DETERMINANTS OF THE ABSORPTION OF THE DIETARY FLAVONOID QUERCETIN IN MAN

Peter C.H. Hollman

Promotoren: Dr. J.G.A.J. Hautvast
Hoogleraar in de leer van de voeding van de mens

Dr. M.B. Katan
Universitair hoofddocent aan de Vakgroep Humane Voeding
Bijzonder hoogleraar in de voedingsleer van de mens aan de
Katholieke Universiteit Nijmegen

Co-promotor: Dr. O. Korver
Hoofd Unilever Nutrition Centre
Unilever Research Laboratorium Vlaardingen

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Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen
dr.C.M. Karssen,
in het openbaar te verdedigen
op woensdag 18 juni 1997
des namiddags om half twee in de Aula

The research described in this thesis was carried out at the State Institute for Quality Control of Agricultural Products (RIKILT-DLO) and the Department of Human Nutrition, Wageningen Agricultural University.

The research was financially supported by the Netherlands Heart Foundation (94.128), the Foundation for Nutrition and Health Research, and the Agricultural Research Department (DLO).

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ISBN 90-5485-691-2

Cover: Frans Hollman
"Still life with onions" (fragment)
Maastricht 1994
Pastel on paper 40 cm x 23 cm

Printing: Grafisch Bedrijf Ponsen & Looijen BV, Wageningen, The Netherlands

Stellingen

1. Chromosomen hebben met betrekking tot hun synaptisch gedrag een geheugen.

Dit proefschrift

2. Homo- of heterozygotie voor de Robertsonische translocatie Rb(11.13)4Bnr (Rb4) heeft niet altijd een reducerend effect op de mannelijke vruchtbaarheid. Afhankelijk van de overige in het karyotype aanwezige chromosoomafwijkingen kan Rb4 ook stimulerend werken.

Dit proefschrift

3. Niet-homologe chromosoom synapsis in afwezigheid van meiotische recombinatie leidt niet tot dominante of recessieve vormen van chromosoom instabiliteit. In aanwezigheid van recombinatie mogelijk wel.

Dit proefschrift

4. Klassieke chromosoom mutanten kunnen een vergrootglas funktie vervullen bij meiotisch cytologisch onderzoek.

Plug *et al.*, Nature Genetics, in press

5. Publiceerbaarheid van gegevens bepaalt niet alleen het plezier in wetenschappelijk onderzoek. Het kan dit wel versterken.

6. Moleculair biologen leven in zwart/wit, cytologen in kleur.

7. Sonore koorzang is het zoeken naar harmonie tussen het individu en het collectief.

8. Helaas zijn veel (vooraanstaande) wetenschappers geen goede koorzangers.

9. Nalatige hooggeplaatsten zijn ware alchemisten: het lukt hen vaak het vuil aan hun handen om te zetten in goud.

10. Email maakt onmogelijke relaties mogelijk.

11. Het betere is de vijand van het goede.

Stellingen behorende bij het proefschrift
"Non-homologous chromosome synapsis during mouse meiosis:
consequences for male fertility and survival of progeny"

Wageningen, 21 november 1997 Antoine H.F.M. Peters

10. De overhead van een onderzoeksorganisatie gedraagt zich als overgewicht. Voorkomen ervan vereist een streng dieet, maar dat blijkt moeilijk vol te houden.

11. De wachtgeldfobie van de overheid wordt duur betaald.

12. Het Kenniscentrum Wageningen werkt.
    Dit proefschrift.

13. Ofschoon niet ontworpen voor gebruik op de elektronische snelweg, is de fiets een onmisbaar hulpmiddel in het Wageningse netwerk.

14. Voor het milieu is nalaten beter dan doen.
    Lyklema H. *Chemisch Weekblad* 1996;**92(48)**:2.

15. 'Natuurlijk' is niet per definitie gezonder.
    Ames BN, Gold LS, Willett WC. The causes and prevention of cancer. *Proc Natl Acad Sci USA* 1995;**92**:5258-5265.

16. Gelukkig zijn er wetenschappers die 'nutteloos' onderzoek verrichten.
    Chemisch Weekblad 1997;**93(11)**:2

17. Als Eva dit proefschrift had kunnen lezen, dan zou zij de verleiding van de appel wellicht hebben weerstaan, maar in plaats daarvan bezweken zijn voor een gefruit uitje.

