Iris Erlund

CHEMICAL ANALYSIS AND PHARMACOKINETICS OF THE FLAVONOIDS QUERCETIN, HESPERETIN AND NARINGENIN IN HUMANS

ACADEMIC DISSERTATION

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ABSTRACT

Flavonoids are phenolic compounds widely present in plants and foods of plant origin. Experimental and epidemiological studies have suggested a protective role for the compounds on human health, but until recently, because methods for their analysis in tissues were lacking, knowledge about their bioavailability, pharmacokinetics and metabolic fate in humans was limited.

The primary aims of the studies presented in this thesis were to develop methods suited for the analysis of the flavonoids quercetin, hesperetin and naringenin in human plasma and urine. Other aims were to investigate their bioavailability, pharmacokinetics and use as biomarkers of intake. The compounds were chosen on the basis of experimental and epidemiological evidence, and dietary intake.

The analytical methods developed allowed the analysis of low concentrations of quercetin in plasma, and hesperetin and naringenin in plasma and urine, with good reproducibility. The methods were based on solid-phase and liquid-liquid extraction and high-performance liquid chromatography with electrochemical detection.

The pharmacokinetics and bioavailability of quercetin were studied in subjects ingesting single doses of quercetin aglycone and rutin, and in subjects consuming berries or their habitual diets for several weeks. In the first human study, healthy volunteers received three different doses of quercetin aglycone or rutin orally in a double-blind, diet-controlled, cross-over setting. The overall kinetic behaviour of quercetin differed after the two treatments, although the mean C_{max} and $AUC_{(0-32)}$ values were similar. Quercetin from quercetin aglycone was absorbed rapidly from the upper parts of the gastrointestinal tract, while quercetin from rutin appeared to be absorbed from the distal parts of the small intestine or the colon. Especially after ingestion of rutin, marked variation in plasma levels occurred. Furthermore, quercetin from rutin was more bioavailable in women than in men. $T_{1/2}$ of quercetin ranged

between 15 and 18 h. In the second human study, middle-aged men consumed 100 g/day in total of lingonberries, black currants and bilberries for two months, or their habitual diets. Plasma quercetin concentrations were 30-50% higher in the berry group than in the control. When the men were still on their habitual diets, the mean plasma concentration was 16 ± 13 µg/l.

The pharmacokinetics and bioavailability of the flavanones hesperetin and naringenin were investigated in a study where healthy volunteers ingested 400-760 ml of orange juice or grapefruit juice once. Relatively high concentrations of flavanones were reached in plasma, but interindividual variation in plasma levels was marked. Both flavanones appeared to be absorbed from the distal parts of the small intestine or the colon, and their plasma half-lifes were similar (1-2 h). The mean urinary recovery of naringenin was 1% from orange juice and 30% from grapefruit juice. The corresponding value for hesperetin from orange juice was 5%. These values are minimum estimates for bioavailability.

The results of the studies indicate that plasma quercetin is a fairly good biomarker of intake. Its plasma concentrations increase with increasing dose, and it has a comparatively long half-life. It is also bioavailable from a typical Finnish diet. Fasting plasma and especially urine flavanone levels, by contrast, appear to be less useful as biomarkers of intake.

In conclusion, methods for the analysis of quercetin, hesperetin and naringenin in human plasma and urine were developed. Quercetin was shown to be bioavailable from capsules, berries and the diet, and hesperetin and naringenin from citrus juices.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals.

I. Erlund I[1], Alfthan G, Siren H, Ariniemi K, Aro A. Validated method for the quantitation of quercetin from human plasma using high-performance liquid chromatography with electrochemical detection. J Chromatogr B 1999;727:179-189.

II. Erlund I[1], Kosonen T, Alfthan G, Mäenpää J, Perttunen K, Kenraali J, Parantainen J, Aro A. Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers. Eur J Clin Pharmacol 2000;56:545-553.

III. Erlund I[1], Marniemi J, Hakala P, Alfthan G, Meririnne E, Aro A. Consumption of black currants, lingonberries and bilberries increases serum quercetin concentrations. Eur J Clin Nutr 2002 (in press).

IV. Erlund I[1], Meririnne E, Alfthan G, Aro A. Plasma kinetics and urinary excretion of the flavanones naringenin and hesperetin in humans after ingestion of orange juice and grapefruit juice. J Nutr 2001;131:235-241.

[1]Author contributed to the design of the study, data collection, chemical and statistical analyses, interpretation of results and writing of manuscript.

ABBREVIATIONS

ATBC	Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study
AUC	area under plasma concentration-time curve
BMI	body mass index
Cl_{ren}	renal clearance
C_{max}	maximum plasma concentration
CYP	cytochrome P450
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
LDL	low-density lipoprotein
MPR	multi drug resistance-associated protein
SGLT	sodium-dependent D-glucose cotransporter
SULT	cytosolic sulfotransferase
$T_{1/2}$	elimination half-life
T_{max}	time to reach C_{max}
UGT	uridine diphosphate glucuronosyltransferase

1. INTRODUCTION

A diet rich in vegetables and fruit has long been recognized to protect against chronic diseases such as cardiovascular disease and cancer. Lifestyle factors, such as sufficient physical activity, abstinence from smoking, and a low-energy diet, probably explain a large part of this protection. Individual constituents of the diet or plants may also play a role. Until recently, nutritional research mainly focused on fats, carbohydrates, proteins, vitamins and minerals. The existence of secondary plant metabolites, often present in high quantities in the fibre fraction of plants, was largely ignored. Today, however, many of these compounds, although they are not essential for maintaining life, are recognized as being potentially beneficial to human health.

Flavonoids comprise one of the largest and most widely distributed groups of secondary plant metabolites (Kühnau 1976, Robards and Antolovich 1997). That they possess promising biological activities has been known for some time, but little information has been available about their bioavailability, metabolic fate and health effects in humans. Flavonoids are found in practically all photosynthesizing plants and therefore all humans consuming foods of plant origin are exposed to them. Flavonoids are divided into several subgroups and the different compounds possess different chemical and biological properties. They also have different dietary sources, bioavailabilities and, most likely, abilities to exert biological actions *in vivo*.

In this work, the flavonoids quercetin, hesperetin and naringenin were examined. They were selected because of their promising biological activities, significant dietary intake and epidemiological evidence. Quercetin is the most studied flavonoid, and *in vitro* and animal studies indicate antioxidative, anticarcinogenic and anti-inflammatory activities. Dietary intake of the compound is significant and several epidemiological studies suggest an inverse association between intake of quercetin and risk of cardiovascular disease. The flavanones hesperetin and naringenin are present in high

concentrations in citrus fruits, and thus, their intake can be rather high in individuals consuming citrus products regularly. Biological activities ascribed to flavanones include anticarcinogenic, antioxidant, phytoestrogenic and blood lipid-altering activities.

In this thesis, methods for the analysis of quercetin, hesperetin and naringenin in human plasma and urine were developed. In addition, their bioavailability and pharmacokinetics from pure compounds, fruit juices, berries and the diet were studied. Information was also obtained about whether their plasma or urine concentrations are reliable as biomarkers of dietary intake. The work was part of a project studying the bioavailability of the most important dietary flavonoids, phenolic acids and lignans in humans, and investigating the associations between their plasma concentrations and the risk of chronic diseases.

2. REVIEW OF THE LITERATURE

2.1. Definitions

2.1.1. Bioavailability

No single definition accurately describing the multiplexity of the term *bioavailability* is available. The definition offered by the Food and Drugs Administration (FDA) is ”the rate and extent to which the therapeutic moiety is absorbed and becomes available to the site of drug action”. Unfortunately, this definition can be misinterpreted in many ways. In this work, the more commonly used definition ”the fraction of an ingested dose of a compound which is taken into the systemic circulation”, is used.

Attempts to use the term bioavailability with quantitative precision or to give exact values for it are likely to fail. In clinical pharmacology, the term *absolute bioavailability* is sometimes used to describe the exact amount of compound that reaches the systemic circulation. It is the fraction of the area under the curve (AUC) after oral ingestion of the AUC after intravenous administration. In nutrition, however, *relative bioavailability*, describing the bioavailability of a compound from one source compared with another, is a more useful term.

A multitude of factors affect the bioavailability of a compound. Such factors include the food matrix, the type of pharmaceutical preparation, the chemical form of the compound, co-ingested compounds and eating itself. However, bioavailability is not only a property of the compound or the food itself, but of the individual as well. Factors varying between individuals, also called subject factors, include mucosal mass, intestinal transit time, rate of gastric emptying and up/down regulation of absorption. Furthermore, biotransformation of a compound, occurring in the intestinal wall during absorption or in the liver, can alter the amount of compound reaching the systemic circulation (= first-pass effect).

Several different methods, none of them lacking shortcomings, are used for the measurement of bioavailability (Heaney 2001). The balance method - measuring the difference between what is ingested and what is found in the faeces – does not take into consideration bacterial transformation or degradation of compounds. The tracer method (using stable or radioactive isotopes) is sensitive and reproducible, but also fails to take into account biotransformation and degradation. Using the biochemical or physiological effect of a compound as a measure of bioavailability is promising in theory, but many problems are associated with this approach. For instance, the effect of a nutrient can depend on the nutritional status of the individual. The amount of compound excreted into the urine is sometimes used as a measure of absolute bioavailability; however, this only describes the minimum amount of compound absorbed since other routes of excretion, such as biliary excretion, are overlooked. Plasma or serum concentrations are often used in bioavailability studies, but it should be kept in mind that a single measurement with no information on time of ingestion and pharmacokinetics is a poor measure of bioavailability, especially at an individual level. Repeated measurements or calculations of AUC values give a much more reliable estimate on bioavailability, although homeostatic factors sometimes limit the use of this approach.

2.1.2. Pharmacokinetics

In the field of pharmacology, pharmacokinetic parameters are calculated to describe the kinetics of drug absorption, distribution and elimination. Information about these processes is important when assessing the time-course of effects of a drug. No universally accepted term describing the corresponding events for nutrients or xenobiotics is available. Terms such as ”biokinetics” or ”kinetics” have been used, but their meanings are broader and they are rather non-descriptive. Therefore, in this thesis, the term pharmacokinetics is used. However, the viewpoint of the thesis is nutritional. Also, although quercetin, hesperetin and naringenin are components of

over a hundred pharmaceutical preparations sold worldwide, it should be emphasized that their pharmacological effects have not been demonstrated in clinical studies, and therefore they cannot be considered to be effective medications.

In the following section, some basic concepts in pharmacokinetics are covered. This section is mainly based on textbooks edited by Rang and Dale (1991) and Rowland and Tozer (1995).

Pharmacokinetic models

Following oral administration, the plasma concentration of a compound rises, but when the rate of elimination exceeds the rate of absorption, its concentration starts to decline. For many compounds, the disappearance from plasma follows an exponential, rather than linear, time-course. Different pharmacokinetic models are used to calculate pharmacokinetic parameters. Important parameters include elimination half-life ($T_{1/2}$), time to reach $T_{1/2}$, maximum plasma concentration (C_{max}), time to reach C_{max} (T_{max}) and renal clearance (Cl_{ren}).

The most commonly used pharmacokinetic model is the two-compartment model. It is an approximation of a situation where the kinetics becomes bi-exponential. The two compartments are the central compartment (plasma) from which the compound can move into (and back from) the peripheral compartment (other tissues). The transfer of a compound to tissues occurs quickly (the fast phase, α, in Figure 1), and the elimination of the compound through excretion or metabolism occurs more slowly (the slow phase, β, can also be distinguished in the graph). The half-lives of the phases are calculated from the equation $T_{1/2}=\ln 2/k$, where k is the slope of each phase.

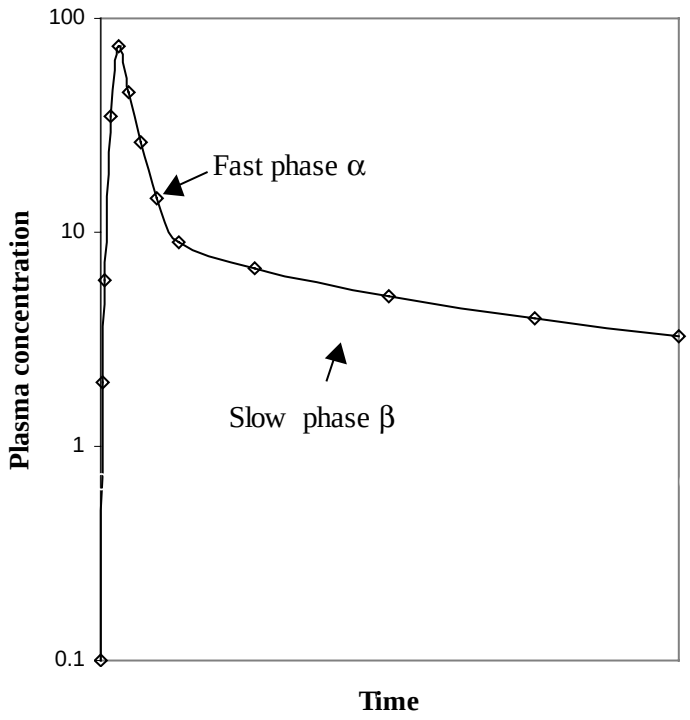


Figure 1. Plasma concentration vs. time curve for a hypothetical compound following a two-component decay.

Linear and non-linear pharmacokinetics

The pharmacokinetics of compounds are often studied after single-dose administration. For compounds exhibiting linear pharmacokinetics, the kinetic behaviour of compounds during multiple dosing is usually predictable based on such single-dose data. Occasionally, although quite rarely, the pharmacokinetic behaviour of compounds is different during long-term administration. Reasons for non-linear kinetics include, for instance, induction or saturation of metabolic pathways (Ludden 1991). Because such phenomena are possible, the pharmacokinetics of compounds should also be studied during long-term administration or steady-state.

Absorption

Absorption means the passage of a compound from its site of administration, usually the gastrointestinal tract, into the bloodstream. Sometimes the term is erroneously used as a synonym for bioavailability, but in that case the first-pass effect is overlooked. Factors affecting gastrointestinal absorption include gastrointestinal motility, chemical factors (ionization, lipid solubility, interaction with gut contents), splanchnic blood flow, particle size and formulation, and competition for carrier-mediated transport. Activity and amount of transport proteins and of secretory proteins, such as P-glycoprotein and multidrug resistance associated protein 2 (MRP2), also affect the net absorption of compounds (Wagner et al. 2001).

Distribution

A number of factors affect the time-course of distribution and the extent of uptake of compounds into tissues or cells. Whether a compound can cross membranes depends on lipid solubility, ionization, molecular weight and the presence of transport systems. Furthermore, binding to blood components, such as plasma proteins and blood cells, and to tissue components, affects distribution.

Elimination

Elimination of a compound occurs through excretion by the kidneys, skin or lung, or in the bile, or by metabolism or degradation. Renal excretion occurs via glomerular filtration, active tubular secretion or passive diffusion across the tubular epithelium. Nearly all molecules with a molecular weight < 20 000 kD can cross the glomerular capillaries. Macromolecules such as albumin, with a molecular weight of 69 000 kD, do not, and therefore glomerular filtration is not the main excretion mechanism of compounds bound to this protein. Tubular secretion is a more effective route of

elimination. The carrier-mediated transport systems eliminate both acids and bases, and compounds bound to plasma proteins.

Biotransformation

Biotransformation is an important step in the elimination of many compounds. Phase I reactions include oxidation, reduction and hydrolysis reactions, which often result in more reactive compounds than the parent compound. Different conjugation reactions, i.e. phase II reactions, usually yield less toxic or less active and more readily excreted metabolites.

