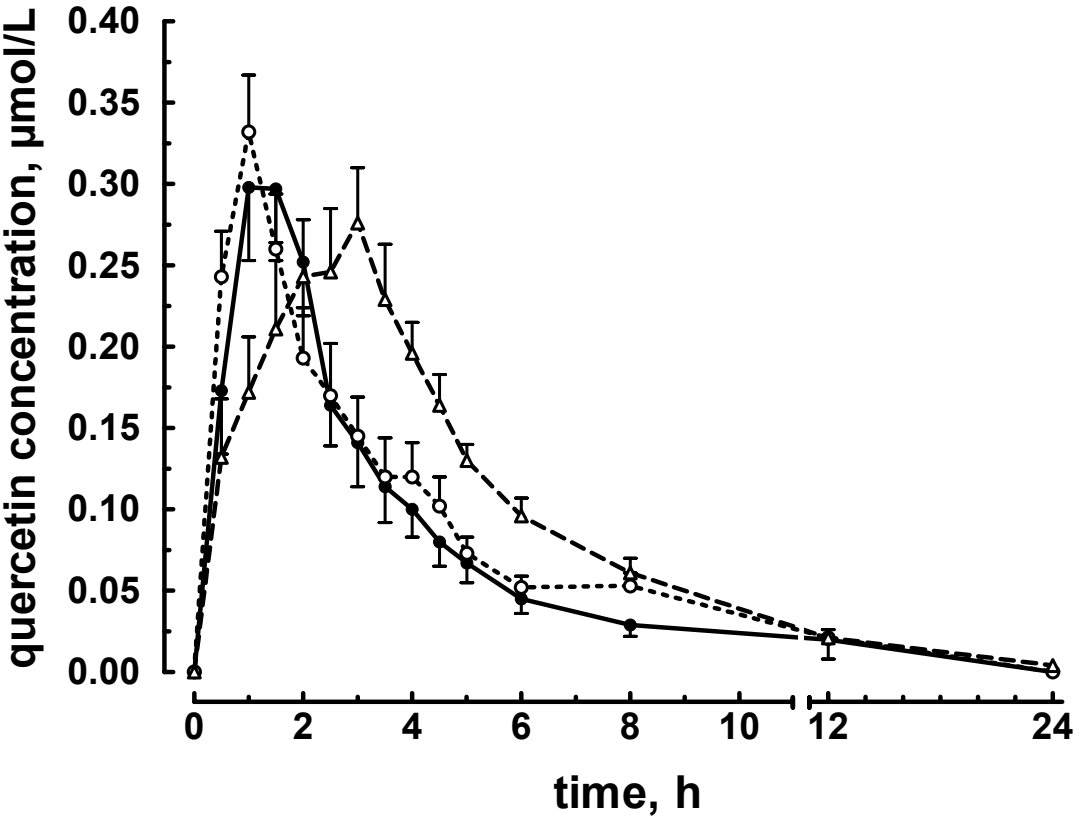

Figure III.1: Plasma concentration-time curves of the main metabolite quercetin after oral administration of quercetin (30 µmol/kg BW) to pigs in test meals that differed in their fat content and/or fatty acid pattern

Values are means for six pigs, with standard errors of the mean represented by vertical bars. For composition of diets, see Table III.1. (-●-), standard diet; (-○-), LCT diet; (-Δ-), MCT diet; LCT, long-chain fatty acid triacylglycerols; MCT, medium-chain fatty acid triacylglycerols.

When the flavonol was administered together with LCT diet, the plasma concentrations of quercetin peaked already after 65 min. This was, however, not significantly different from intake with standard diet (Table III.2). At 8 h after meal intake, the plasma quercetin concentrations were 0·053 (SEM 0·008) and 0·029 (SEM 0·007) µmol/L for LCT and standard diet, respectively.

After administration of quercetin together with the MCT diet, flavonol plasma concentrations rose steadily over 170 min until peak level (Table III.2), and thereafter declined continuously. At 8 h after intake, the mean plasma concentration was still higher (0·061 (SEM 0·009) µmol/L) than after intake with standard diet ($P<0·05$). At 12 h after meal intake, mean plasma

concentrations in all groups were equally low (Figure III.1). Maximum plasma concentrations of quercetin after intake with the three different diets were not significantly different. Total oral bioavailability (AUC_{total}) of quercetin, however, was significantly higher after intake of quercetin with MCT diet than after intake with standard and LCT diet (Table III.2).