Little pieces in a giant jigsaw

Voor Paula, Pascal en Danielle

Abstract

Determinants of the absorption of the dietary flavonoid quercetin in man

PhD thesis by Peter C.H. Hollman, State Institute for Quality Control of Agricultural Products (RIKILT-DLO), Wageningen, the Netherlands. June 18, 1997

Oxidation of low density lipoprotein is hypothesised to play an important role in the development of cardiovascular disease. It might be prevented by dietary antioxidants. Quercetin is a dietary flavonoid antioxidant and its intake was inversely associated with cardiovascular disease in some studies. Absorption from the diet is a prerequisite for its potentially beneficial role. This thesis describes studies on the absorption and elimination kinetics of dietary quercetin in humans.

To perform these absorption studies, we developed a postcolumn chelation technique for quercetin in HPLC with fluorescence detection using aluminum. Only flavonols that contain a free 3-hydroxyl and 4-keto oxygen binding site formed fluorescent complexes with Al^{3+}. This method improved detectability of quercetin 300 fold as compared to conventional UV detection.

We studied the absorption of quercetin in healthy ileostomy subjects so as to avoid losses caused by colonic bacteria. Absorption of quercetin was 52 $\pm$ 15% for quercetin glucosides found in onions, 17 $\pm$ 15% for quercetin rutinoside a major quercetin glycoside of tea, and 24 $\pm$ 9% for free quercetin aglycone.

The time course of the plasma quercetin concentration was studied in normal subjects with an intact colon who ingested major dietary sources of quercetin, viz. fried onions containing glucose conjugates of quercetin, apples containing both glucose- and non-glucose quercetin glycosides, and of quercetin-3-rutinoside. Peak plasma levels of quercetin were reached <0.7 h after ingestion of the onions, 2.5 h after the apples, and 9 h after the rutinoside. Bioavailability of both quercetin from apples and of pure quercetin rutinoside was 30% relative to onions. Half-lives of elimination were independent of the quercetin source and were about 24 h.

We confirmed that the sugar moiety of the glycoside is an important determinant of absorption in a study with volunteers who ingested solutions of pure quercetin-4'-glucoside and of pure quercetin-3-rutinoside.

In conclusion, absorption of dietary quercetin glycosides can be appreciable and depends on the type of sugar moiety of the glycoside. We propose that the sodium-glucose cotransporter is involved in the absorption of quercetin-4'-glucoside. Elimination of quercetin from the blood is slow. Repeated consumption of quercetin-containing foods therefore will lead to accumulation of quercetin in plasma.

Contents

1

GENERAL INTRODUCTION

Background

Flavonoids comprise a large class of polyphenolic components and are common ingredients in foods of plant origin. The basic structure of flavonoids (*Figure 1*) allows a multitude of substitution patterns in the benzene rings A and B, and variations also can occur in the heterocyclic ring C, giving rise to flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids. Over 4000 different naturally occurring flavonoids have been described[1] and this list is still growing. Recently, much attention has been paid to their antioxidant properties[2] which may protect tissues against oxygen free radicals and lipid peroxidation. Oxygen free radicals and lipid peroxidation are thought to play a role in several pathological conditions such as atherosclerosis, cancer, and chronic inflammation.[3]

Cardiovascular disease is the major cause of death in Western societies. In 1994, 20 700 people died from coronary heart disease and 12 600 people from stroke in the Dutch population which comprises more than 15 million people.[4] Coronary heart disease and stroke were placed number one and two in the top ten of causes of death in the Netherlands. The impact of cardiovascular disease on the nations health is even more pronounced when one considers that the number of people suffering from this disease is 10-fold higher than the number of mortalities. The quality of life of this group of people is reduced and involves reliance on medical care and hospitalization, sick leave, disablement, health impairment and handicaps.[5]

A number of risk factors for coronary heart disease have been described.[5] These risk factors include high blood pressure, high total cholesterol, low cholesterol in high density lipoprotein particles, obesity and diabetes. Lifestyle factors, such as smoking and physical inactivity, also play an important role.[5]

Atherosclerosis is the prime cause of coronary heart disease and is also important in thrombotic stroke. It is characterized by thickening and narrowing of arteries because of the formation of fibrofatty and fibrous lesions which obstruct the blood flow. A major hypothesis proposes that oxidised low density lipoprotein (LDL) particles, which constitute one of the carriers of cholesterol, play a key role in the development of atherosclerosis.[6-10] Consequently, the avoidance or delay of LDL oxidation by dietary antioxidants might provide a promising strategy for preventing atherosclerosis and consequently coronary heart disease. However, the ability of dietary antioxidants to inhibit LDL *in vivo* still has to be established. Flavonoids are such dietary antioxidants and epidemiological data support a potential role for

the subclass of flavonols, with quercetin as the major representative, in the prevention of cardiovascular disease.[11] However, the role of flavonoids in human physiology is largely unknown and hampers an evaluation of their potential health effects. Animal studies suggested that gastro-intestinal absorption from foods of most flavonoids will be marginal, because they are linked to sugars.[12] These so-called glycosides are reported to be non-absorbable and require hydrolysis by bacteria in the colon, where absorption is inefficient and degradation immanent.[13]