The liver has traditionally been considered to be the major site for biotransformation reactions. However, during the past few years it has become increasingly evident that extrahepatic tissues, especially the gastrointestinal tract, possess considerable metabolic potential as well. From the standpoint of first-pass extraction, the most important biotransformation reaction is oxidation by enzymes belonging to the cytochrome P450 superfamily. Conjugation with glucuronic acid or sulfate groups also occur for many xenobiotics. Glucuronidation is mediated by the UGT (UDP-glucuronosyltransferase) multigene family and sulfation by cytosolic sulfotransferases (SULT). To date, at least 17 UGT (Meech and McKenzie 1997) and 11 SULT (Glatt 2000) forms have been identified in humans. Interestingly, the isoenzymes differ in both their substrate specificities and tissue distribution (Her et al. 1996, Thummel et al. 1997, Cheng et al. 1999, Fisher et al. 2000, Glatt 2000). Furthermore, marked interindividual variation in the synthesis or activity of these enzymes has been reported.

2.1.3. Biomarkers

Biomarkers can reflect exposure, status, disease susceptibility, metabolic effects, disease occurrence and compliance (Kohlmeier 1991). Nutritional epidemiologists are mainly interested in biomarkers as measures of dietary intake, which can serve as quantifiable determinants of disease risk. For more comprehensive reading on biomarkers in nutritional epidemiology, refer to Bates et al. (1997) and Hunter (1998). The following section is mainly based on these textbooks.

Assessment of dietary intake by dietary survey methods is associated with many problems such as under-reporting, inaccurate or lacking food composition data and variation in nutrient composition of individual foods. Furthermore, in epidemiological studies, relevant questions sometimes go unanswered because at the time of data collection, the question had not yet been formulated. These problems can in some cases be avoided by using the concentrations of specific compounds in human tissues, mainly plasma, as biomarkers of intake. Plasma concentrations of nutrients do not, however, always reflect dietary intake because metabolism and pharmacokinetic properties also have an impact. Therefore, such biomarkers should be carefully validated before they are used. Important requirements of a tissue biomarker are that it be sensitive to intake and measureable in tissues. Optimal biomarkers are also readily available and reflect long-term intake. In reality, few good tissue biomarkers of intake are available. Exceptions include certain adipose tissue fatty acids and toenail selenium. Examples of compounds, the plasma concentrations of which reflect intake poorly, are retinol and calcium.

Several types of studies are informative when evaluating whether a biomarker is sensitive to changes in intake. Small-scale feeding studies yield information on pharmacokinetics (including half-life, dose-response, and steady-state concentrations) and bioavailability. In cross-sectional studies, different intake levels between countries

can be compared. Another approach is to compare the biomarker with intake directly (e.g. when intake is measured from duplicate portions).

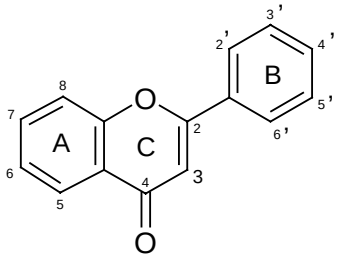
2.2. Flavonoids

2.2.1. Chemistry and classification

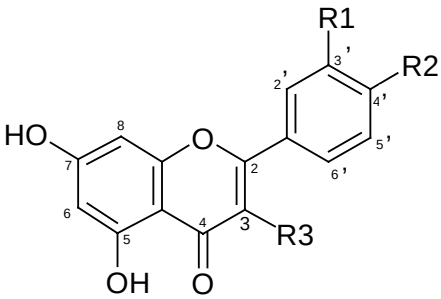
Flavonoids consist of two benzene rings (A and B), which are connected by an oxygen-containing pyrane ring (C) (Figure 2). Flavonoids containing a hydroxyl group in position C-3 of the C ring are classified as 3-hydroxyflavonoids (flavonols, anthocyanidins, leucoanthocyanidins and catechins), and those lacking it as 3-desoxyflavonoids (flavanones, flavones). Classification within the two families is based on whether and how additional hydroxyl or methyl groups have been introduced to the different positions of the molecule. Isoflavonoids differ from the other groups; the B ring is bound to C-3 of ring C instead of C-2. Anthocyanidins and catechins, on the other hand, lack the carbonyl group on C-4 (Kühnau 1976).

Flavonoids are mainly present in plants as glycosides. Aglycones (the forms lacking sugar moieties) occur less frequently. At least 8 different monosaccharides or combinations of these (di- or trisaccharides) can bind to the different hydroxyl groups of the flavonoid aglycone (Williams and Harborne 1994). The most common sugar moieties include D-glucose and L-rhamnose. The glycosides are usually O-glycosides, with the sugar moiety bound to the hydroxyl group at the C-3 or C-7 position.

To date, over 6000 flavonoids have been identified in plants (Harborne and Williams 2000). The large number is a result of the many possible combinations of flavonoid aglycones and different sugars. The number of aglycones and flavonoid glycosides commonly found in edible plants or foods, is much smaller.



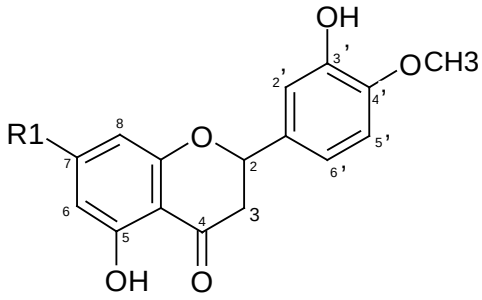
Flavane nucleus



Quercetin: R1=OH, R2=OH, R3=OH

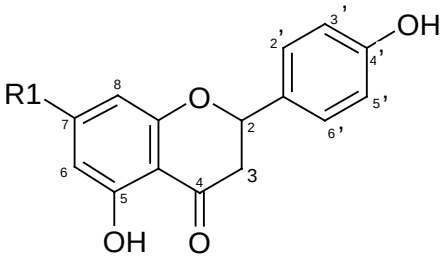
Rutin: R1=OH, R2=OH,
R3=6-O-α-L-rhamnosyl-D-glucoside

Spiraeoside: R1=OH,
R2=O-ß-D-glucoside, R3=H



Hesperetin: R1=OH

Hesperidin:
R1=6-O-α-L-rhamnosyl-D-glucoside



Naringenin R1=OH

Naringin
R1=2-O-α-L-rhamnosyl-D-glucoside

Narirutin
R1= 6-O-α-L-rhamnosyl-D-glucoside

Figure 2. Chemical structures of the flavane nucleus, quercetin (M_W=302 g/mol), hesperetin (M_W=302 g/mol) and naringenin (M_W=272 g/mol), and some of their most common glycosides.

2.2.2. Occurrence in foods

Flavonoids are present in most edible fruits and vegetables, but the type of flavonoids obtained from different dietary sources varies. The main dietary flavonoids and their sources are shown in Table 1. Intake estimates for flavonoids are only available for a few flavonoid subclasses such as flavonols, flavanones and isoflavones.

Flavonols

The most common flavonol in the diet is quercetin. It is present in various fruits and vegetables, but the highest concentrations are found in onion (Table 1) (Hertog et al. 1992a). The importance of different foods as quercetin sources varies between countries. Hertog et al. (1995) calculated flavonol intakes from the Seven Countries Study, which was started in the late 1950s, and reported that tea was the predominant source of quercetin in the Netherlands and Japan. Wine was the major source of quercetin in Italy, while onion and apples contributed most in the US, Finland, Greece and former Yugoslavia. More recently, Häkkinen et al. (1999) estimated that onions, followed by tea, apples and berries are the major sources of quercetin in Finland. It should be noted that onion is usually not consumed in high quantities, but it is an important source because of its high quercetin content. Tea and especially wine, on the other hand, contain relatively low amounts of quercetin but are consumed, at least in some countries, in rather high quantities. The daily intake of quercetin was estimated to range between 3 and 38 mg in the Seven Countries Study (Hertog et al. 1995), and in Finnish male smokers, the intake estimate (from the 1980s) was 7.4 mg (Hirvonen et al. 2001a).

Quercetin is present in plants in many different glycosidic forms (Kühnau 1976) with quercetin-3-rutinoside, also called quercetin-3-rhamnoglucoside or rutin, being one of the most widespread forms. In onions, the compound is bound to one or two glucose molecules (quercetin-4’-glucoside, quercetin-3,4’-glucoside). Other quercetin glycosides present in the diet are, for instance, quercetin galactosides (apples) and

quercetin arabinosides (berries). Other flavonols in the diet include kaempferol (broccoli), myricetin (berries) and isorhamnetin (onion). The chemical structures of quercetin and some quercetin glycosides are shown in Figure 2.

Flavanones

Flavanones occur almost exclusively in citrus fruits. The highest concentrations are found in the solid tissues, but concentrations of several hundred mg per litre are present in the juice as well (Tomás-Barberán and Clifford 2000). Hesperidin (hesperetin-7-rutinoside) and narirutin (naringenin-7-rutinoside) are the major flavonoids of oranges and mandarines. The main flavonoids of grapefruit are naringin (naringenin-7-neohesperoside) (70%) and narirutin (20%) (Kawaii et al. 1999). Low concentrations of naringenin are also found in tomatoes and tomato-based products. Fresh tomatoes, especially tomato skin, also contain naringenin chalcone, which is converted to naringenin during processing to tomato ketchup (Krause and Galensa 1992). In Finland, the average intake of naringenin has been estimated to be 8.3 mg/day, and for hesperetin 28.3 mg/day (Kumpulainen et al. 1999). The structures of hesperetin, naringenin and their most important glycosides are shown in Figure 2.

Catechins

Catechins usually occur as aglycones or are esterified with gallic acid. (+)-Catechin and (-)-epicatechin are found in various fruits and vegetables such as apples, pears, grapes and peaches (Arts et al. 2000a). The highest concentrations of catechins are found in tea and red wine (Arts et al. 2000b).

Flavones

The main flavones in the diet are apigenin and luteolin. Their dietary intake is rather low because they occur in significant concentrations in only a few plants, such as red pepper (Hertog et al. 1992a) and celery (Hertog et al. 1992b) .

Anthocyanidins

Anthocyanins (=anthocyanidin glycosides) are responsible for the red, blue or violet colour of such edible fruits as plums, apples, aubergine and many berries. The most common anthocyanidins include pelargonidin, cyanidin, delphinidin and malvidin (Kühnau 1976).

Isoflavonoids

The predominant isoflavonoids are the isoflavones genistein and daidzein, which occur mainly in legumes. The highest concentrations are found in soy bean and soy products, and much lower concentrations are present in other legumes (Mazur 1998, Liggins et al. 2000), not to mention other vegetables and fruit.

Table 1. Main dietary flavonoids and their sources in the diet.

Flavonoid[1]	Source	Content of aglycone (mg/kg)
Flavonol		
Quercetin-3,4'-glucoside Quercetin-3-glucoside	onion	284-486[2]
Quercetin-3-rhamnoglucoside (rutin)	black tea	10-25[3]
Quercetin-3-galactoside Quercetin-3-rhamnoside Quercetin-3-arabinoside Quercetin-3-glucoside	apple	21-72[2]
Quercetin-3-rhamnoglucoside Quercetin-3-rhamnoside Quercetin-3-galactoside	black currant	44[4]
Myrisetin-3-glucoside		71[4]
Flavone		
Luteolin-7-apiosylglucoside	red pepper	7-14[2]
Flavanone		
Hesperetin-7-rhamnoglucoside (hesperidin)	orange juice	116-201[5]
Naringenin-7-rhamnoglucoside (narirutin)		15-42[5]
Naringenin-7-rhamnoglucoside (naringin) Naringenin-7-rhamnoglucoside (narirutin)	grapefruit juice	68-302[5]
Flavanols		
(+)-Catechin	apple	4-16[6]
(-)-Epicatechin		67-103[6]
(+)-Catechin	red wine	16-53[7]
(-)-Epicatechin		9-42[7]
(Epi)catechin and their gallates	black tea	102-418[7,8]
Anthocyanins		
Cyanidin-3-glucoside Cyanidin-3-rutinoside	black currant	760[9]
Delphinidin-3-glucoside Delphinidin-3-rutinoside		590[9]
Isoflavones		
Genistein-7-glycoside	soy beans	480[10]
Daidzein-7-glycoside		330[10]

[1]Kühnau 1976, [2]Hertog et al. 1992a, [3]Hertog et al. 1993a, [4]Häkkinen et al. 1999, [5]Mouly et al. 1994, [6]Arts et al. 2000a, [7]Arts et al. 2000b, [8]sum of aglycones and gallates, [9]Nyman and Kumpulainen 2001,[10]Mazur et al. 1998

2.3. Quercetin, hesperetin and naringenin

2.3.1. Biological activities

A wide range of biological activities have been reported for different flavonoids (for reviews, see Formica and Regelson 1995, Cook and Samman 1996, Di Carlo et al. 1999, Nijveldt et al. 2001). The most studied flavonoid is quercetin, for which over 2700 citations are listed in PubMed.

Quercetin has been reported to exhibit biological effects such as antioxidant (Hayek et al. 1997, Chopra et al. 2000), anticarcinogenic (Verma et al. 1988, Deschner 1991, Pereira 1996), anti-inflammatory (Ferry et al. 1996) and antiaggregatory (Pignatelli et al. 2000) effects. The mechanisms behind the effects are largely unknown, but it is possible that several different types of biochemical events precede a biological effect. The antioxidant effect, for instance, could be a result of metal chelation (Ferrali et al. 1997, Sestili et al. 1998), scavenging of radicals (Huk et al. 1998, Aherne et al. 2000) and/or enzyme inhibition (Da Silva et al. 1998, Nagao et al. 1999). Anticarcinogenesis, on the other hand, could result from enzyme inhibition (Agullo et al. 1997, Huang et al. 1997), antioxidation or effects on gene expression (Hansen et al. 1997, Piantelli et al. 2000, Xing et al. 2001). Altered gene expression could lie behind the anti-inflammatory effect (Kobuchi et al. 1999). Regarding anticarcinogenesis, it should be noted that in the 1970s, quercetin was actually considered to be a carcinogen because the compound showed mutagenicity in the Ames test (Bjeldanes and Chang 1977). However, a number of long-term animal studies subsequently performed with different species have indicated that this is not the case. On the contrary, quercetin has been shown to inhibit carcinogenesis in laboratory animals (Stavric 1994).

Hesperetin has also attracted the attention of cancer researchers. This compound (and orange juice) has been shown to inhibit chemically induced mammary (So et al. 1996), urinary bladder (Yang et al. 1997) and colon (Tanaka et al. 1997, Miyagi et al. 2000)

carcinogenesis in laboratory animals, and to have antioxidative effects (Miyake et al. 1998). Other possible effects of hesperetin, as well as the other major citrus flavanone, naringenin, are on lipid metabolism. They have been reported to regulate apolipoprotein B secretion by HepG2 cells, possibly through inhibition of cholesterol ester synthesis (Borradaile et al. 1999), and to inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and acyl coenzyme A:cholesterol O-acyltransferase (ACAT) in rats (Lee et al. 1999a, 1999b). Furthermore, a decrease in plasma low-density lipoprotein (LDL) levels and hepatic cholesterol levels in rabbits fed a high-cholesterol diet has been observed (Kurowska et al. 2000a). An increase of high-density lipoprotein (HDL) levels in hypercholesterolemic human subjects after consumption of orange juice was also reported (Kurowska et al. 2000b).