Gastric emptying in rats

Within 15 min, the rats consumed 5 g of the experimental diets which contained the same dietary concentration of quercetin as in the original pig test meals. This resulted in an intake of about 100 µmol quercetin/kg BW. One hour after consumption of the meals, blood samples and gastric contents were collected. Dry matter of gastric contents did not differ in weight (Figure III.2). About 80% of dry matter consumed remained in the stomach one hour after meal intake.

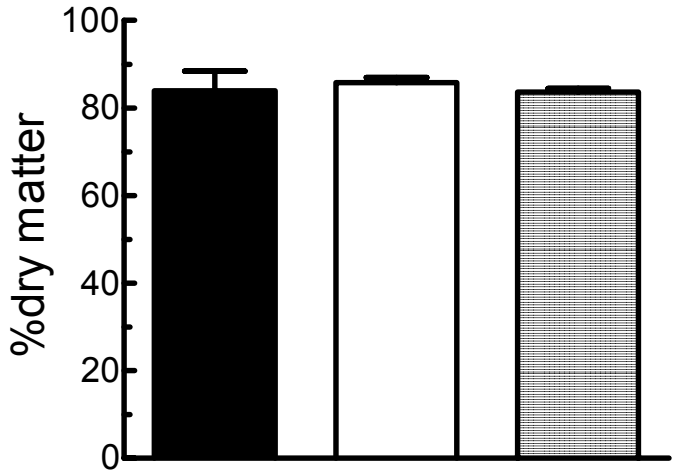

Figure III.2: Gastric content of rats 1 h after administration of 5 g test meals that differed in their fat content and/or fatty acid pattern (dry matter expressed as percentage of intake)

All test meals contained approx. 100 µmol quercetin/kg BW. Values are means for six rats, with standard errors of the mean represented by vertical bars. For composition of diets, see Table III.1. (■), standard diet; (□), LCT diet; (≡), MCT diet; LCT, long-chain fatty acid triacylglycerols; MCT, medium-chain fatty acid triacylglycerols.

The flavonol plasma concentrations (sum of quercetin and methylated metabolites) 1 h after intake of quercetin were 3·736 (SEM 0·226), 3·619 (SEM 0·190), and 3·010 (SEM 0·243) µmol/L with the standard, LCT and MCT diet, respectively (P=0·073). The pattern of deconjugated quercetin metabolites in rats plasma differed from that found in pigs. In rats, the relative proportions of quercetin, isorhamnetin and tamarixetin in plasma were 52·3 (SEM 1·3), 45·0 (SEM 1·4), and 4·9 (SEM 0·1) %, respectively (n = 18). As observed in pigs, dietary treatment did not influence proportions of methylated metabolites.

DISCUSSION

Effects of dietary co-ingestion of either LCT or MCT compared to a low-fat diet on the relative systemic availability of quercetin aglycone were investigated in the current pig study. In principle, the results obtained in the present study agree with the results of our previous study (Lesser *et al.* 2004). Again, the co-administration of dietary fat enhanced the bioavailability of quercetin in pigs. Compared to the previous study, however, the stimulatory effect of the LCT diet was less pronounced and failed to meet the level of significance. The AUC obtained for quercetin applied with MCT fat was significantly higher than with LCT or standard diet. Interestingly, quercetin absorption from the MCT containing test meal was substantially delayed, indicated by the relatively slow rise in plasma levels (Figure III.1) and the late t_{max} (Table III.2).

One possible cause for this delay in quercetin absorption from MCT diet could have been a delayed gastric emptying of the MCT diet. In the literature, the mode of action of MCT fat on gastric emptying is discussed controversially. Some studies found that gastric emptying after intake of MCT or medium-chain fatty acids was more rapid than after intake of LCT or long-chain fatty acids in humans (Hunt & Knox 1968) and rats (Harkins *et al.* 1964). Another study observed no difference in gastric emptying between LCT and MCT in rats (Maggio & Koopmans 1987), while other groups reported a retardation of gastric emptying by MCT in comparison to LCT. In rats, the gastric emptying of tridecanoylglycerol was found to be significantly slower than that of cocoa butter or rapeseed oil (Porsgaard *et al.* 2003). Pirk & Skala (1970) observed retarded gastric emptying in both rats and man by x-ray observation after administration of a MCT mixture compared to a lard mixture. Additionally, they found a delay in complete evacuation time of the small intestine after administration of MCT.