This thesis describes studies on the absorption and elimination kinetics of the major dietary flavonol quercetin in healthy humans. Epidemiological studies on the relation between dietary flavonol intake and the risk of cardiovascular disease are summarised in this introduction, and the usefullness of kinetic studies is briefly discussed. Finally, the purpose of the research of this thesis and an overall outline of the thesis are given.

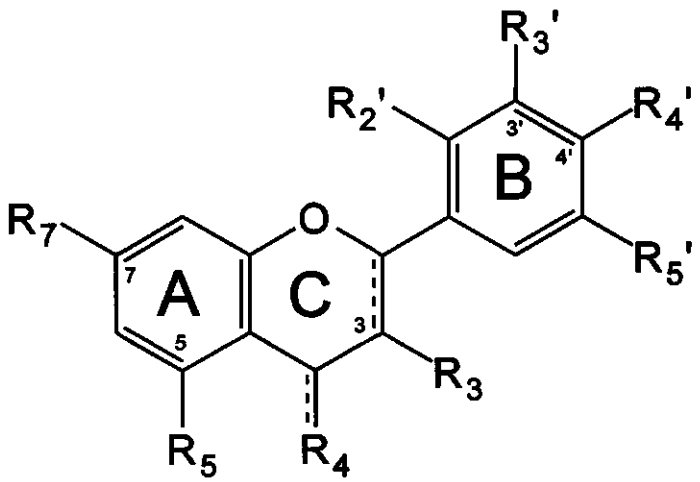

Figure 1. *General structure of flavonoids. Classification into flavones, flavonols, flavanones, catechins, anthocyanidins, isoflavones is based on variations in the heterocyclic C-ring. A multitude of substitutions in the ring systems occur:*

R_i $R_{i'}$ = H, OH, O-sugar (glycosides), O-CH_3, sulfate, O-glucuronic acid, or O (only R_4)

Quercetin: R_3 R_5 R_7 $R_{3'}$ $R_{4'}$ = OH; R_4 = O ; $R_{2'}$ $R_{5'}$ = H; double bond between C_2 and C_3

Flavonoids and cardiovascular disease risk

The only studies relating the intake of dietary flavonoids to risk of disease have been observational in nature. These studies were only possible after development of quantitative methods of analysis and their application to the quantification of flavonoids in foods. Until the end of the 1980s, the methods of flavonoid analysis were qualitative since they had been primarily developed for taxonomic studies. The aim of the taxonomic studies was to unequivocally characterise the flavonoids present in individual plant species. To date, quantitative data for only two subclasses of flavonoids, the flavonols and flavones, have been published.[14-16] These compounds are present in plants bound to sugars, the so-called glycosides (*Figure 1*). Flavonols are by far the most important of these two classes of flavonoids since flavones were only found in a few foods. Quercetin is the most important flavonol found in plant foods.

We determined the average dietary flavonol and flavone intake as it was around 1960 in 16 cohorts participating in the Seven Countries Study. The average flavonol and flavone intake was inversely correlated to mortality rates of coronary heart disease after 25 years of follow-up (*Table 1*).[17] The intake of flavonols and flavones, together with smoking and the intake of saturated fat, explained about 90% of the variance in coronary heart disease mortality rates across the 16 cohorts.

Five prospective within-population cohort studies have been carried out (*Table 1*). Coronary heart disease mortality was strongly inversely associated with flavonol and flavone intake in the Zutphen Elderly Study[18] with a reduction in mortality risk of more than 50% being recorded (*Table 1*) in the highest tertile of flavonol intake. Average flavonol intake in the highest tertile was 42 mg/day, and in the lowest 12 mg/day. Recently, the ten year follow-up of the Zutphen Elderly Study was completed with results strengthening the findings of the five year follow-up (M.G.L. Hertog, personal communication). Unlike the findings of the five year follow-up, a clear dose-response relationship between flavonol intake and coronary heart disease mortality was now recorded.

The association between flavonol and flavone intake and risk of stroke was studied in a cohort of 550 middle-aged men[19] (*Table 1*). These men were followed for 15 years, and the men in the highest quartile of flavonol and flavone intake (> 30 mg/ day) showed a considerably reduced risk of the disease of about 60%.