Naringenin has mainly been studied for its possible role in grapefruit juice – drug interactions (Fuhr 1998, Bailey et al. 2000). The considerable increase in plasma concentrations of many drugs metabolised by intestinal cytochrome P450IIIA (CYP 3A4) when administered concominantly with grapefruit juice is well documented and is of clinical relevance (Dresser et al. 2000, Lilja et al. 2000). Naringenin is an inhibitor of the enzyme (Ghosal 1996) and could be one of the compounds causing the interaction. Other biological activities attributed to naringenin include antioxidative (van Acker et al. 2000, Jeon et al. 2002) and anti-inflammatory (Manthey et al. 2001) actions. Different types of effects for naringenin on sex-hormone metabolism have also been suggested (Ruh et al. 1995, Rosenberg et al. 1998, Déchaud et al. 1999, Yoon et al. 2001). The compound has, for instance, been shown to bind to estrogen receptors (Kuiper et al. 1998).

In vitro studies have usually been performed with flavonoid aglycones or glycosides. Flavonoid metabolites have rarely been used, mainly because data about their identity is very scarce and chemical standards for only a few potential metabolites are commercially available. Recently, a few reports on the biological activities of possible quercetin metabolites have been published. Manach et al. (1998) tested the effect of

quercetin and isorhamnetin conjugates, obtained by enzymatic synthesis *in vitro*, on copper-induced LDL oxidation. A mixture of four quercetin glucuronides (0.5 M concentration) and quercetin-3-sulfate (0.5 M concentration) resulted in a similar inhibition of conjugated diene appearance as quercetin aglycone. The inhibitory effect was weaker for the individual metabolites than for the aglycone. Moon et al. (2001) also studied the antioxidant effect of quercetin glucuronides; metabolites were identified from rat plasma obtained 30 min after administering quercetin aglycone (250 mg/kg) intragastrically. The metabolites in question, i.e. quercetin-3-glucuronide and quercetin-4'-glucuronide, were then synthesized enzymatically for *in vitro* testing. Quercetin-3-glucuronide was found to inhibit LDL oxidation and to possess substantial 1,1-diphenyl-2-picrylhydrazyl radical-scavenging activity, but the effect was less pronounced than for the aglycone. Quercetin-4'-glucuronide was ineffective. Day et al. (2000a) produced quercetin glucuronides by incubating quercetin with human liver cell-free extracts. The compounds were identified by studying their absorbance spectra and their response to the addition of shift reagents. After this, their ability to inhibit xanthine-oxidase and lipoxygenase was assessed. Quercetin glucuronized at the 4' or 3' positions was able to inhibit xanthine oxidase at low micromolar concentrations, but 80- to 500-fold higher concentrations were required by quercetin-3-sulfate and 3- or 7-glucuronide. Quercetin-4'-glucuronide was as effective as quercetin aglycone. Quercetin aglycone, quercetin-7-glucuronide, quercetin-4'-glucuronide and quercetin-3'-glucuronide all inhibited soy bean lipoxygenase, but quercetin-3-glucuronide was ineffective.

On the whole, with the available scientific evidence, it is difficult to form an opinion on the significance of flavonoids on human health. Most studies have been performed *in vitro*, and flavonoid aglycones or flavonoid glycosides have been investigated. Many animal studies have also been performed, but results from human studies are very limited. Knowledge about flavonoid metabolism is scarce and until more information on the identity of flavonoid metabolites is gained, evaluating whether results from in vitro studies can be extrapolated to humans, is difficult. This is also the

case for animal studies because metabolism may be species-dependent. Furthermore, the concentrations or amounts used in experimental studies have been high compared with the amounts possibly present in human tissues or the diet. Therefore, in the future, it is important that human studies using intakes within the range of typical dietary intakes be performed. However, because the sensitivity of methods capable of detecting changes in physiological or biochemical responses is limited, human studies performed with high/pharmacological doses are also warranted. Nevertheless, the possibility that the compounds may also have adverse effects, although it may seem remote, should not be forgotten.

2.3.2. Analysis from human plasma and urine

The advent of chromatography revolutionized the analysis of natural compounds. In the 1950s and 1960s, many paper chromatographic methods were developed for the analysis of flavonoids from plants (Robards and Antolovich 1997). Today, the extraction of flavonoids from plant material typically involves acid hydrolysis, followed by high-performance liquid chromatography (HPLC) (Häkkinen et al. 1998, Mattila et al. 2000). Analysis of flavonoids from human plasma or tissues is much more difficult because the compounds are usually bound to proteins and are present in much lower concentrations. When concentrations are very low, extraction methods causing degradation of the flavonoid molecule and yielding low recoveries are unacceptable. Moreover, binding to metals and silica poses a problem when analysing low levels of the compounds with chromatographic methods.

Several methods for the analysis of quercetin from human plasma and urine have been developed. Many of them utilize UV detection, which is not sufficiently sensitive to allow analysis of the compound in plasma when intakes lie within the normal dietary range. The method of Liu et al. (1995), for instance, has a detection limit of 100 µg/l, a concentration found in a minority of plasma samples of subjects consuming their habitual diets. Moreover, several groups have used liquid-liquid extraction methods,

which do not release the compound from protein very effectively. The method of Hollman et al. (1996) is based on acid hydrolysis and HPLC with post-column derivatization. Quercetin is released from protein and conjugates by heating plasma with a mixture of hydrochloric acid and methanol. Quercetin aglycone is then separated by HPLC and detected by fluorescence after complexing the compound with aluminum ions. The limit of detection is 2 µg/l.

No methods suitable for the analysis of hesperetin and naringenin in human plasma after consumption of citrus have been published previously. A method for the analysis of naringenin from plasma after addition of the compound to plasma was developed by Ishii et al. (1996), but endogenous naringenin was not measured. For the analysis of hesperetin or naringenin in urine, methods based on solid-phase or acetonitrile extraction and ultraviolet or mass-spectrometric detection are available (Weintraub et al. 1995, Ishii et al. 1997, Lee and Reidenberg 1998). Partly because of the high detection limits, attempts to analyse flavanones in plasma with methods utilizing UV detection have failed.

2.3.3. Bioavailability and pharmacokinetics

Data on the bioavailability and pharmacokinetics of flavonoids mainly concerns quercetin, catechins and isoflavones. Both seem to vary greatly between different flavonoid classes and different compounds. This is hardly surprising, considering the differences in chemical properties such as polarity. In this section, studies on the bioavailability and pharmacokinetics of quercetin (Table 2), hesperetin and naringenin are reviewed.

Quercetin

Data regarding the bioavailablity and pharmacokinetics of quercetin mostly originate from studies conducted by P. Hollman and co-workers. Previously, in part because Gugler et al. (1975) failed to detect quercetin in plasma and urine of subjects receiving 4 g of quercetin orally, quercetin was thought not to be absorbed. Hollman et al. (1995, 1996, 1997, 1999, 2001), however, showed that the compound is bioavailable from various quercetin-containing foods, and supplements containing quercetin glycosides. In their studies, bioavailability was examined after either single ingestion of relatively high amounts of the foods or compounds, or ingestion over a few days. The pharmacokinetics of quercetin after consumption of onions, quercetin-3-rutinoside, quercetin-4'-glucoside and quercetin-3-glucoside were also studied. The pharmacokinetic parameters calculated are shown in Table 2. The pharmacokinetics of quercetin after intravenous dosage have been investigated in two studies (Gugler et al. 1975, Ferry et al. 1996). In both of these studies, the analytical methods used had rather high detection limits (100 µg/l) and only unconjugated quercetin was measured. Therefore, the results will not be discussed here.

The urinary excretion of quercetin has been investigated in several studies (Table 2). In these studies, the urinary recovery, as a percentage of the ingested dose, ranged between 0.07% and 1.4%. Furthermore, it was lower after ingestion of quercetin-rutinoside than after ingestion of onion, although some variation is present in results of

different studies. From the urinary excretion data, it cannot be concluded that approximately 1% of quercetin is bioavailable. Biliary excretion cannot be ruled out and has been shown to be a major route of quercetin elimination in rats (Ueno et al. 1983, Manach 1996). In rats fed a diet containing 0.25% quercetin, the concentrations of quercetin and methylated metabolites were approximately threefold in bile compared with urine. The high molecular weight of quercetin glucuronides and sulfates and their extensive binding to protein (Spencer et al. 1988, Manach et al. 1995, Boulton et al. 1998) could favour their biliary excretion (Fleck and Bräunlich 1990).

Little is known about the bioavailability of quercetin from diets resembling those of the general population. In one study performed in Glasgow, Scotland, plasma quercetin values of 23 ± 4 µg/l were reported in 10 diabetic subjects following their habitual diets (Noroozi et al. 2000).

Table 2. Bioavailability and pharmacokinetics studies on quercetin (Q).

STUDY DESIGN	SOURCE	DOSE[a] (mg/d)	FASTING PLASMA (µg/l)	URINARY RECOVERY (%)	PLASMA PHARMACOKINETICS	BIOAVAILA-BILITY [b] (%)
Ileostomy subjects (n=9)[1,c]	onion (150 g)	89		0.31 ± 0.14		52 ± 15
Random cross-over	Q-3-rutinoside	100		0.07 ± 0.19		17 ± 15
Single ingestion	Q aglycone	100		0.12 ± 0.08		24 ± 9
Healthy subjects (n=9)[2,c]	onion	68		1.4 ± 0.5	C_{max} = 224 ± 44 µg/l $T_{1/2}$ = 28 ± 92 h T_{max} = 0.7 ± 1.1 h $AUC_{(0-36h)}$ = 2330 ± 849 µgh/l	
Random cross-over Single ingestion	apple sauce	98		0.4 ± 0.2	C_{max} = 92 ± 19 µg/l $T_{1/2}$ = 23 ± 32 T_{max} = 2.5 ± 0.7 $AUC_{(0-36h)}$ = 1061 ± 375 µgh/l	
	Q-3-rutinoside	100		0.3 ± 0.4	C_{max} = 90 ± 93 µg/l $T_{1/2}$ = not calculated T_{max} = 9.3 ± 1.8 $AUC_{(0-36h)}$ = 983 ± 978 µgh/l	
Healthy subjects (n=15)[3,c]	1600 ml black tea	49	22 ± 5	1.1 ± 0.5		
Random cross-over	129 g fried onions	13	22 ± 7	1.0 ± 0.6		
(3*3 days)	129 g fried onions	13	22 ± 5	1.1 ± 0.5		
3 servings/d						
Healthy subjects (n=27)[4,d]	Q-aglycone +	1000	427 ± 89			
Randomized parallel	Q-3-rutinoside	100				
28 days						
4 ingestions/d						

Healthy subjects (n=5)[5] Random cross-over (3*1 week) Several ingestions/d	750 ml fruit juice[e] 1000 ml fruit juice 1500 ml fruit juice	4.8 6.4 9.6	15 32 9	0.3 – 0.5 0.3 – 0.5 0.3 – 0.5	
Healthy subjects (n=9)[6,d] Random cross-over Single ingestion	Q-3-rutinoside	94			C_{max} = 54 ± 12 µg/l $T_{1/2}$ = 28.1 ± 6.4 h T_{max} = 6.0 h ± 1.2 h $AUC_{(0-\infty)}$ = 1117 ± 211 µgh/l
	Q-4'-glucoside	94			C_{max} = 1057 ± 181 µg/l $T_{1/2}$ = 21.6 ± 1.9 h T_{max} = <0.5 h $AUC_{(0-\infty)}$ = 5678 ± 725 µgh/l
Diabetic subjects (n=10)[7,d] Random cross-over (2*2 weeks) 3 servings/d	tea (1500 ml) + onion (400 g)	11+ 90	87 ± 27	0.26	
	tea (1500 ml) + onion (400 g)	11+ 57	48 ± 12	0.27	
Healthy subjects (n=9)[8,d] Random cross-over Single ingestion	Q-3-glucoside	98		3.0 ± 0.3	C_{max} = 1526 ± 315 µg/l $T_{1/2}$ = 18.5 ± 0.8 h T_{max} = 0.6 h ± 0.2 h $AUC_{(0-36h)}$ = 5775 ± 876 µgh/l
	Q-4'-glucoside	100		2.6 ± 0.4	C_{max} = 1345 ± 212 µg/l $T_{1/2}$ = 17.7 ± 0.9 h T_{max} = 0.45 ± 0.08 h $AUC_{(0-36h)}$ = 5276 ± 730 µgh/l

[a]Dose given as quercetin equivalents ingested during one day
[b]Bioavailability defined as ingested amount minus amount recovered in ileostomy effluent
[c]Results given as mean ± SD, [d]Results given as mean ± SEM, [e]Apple juice and black currant juice (1:1)

[1]Hollman et al. 1995, [2]Hollman et al. 1997, [3]DeVries et al. 1998, [4]Conquer et al. 1998, [5]Young et al. 1999, [6]Hollman et al. 1999, [7]Noroozi et al. 2000,[8]Olthof et al. 2000

Hesperetin and naringenin

Previously, data on flavanone bioavailability and pharmacokinetics relied on animal studies and a few human studies where their urinary excretion was investigated. No information on plasma pharmacokinetics of flavanones was available because methods allowing their analysis in plasma had not yet been developed.

In one interesting animal study (Honohan et al. 1976), low amounts (150-290 µg) of ^{14}C-labelled hesperetin were administered orally to rats. The results indicated that biliary excretion is the main route of elimination (57% was recovered in bile). Furthermore, a substantial portion was expired as carbon dioxide, enterohepatic circulation seemed to occur and total absorption was over 90%. It should be noted, however, that the amount of radioactivity measured was probably a sum of intact hesperetin and degradation products formed in the gastrointestinal tract prior to absorption. Therefore, the bioavailability of intact hesperetin was most likely less than 90 %.

In a human study, the urinary recovery of hesperetin was 3% in a subject ingesting 500 mg of naringin and 500 mg of hesperidin once, and 24% in four subjects ingesting 1250 ml of grapefruit juice and 1250 ml of orange juice daily for four weeks. For naringenin, individual urinary recoveries of 5-59% (six subjects; Fuhr and Kummert 1995), 5% (one subject; Ameer et al. 1996), 14-15% (two subjects; Lee and Reidenberg 1998) and 1-6% (six subjects; Ishii et al. 2000) have been reported after single ingestion of between 214-700 mg of naringin as a supplement or in juice. The half-life for naringenin conjugates in urine has been estimated as 2.6 h (Fuhr and Kummert 1995).

2.3.4. Metabolism

Important sites of flavonoid metabolism are the gastrointestinal lumen, cells of the intestinal wall, and the liver. The metabolism of flavonoids is a matter of interest because metabolism often affects the biological activity of a compound and its ability to enter cells. Data regarding flavonoid metabolism mainly concerns quercetin and the catechins. The metabolism of flavanones is poorly known. One common characteristic of the flavonoids is that they occur as glucuronide or sulfate conjugates in the bloodstream.