Thus, we investigated gastric emptying in rats after consumption of the specific experimental diets. Rats are a frequently used animal model for investigations on gastric emptying (see above) and were chosen in this experiment because of the easier handling of the greater number of animals required (here n = 18) compared to the pigs. Regulation of gastric emptying in rats and pigs in response to dietary fat seems to function similarly. Plasma levels of cholecystokinin (CCK), one major mediator in the complex interplay of nutritional, hormonal and neural factors regulating gastric emptying, were shown to elevate, among other triggers, in response to ingestion or intraduodenal administration of LCT or long-chain fatty acids in rats and pigs (Lilja *et al.* 1984; Cuber *et al.* 1990; Lewis & Williams 1990; Hölzer *et al.* 1994). Intravenous injection of active CCK-8 peptide dose-dependently evokes pylorus contraction and reduces intragastric pressure in rats, thus slowing gastric emptying down (Adelson *et al.* 2004). In pigs, gastric emptying was slowed in response to i.v. application of CCK-8 (Gregory *et al.* 1995), too, and i.v. application of a specific CCK peripheral receptor inhibitor reduced inhibition of gastric emptying triggered by duodenal fat infusion (Rayner & Miller 1993). In our experiment, no diet-related differences in gastric emptying were observed 1 h after intake of the test meals. Thus, the delayed quercetin absorption from the MCT diet can probably not be explained by a slower gastric emptying. Some authors (Hopman *et al.* 1984; Vu *et al.* 1999) observed that, in contrast to LCT, dietary MCT do not induce CCK release in man. Because CCK inhibits gastric motility, a lack in CCK secretion would result in an enhanced gastric emptying and thus cannot explain the results obtained in the present study.

We also analysed the plasma flavonol levels at 1 h after meal intake. Interestingly, flavonol levels in rat plasma tended to be lower with the MCT diet. This was in accordance with the respective 1 h values in pigs, where we observed a significant difference (Figure III.1; $P<0{\cdot}05$). This might indicate that absorption of quercetin was probably also delayed from the MCT diet in rats, despite the fact that quercetin absorption may already occur from rat stomach (Crespy *et al.* 2002).

Although the MCT and LCT fat differ somewhat regarding their melting temperature (lard 26-39 °C, C8:0 16·3 °C, and C10:0 31·3 °C), it can be assumed that both fats are fluid at body temperature. Conjugated bile acids are essential for the formation of mixed micelles which enhance the absorption of fat soluble substances including molecules deriving from luminal triacylglycerol hydrolysis. The rather lipophilic quercetin molecule might dissolve in the lipid phase of the chyme and mucosal uptake seems to be promoted in the presence of micelles

(Azuma *et al.* 2002) as presumably indicated by the accelerated absorption of quercetin from a lard containing diet, compared with a low-fat diet, found in our previous study (Lesser *et al.* 2004). As already mentioned above, some authors (Hopman *et al.* 1984; Vu *et al.* 1999) observed that, in contrast to LCT, dietary MCT do not induce CCK release with subsequent gallbladder contractions in man. Thus, it can be assumed that in the pigs after ingestion of the MCT containing diet an emulsion without formation of mixed micelles will be present in the small intestinal contents. This might be one possible reason for a delayed absorption of quercetin from the MCT diet compared with the LCT-containing meal.

An explanation for delayed absorption of quercetin from MCT diet compared to LCT or standard diet could be a shift of quercetin absorption from the duodenum to more distal parts of the small intestine, due to physico-chemical factors. Some authors reported an acceleration of duodenocecal transit time during administration of MCT fat (Ledeboer *et al.* 1995; Verkijk *et al.* 1997). As suggested by Vu *et al.* (1999) MCT, other than LCT, might not be rapidly absorbed in the proximal gut, but probably reach the ileocolonic region. An accelerated transit of quercetin containing chyme through the duodenum and proximal jeunum after ingestion with MCT diet, combined with a lower absorption from the proximal small intestine (due to solution in the lipophilic phase), could have shifted absorption of the flavonol to more distal segments of the small intestine. Assuming a more lipophilic nature of the chyme in the distal small intestine after MCT intake, a prolonged and intensified absorption of quercetin which resulted in higher accumulation in plasma could be feasible. In this context it is of interest that another lipid soluble substance, namely vitamin E, showed an enhanced bioavailability when administered with MCT in comparison to LCT (Gallo-Torres *et al.* 1978).