Mortality from coronary heart disease was weakly inversely associated with flavonol and flavone intake in a cohort of 5130 Finnish men and women aged 30 -

69 years followed over a 20 years period[20] (*Table 1*). The relative risks of mortality from coronary heart disease between the highest (>5 mg/day) and lowest quartiles (<2.5 mg/day) of flavonol and flavone intake were 0.73 for women and 0.67 for men.

Table 1. *Summary of epidemiological prospective studies on flavonol and flavone intake and coronary heart disease (CHD) and stroke risk*

Population	Age (y)	Follow-up (y)	Relative Risk[1] (95% Confidence Interval)
Cohort studies			
CHD, 805 men; Zutphen (The Netherlands)[18]	65 - 84	5	0.32 (0.15 - 0.71)
CHD, 5133 men + women; Finland[20]	30 - 69	20	♀: 0.73 (0.41 - 1.32) ♂: 0.67 (0.44 - 1.00)
CHD, 34 789 men Health Professionals (U.S.A.)[21]	40 - 75	6	1.08 (0.81 - 1.43)
CHD, 1900 men Caerphilly (U.K.)[22]	49 - 59	14	1.6 (0.9 - 2.9)
Stroke, 552 men; Zutphen (The Netherlands)[19]	50 - 69	15	0.27 (0.11 - 0.70)
Cross-cultural study			
CHD, 12 763 men Seven Countries Study[17]	40 - 59	25	r = -0.50 (*P* = 0.01)

[1] Relative risk of highest versus lowest flavonol intake group, adjusted for age, diet and other risk factors for coronary heart disease

Recently, in male US health professionals a modest, but non-significant, inverse association between flavonol and flavone intake and coronary mortality was found only in men with previous history of coronary heart disease[21] (*Table 1*). Median flavonol intake in the highest quintile was 40 mg/day and 7 mg/day in the

lowest.

In contrast to the above studies, increased mortality of ischaemic heart disease was found in Welsh men[22] in all quartiles of high flavonol intake compared to the lowest quartile (*Table 1*). Mean flavonol intake in the highest quartile was 43 mg/day, and 14 mg/day in the lowest quartile.

To summarise, a protective role for flavonols in cardiovascular disease was found in 3 out of 5 prospective cohort studies, in addition to one cross-cultural study. One prospective cohort study showed no association, and one a weakly positive association between flavonol intake and coronary heart disease. So far, the epidemiological evidence points to a protective effect of antioxidant flavonols in cardiovascular disease but it is not conclusive.

Kinetic studies on absorption and elimination

For quercetin to act as a potential antioxidant in humans it is required to enter the blood circulation. Thus, after consumption, quercetin has to pass across the intestinal wall and resist metabolism in the liver. The bioavailability of quercetin in various foods, i.e. the fraction of quercetin molecules that successfully survives these barriers,[23] will be an important determinant of exposure. This fraction, called 'absolute bioavailability', is determined experimentally by administrating quercetin either orally or intravenously and then comparing the ratio of the areas under the plasma quercetin versus time curve. The studies in this thesis did not include intravenous administration of quercetin glycosides. The approach used in this thesis only gave data on the relative bioavailability of quercetin present in quercetin-rich foods and of selected pure quercetin glycosides. Thus, a comparison of the potential of various foods to increase plasma levels of quercetin could be made.

As all tissues are supplied with plasma, there will be a relationship between the concentration of quercetin in the plasma and that at its site of action. The plasma concentration of quercetin will change with time, and as a consequence also its effect in the body. The time course of the quercetin concentration in plasma after oral ingestion of a known amount of quercetin present in foods or in some glycosidic form, is used to calculate kinetic parameters of absorption and elimination, such as half-lives. Absorption kinetics may give information on the mechanisms of absorption involved and consequently on determinants which affect absorption.

Oxidants, such as free radicals, are continuously generated by cells and can potentially cause damage to these cells. A steady-state antioxidant concentration which is sufficiently high to prevent this deleterious oxidation, would probably be beneficial. Because food is not consumed continuously over 24 hours, the input of dietary antioxidants such as quercetin to the body may occur as a series of pulses separated by many hours. Rapid elimination of these antioxidants from the body after a meal would be undesirable. Accumulation of dietary antioxidants in the body may occur, but that depends on their elimination half-lives and consumption intervals. The average steady-state level of a dietary antioxidant achieved will additionally depend both on the amount ingested and its bioavailability.[23] It is feasible that kinetic parameters vary between foods. Thus, kinetic studies on absorption and elimination will enable us to compare the supply of quercetin to the body from the habitual consumption of various foods and to predict plasma steady-state levels of these potentially beneficial antioxidants in the diet. As a second step, the efficacy of these quercetin concentrations *in vivo* has to be determined.