Metabolism prior to absorption

The mechanisms of and the events preceding flavonoid absorption have been a matter of much debate. Enzymes capable of cleaving flavonoid glycosides were previously assumed not to be present in the small intestine, and uptake was thought to occur only in the large intestine, where cleavage of flavonoid glycosides by enterobacterial enzymes would precede absorption. In the 1990s, however, quercetin or quercetin conjugates were shown to rapidly appear in plasma after consumption of foods containing quercetin glycosides, indicating absorption from the small intestine. The sodium-glucose cotransporter (SGLT1) was hypothesized to transport quercetin glucosides across the enterocytes (Hollman et al. 1999). Later, transport of quercetin-4'-glucoside was actually demonstrated in a cell model of human intestinal absorption (Caco-2 cell line), but the compound was effluxed from the cells by the secretory protein MRP2, and transcellular absorption did not occur (Walgren et al. 2000). In fact, despite numerous attempts, quercetin glycosides have not yet been found in the circulation. Exceptions are two studies using HPLC retention times and/or UV spectra for identification (Paganga and Rice-Evans 1997, Aziz 1998). These methods of identification are, however, rather unreliable because the retention times of flavonoid glucuronides and glycosides are very similar and their UV spectra not very specific (Manach et al. 1998). Since the presence of enzymes capable of cleaving flavonoid glycosides in the small intestine has now been demonstrated, it appears likely that the

flavonoid glycosides are cleaved, either in the lumen or in the cells of the gut, prior to absorption.

Enzymes present in the small intestine capable of cleaving flavonoid glycosides are lactase-phloridzin hydrolase (Day et al. 2000b) and another, less well-characterized β-glycosidase with a broad substrate specificity (Day et al. 1998). *In vitro*, the former enzyme has been shown to cleave quercetin-4'-glucoside, quercetin-3-glucoside, quercetin-3,4'-glucoside, 3'-methylquercetin-3-glucoside, genistein-7-glucoside and daidzein-7-glucoside. Quercetin-3-rhamnoglucoside (rutin) and naringenin-7-rhamnoglucoside (naringin) were not substrates for the enzyme. Cell-free extracts of human small intestine and liver, containing the latter enzyme, hydrolysed several flavonoid glucosides with the sugar moiety attached to the 4'-OH or 7-OH moieties. Compounds such as quercetin-3,4'-glucoside, quercetin-3-glucoside, quercetin-3-rhamnoglucoside (rutin) and naringenin-7-rhamnoglucoside (naringin) were not hydrolysed by the enzyme.

Which enzymes in the large intestine are responsible for the hydrolysis of flavonoid glycosides remains to be elucidated. Enzymes (β-glucosidase, α-rhamnosidase) produced by gastrointestinal bacteria, such as *Bacteroides* JY-6 (Jang and Kim 1996), *Streptococcus faecium* VGH-1 and *Streptococcus sp*. strain FRP-17 (MacDonald et al. 1984), have been shown to hydrolyse some flavonoid glycosides, but other unknown enzymes/bacteria could also be important. Cleavage of the flavonoid ring also occurs in the large intestine, yielding ring fission products such as phenylacetic acids and phenylpropionic acids (Nakagawa 1965, Baba 1981).

Conjugation and methylation in the intestinal wall and the liver?

Incubation of human plasma with a mixture of β-glucuronidase/sulfatase releases quercetin aglycone, which shows indirectly that quercetin is present in plasma as glucuronides, sulfates, or both (Manach 1998). Perfusion studies performed with rat intestines indicate that at least a part of the formation of quercetin glucuronides and sulfates occurs in the intestinal wall (Crespy et al. 1999, Spencer et al. 1999).

High concentrations of methylated quercetin, such as 3'-methylquercetin (isorhamnetin) and 4'-methylquercetin (tamarixetin), have been measured in plasma, urine and bile of rats kept on a high-quercetin diet (Manach et al. 1996), and in a human hepatoma cell line (HepG2) (Boulton et al. 1999). In humans, they occur in plasma at very low concentrations, if any (Manach et al. 1998, Erlund et al., unpublished). In rats, they appear to be formed mainly in the liver, not the intestinal wall (Crespy et al. 1999). Whether the formation of methylated quercetin metabolites is species-dependent or whether a concentration threshold exists, is not known. In the studies where they have been found, rather high amounts of quercetin, typically 0.25% of the diet, have been given to laboratory animals.

2.3.5. Quercetin as a biomarker of intake

Few studies have attempted to assess the use of plasma or urine quercetin levels as biomarkers of intake. Noroozi et al. (2000) studied the effect of two high-flavonol diets on plasma quercetin concentrations in 10 diabetic subjects receiving daily either a fried onion dish containing 90 mg of quercetin (prepared from 400 g of white onions) or plain fried onions containing 57 mg of quercetin (also prepared from 400 g of white onions). After the two-week study period, the mean (±SD) fasting plasma concentration in subjects receiving 90 mg of quercetin was 87 ± 27 µg/l (n=5), and in those receiving 57 mg of quercetin 48 ± 12 µg/l (n=5). The mean baseline value was 23 ± 4 µg/L whereas during a two-week low-flavonoid diet, when no foods known or

suspected to contain flavonoids were consumed, the concentration was 6 ± 3 µg/l. These findings indicate that plasma quercetin concentrations increase with increasing intake.

de Vries et al. (1998) studied the use of quercetin as a biomarker of intake in a setting where 15 subjects consumed 1.6 l of concentrated tea or 129 g of fried onions per day for three days. The onion treatment was given twice. Quercetin was bioavailable from both sources. Furthermore, the results indicated that the reproducibility of plasma quercetin concentrations are such that they are suitable for biomarker use in epidemiological studies.

Young et al. (1999) studied the use of urine quercetin as a biomarker of intake in five subjects consuming 1:1 black currant/apple juice for one week. There were three one-week intervention periods (separated by two weeks) and three doses of fruit juice (750, 1000 and 1500 ml), corresponding to daily intakes of 4.8, 6.4 and 9.6 mg of quercetin. Between 0.3% and 0.5% of the ingested amount of quercetin was recovered in the urine. A significant increase in urinary quercetin was found with dose and time, but the fraction of quercetin excreted, of the ingested dose, appeared to be constant.

In light of the results on quercetin bioavailability, pharmacokinetics and excretion, it would seem reasonable to recommend that plasma quercetin be used as a biomarker of intake rather than urine concentrations. Sometimes, the errors caused by fluctuating plasma levels can be overcome by using 24-h urinary recovery results instead of plasma concentrations as biomarkers of intake. However, because the urinary recovery of quercetin is very low, and data from animal studies indicate substantial biliary excretion, more data on other routes of quercetin excretion and the factors regulating them are needed before urinary recovery is used as a biomarker of quercetin intake. Moreover, 24-h urine has seldom been, for practical reasons, collected in population studies, making it a less attractive tissue for development of intake measures.

2.3.6. Association between flavonoid intake and risk of chronic diseases

The association between flavonoid intake and the risk of cardiovascular disease and cancer has been investigated in several epidemiological studies. In most studies, the term flavonoid refers to flavonoles and flavones, with quercetin being quantitatively the most important flavonoid.

Most, but not all, prospective cohort studies have indicated some degree of inverse association (from borderline to modest) between flavonoid intake and coronary heart disease. An inverse association was found in the Zutphen Elderly Study (Hertog et al. 1993b), the Finnish Mobile Clinic Study (Knekt et al. 1996, 2002), the Iowa Women's Health Study (Yochum et al. 1999) and the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study (Hirvonen et al. 2001b). No association was found between flavonoid intake and risk of coronary heart disease in subjects free of disease at baseline in the Health Professionals Follow-up Study (Rimm et al. 1996). Interestingly, in the Caerphilly Study (Hertog et al. 1997), flavonol intake in 1900 men was directly associated with the risk of ischaemic heart disease and all-cause mortality. The result may be explained by tea being a very important source of flavonoids in Scotland; its consumption was associated with lower social class, smoking and higher fat intake (Hertog et al. 1997). In the above-mentioned ATBC Study, no association was found between flavonoid intake and risk of stroke (Hirvonen et al. 2000)

The epidemiological evidence regarding the cancer protecting effects of flavonoids is conflicting. Some case-control studies have indicated an inverse association between intake of flavonoids and risk of lung cancer (de Stefani et al. 1999a, Le Marchand et al. 2000), upper aerodigestive tract cancer (de Stefani et al. 1999b) and gastric cancer (Garcia-Closas et al. 1999), while no association was found in other studies for lung cancer (Garcia-Closas et al. 1998) or bladder cancer (Garcia et al. 1999). In two cohort studies, no association between intake of flavonoids and cancer risk was present

(Hertog et al. 1994, Hertog et al. 1995), and in two other cohort studies, an inverse association was shown for lung cancer (Knekt et al. 1997, Hirvonen et al. 2001c).

On the whole, the epidemiological evidence concerning flavonoids and chronic diseases is very difficult to interpret. One reason is that confounding factors (a common problem of epidemiological studies), probably affect the outcome of the studies. In many countries, drinking tea and consuming high quantities of vegetables and fruit are merely indicators of a generally healthy lifestyle or a high level of education. This may not be the case for onion, but this source of flavonoids is perhaps the most problematic in epidemiological studies. Onion is qualitatively, and in many countries, quantitatively, the most important source of quercetin (Hertog and Hollman 1996, Rimm 1996, Häkkinen et al. 1999, Arai et al. 2000). The accurate assessment of an individual's onion consumption is difficult with dietary survey methods. Onion is a commonly used "hidden" ingredient of many home-made and processed foods; it is added to meatballs, hamburger meet, tv-dinners, soups, salads, sausages, etc. Thus, it is likely that intake estimates of onion contain a large margin of error. Assessment of tea intake, by contrast, an important source of quercetin in some countries, is probably fairly accurate. However, the bioavailability of quercetin from tea is poorer than from onion. Considering the possible impact on the results of epidemiological studies, it is rather surprising that these problems have not been discussed in the reports. With the exception of Hirvonen et al. (2001c), little or no information about how onion intake was estimated has usually been given.

In summary, the epidemiological evidence concerning the association between flavonoid intake and the risk of chronic diseases is conflicting. Whether or not flavonoids protect against chronic diseases may be difficult to show using traditional epidemiological methods. An alternative, which may help to overcome some of the problems associated with intake assessment, is to use tissue concentrations as biomarkers of intake.

3. AIMS OF THE STUDY

The work presented in this thesis is part of a project with the following goals: to develop analytical methods for the analysis of the most important dietary flavonoids and other phenolic compounds, to study their bioavailability, pharmacokinetics and metabolism, to evaluate their use as biomarkers of intake, and finally, to study whether their serum concentrations are associated with the risk of chronic diseases, such as cardiovascular disease, in population studies.

The specific aims of the thesis were:

1. To develop methods for the analysis of quercetin, hesperetin and naringenin from plasma and/or urine (I, IV).
2. To investigate the pharmacokinetics and bioavailability of quercetin from quercetin aglycone and rutin after single ingestion of the compounds as pure substances (II).
3. To determine plasma quercetin concentrations after long-term consumption of berries and a habitual Finnish diet (III).
4. To investigate the pharmacokinetics and bioavailability of hesperetin and naringenin after single ingestion of citrus juices (IV).
5. To evaluate plasma quercetin, hesperetin and naringenin as biomarkers of intake (II, III, IV).

4. SUBJECTS AND STUDY DESIGNS

Study II

Sixteen apparently healthy young men and women with no diseases of the gastrointestinal tract were recruited. Their baseline characteristics are shown in Table 3. The study was performed in a double-blind, diet-controlled, cross-over design. It consisted of two 14-day study periods, two treatments (quercetin and rutin) and three doses (8, 20 and 50 mg as quercetin equivalents) within both treatments. Each subject received each treatment and dose once (6 combinations altogether). The subjects were randomized into two groups with 4 females and 4 males in one group and 3 females and 5 males in the other group. One group received the quercetin treatment first (Period 1), followed by the rutin treatment (Period 2). The other group had the order of the treatments reversed. The compounds were given in ascending dosages. The dosing schedule is presented in Figure 3. The subjects consumed the capsule with 200 ml of water. Two hours after ingesting the capsule, the subjects drank 200 ml of water, and 4 hours from ingestion they ate lunch. Blood samples were collected 15-20 min before, and 15 and 30 min, and 1, 2, 4, 6, 8, 12, 24 and 32 h after each dose/treatment.

Table 3. Baseline characteristics of subjects (mean ± SD).

Study	N (women/men)	Age (years)	Height (cm)	Weight (kg)	BMI (kg/m^2)
II	16 (8/8)	22 ± 4	173 ± 7	65 ± 9	22 ± 2
III	60 (0/60)	60 ± 0	176 ± 6	78 ± 7	25 ± 2
IV	13 (7/6)	28 ± 5	175 ± 7	71 ± 15	23 ± 3

Altogether 12 subjects completed all doses of both treatments. Two subjects (one female and one male) in both groups discontinued the study. Two of them discontinued without finishing any treatment or dose, and two after receiving all doses of one treatment and one dose of the other treatment. The reasons for discontinuing were personal, and no side-effects attributed to the flavonoids were encountered.

During both study periods the subjects followed a low-quercetin diet. Because the doses of quercetin used in the study were similar to those attainable from the diet, it was crucial that the dietary intake of quercetin be kept as low as possible. Lunch and dinner were provided at the study site, and additional meals were eaten elsewhere, except on days of sample collection, when all foods were obtained at the study site. The subjects were given a list of allowed and forbidden foods, and they kept a record of all foods eaten outside the study site. The diet consisted mainly of unprocessed meat and poultry, milk products, white wheat bread and a few vegetables. All vegetables, fruit, beverages or other foods known or suspected to contain quercetin or quercetin glycosides (Kűhnau 1976, Hertog et al. 1992a, 1993a) were forbidden.

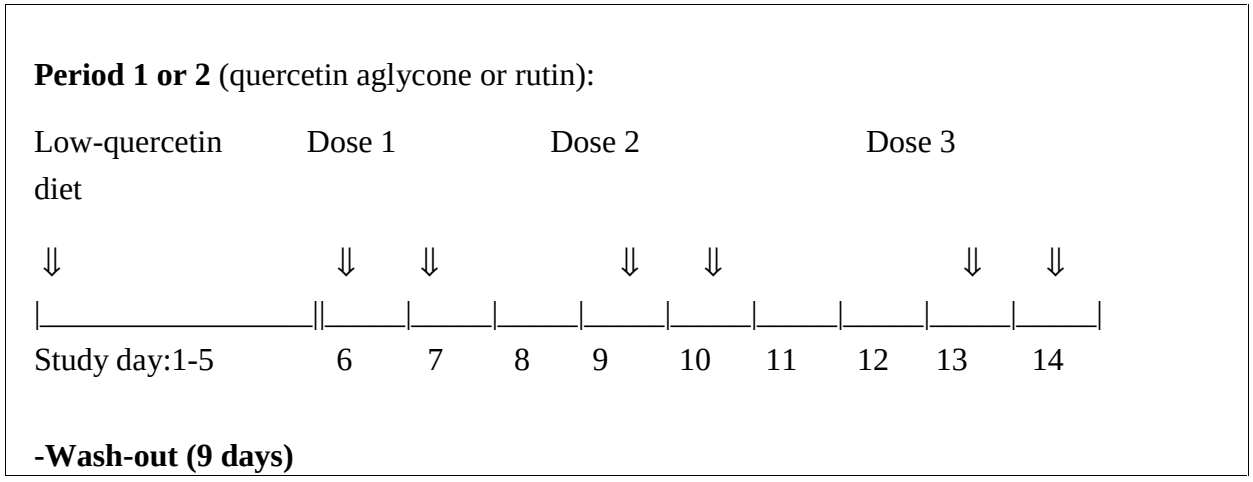


Figure 3. Dosing schedule in Study II.

Study III

The participants comprised 60 apparently healthy middle-aged men. They were recruited among 523 men, who in 1994 participated in a health survey for men born in 1935 and living in the city of Turku, conducted by the Research and Development Centre of the Social Insurance Institution in Turku. Exclusion criteria were use of regular medication, use of dietary supplements during the past month, and overweight (BMI>30 kg/m^2).