Although the rats ingested a quercetin dosage only about three times higher than that applied to pigs, plasma levels after 1 h were at least ten times higher in rats. In addition, the pattern of methylated metabolites was also different between the two species. The higher proportion of isorhamnetin found in rat plasma in comparison to pig plasma might be due to a higher methylation activity exerted by catechol-*O*-methyltransferase (EC 2.1.1.6). These observations are in good agreement with findings from other groups (Manach *et al.* 1996; de Boer *et al.* 2005). Thus, the methylation pattern of quercetin metabolites in pigs seems to be more similar to humans (Hubbard *et al.* 2003) than to rats.

The unexpected effect of delayed quercetin absorption with the MCT diet hampered the interpretation of the data with regard to the impact of either micelles or chylomicrons on quercetin bioavailability, as was the original intention of our study. However, we observed an

enhanced bioavailability of the flavonol quercetin when administered with a MCT containing diet. The absorption of quercetin was significantly delayed with that diet compared to a LCT containing diet or the low-fat standard pig ration. However, this was probably not due to slower gastric emptying of the MCT diet but may be caused by differences in the physico-chemical nature of the chyme. The observed effect of MCT might be attributable to a shift of the absorption of fat soluble substances, including quercetin, to more distal parts of the small intestine.

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CHAPTER FOUR

General Discussion

Flavonoids, including the abundant flavonol quercetin, are bioactive plant components present in our daily diet. A wide spectrum of health promoting activities has been claimed for flavonols. These claims, however, are mainly deduced from *in vitro* studies. A prerequisite for potential *in vivo* effects of quercetin is of course a sufficient bioavailability of this polyphenol. Bioavailability in this context summarizes gastrointestinal absorption, distribution, and metabolism within the organism as well as excretion. The present work focuses on the flavonol quercetin, one of the most important flavonoids in terms of quantity of intake with Western-style human diets as well as with respect to potential biological activities. The aim of this thesis is to contribute to the knowledge on effects of dietary factors that influence bioavailability of quercetin.

In the first study of this thesis (chapter II), the influence of the amount of dietary fat on the bioavailability of the flavonol quercetin was investigated in pigs. Thereby, the systemic availability of quercetin, applied as pure substance either as the aglycone or as the 3-*O*-monoglucoside (Q3G) was monitored after intake with isoenergetic test meals that differed in fat content (3, 17, and 32%, wt/wt). In the second study (chapter III), possible influences of the fatty acid chain length of dietary triglacylglycerols (long- or medium-chain triacylglycerols) on quercetin bioavilability were examined. In this study, quercetin aglycone was again added as pure substance to test meals. The aglycone was applied in this study, because it has become increasingly clear during the last years, that flavonols have to be deglycosilated before absorption into the systemic circulation (see chapter I.3.2.). In addition, the enhancing effect of dietary fat on the bioavailability of quercetin observed in the first study was more pronounced when quercetin was applied as the aglycone compared to Q3G.

The pig was chosen as model animal because of the high similarity between humans and pigs concerning the anatomy and physiology of the digestive and circulatory system (Almond, 1996). In addition, larger volumes of blood can be obtained from a pig compared to a rat or mouse, allowing investigation of pharmacokinetics in each single animal. In general, there are various species differences with respect to morphology and physiology of the gastrointestinal tract (GIT). One example are differences in gastric mucosa types and distributions (Kararli, 1995). Whereas in rats and pigs the cardia is lined by a non-secreting epthelium, such a pars proventricularis is absent in humans. Another example is, that pigs like humans have a gall bladder which functions as a contractible reservoir for bile. In contrast, rats lack a gall bladder, and, therefore, secrete bile more or less continuously (DeSesso & Jacobson, 2001) and independent from food intake (Edwards *et al.*, 2001). The rat common bile duct is divided

into two morphologically and physiologically distinct parts. The hepatic part was found to be richly vascularized and contractile, and thus might partially replace a gall bladder (Carrier & Connat, 1995), whereas the duodenal part is less vascularized and not contractile, presumably acting as pancreatic duct (Carrier & Connat, 1995). From an anatomical study using scanning electron microscopy of rat intestine, bile storage in periportal plexi of bile ductules was suggested (Murakami *et al.*, 2001). Nevertheless, the concentration of conjugated bile acids in rat bile was found to be lower compared to man (Andrews & Andrews, 1979). Therefore, although parts of the biliary duct system in rats may substitute for the missing gall bladder, a better comparability of fat digestion between pigs and humans can be assumed.