Purpose of the studies and outline of this thesis

The aim of this thesis was to determine whether the proposed role of flavonols in the protection against cardiovascular disease, is supported by their physiological behaviour. To this purpose, the rate and extent of absorption and elimination of dietary quercetin and of quercetin glycosides were studied in human volunteers.

Chapter 2 reviews studies on the absorption, metabolism and bioavailability of flavonoids reported in the literature. This review is updated with the main results of our research presented in the subsequent chapters. *Chapter 3* describes the development of a postcolumn derivatization method for the analysis of flavonols by HPLC using fluorescence detection. This technique was essential since the methods available lacked sufficient sensitivity to allow the determination of quercetin in blood plasma of subjects after consumption of flavonoid-rich supplements. We performed a mass balance study with ileostomy volunteers, reported in *Chapter 4*, to quantify the absorption of quercetin from fried onions (rich in quercetin glucosides), pure quercetin rutinoside (a major quercetin glycoside of tea), and pure quercetin aglycone. In *Chapter 5*, we present a pilot study on the plasma kinetics of quercetin after consumption of a single dose of fried onions by two normal healthy human subjects. The bioavailability of quercetin glycosides from onions and

apples, and of the pure quercetin rutinoside in nine healthy subjects is compared in *Chapter 6*. The role of the sugar moiety of quercetin glycosides as a potential determinant of absorption is studied in *Chapter 7*. Finally, *Chapter 8* discusses the main results.

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2

ABSORPTION, METABOLISM and BIOAVAILABILITY of FLAVONOIDS

Hollman PCH, Katan MB.
In: Rice-Evans C, Packer L, eds. *Flavonoids in health & disease*. Marcel Dekker Inc. New York. 1997; in press

Abstract

Flavonoids are dietary polyphenols with potential health benefits. This review discusses results of studies on the absorption, metabolism and pharmacokinetics of flavonoids. Most studies have been carried out with aglycones in rodents using unphysiologically high doses. Absorption depended on the type of flavonoid and was between 4 and 58%. The absorption from dietary sources is largely unknown. In the metabolism of flavonoids the liver and the colon are important. Evidence for phase II biotransformation reactions in the liver is abundant. Colonic bacteria are involved in hydrolysis of glycosides and conjugates and in ring fission producing phenolic acids which are absorbed. Three types of ring fission depending on the structure of the flavonoid C-ring have been described. The susceptibility to ring cleavage is determined by the pattern of free hydroxyl groups in the flavonoid molecule. Very few quantitative data on metabolism are available. Pharmacokinetic data on flavonoids are scarce and absolute bioavailability of dietary flavonoids has not been determined.

INTRODUCTION

Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. They comprise 2-phenylbenzo-γ-pyrones, -dihydropyrones, -dihydropyrans, and -pyryliums. Variations in the oxygen-containing heterocyclic ring give rise to catechins (dihydropyrans), flavonols and flavones (γ-pyrones), flavanones (dihydropyrones), and anthocyanidins (pyryliums) (*Figure 1*). Attachment of the second benzene ring to the 3 instead of the 2 position creates isoflavonoids (*Figure 1*). In addition, the basic structure of flavonoids allows a multitude of substitution patterns in the two benzene rings within each class of flavonoids. Over 4000 different naturally occurring flavonoids have been described[1] and this list is still growing.