The subjects were randomized into three groups (n=20 in each group) (Marniemi 2001). One group received berries, one group vitamin supplements (containing no quercetin), and one group served as a placebo group. Serum samples from the berry and the control group, but not from the supplement group, were analysed for quercetin. The samples were also analysed for indices of antioxidant capacity, but those results have been published elsewhere (Marniemi et al. 2000) and will not be discussed in this thesis. The baseline characteristics of the subjects are shown in Table 3.

The subjects in the berry group were given 2 kg each of deep-frozen black currants, lingonberries and bilberries. The berries were packed in 100-g portions in plastic bags. The subjects were instructed to take one bag out of the freezer each day and eat one portion of berries per day. They were also instructed to eat the different berries in turns to ensure an even distribution over the 8-week intervention period. The berries were eaten fresh and heating of the berries was not allowed. The control group received 500 mg daily of calcium gluconate as placebo. All subjects were instructed not to change their usual dietary habits during the study. Dietary records (3-day) were kept at the beginning of the study and at 8 weeks. Compliance was asked about and was emphasized at each blood sampling. Blood samples were taken after an overnight fast two weeks prior to the study, at baseline, and at weeks 2, 4 and 8.

Study IV

The study population consisted of young healthy volunteers, mainly summer students/trainees at the National Public Health Institute. The baseline characteristics of the subjects are presented in Table 3. Eight subjects (5 women and 3 men) were allocated into the orange juice group and 5 subjects (2 women and 3 men) into the grapefruit juice group. Exclusion criteria were use of medication, or a history of diseases or symptoms of the gastrointestinal tract (e.g. lactose intolerance or coeliac disease).

The subjects ingested 8 ml/kg of body weight of either orange juice or grapefruit juice in the morning after an overnight fast. The ingested amounts ranged between 400 ml and 760 ml. The subjects were allowed to eat for the first time 4 h after ingestion of the test juice. Blood samples were collected at 1, 2, 3, 4, 6, 8, 10, 12, 14 and 24 h after drinking the juice. Urine was collected in 4 fractions (0-4 h, 4-8 h, 8-14 h and 14-24 h) over 24 h. Baseline urine and blood samples were obtained 10-20 min before juice administration.

The subjects followed a citrus-free diet for one week prior to the study and on the study day. They were given oral instructions on the diet and a list of prohibited foods, which included all foods and beverages known or suspected to contain citrus ingredients. The subjects were also asked to restrain from using dietary supplements during this period. Compliance with the one-week citrus-free diet was confirmed by a questionnaire, which the participants filled out during the study day. According to the questionnaires, only a few minor deviations occurred during the first days of the citrus-free diet.

5. METHODS

5.1. Analytical methods

Standards

Rutin, hesperetin and naringenin were obtained from Sigma Chemical Co. (St. Louis, MA, USA). All other flavonoid standards were purchased from Extrasynthese (Genay, France).

Equipment

Chromatographic analysis was performed with a system consisting of an HP 1090 liquid chromatograph, an HP 3396 II integrator with a 9122 C/D disc drive (Hewlett-Packard, Palo Alto, CA, USA) and a Coulochem 5100A electrochemical detector with a model 5011 analytical cell (ESA Inc., Chelmsford, MA, USA). The following HPLC columns were tested: Inertsil pH (250*4 mm, 5 µm, Gl Sciences Inc.), Supelco LC-CN (250*2.6 mm, 5 µm, Supelco Inc.), YMC carotenoid (150*4.6 mm, 3 µm, YMC), Phenomenex LUNA-CN (250*4.6 mm, 5 µm, Phenomenex), POLY RPCO (150*4.6 mm, 4 µm, Interaction Chromatography Inc.), Hypersil-ODS (125*4 mm, 5 µm, Hewlett Packard Inc.), Symmetry C18 (250*4.6 mm, 4 µm, Waters Assoc.), LiChrosorb Hypersil ODS (250*4 mm, 5 µm, Merck) and Inertsil ODS-3 (250*4 mm, 5 µm, Gl Sciences Inc.). In the final method, the last-mentioned column was used.

Extraction

For extraction of quercetin from plasma, various extraction techniques were tested including solid-phase extraction, liquid-liquid extraction and complexation with metals or other derivatization agents. The following Bond Elut solid-phase extraction (SPE) columns were tested: C18, CN, NH4, silica, diol, PBA (Varian, Harbour City, CA, USA). The following SPE columns from International Solvent Technology were

tested: C18, CN, C8 and ENV (polystyrene divinyl benzene) (International Solvent Technology, Hengoed, UK).

Mobile phases and detector potentials

For the separation of quercetin by HPLC, different combinations of the following solvents and buffers were tested: methanol, acetonitrile, tetrahydrofurane, acetic acid, orthophosphoric acid and monochloroacetic acid. Detector potentials between 50 and 500 mV were tested for quercetin, and between 200 and 700 mV for naringenin and hesperetin.

Validation of methods

The analytical methods were validated for the following parameters: intra-assay precision, inter-assay precision, recovery, linearity and stability. The quercetin peak was identified by hydrodynamic voltammetry. The plasma samples used in validation were obtained from persons who had either been instructed to consume quercetin-containing foods (=high-quercetin plasma, 80 µg/l), or to exclude the compound from their diets as far as possible (=low-quercetin plasma, 3.5 µg/l).

Intra-assay precision of the quercetin method was assessed by analysing high-quercetin plasma (n=6) and spiked and non-spiked low-quercetin plasma (n=6 per group). Inter-assay precision was determined by analysing high-quercetin plasma (n=6) on 7 separate days. Recovery was determined by comparing the peak height of hydrolysed (n=6) spiked low-quercetin plasma (n=6) to the peak height of standards (n=6). The height of the quercetin peak in non-spiked low-quercetin plasma was subtracted. Spiked samples were prepared by addition of 60 or 200 ng of quercetin to 1-ml aliquots of low-quercetin plasma. Linearity of the assay was evaluated by plotting the peak height of standards in the range of 3.5-320 µg/l against the

corresponding concentration. The standards were made by spiking low-quercetin plasma and they were treated like the other samples.

The intra-assay precisions of the flavanone methods were assessed by analysing low-quercetin plasma spiked with 200 or 50 µg/l of naringenin or hesperetin (n=4 each). Inter-assay precision was determined by analysing the same spiked samples on 4 days. Linearity was checked in the range of 10-1000 µg/l for hesperetin and 20-1000 µg/l for naringenin. Recovery was determined by the addition of 125 µg/l of flavanones to low-quercetin plasma.

5.2. Pharmacokinetic methods

In Study II, the pharmacokinetic parameters were determined from total plasma quercetin concentrations using a two-compartment model. Pharmacokinetic variables were area under the concentration versus time curve (AUC_{0-24}), calculated using the trapezoidal rule, and maximum plasma quercetin concentration (C_{max}). Other variables were time to maximum plasma concentration (T_{max}) and elimination half-life ($T_{½}$). The pharmacokinetic parameters were calculated using a validated commercial software package SIPHAR/PC version 4.0 obtained from SIMED, Créteil, France.

In Study IV, the pharmacokinetic parameters were calculated by model-independent methods. The peak concentration (C_{max}) and the time to reach it (T_{max}) were taken directly from the data. The elimination half-life $T_{½}$ was calculated from the equation $T_{½} = \ln 2 / k$, using the terminal monoexponential log-linear slope of the time vs. concentration curve of each subject for the estimation of k by the least-squares method. AUC_{0-24} was calculated using the trapezoidal method. Renal clearance CL_{ren} was obtained by dividing the total amount of flavanone excreted in the urine in 24 h with AUC_{0-24}. All data are expressed as mean ± SD.

5.3. Assessment of flavonoid intake

In Study III, the average daily intakes of quercetin, energy and nutrients were calculated from 3-day dietary records with the Nutrica computer program. The database of this program has been validated by Hakala et al. (1996). Quercetin data from the Fineli database (kindly provided by M-L Ovaskainen from the National Public Health Institute) were added to the Nutrica database before calculations were made.

In Study IV, the intake of hesperetin and naringenin was assessed by multiplying the amount of juice ingested with their concentrations in the citrus juices. The naringenin and hesperetin concentrations of the juices were analysed by HPLC with electrochemical detection (IV) .

5.4. Statistical methods

Study II

The main approach was the intention-to-treat analysis in which all randomized subjects were included in the analyses. Baseline comparisons between sequences were made using a t-test for two independent samples. Pharmacokinetic parameters (except t_{max}) were examined using variance analysis for cross-over design when there were repetitions within the period. T_{max} was analysed using the Wilcoxon rank sum test. Single-dose comparisons between treatments were carried out with linear contrasts or pair-wise comparisons. For calculations of $AUC_{(0-24)}$, $AUC_{(0-32)}$ and C_{max}, the data was ln-transformed. Comparisons between gender of body weight-adjusted $AUC_{(0-32)}$ values were performed by analysis of variance (ANOVA) for repeated measures; post-hoc comparisons were performed using contrast analysis. Statistical analyses were performed by using SAS System 6.12.

Study III

Statistical significance of the difference between serum quercetin concentrations of the two dietary groups was assessed by analysis of covariance (ANCOVA) for repeated measures; post-hoc comparisons were performed using contrast analysis. Whether the intake of quercetin differed between the groups at 8 weeks was tested by ANCOVA; post-hoc comparisons were performed by Tukey's test. Differences in baseline values of serum quercetin or quercetin intake between the two groups were assessed by the Student's t-test. The paired t-test was used to test the difference in quercetin intake between baseline and 8 weeks within the two groups. Statistical analyses were performed by using SPSS 10.0 for Windows.

Study IV

Differences between the means of selected pharmacokinetic indices (T_{max}, $T_{1/2}$, renal clearance, relative urinary excretion and C_{max} to ingested dose ratio) for naringenin from grapefruit and naringenin from orange juice were tested by the Mann-Whitney U-test. Spearman's correlation was used to study the association between plasma flavanone $AUC_{(0-24)}$ and relative urinary excretion values. Statistical analyses were performed by using SYSTAT 10.0 for Windows.

A p-value of less than 0.05 was considered statistically significant in all studies.

6. RESULTS

6.1. Analysis of quercetin, hesperetin and naringenin (I, IV)

The methods developed for the analysis of quercetin, hesperetin and naringenin are shown in Figure 4. The same method of hydrolysis was used for all three compounds. The most efficient hydrolysis was obtained by using a crude extract of *Helix pomatia*. Quercetin, in particular, is easily degraded and much work was done to stabilize it during the different steps of analysis. Flavonoid degradation was minimized by using ascorbic acid during hydrolysis and oxalic acid after extraction. For extraction of quercetin, solid-phase extraction followed by additional purification by liquid-liquid extraction was used. For extraction of flavanones, the liquid-liquid step was omitted because part of the compounds were extracted into the wrong phase.

For separation of analytes, an Inertsil ODS-3 analytical HPLC column (GL Sciences, Tokyo, Japan) was used. This column yielded the best separation of the flavonoids from interfering compounds in the extracts. Also, with this column, the narrowest and most symmetrical peaks were obtained. The superiority of this column compared with the others is probably the result of efficient endcapping of residual silanol groups and the use of inert low-metal silica.

Electrochemical detection was used for the analysis of flavonoids. Its sensitivity for quercetin, in particular, is superior compared to that of ultraviolet detection. A detector potential as low as 100 mV could be used for quercetin. Since few compounds in plasma are oxidized at such a low potential, the problem with interfering compounds disappeared, and very low levels of quercetin could be detected. Higher potentials are required for the analysis of hesperetin and naringenin. Therefore, it is not surprising that the problem with coeluting compounds could not be entirely overcome, and the detection limits were higher for flavanones than for quercetin.

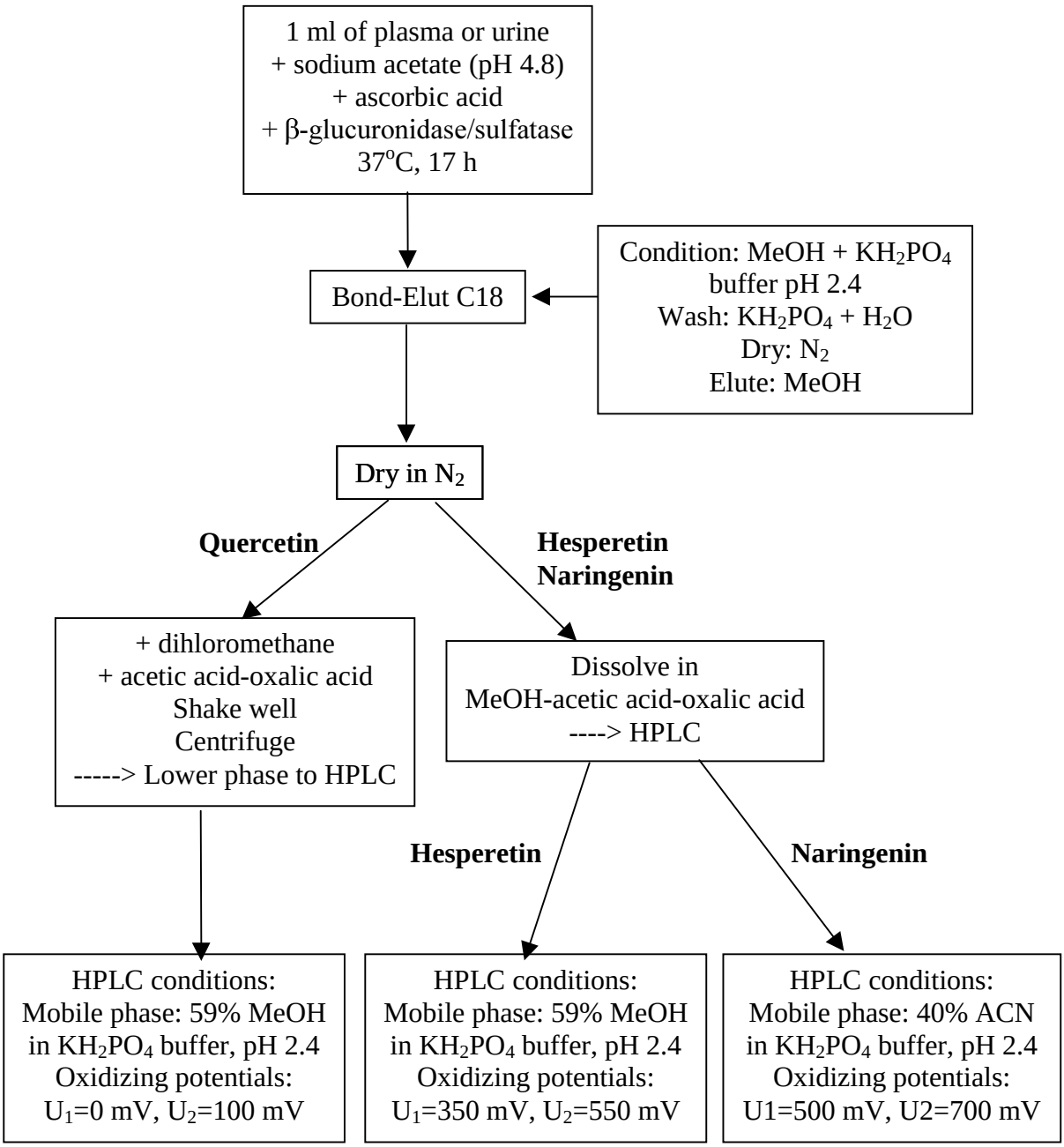


Figure 4. Flow-chart of the analytical methods used (I, IV).