In the second study an additional experiment using rats was performed, aimed to investigate a possible influence of medium-chain triacylglycerols (MCT) on gastric emptying (chapter III). To our best knowledge, there are no known species differences that would hamper an extrapolation of the results obtained in rats on gastric emptying in pigs or man, although the regulation of gastric emptying is very complex and yet unknown species differences might exist. In the rat study, no diet-related difference with respect to the remaining gastric contents were detected one hour after meal intake. This time point was chosen, because in pigs a significantly lower plasma quercetin concentration was observed with the MCT-containing test meal as compared to the long-chain triacylglycerols (LCT) diet (Figure III.1). The plasma quercetin concentration after one hour in the rats fed the same test meals as in the respective pig study tended to be lower. This must be, however, interpreted with some caution, because no complete pharmacokinetic study was conducted in the rats. Although the results did not indicate a delayed gastric emptying after intake of the MCT-containing meal, a delay in gastric emptying cannot be completely ruled out as one possible reason for the later appearance of quercetin in the systemic circulation. Because gastric emptying in pigs has been observed to be somewhat faster than in other species (Gregory *et al.*, 1995), and because only ~20% of gastric content have been emptied from the rat stomachs one hour after meal intake (Figure III.2), a later time point might have been more appropriate.

The amount of diet administered was adjusted to a meal size of 5 g per serving for the rats. No differences in gastric emptying were observed after intake of the different diets. Compared with the situation in the pig trial, it must be kept in mind, that the relative meal size in the rat study (5 g, ~250 g BW) was much larger than in the pig study (162 or 200 g, ~35 kg BW). Thus, gastric distention which also modifies gastric emptying was different in rats and pigs.

With regard to the known mechanisms of gastric emptying, which include, among others, the gastrointestinal peptide hormone cholecystokinin (CCK) as one major player, it was somewhat surprising that consumption of the LCT diet did not result in a slower gastric emptying than the other two diets, because LCT or long-chain fatty acids are known to stimulate CCK release from the duodenal mucosa (Lewis & Williams, 1990). CCK in turn, is known to retard gastric emptying (Adelson *et al.*, 2004; Rayner & Miller, 1993). Again, one hour after meal intake might have been to early to detect any differences.

In both studies of this thesis, quercetin was supplemented to pigs (~35 kg BW) at a level of 30 μmol kg^{-1} BW, which equals 10 mg kg^{-1} BW. This resulted in a single dose of ~350 mg quercetin (either as the aglycone or Q3G) consumed with the test meals. Thus, the amount of quercetin ingested by the pigs in these trials exceeded estimated mean daily dietary quercetin intake in humans ~20fold (Hertog *et al.*, 1993). The main reason for choosing this dosage was to ensure that measurable plasma concentrations were achieved in pigs by the single test meal. Although the dose applied in these studies was clearly above the average dietary intake of quercetin in humans, one has to keep in mind that intake of flavonoid enriched food supplements (functional food) and a more selective intake of food components with high quercetin content, e.g. onions, might result in a considerably higher quercetin intake in humans.

To investigate effects of food on flavonoid pharmacokinetics, Manach & Donovan (2004) recently proposed to firstly administer the flavonoid containing food by itself, and then to test the hypothesis that certain macronutrients consumed together with that food significantly alter flavonoid pharmacokinetics, in order to facilitate comparisons between studies, different flavonoids and food sources. In the current studies, quercetin was always applied as pure substance (aglycone or Q3G) mixed among test meals varying in composition. Thereby, effects of co-ingested nutrients were investigated to evaluate their influence on the bioavailability of quercetin. Thus, this approach does not take into account possible effects of the food matrix and therefore might not truly reflect bioavailability of quercetin from natural food sources. Nevertheless, this approach enables the investigation of principle influences of food components on the bioavailability of quercetin.

In chapter I.3.2., species differences in the activity of flavonol metabolising enzymes, which may result in different metabolite patterns, as well as species differences in the binding affinity of serum albumin against flavonoids, influencing their terminal elimination half-life, as well as potential variations in urinary excretion have been shortly addressed. In addition to

such species differences, striking inter-individual differences within a species in quercetin bioavailability were reported in the majority of the respective studies (Németh *et al.*, 2003; Graefe *et al.*, 2001; da Silva *et al.*, 1998; Hollman *et al.*, 1995). These inter-individual variations might be attributable to a variety of causes, like individual enzyme expression and activities (Németh *et al.*, 2003), bacterial colonisation of the intestine, and/or variations in levels of transporters, enabling more efficient absorption or secretion of flavonols (Manach *et al.*, 2005). Manach *et al.* (2005) recently compiled data on the bioavailability of quercetin from 14 human studies after oral intake of quercetin from different sources and calculated mean pharmacokinetic parameters. Thereby, the authors assumed a linear dose-response relationship of c_{max}, which, however, can only be assumed to occur at low dietary concentrations of quercetin and which is not confirmed by the majority of respective studies. An overview on quercetin bioavailability parameters obtained in human bioavailability studies with a comparable amount of quercetin intake originating from different sources is presented in Table IV.1.