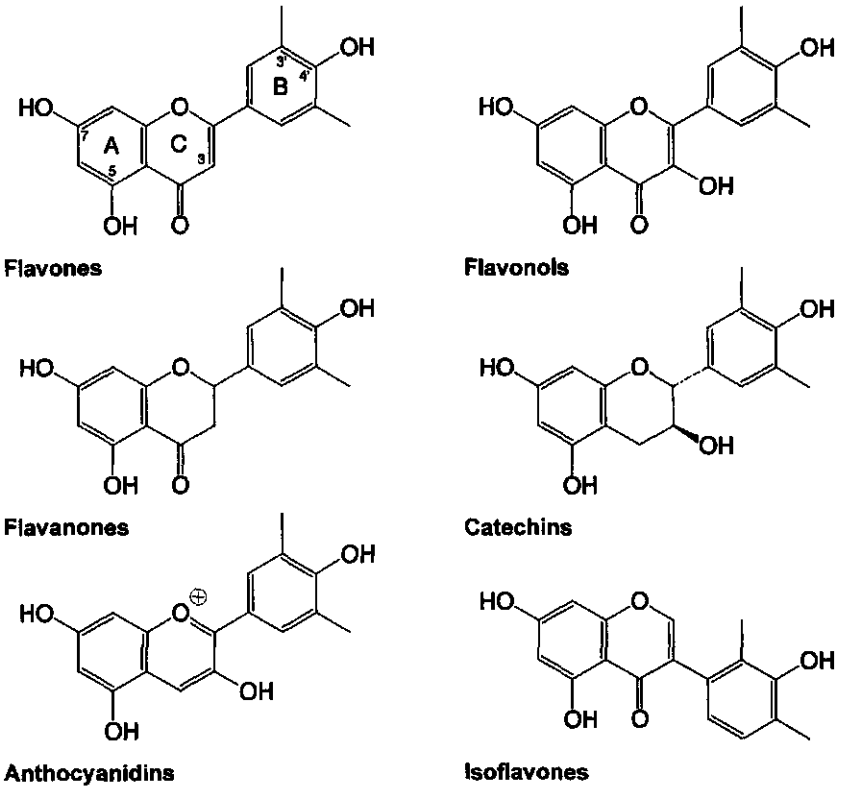

Figure 1. *Subclasses of flavonoids. Classification is based on variations in the heterocyclic C-ring.*

Major dietary sources of flavonoids are vegetables, fruits, and beverages such as tea and red wine.[2-7] Kühnau [4] estimated that the total flavonoid intake in the U.S.A. was 1 g/day expressed as glycosides or 650 mg/day expressed as aglycones, but most likely this estimate is too high. New, more specific food analyses[2,3] suggested that the Dutch intake of flavonols and flavones was 23 mg/day (expressed as aglycones),[8] as opposed to Kühnau's estimate of 115 mg/day (expressed as aglycones) in the U.S.A.[4]

History

In 1936 it was observed that a mixture of two flavanones decreased capillary permeability and fragility in humans. This gave rise to a claim for vitamin action of flavonoids (Vitamin P). Doubts about the evidence for these claims prompted the US Council on Foods and Nutrition to issue a report on the absorption and excretion of flavonoids.[9] It was concluded that flavonoids are probably largely destroyed in the mammalian gastro-intestinal tract, thus strengthening the scepticism about "Vitamin P". The possibility was suggested that one or more metabolites were responsible for the potential therapeutic effects.

In the 1950s and 1960s research on absorption and metabolism of flavonoids was advanced by Booth, Das, and Griffiths who published many articles exploring the metabolic routes of various flavonoids in animals. Several review articles describe these achievements.[4,10,11] However, most of these studies used high doses, mainly because the analytical techniques used for identification of metabolites lacked sensitivity. Details of the metabolic pathway still had to be unravelled.

Recent developments

A multitude of *in vitro* studies suggested that flavonoids inhibited, and sometimes induced, a large variety of mammalian enzyme systems.[1] Some of these enzymes are involved in cell division and proliferation, platelet aggregation, detoxification, and inflammatory and immune responses. Thus, it is not surprising that effects of flavonoids on different stages in the cancer process, on the immune system, and on haemostasis were reported in cell systems and animals.[1,12] Recently, much attention has been paid to the antioxidant properties of flavonoids, caused by their ability to scavenge oxygen free radicals.[13-15] Oxygen free radicals and lipid peroxidation might be involved in atherosclerosis, and a role has also been suggested in cancer and chronic inflammation.[16] There is indeed some epidemiological evidence for an inverse association of the intake of flavonols and flavones with subsequent coronary

heart disease,[17] although the association is still controversial.[18] No association of flavonoid intake and cancer risk in man has been established.[17]

Scope of this review

The increasing awareness of a potential beneficial role of flavonoids in human health provided new perspectives for flavonoid research. Knowledge of the pharmacokinetics and bioavailability of flavonoids in man is indispensable to fully evaluate this role. Metabolic transformations of flavonoids in the human system may be crucial for their biological effect. This chapter focuses on the fate of dietary flavonoids, except isoflavonoids, in mammals, and thus updates the reviews published.[4,10,11] Pharmaceutical preparations are dealt with only where that helps to understand general principles of bioavailability.