Quantification of flavonoids was based on the standard additions method. The standards, containing known amounts of added quercetin, were treated exactly the same way as samples; thus the final results could be read directly from the standard curves.

The most important validation results are presented in Table 4. The results indicate good reproducibility for the methods, especially the quercetin method. An exact detection limit could not be calculated for quercetin because a plasma sample containing no quercetin could not be obtained.

Table 4. Main validation results for the analytical methods used (I, IV).

	Quercetin	Hesperetin	Naringenin
Intra-assay precision (CV%)	<4	<6	<6
Inter-assay precision (CV%)	<8	<10	<10
Recovery (%)	>70	>70	>70
Limit of detection (µg/l)	<3.5	10	20

6.2. Pharmacokinetics and bioavailability of quercetin from quercetin aglycone and rutin (II)

Quercetin was bioavailable from both quercetin aglycone and rutin, but the time taken for plasma quercetin concentrations to increase differed for the two forms of quercetin. After ingestion of quercetin in the aglycone form, the compound was absorbed rapidly; quercetin could be measured in plasma 15 min after ingestion, and the T_{max} values ranged between 1.9 and 4.9 h. The concentration vs. time curves were, however, biphasic and the second concentration peak increased (relative to the first one) with increasing dose of quercetin aglycone. After ingestion of rutin, on the other hand, quercetin concentrations did not increase in plasma until after 3 to 6 h, and the mean T_{max} values ranged between 6.5 and 7.5 h. The individual plasma concentration vs. time curves for quercetin after ingestion of dose 3 of quercetin aglycone or rutin are shown in Figures 5A and 5B.

The mean C_{max} and $AUC_{(0-32)}$ values increased linearly for both quercetin and rutin (Figures 6A-6D), and the values did not differ significantly for the corresponding doses. The C_{max} and $AUC_{(0-32)}$ values increased with increasing dose for practically all individuals between doses 1 and 2 of both quercetin aglycone and rutin. Between doses 2 and 3, the increase was less pronounced for all individuals after ingestion of quercetin aglycone and for some individuals after ingestion of rutin.

Marked interindividual variation occurred in C_{max} and $AUC_{(0-32)}$ values after ingestion of rutin, especially at higher doses. Furthermore, quercetin from rutin was more bioavailable in women than in men (Figure 7). The highest concentrations of quercetin were found in women using oral contraceptives (4 women receiving dose 1, and 3 women receiving doses 2 and 3). Gender or use of oral contraceptives did not affect T_{max} and $T_{1/2}$ after either treatment.

$T_{1/2}$ for quercetin from quercetin aglycone ranged between 15 and 18 h. The $T_{1/2}$ values for quercetin from rutin are not given because the late absorption resulted in too few time-points in the elimination phase to allow an accurate estimation of the parameter. However, $T_{1/2}$ did not appear to differ for quercetin from rutin compared with quercetin from quercetin aglycone, as judged from the plasma curves of subjects absorbing quercetin from rutin rapidly enough to allow a rough estimation of $T_{1/2}$.

The method originally developed for the analysis of total quercetin in plasma (I) was developed further to allow analysis of rutin and unconjugated quercetin, but no traces of rutin were detected after ingestion of the highest dose of rutin (II). The proportion of unconjugated quercetin aglycone of total quercetin was measured for the 4-, 6-, 8- and 12-h time-points, being 38 ± 19%, 15 ± 12%, 10 ± 13% and 11 ± 13% (mean ± SD), respectively.

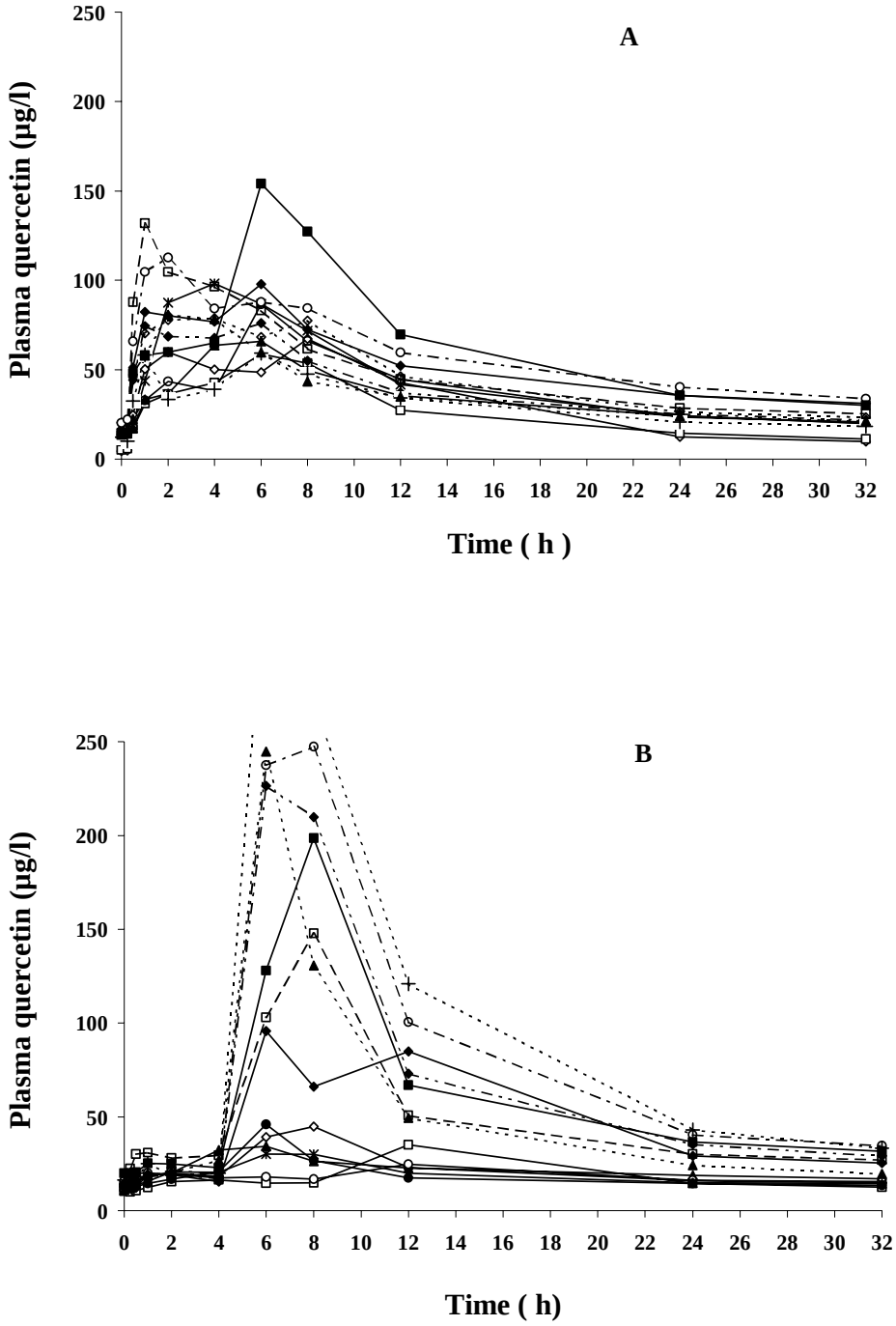


Figure 5. Individual plasma quercetin concentration vs. time curves after ingestion of dose 3 of quercetin aglycone (A) or rutin (B). Straight lines represent men and broken lines women.

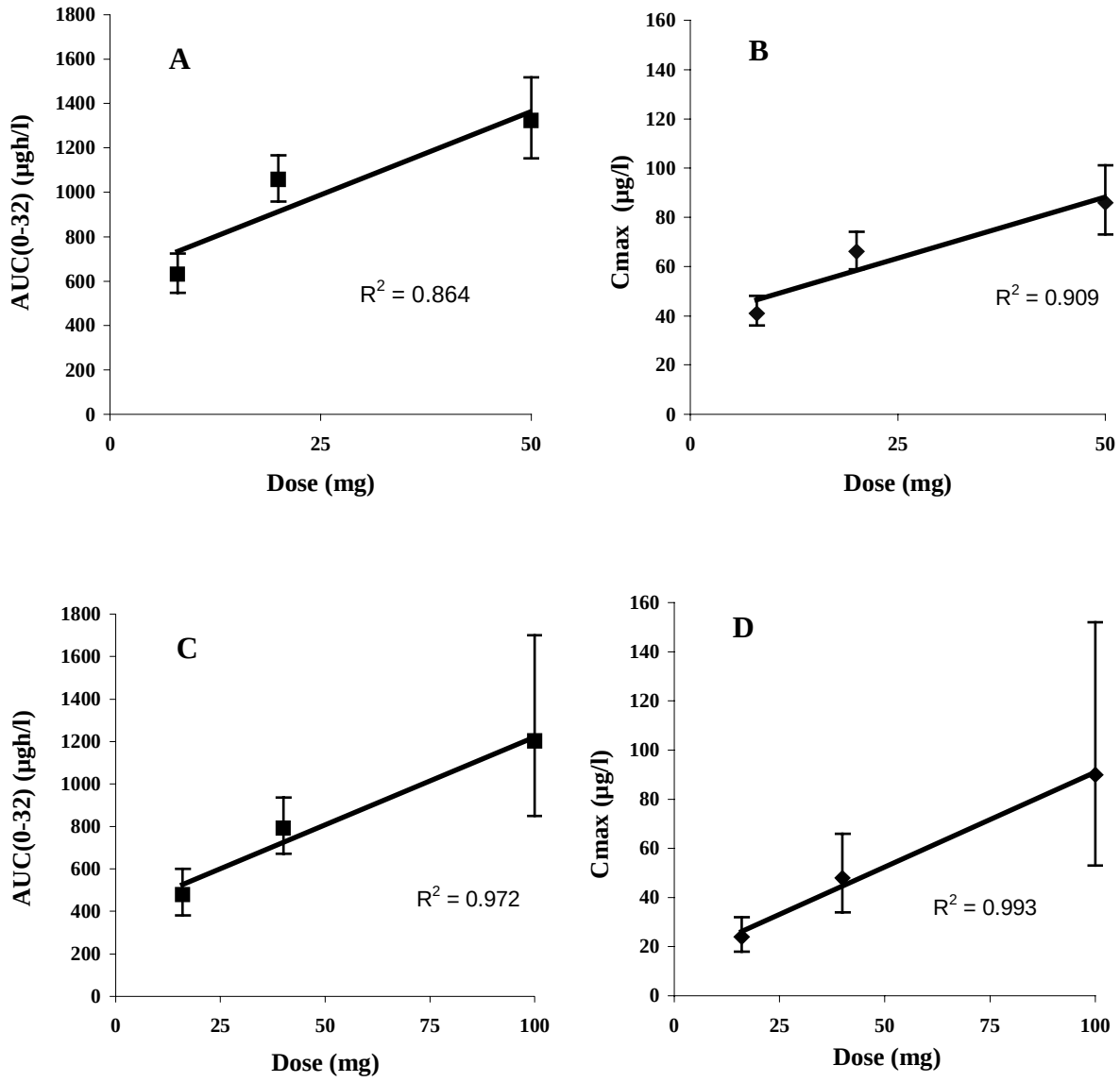
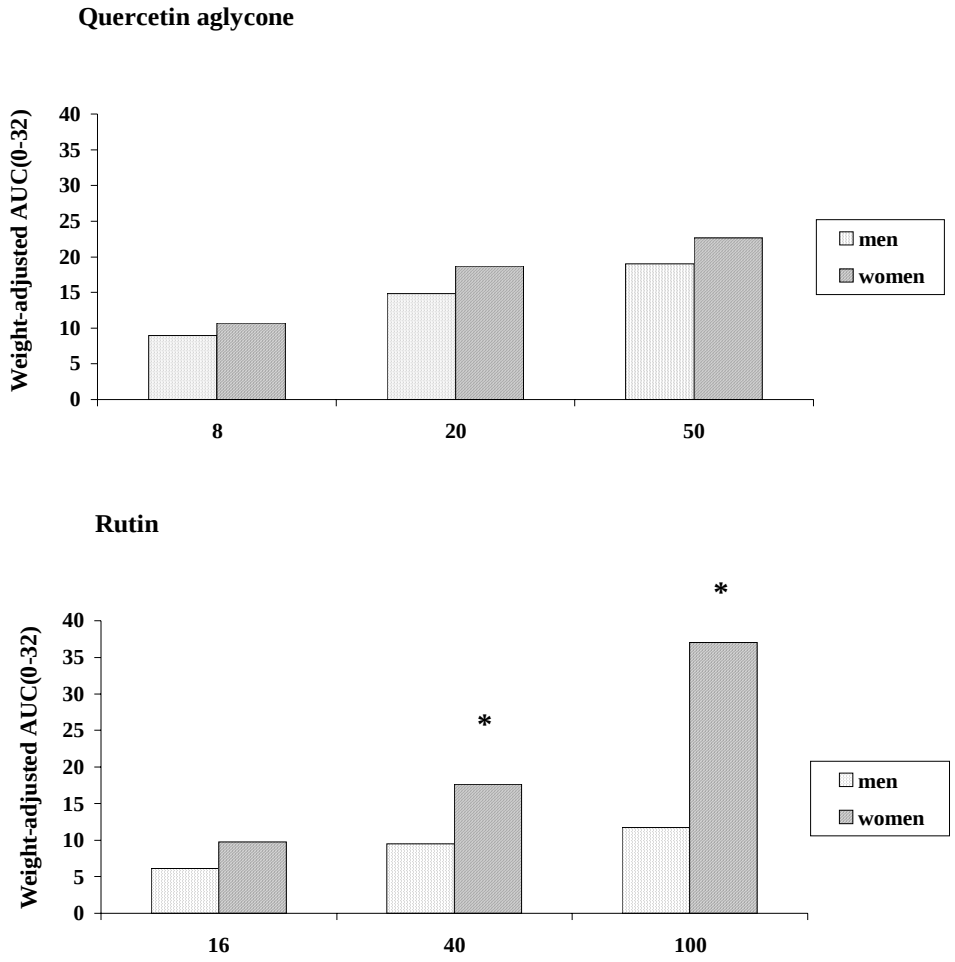


Figure 6. Dose-response curves for quercetin after ingestion of quercetin aglycone (A, B) or rutin (C, D). Values are geometric means with 95% confidence intervals.



[a]AUC(0-32) was divided with body weight

[b]Values are geometric means of body weight adjusted $AUC_{(0-32)}$

[c]Unit is µgh/lkg

Figure 7. Body weight-adjusted relative bioavailability of quercetin for men and women (II). Women and men differed significantly (p=0.002) for the rutin, but not the quercetin aglycone treatment. The asterisk indicates the doses for which women and men were significantly different[a,b,c].

6.3. Pharmacokinetics and bioavailabilty of hesperetin and naringenin from citrus juices (IV)

The plasma pharmacokinetics of hesperetin and naringenin from orange juice and grapefruit juice appeared to be similar (Table 5). For both compounds, relatively high concentrations (up to 4 mg/l) were measured in plasma after ingestion of 400-760 ml of the juices (Figures 7A-7C). There was, however, marked interindividual variation in the pharmacokinetic parameters describing bioavailability. Maximum plasma concentrations were obtained in about 5 h after ingestion of the juices. The half-lives of the compounds were rather short and they were rapidly cleared from plasma. No signs of separate distribution and elimination phases could be seen in the plasma concentration vs. time curves.