Table IV.1: Pharmacokinetic parameters and urinary fractional excretion of intact quercetin in humans after consumption of ~100 mg quercetin equivalents as aglycone, glycosides, or with food[a]

Quercetin source	**c_{max} (plasma)**	**t_{max} (plasma)**	**Elimination half-life $t_{1/2}$**	**Excretion (urine)**	**n**
Quantity of ingestion: ~100 mg[b]	*µmol L^{-1}*	*min*	*h*	*% of dose*	
Apple meal	0.3	150	23	0.4	9[1]
Onion supplement	7.6	41	10.9	6.4[d]	12[3]
Onion supplement (68 mg quercetin)	0.7	42	28	1.4	9[1]
Q3G, pure compound	5.0	37	18.5	3.6[d]	9[4]
Q4'G, pure compound	3.5	<30	21.6	n.d.[c]	9[2]
Q4'G, pure compound	6.9	42	11.9	4.5[d]	12[3]
Q4'G, pure compound	4.5	27	17.7	3.1[d]	9[4]
Rutin, pure compound	0.3	558	-	0.4	9[1]
Rutin, pure compound	0.2	360	28.1	n.d.	9[2]

(modified from Day & Williamson, 2003, and Scalbert & Williamson, 2000)

[a]Flavonol conjugates in plasma and urine were hydrolysed by acid or enzymes before chromatographic or colorimetric analysis, [b]quercetin aglycone equivalent, [c]n.d. = not determined, [d]sum of excreted flavonols, [1](Hollman *et al.*, 1997), [2](Hollman *et al.*, 1999), [3](Graefe *et al.*, 2001), [4](Olthof *et al.*, 2000).

Although the pigs used in each trial of the current studies were taken from the same litter, inter-individual variations were also observed. Therefore, each animal was subjected to each treatment, thus serving as its own 'control', to avoid masking of effects of food components, e.g. dietary fat, by inter-individual variations in the overall handling of quercetin.

In the first trial presented in this thesis (chapter II), a significant enhancing effect of LCT (~54%) on the bioavailability of quercetin could be documented. Some of the factors mentioned above might also have been responsible for the failure in reaching the level of significance with respect to the influence of LCT on the bioavailability of quercetin in the second trial (non-significant increase of 12%, chapter III). Such problems might be eliminated by using a larger number of animals per trial.

In the first study, 10 mg quercetin kg^{-1} BW was administered to the pigs in the form of Q3G, yielding maximum plasma concentrations within the range of 0.9-0.6 µmol L^{-1}. Compared to the studies cited in Table IV.1, where approximately 1.4 mg quercetin equivalent kg^{-1} BW have been administered, the maximum plasma levels reached in humans seem to be considerably higher than in pigs. Similarly, in the second study, comparison of the post-absorptive plasma levels at 1 h after intake in pigs and rats revealed that plasma levels (at least at this time point) in rats are considerably higher than in pigs (chapter III). One possible explanation for the substantially lower quercetin concentrations in pigs as compared to humans and rats (normalized to an equivalent dietary dose of quercetin) might be a more efficient first pass elimination (intestinal mucosa, liver) in pigs.

In general, c_{max} of plasma quercetin after application of quercetin monoglucosides is reached within ~1.1 h (0.5-2.9 h, t_{max}) after oral intake in humans, rats and pigs (Manach *et al.*, 2005; Cermak *et al.*, 2003; Aziz *et al.*, 1998; Hollman *et al.*, 1996). Thus, the results obtained with low fat and LCT diets in the present experiments are well within this range (chapters II and III). In both current studies, a higher plasma level at later time points (~8 h after intake) were consistently observed after intake of quercetin with the LCT-containing meal as compared to the low fat test meal (although not significant in the second study). This phenomenon might be due to some enterohepatic recycling of quercetin (chapter I.3.3.4.), which might be intensified in the presence of LCT within the chyme. Hints for an enterohepatic recycling of quercetin in pigs have also been observed in earlier studies (Ader *et al.*, 2000).