ABSORPTION

The major questions here are to what extent flavonoids are absorbed from the gastro-intestinal tract, and which factors affect absorption. Absorption of flavonoids from the diet was long considered to be negligible, as most of the flavonoids, except catechins, are present in plants bound to sugars as glycosides, and these were considered non-absorbable. Studies with germ-free rats indeed showed that large amounts of unchanged glycosides were excreted with faeces, whereas only small amounts of glycosides were found in faeces of rats with a normal microflora.[19] Evidently, enzymes that can split these predominantly ß-glycosidic bonds were not secreted into the gut or present in the intestinal wall. Bacteria in the colon were able to hydrolyse flavonoid glycosides[20-22] but at the same time degraded the liberated flavonoid aglycones (see page 30). In addition, the absorption capacity of the colon is far less than that of the small intestine. The assumption that only free flavonoids (aglycones) are absorbed by the gut and that glycosides are not is a classic example of "conventional wisdom". It was never seriously questioned even though there was little evidence to support it.

Absorption of pure compounds

Balance studies with radioactively labelled flavonoid aglycones were used in the 1970s and 1980s to quantify the absorption of (+)-catechin, quercetin, and flavanones in rodents, monkeys and man (*Table 1*), always without their attached sugars.

In these studies total radioactivity was measured in urine, faeces, expired air, and sometimes also in tissues.[28,31] As a consequence, the excreted radioactivity included the intact administered compound, if any, and metabolites that contained radioactive atoms. Catechins and their microbial degradation products were well absorbed as judged by excretion of 47% to 58% of the total administered radioactivity into urine. The administered dose did not seem to be an important variable, and absorption in rodents, monkeys and man was similar. It was suggested[23] that the radioactivity not accounted for, some 20%, had possibly been incorporated into tissues. Excretion of unchanged catechin aglycones in urine was only 0.1 - 2% of the dose.

Radioactive quercetin aglycone was less well absorbed than catechins, with only 4 - 13% recovered in urine (*Table 1*). About 40% was excreted with faeces. The high excretion of radioactivity associated with CO_2 could originate to some extent from absorbed quercetin metabolites through ß-oxidation of phenylpropionic acids (*see page 49*). In rats, 1% of the administered dose of quercetin was excreted as quercetin(conjugates).[28] However, after oral administration of quercetin aglycone to humans the aglycone or its conjugates could not be detected in urine.[29] These investigators concluded that less than 1% of the administered quercetin could have been absorbed.[29] This conclusion was based on the limit of detection of their analytical method. Absorption of the flavanone aglycones and their metabolites was somewhat higher than that of quercetin aglycone: about 30% was excreted with urine (*Table 1*).

Absorption of flavonoids from foods

Previous studies did not address the absorption from foods, but only of pure aglycones. We ourselves were interested in the absorption of flavonoids from regular foods, and in man rather than in animals. To circumvent the problem of microbial degradation we employed ileostomy subjects.[30] To our surprise, the quercetin glycosides from onions were absorbed far better than the pure aglycone (*Table 1*). Absorption from onions was 52% of the ingested amount, while only 24 % of the aglycone, and 17% of rutin (quercetin-3-O-rutinoside) were absorbed. A small percentage (< 0.5%) of the absorbed quercetin was excreted into urine as the intact quercetin molecule, conjugated or otherwise. Thus, glycosides can be absorbed in man as such without prior hydrolysis by microorganisms. Evidence for direct absorption of glycosides was also found in rats; oral administration of naringin (5,7,4'-trihydroxyflavanone-7-neohesperidoside) and hesperidin (5,7,3'-trihydroxy-

4'-methoxyflavanone-7-rutinoside) showed that the parent glycosides were secreted with bile,[33] which implied that glycosides were transported across intestinal membranes.

As far as the catechins are concerned, epigallocatechins present in green tea were shown to be absorbed in rats. The following compounds were identified in the portal vein after their oral administration: (-)-epigallocatechin-3-gallate (EGCg), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECg) and (-)-epicatechin (EC).[34,35] It was reported that 2% of ingested green tea catechins were excreted into urine in humans.[36]

Data on mechanisms of flavonoid absorption across the intestinal membrane itself are scarce. The absorption of (+)-catechin, epicatechin-2-sulfonate, and 7,3',4'-tri-*O*-(ß-hydroxy-ethyl)quercetin-3-rutinoside was studied in the rat everted sac model, and the rate of passive transport was in the order epicatechin-2-sulfonate > (+)-catechin > trihydroxyrutinoside.[37] However, of these three flavonoids only (+)-catechin was absorbed in an *in situ* perfused small intestine segment of the rat.[38] This demonstrated the limitations of the everted sac model.

In conclusion, the absorption of flavonoid aglycones in rats was estimated at 4 - 58% of ingested radioactive aglycones as judged by the amount of radioactivity excreted into urine. Contrary to the common belief that only aglycones can be absorbed, flavonol glycosides were well absorbed in man without prior hydrolysis by microorganisms, and similar observations have been made in rats. Only a small fraction of the flavonols subsequently excreted with urine had an intact flavonoid structure.