The time-course of urinary excretion was similar for naringenin and hesperetin from orange juice and grapefruit juice. Most (73-87%) of the total amount excreted into the urine was recovered within 8 h. However, the renal clearance of flavanones, at least naringenin, appeared to be dose-dependent. For naringenin from orange juice, the renal clearance was 0.4 l/h (ingested amount 23 mg), and when the compound was obtained from grapefruit juice (ingested amount 199 mg), the value was 8.4 l/h. Furthermore, correlations between $AUC_{(0-24)}$ and urinary recovery values were found for hesperetin from orange juice and naringenin from grapefruit juice, but not for naringenin from orange juice.

The individual urinary recovery values for hesperetin from orange juice ranged between 1% and 11%. The corresponding values for naringenin from orange juice ranged between 0.2% and 3 %, and for naringenin from grapefruit juice between 4% and 69%. These values are minimum estimates for bioavailability.

Table 5. Pharmacokinetics of naringenin and hesperetin after single ingestion of orange juice or grapefruit juice (IV)[1,2].

	NARINGENIN		HESPERETIN
	Grapefruit juice	Orange juice	Orange juice
Ingested dose of flavanone (mg)	199 ± 42	23 ± 2	126 ± 26
AUC_{0-24} (µg·h/l)	7534 ± 7151	719 ± 437	3099 ± 2464
C_{max} (µg/l)	1628 ± 1459	175 ± 110	655 ± 479
T_{max} (h)	4.8 ± 1.1[2]	5.5 ± 2.9[2]	5.4 ± 1.6
$T_{1/2}$ (h)	2.2 ± 0.1[2]	1.3 ± 0.6[2]	2.2 ± 0.8
Renal clearance (l/h)	8.4 ± 1.5 [2]	0.4 ± 0.2[2]	2.4 ± 0.5
Urinary recovery (%)	30.2 ± 25.5[2]	1.1 ± 0.8[2]	5.3 ± 3.1

[1]Values are means ± SD.
[2]Values are significantly different for grapefruit juice and orange juice. Only selected variables were compared statistically.

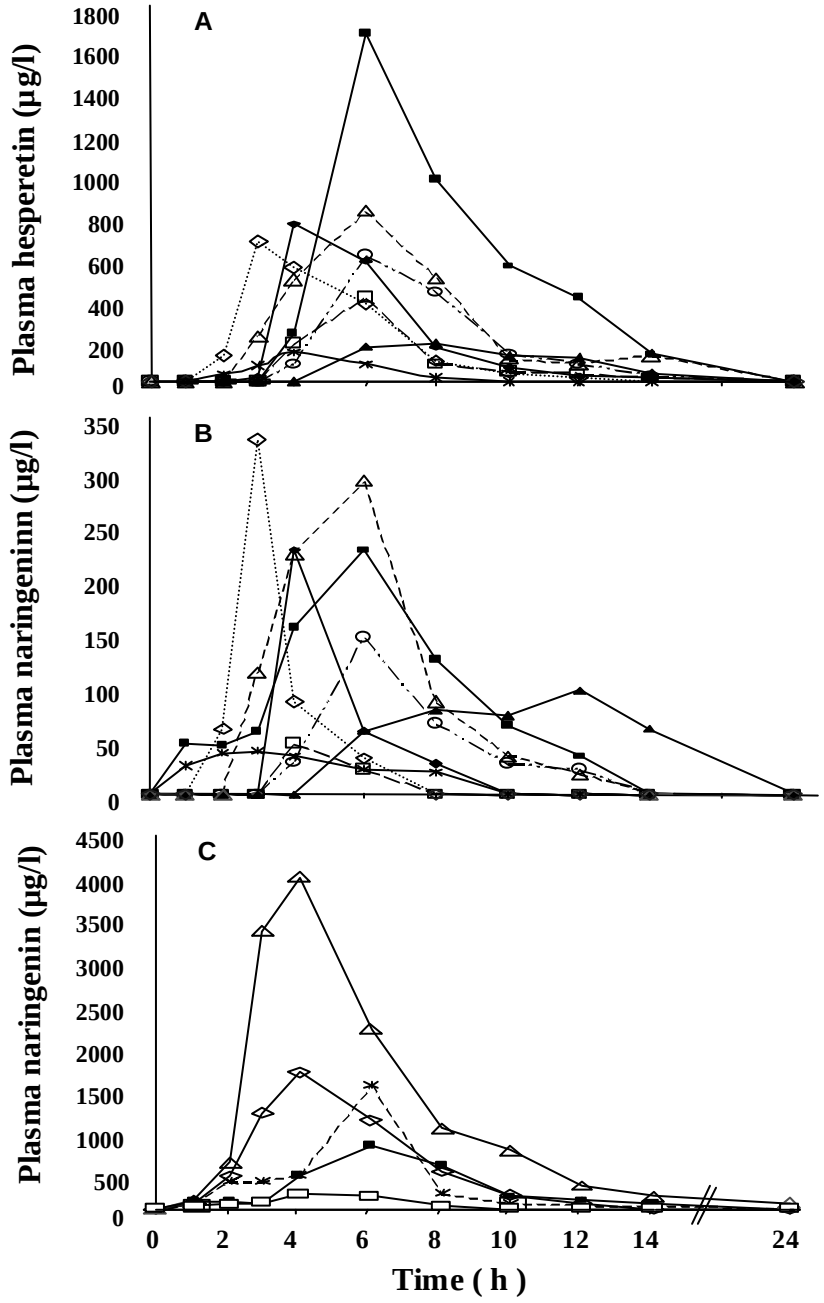


Figure 8. Individual plasma-concentration vs. time curves for hesperetin (A) and naringenin (B) after ingestion of orange juice and for naringenin (C) after ingestion of grapefruit juice.

6.4. Bioavailability of quercetin from berries and the diet (III)

Serum quercetin concentrations were significantly higher during the berry consumption period in the subjects consuming 100 g/day of berries compared with the subjects consuming their habitual diets ($p=0.039$). In the berry group, the mean serum quercetin concentrations ranged from 21 to 25 µg/l between weeks 2 and 8. The values were 32-51% higher than in the control group at corresponding time-points. Two weeks prior to the study and at baseline, the mean concentrations of the two groups were similar. At these two time-points, when the men still followed their habitual diets, the mean quercetin concentration for all subjects was 16 ± 13 µg/l (mean ± SD).

The mean calculated intake of quercetin was significantly higher in the berry group at the end of the 8-week intervention (12 ± 6 mg) compared with baseline (8 ± 5 mg) ($p=0.001$). In the control group, the daily intake did not change (6 mg at baseline and at 8 weeks). The intake of quercetin from the background diet (when intake from berries was disregarded) did not change within groups during the study. In the berry group, the mean estimated intake of quercetin from the berries was 6 mg/day.

The mean calculated intake of energy and nutrients for all subjects at baseline were as follows: energy 2125 kcal, fat 37 E%, protein 15.5 E%, carbohydrate 40 E%, saturated fat 15.5 E%, fibre 21 g, cholesterol 329 mg, β-carotene 1.6 mg, vitamin C 52 mg and vitamin E 10.6 mg. Intakes of energy and nutrients from the background diet did not change significantly during the intervention.

7. DISCUSSION

7.1. Analytical methods

The main goals of the flavonoid project at the Biomarker Laboratory were to analyse the most important flavonoids, phenolic acids and lignans in plasma samples from population studies, and to examine the association between plasma concentrations of the compounds and risk of chronic diseases. Because the amount of sample available from such studies is usually limited and analysis of large amounts of samples can be expensive, it was of interest to measure as many compounds as possible from the same sample. The main requirements of an analytical method used for the above-mentioned purpose are good reproducibility over a long period of time and low detection limits. However, the analysis of many of these compounds is difficult even individually and at much higher concentrations. The approach used in this work was to first develop a method for the analysis of an analytically demanding compound of interest, and then test whether less demanding compounds could be analysed with the same method. Quercetin was considered to be one of the most promising compounds with regard to biological activity, dietary intake and epidemiological evidence. This compound is, however, very difficult to analyse from human plasma because it forms strong bonds with plasma albumin and is chemically unstable under various conditions. Therefore, a method was first developed to allow analysis of quercetin from plasma and was later applied to the analysis of other phenolic compounds such as flavanones, isoflavones, lignans and phenolic acids. In this thesis, methods for the analysis of quercetin, hesperetin and naringenin are presented.

The method presented in Study I was suitable for the analysis of physiological levels of quercetin in plasma, i.e. the levels found in subjects consuming typical Finnish diets or diets low in quercetin. Most importantly, the method had a good long-term reproducibility even at low concentrations, as shown by the low coefficient of variation of quality control samples in Study II. No information about the long-term

reproducibility of previously published quercetin methods is available. In addition to good reproducibility, the method was linear in the examined concentration range and had an acceptable recovery. The degradation of quercetin was minimized by the use of ascorbic acid during hydrolysis and oxalic acid after extraction. Quercetin aglycone is a compound which can be stable for a long time under the right conditions, but in the conditions typically used during the extraction of many nutrients or drugs, the compound is degraded rapidly (Nordström and Majani 1965). The advantage of the solid-phase extraction method used in this work is that it is less harsh than extraction with hydrochloric acid/methanol, which was used by Hollman et al. (1996). Another advantage is that the risk of cleaving quercetin conjugates is smaller than when using hydrochloric acid/methanol extraction. Therefore, this extraction method can be used in the search for quercetin or flavonoid conjugates. The quercetin method was also applicable for analysing the compound in urine; the method was, however, not validated for that purpose.

The analytical method presented in Study IV was the first published method suitable for the analysis of hesperetin and naringenin in human plasma. It was also suited for the analysis of the flavanones in urine. The solid-phase extraction method was the same as that originally developed for the extraction of quercetin from plasma. The second extraction step used in the quercetin method had to be omitted because some hesperetin and naringenin was extracted into the wrong phase. Further clean-up attempts of the solid-phase extract were unsuccessful, and therefore, the extract was, after concentration, injected directly into the HPLC system. Because hesperetin and naringenin are oxidized at higher potentials than quercetin, interfering compounds present a bigger problem in their HPLC analysis. The sample could not be purified of all interfering compounds, and thus, the detection limits for the flavanones were higher than for quercetin. The recovery, precision and reproducibility of the flavanone methods were good. Hesperetin and naringenin are not as prone to degradation as quercetin, and the compounds were stable under the same conditions as quercetin.

7.2. Pharmacokinetics of quercetin, hesperetin and naringenin

Absorption and elimination

Whether the flavonoids were obtained as aglycone or as glycosides affected the time it took for them to appear in plasma but did not appear to affect the rate of elimination. In the absorption phase, the shape of the plasma concentration vs. time curves for quercetin from rutin, and hesperetin and naringenin from citrus juices, were rather similar, whereas in the elimination phase, the curves for quercetin and the flavanones were clearly different. The late time-point of absorption, with mean T_{max} values ranging between 4.8 and 7.5 h, indicates that from the above-mentioned sources the compounds are absorbed from the distal parts of the small intestine or from the colon. Orocecal transit times of 50 min (Lorena et al. 2000), 1.8 h (van Nieuwenhoven et al. 1999), 2.3 h (Boekema et al. 2000), 3.2 h (Kagaya et al. 1997) and 5 h (Bennik et al. 1999) have been reported, supporting this assumption. Quercetin from quercetin aglycone, by contrast, was absorbed rapidly, probably from the duodenum. Absorption of quercetin from the stomach may also be possible, as indicated by a recent rat study (Crespy et al. 2002). The plasma quercetin curve after ingestion of quercetin aglycone was biphasic and the second absorption peak increased with increasing dose. This indicates that the compound was absorbed farther down the gastrointestinal tract as well. Whether eating lunch after the 4-hour blood sampling increased absorption is impossible to say. Eating is known to increase splanchnic blood flow. Furthermore, eating stimulates emptying of the gall bladder and the bile could have enhanced absorption of the fraction of quercetin which remained unabsorbed in the upper small intestine. Alternatively, the second concentration peak, usually occurring at 6 h, could be a result of enterohepatic circulation.

The results indicate that rutin and flavanones are cleaved in the distal parts of the gastrointestinal tract prior to absorption. This is in line with the findings of Day et al., who reported that rutin and naringin are not hydrolysed by cell-free extracts of the human small intestine (Day et al. 1998) or lactase from lamb small intestine (Day et al.

2000b). Quercetin-3-glucoside and naringenin-7-glucoside, on the other hand, were cleaved by enzymes from the small intestine, and therefore, it appears likely that the limiting step of hydrolysis in the small intestine is the α bond between the glucose and the rhamnose molecules. Rutin (quercetin-3-rutinoside), narirutin (naringenin-7-rutinoside) and naringin (naringenin-7-neohesperoside) all contain a glucose molecule, which is bound to the flavonoid aglycone with a β-linkage, and a rhamnose molecule, which is bound to the glucose moiety with an α bond. Bacterial enzymes capable of hydrolysing both types of bonds are present in the colon, but enzymes cleaving the α bond have not been identified in the small intestine.

The urinary excretion of quercetin was not investigated in this work. According to many studies, the urinary recovery of quercetin from quercetin glycosides is rather low (0.07-1.4% of ingested dose) (Table 2). Animal studies indicate that a substantial portion of ingested quercetin is excreted in bile (Ueno et al. 1983, Manach et al. 1996).

The urinary excretion of flavanones was studied in the citrus juice study (IV), and it seemed to depend on the source and/or the obtained dose. Only 1% of naringenin from orange juice (where present as narirutin) was recovered in urine, but when the compound was obtained from grapefruit juice (where present as naringin) the urinary recovery was 30%. The ingested doses were 23 mg from orange juice and 199 mg from grapefruit juice. The values for hesperetin from orange juice were between the values for naringenin from the two sources. The results were most likely caused by dose-dependent renal clearance rather than differences in bioavailability. This interpretation is supported by the fact that the C_{max}-to-ingested dose ratios did not differ for naringenin depending on the dose and source, and the fact that the half-life of naringenin was shorter when its intake was high (as from grapefruit juice).

The pharmacokinetics of quercetin, hesperetin and naringenin were in Studies II and IV investigated after single-dose administration. The kinetic behaviour of compounds during long-term administration is usually predictable based on single-dose data.

Steady-state concentrations of a compound are generally reached after administration of a compound daily for 4-5 times its half-life. The results of Study II indicate that steady-state concentrations of quercetin should be reached within 3-4 days. The half-life of flavanones, on the other hand, was only 1-2 hours, suggesting that no substantial accumulation occurs during once daily consumption of citrus fruit or juices. These assumptions should, however, be confirmed during long-term administration. For some compounds following non-linear kinetics, the kinetic behaviour changes during long-term administration or is disproportional to what is expected based on single-dose studies (Ludden 1991). Furthermore, sometimes the development of more sensitive analytical techniques has revealed new, longer elimination phases for compounds, thus explaining the effect of a compound after its apparent disappearance from plasma.

Few studies on the pharmacokinetics of flavonoids have been performed. The results obtained in this study regarding the T_{max} values of quercetin from rutin are similar to those reported previously by Hollman et al. (1997, 1999). The plasma concentrations in this study were somewhat lower, which is not surprising considering the fact that the ingested doses were lower. The C_{max} and AUC values after ingestion of 100 mg of rutin (containing 50 mg of quercetin) were, however, almost equal to the values reported by Hollman et al., although, in our study, the dose ingested was only half of that used by Hollman et al. Recently, pharmacokinetic results similar to those presented in Study II were reported by Graefe et al. (2001), who studied the pharmacokinetics of a 100- to 200-mg dose of quercetin from onion, buckwheat tea, quercetin-3-rutinoside and quercetin-4'glucoside.