As pointed out earlier, one aim of the second study of this thesis was to get some insight into the possible involvement of lymphatic absorption of quercetin after co-administration with dietary lipids and to further elucidate the mechanisms responsible for the higher

bioavailability of quercetin from lard-enriched test meals as compared to low-fat control. Two reasons were theoretically considered to explain this finding: (1) A better solubility of the relatively lipophilic quercetin aglycone in the intestinal tract in the presence of fat and/or (2) the integration of quercetin into chylomicrons with subsequent lymphatic absorption. In the first case, an increased absorption from fat containing diets should be largely independent of the type of dietary fat. On the other hand, formation of chylomicrons is largely restricted to the absorption of LCT or long-chain fatty acids, because MCT or medium-chain fatty acids are absorbed directly into the portal vein (Harkins & Sarett, 1968; Bloom *et al.*, 1951). Lymphatic absorption of quercetin could enhance quercetin bioavailability due to bypassing of the liver, which might reduce biliary elimination during the first liver passage (*first pass* effect). If this was true, only fats containing LCT should increase the bioavailability of flavonols but not fats comprised of MCT due to the different handling of LCT and MCT during intestinal absorption. As already discussed (chapter III), the interpretation of the results was somewhat hampered by the unexpected finding, that the MCT-containing meal significantly delayed the absorption of quercetin. Nevertheless, both types of dietary fat enhanced the bioavailability of quercetin. Thus, both mechanisms pointed out above may be of relevance with respect to the influence of dietary lipids on the bioavailability of quercetin. It appears, that the formation of chylomicrons accelerates absorption of quercetin from chyme (only relevant for LCT) and that the more lipophilic nature of chyme after ingestion of both types of dietary lipids (LCT and MCT) may promote a more extensive absorption of dietary quercetin. Further investigations on flavonoid content of lymph are needed to get conclusive information on this potential uptake pathway. In a very recent study, quercetin was detected in lymph of rats (Murota & Terao, 2005). In this study, quercetin was administered within a propylene glycol/water mixture, and no fat was administered. Quercetin appeared in the lymph within 30 min, similar to its appearance in blood. In a proceeding study of the same group conducted under similar conditions, administration of the same amount of quercetin to rats resulted in clearly higher plasma flavonol levels (da Silva *et al.*, 1998) compared to the lymphatic concentrations of quercetin reported in the later study (c_{max} = 3.1 µM, t_{max} = 30 min) (Murota & Terao, 2005).

Methylation of quercetin

Methylation of quercetin occurs in different species as part of the detoxification reactions in gut and liver (Donovan *et al.*, 2001; Moon *et al.*, 2000; Manach *et al.*, 1999; Piskula & Terao,

1998; Manach *et al.*, 1997; Hollman *et al.*, 1997). Main molecular sites for *O*-methylation of quercetin are the 3'- and 4'-hydroxyl groups, yielding isorhamnetin or tamarixetin, respectively. Differences in the pattern of methylated metabolites of quercetin have been observed between species. Morand *et al.* (2000) reported that the methylated quercetin derivatives isorhamnetin and tamarixetin accounted for ~50% of flavonols (with only minor amounts of tamarixetin) 3.5 h post dosing of 20 mg of quercetin equivalents in rats. At time points ≥7 h, the concentration of these monomethylated quercetin derivatives reached 70-77%. Similar plasma concentrations of isorhamnetin were also observed in another rat study (Manach *et al.*, 1997). In the own rats experiment (chapter III), ~50% of total flavonols were present as quercetin, ~45% isorhamentin and only ~5% tamarixetin were observed at 1 h post-dosing (100 μmol kg^{-1} BW) which is in good agreement with other rat studies. In humans, ~20-40% of absorbed quercetin is methylated in the 3'-position, yielding isorhamnetin (Graefe *et al.*, 2001; Olthof *et al.*, 2000; Erlund *et al.*, 2000). In human blood samples, after a 5 d period with intake of flavonoids-rich fruits and vegetables, using the same method applied for analysis of pig plasma samples in the current studies, ~80% of quercetin and only ~20% of both isorhamnetin and tamarixetin were detected (Hubbard *et al.*, 2003). In pigs, consistently a fraction of ~6-11% of isorhametin and of 5-18% of tamarixetin with the remainder being present as non-methylated quercetin were found (chapter II and III) (Cermak *et al.*, 2003; Ader *et al.*, 2000). Thus, methylation seems to be species-dependent but may also vary depending on the dose of quercetin applied.