Study II was the first report on plasma pharmacokinetics of quercetin after ingestion of quercetin aglycone. Several authors have previously suggested that the aglycone form is not absorbed. Study II and another recent report (Walle et al. 2001) show that this is not the case. Walle et al. (2001) indicated absolute bioavailability of 36-53% for quercetin aglycone. They also demonstrated that a substantial portion of quercetin is

excreted by the lungs as CO_2. Until more information is published on this very interesting study, conclusions should be made with caution, since radioactive quercetin was used and the results have been based on recovery of radioactivity. Therefore, the findings may reflect bioavailability and pharmacokinetics of degradation products, which could have been formed, at least partly, prior to absorption.

Plasma hesperetin and naringenin concentrations in humans have not been reported previously. Attempts to analyse them have been made, but the detection limits of the analytical methods have been too high. Regarding urinary excretion, the results of Study IV were similar to a previous report (Fuhr and Kummert 1995). The individual relative urinary recovery values for the two flavanones from the two different juices ranged between 0.2% and 69%. This suggests that bioavailability ranged between these values. However, it is also possible that it was much higher. In rats, biliary excretion of hesperetin appears to be a more important route of elimination than urinary excretion (Honohan et al. 1976).

Interindividual variation in bioavailability

The most interesting findings of the pharmacokinetic studies were the marked interindividual variations in plasma concentrations of flavonoids after ingestion of rutin and citrus juices, and the bioavailability of quercetin from rutin being affected by gender.

In general, variation in pharmacokinetics can be caused by physiological factors, such as differences in body weight, body composition and gastric motility, or molecular factors, including differences in activity or synthesis of different transporters, or enzymes involved in biotransformation (Meibohm 2002). Variation has been reported to occur for secretory transporter, such as P-glycoprotein (Lown et al. 1995, Kerb et al. 2001) and MRPs (van der Kolk et al. 2000), and biotransformation enzymes, such as CYP3A4 (Hall et al. 1999, Dai et al. 2001), UDP-glucuronosyltransferases (Fisher et

al. 2000) and sulfotransferases (Her et al. 1996). All of these proteins have been associated with flavonoids; quercetin interacts *in vitro* with P-glycoprotein (Shapiro and Ling 1997), MRP1 (Leslie et al. 2001), MRP2 (Walgren et al. 2000), and is a substrate for UGTs and sulfotransferases. Little is known about the factors that lie behind the variation in activity or amount of these proteins (Thummel et al. 1997). Genetic and environmental factors are probably differentially important for different systems. Gender differences, due to hormonal influence, have been implied in some cases (Back and Orme 1990, Harris et al. 1995, Kashuba and Nafziger 1998, Meibohm et al. 2002). However, gender disparities in pharmacokinetics are usually small and are rarely of clinical relevance. Still, gender differences have been reported for several transporters and enzymes involved in biotransformation. For instance, men seem to have higher P-glycoprotein and CYP1A2 activities. Furthermore, glucuronization and sulfation by certain UGT and SULT isoforms have been reported to be higher in men.

In Study IV, but not Study II, the doses were corrected for weight. Differences in body weight and possibly body composition may therefore explain some of the variation seen for rutin. However, since the variation was so marked, it was more pronounced at higher doses of rutin, and, in addition, less variation was seen for quercetin aglycone, it is concluded that the variation was likely caused by molecular factors, which depended on the site of absorption, rather than physiological factors. Furthermore, variation in rutin data was affected by gender and the highest concentrations were found for subjects using oral contraceptives. Due to the small number of subjects using oral contraceptives, the latter finding may, however, be coincidental. Since the site of absorption seems to have determined whether variation in plasma levels occurred, gender-specific variation in microflora is hypothesised to be one factor causing variation in bioavailability. Cleavage of flavonoids by bacterial enzymes is, as mentioned earlier, a prerequisite of absorption.

Circulating forms of flavonoids – does it matter what they are and where absorption occurs ?

In this work, mainly total plasma and urine flavonoids (=unconjugated flavonoid + glucuronized/sulfated flavonoid) were analysed after enzymatic hydrolysis of conjugates. Flavonoid conjugates could not be analysed separately because they are unknown and standards are not commercially available. The number of possible glucuronosyl and/or sulfate conjugates is high due to the large number of hydroxyl groups on the aglycones. In Study II, unconjugated quercetin and rutin were analysed in addition to total quercetin in samples taken 4-12 h after ingestion of the highest dose of rutin. Contrary to previous reports, we could detect unconjugated quercetin in plasma. The reasons for this discrepancy could be use of different analytical methods, or that in the other studies, the aglycone was measured after the ingestion of quercetin glycosides that are absorbed in different parts of the intestines than rutin. Another noteworthy finding in Study II was that rutin was not detected in plasma in its original glycosidic form after ingestion of rutin, which further confirms the view that the compound is hydrolysed prior to absorption of the aglycone. This result contradicts a previous report indicating that high amounts of rutin circulate in plasma (Paganga and Rice-Evans 1997). In that study, retention time in HPLC and UV spectra were used for identification. These are similar for quercetin glucuronides and glycosides, and they could have been confused with each other (Manach et al. 1998).

The question about which form of quercetin circulates in plasma is not only of academic interest. Which hydroxyl groups on the flavonoid molecules are conjugated is of relevance because this probably affects the biological activities of the compounds. Often metabolism reduces activity, but in some cases, metabolites are even more potent than the parent compound (Glatt 2000, Osborne et al. 2000). The site of absorption of flavonoids could be important because the degree or type of biotransformation might vary in different parts of the gut. Therefore, in theory, it is possible that different metabolites or different amounts of metabolites are formed when a compound is absorbed from different parts of the gastrointestinal tract. One

phenomenon resulting in this situation is saturable first-pass metabolism, although, at first thought, it would appear unlikely at the concentrations at which nutrients occur in the diet. However, at least the sulfotransferase pathway appears to be readily saturated (Rogers et al. 1987). Another interesting factor is that the activity and amount of many biotransformation enzymes decrease from the duodenum to the colon (Peters et al. 1991). Furthermore, some enzymes are only expressed in certain tissues or parts of the intestines. For instance, UGT 1A8, which has been shown to glucuronidate both quercetin and naringenin (Cheng et al. 1999), is predominantly expressed in the colon (Mojarrabi and MacKenzie 1998). Transcripts of the enzyme have also been detected in the jejunum and the ileum, but not in the duodenum, stomach or liver (Cheng et al. 1998). On the other hand, UGT 1A9, which also glucuronidates flavonoids (Oliveira and Watson 2000), is expressed in the liver, but not in the small intestine or colon. How likely it is that different metabolites are formed depending on the flavonoid glycoside ingested, or that individuals ingesting the same flavonoid glycoside have different metabolite profiles, is difficult to speculate upon. However, the possibility that health effects occur after ingestion of one type of bioavailable flavonoid glycoside or the aglycone, but not after ingestion of another, should be kept in mind when evaluating the results of studies on the health effects of flavonoids or when planning clinical trials.

7.3. Bioavailability of quercetin from berries and the diet

Serum quercetin concentrations were significantly higher in subjects consuming berries (III) than in those consuming their habitual diets (III) or a low-quercetin diet (II). Therefore it is concluded that quercetin is bioavailable from berries. Compared with the control group, the concentrations were 30-50% higher in the berry group during the berry consumption period. The corresponding increase in intake was 62%. The increase in serum quercetin was similar to or higher than what was previously found in 12 men consuming 375 ml of black tea or 750 ml of red wine for four days, but less than half of what was found when the subjects consumed 50 g of fried onions

(de Vries et al. 2001). However, the serum samples were taken after an overnight fast, while de Vries et al. (2001) collected samples twice on the last day that quercetin-containing foods were consumed. The results are therefore not directly comparable, but they do suggest that more quercetin reaches the circulation after consuming of 100 g of these berries than does after consuming 750 ml of red wine or 375 ml of black tea.

Which berries contributed most to the increase in plasma quercetin levels in Study III is not known, but according to pilot studies from our laboratory (unpublished), quercetin is bioavailable from all of the berries consumed in this study (lingonberries, bilberries and black currants). The absolute bioavailability of quercetin from different berries probably varies. Lingonberries, black currants and bilberries contain partly different quercetin glycosides, and no information is available on the bioavailability of, for instance, quercetin arabinosides. Moreover, differences in the distribution of quercetin in the different compartments of berries and the thickness of the skin of the berries could affect the availability of quercetin.

Limited data are available on plasma quercetin concentrations in subjects consuming their habitual diets. In most studies, the baseline plasma samples have been collected from subjects following a flavonoid-restricted diet. The mean baseline plasma quercetin concentrations were 16 ± 13 µg/l in Study III. Quercetin was detected in plasma of all subjects. The results indicate that the compound is bioavailable from a rather typical Finnish diet. Similar findings have recently been made in other studies performed at the Biomarker Laboratory (not included in this thesis). For instance, in 37 female health professionals following their habitual diets, the mean plasma quercetin concentration was 16 ± 24 µg/l (Erlund et al. 2002). In another study, in 77 female and male subjects following either high- or low-vegetable diets, quercetin concentrations were 24 ± 17 µg/l during habitual diets, 15 ± 7 µg/ after a low-vegetable diet and 42 ± 24 µg/l after a high-vegetable diet (Freese et al. 2002). Similar results were obtained by Noorozi et al. (2000), who reported baseline plasma quercetin values of 23 µg/l in 10 diabetic subjects.

7.4. Plasma and urine quercetin, hesperetin and naringenin as biomarkers of intake

Few excellent biomarkers of nutrient intake on an individual level are available. Factors such as under-reporting, poor memory, inaccurate intake assessment, and lacking or inaccurate food composition data make the accurate assessment of an individual's nutrient intake by dietary survey methods difficult. Plasma or urine (the tissues most often available in population studies) nutrient concentrations, on the other hand, can vary considerably during the day, week or season. Fortunately, there is seldom a need to assess intake accurately on an individual level, but rather on a group basis.

The work included in this thesis indicates that plasma quercetin is a fairly good biomarker of intake. Study II showed that plasma quercetin concentrations increase with increasing dose and that the half-life of quercetin is relatively long. The compound is also bioavailable from the diet (Study III). However, the fluctuation in plasma levels weakens the value of plasma quercetin, as well as most other dietary compounds, as a biomarker of intake. Yet, several factors advocate its use. Firstly, the extent of exposure, the strength of the experimental evidence regarding effects, and the prevalence and severity of the medical conditions in question are such that the subject is considered to be important. Secondly, the only other biomarker of quercetin intake available, i.e. intake assessment from food records or questionnaires, has many disadvantages. An accurate assessment of the intake of onion, in particular, qualitatively and quantitatively the most important source of quercetin, is problematic because onion is a commonly used ”hidden ingredient” of many home-made and processed foods. Thirdly, the use of plasma concentrations instead of calculated intake as a measure of ”bioavailable intake” may correct some of the errors caused by between-individual and between-source differences in bioavailability.

Study IV indicated that the pharmacokinetics of the flavanones hesperetin and naringenin are such that their concentrations in single plasma and urine samples are not well suited for biomarker use. Because their half-lives are short (1-2 h) variations in plasma concentrations are expected to be great. This assumption is supported by results from a more recent study, in which a citrus-containing diet was consumed by healthy women for five weeks (Erlund et al. 2002). In that study, using the method developed in Study IV, ingestion of one glass of orange juice, half an orange and half a mandarin daily resulted in measurable levels of hesperetin and naringenin in fasting plasma of 54% and 21% of all subjects, respectively (Erlund et al. 2002). Study IV also indicated that the urinary clearance of naringenin is dose-dependent, although more studies regarding this issue are needed because there could be a threshold concentration for the phenomenon. Because of the marked interindividual variation in bioavailability, it is important to monitor bioavailability in clinical studies investigating the effects of flavanones. This can be done by taking multiple plasma samples after a suitable timeperiod following flavanone intake. However, in epidemiological studies, a more sensible approach would be to assess intake from food records or questionnaires (if citrus consumption was asked). After all, flavanones are mainly obtained from citrus fruits and juices, and the errors in estimating their intake are probably relatively small.

7.5. Future directions

Most humans are exposed to flavonoids daily and therefore their impact on human health is of relevance. The health effects of flavonoids are, however, largely unknown, and interpretation of the results of the studies performed so far problematic. Several types of studies could yield more information on the issue. For instance, studies are needed on the metabolism of flavonoids to enable *in vitro* studies to be performed with metabolites actually present in human tissues. In epidemiology, the use of plasma flavonoid concentrations as biomarkers of intake may help to overcome some of the problems associated with intake assessment by dietary survey methods. This approach would probably be useful for compounds such as quercetin, but less so for hesperetin and naringenin. More clinical studies on the health effects of both high and low doses of flavonoids are also needed. The lack of methods detecting small changes in biological or physiological responses is, however, a problem. Long-term and large-scale supplementation studies with humans are not warranted at this time because they are too costly and require a more solid evidence of benefit before they can be ethically justified. This applies not only to flavonoids, but to most ”potentially beneficial” dietary components.

Cell studies aimed at identifying quercetin metabolites in humans are currently underway at the Biomarker Laboratory. The analytical methods presented in this thesis have been further developed to cover other phenolic compounds in plasma, such as lignans, isoflavones and phenolic acids, as well as their metabolites. Their plasma concentrations are being measured in subjects selected from Finnish population cohorts. Hopefully, these studies will shed some new light on the associations between flavonoids and other plant phenolics, and chronic diseases such as cardiovascular disease and cancer.

8. SUMMARY AND CONCLUSIONS

Results of the studies can be summarized as follows:

1. Methods were developed for the analysis of quercetin, hesperetin and naringenin in plasma and urine. The quercetin method had a low detection limit and it is suitable for the analysis of quercetin in plasma of individuals with low quercetin intakes. The method had excellent reproducibility over a long period, indicating that it can be used to reliably measure quercetin levels in large sets of samples with low quercetin concentrations. The hesperetin and naringenin method was suitable for the analysis of flavanones in human plasma and urine. The method had good reproducibility and is well suited for analysing hesperetin and naringenin in plasma and urine of human subjects after consumption of citrus fruits or juices.

2. All of the studied flavonoids were bioavailable. The bioavailability and pharmacokinetic properties differed for the compounds depending on the subgroup and the glycosidic form. Especially after ingestion of quercetin and the flavanones as rutinosides or neohesperosides, marked between-subject variation occurred in plasma levels. Furthermore, the bioavailability of quercetin from rutin was better in women than in men. The elimination half-life ($T_{1/2}$) of quercetin in plasma was 15-18 h, which is rather long compared with the half-lives of naringenin and hesperetin (1-2 h).

3. Berries appear to be a good source of bioavailable quercetin. The compound is also bioavailable from a typical Finnish diet. The mean fasting plasma quercetin concentration of middle-aged men consuming their habitual diets was 16 ± 13 µg/l (mean ± SD).

4. Hesperetin was bioavailable from orange juice, and naringenin was bioavailable from orange juice and grapefruit juice. After ingestion of citrus juices, plasma

flavanone concentrations were relatively high (up to 4 mg/l), corroborating the need for more studies on their putative health effects.

5. Fasting plasma quercetin concentration appears to be a fairly good biomarker of dietary quercetin intake. The compound has a comparatively long elimination half-life and its plasma concentrations increase with increasing dose. Fasting plasma and urine flavanone concentrations, on the other hand, are expected to be less useful biomarkers of intake.

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