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PREPARATION OF PLASMA SAMPLES AND HPLC CONDITIONS

Flavonol compounds were extracted from plasma samples according to a method used by Morand *et al.* (1998). Pig plasma (980 µL) was spiked with 20 µL rhamnetin (50 µg/mL), used as an internal standard, and acidified to pH 4.9-5.0 with 130 µL acetic acid (0.583 M). This is the optimal pH for the enzyme glucuronidase/sulfatase. Probes were vortexed thoroughly. For deconjugation of flavonols circulation in plasma, 75 µL of β-glucuronidase/sulfatase (crude enzyme extract type H-2 of *Helix pomatia*, ~130 000 U/mL glucuronidase and ~400 U/mL sulfatase, Sigma-Aldrich) were added and samples were incubated for 60 min while gently shaking in a water bath. The reaction was stopped by adding 5.5 mL acetone, the resulting mixture was shaken horizontally for 20 min, and centrifuged for 45 min (4 °C, 3700 g). Supernatants were evaporated under partial vacuum for ~5 h at 45 °C (Speed-Vac AES 1010, Savant Instruments Inc.) until dryness. The residual pellet was resuspended in 200 µL methanol by vortexing and ultrasonication (15 min). Aqua bidest. (77.5 µL) and 22.5 µL HCl aq. (10 M) were added, and the mixture was vortexed and centrifuged again at room temperature for 10 min at 16000 g. 200 µL of the supernatant were used for analysis and placed in the cooled autosampler (4 °C) of the HPLC apparatus.

CHROMATOGRAPHIC CONDITIONS

HPLC analysis was performed according to the method of Hollman *et al.* (1996). In principal, chelate complexes of intact flavonols with aluminium ions were detected after postcolumn derivatisation using a fluorescence detector. This method is restricted to analysis of intact flavonols containing a free 3-hydroxyl and 4-keto group at the C-ring which forms a fluorescent complex with Al^{3+}.

The HPLC system used (Jasco Labor- und Datentechnik GmbH Deutschland) included an autosampler (AS-2057 Plus), a degasser (DG-1580-54) and an isocratic pump (PU-1580) for the solvent, a second isocratic pump for the postcolumn reactant (PU-980), a column oven, and a fluorescence detector (FP-920). For HPLC analysis, 30 µL of the sample were injected onto a reversed phase C-18 Kromasil 100 column (250 x 4 mm, 5 µm particle size, Jasco Labor- und Datentechnik GmbH Deutschland), protected by a C-18 Inertsil ODS-2 column (10 x 4 mm, 5 µm particle size, Jasco Labor- und Datentechnik GmbH Deutschland). As the mobile phase, a buffered solution of 0.025 M sodium-dihydrogene-phosphate (pH 2.4), acetonitrile, and methanol (68:27:5 v/v), pH 2.96 was used at a flow rate of 1 mL/min. The

column effluent was mixed with the reagent (1 M $Al(NO_3)_3$ in methanol containing 7.2% (v/v) acetic acid) at a flow rate of 0.4 mL/min in a postcolumn reactor (6 m, 0.5 mm inner diameter, teflon tubing, plaited) connected to the HPLC column via a low-dead-volume tee. The column and the reactor were placed in the column oven set at 30 °C. The fluorescence of the ensuing flavonol-metal complex was measured at 485 nm, with an excitation wavelength of 422 nm. Flavonols were identified by comparison of retention time to that of standard substances.

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FATTY ACID COMPOSITION OF LARD

In the experiments of this thesis, lard is used as a typical dietary source of long-chain triacylglycerols (LCT) for creating the test meals. In lard triacylglycerols of the types SUU, USU and UUU (with U = unsaturated fatty acid, and S = saturated fatty acid) are abundant. The typical fatty acid composition of lard is given in Table A.

Table A: Mean fatty acid composition of lard (Belitz & Grosch, 1992)

fatty acid	% (wt/wt)	fatty acid	% (wt/wt)
12:0	0.0	18:0	14.0
14:0	2.0	18:1 (9)	43.0
14:1 (9)	0.5	18:2 (9, 12)	9.0
16:0	24.0	18:3 (9, 12, 15)	1.0
16:1 (9)	4.0	20:0	0.5
		20:1, and 20:2	2.0

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