Table 1. *Summary of studies on absorption of flavonoids*

Compound	Species	Dose (mg/kg body weight)	Excretion (% of dose)				Reference
			Urine	CO_2	Feces	Total	
(+)-[U-^{14}C]catechin	rat, guinea pig	200	58	18	1	77	23
	monkey	125	54	-	2	56	24
	man	25	55	-	-	-	25
3-[^{14}C]methoxy-(+)-catechin	man	30	47	-	-	-	26
[random-^{14}C]quercetin aglycone	rat	15	4	12	33	79	27
[4-^{14}C]quercetin aglycone	rat	630	13	41	47	100	28
	rat, bile duct cannulated	630	21[¶]	34	13	68	28
quercetin aglycone	man	60	<1[#]	-	53	-	29
quercetin aglycone	man	1.4	0.1[#]	-	76	100	30
rutin	man	1.4[§]	0.1[#]	-	83	100	30
quercetin glucosides from onions	man	1.2[§]	0.3[#]	-	48	100	30
[3-^{14}C]hesperetin aglycone	rat	1.5	33	39	15	89	31
[2-^{14}C]flavanone aglycone	rat	100	28	0	71	99	32

- not determined

[¶] urine + bile

[§] expressed as quercetin equivalents

[#] total quercetin including conjugates

METABOLISM

Introduction

Metabolism of flavonoids is relevant because a major part of administered flavonoids is excreted in urine only after more or less extensive modification in the body. Thus, a potential biological effect predicted from *in vitro* studies may be modulated *in vivo* due to metabolism after ingestion of the parent compounds. The major questions are which products are formed and to what extent, and what is their potential biological effect.

In the metabolism of flavonoids, two compartments are important. The first consists of tissues in the body, such as the liver where biotransformation enzymes act upon absorbed flavonoids and their absorbed colonic metabolites. The second metabolically active compartment is the colon where microorganisms degrade unabsorbed flavonoids and flavonoids absorbed and then secreted with bile.

Metabolism by the liver.

Flavonoids absorbed as such as well as their degradation products absorbed from the colon after bacterial action are subsequently metabolised by enzymes located mainly in the liver. The kidney and the small intestine might also contain enzymes capable of biotransformation of flavonoids.[39] The general phase I biotransformation reactions introduce or expose polar groups.[39] These may be less relevant to naturally occurring flavonoids and their colonic degradation products because these already contain several polar hydroxyl groups. Indeed, phase I transformations have been reported almost exclusively for synthetic flavonoids lacking hydroxyl groups.[11] Conjugation of these polar hydroxyl groups with glucuronic acid, sulfate, or glycine constitutes phase II biotransformation reactions,[39] and these have been reported both for flavonoids and for their colonic metabolites. The water-soluble conjugates thus formed can be excreted into urine. In addition, the molecular weight increases which promotes secretion into bile.[40] Finally, O-methylation by the enzyme catechol-O-methyltransferase plays an important role in the inactivation of the catechol moiety,[41] i.e. the two adjacent (*ortho*) aromatic hydroxyl groups, of flavonoids and their colonic metabolites.

Metabolism by the colonic flora.

Flavonoids can reach the colon by two different ways; unabsorbed flavonoids

passing through the small intestine, and absorbed flavonoids secreted as conjugates into the duodenum via the gall bladder. In the colon both are stripped of their sugar moieties, glucuronic acids and sulfates by glycosidases, glucuronidases and sulfatases of colonic bacteria.[42] Hydrolysis by bacterial enzymes enables absorption in the colon because the aglycones formed are less polar.[39,43] As a result, secreted glucuronides and sulfates can be reabsorbed thus entering an enterohepatic cycle.

Another possibility exists in that the heterocyclic oxygen-containing ring is split. The subsequent degradation products can evidently be absorbed because they are found in urine. These include a variety of phenolic acids which, depending on the hydroxylation pattern, are antioxidants themselves[44] and may thus contribute to the biological effects of dietary flavonoids. The type of ring fission depends on the type of flavonoids; as a result, primary ring fission products of catechins, flavonols, and flavones and flavanones are all different. Hydroxyl groups are necessary for ring cleavage, and the hydroxylation pattern of the flavonoids determines their susceptibility to microbial degradation in the colon.[21] Free hydroxyl groups at positions 5 and 7 together with a free hydroxyl group at the 4' position are necessary for ring fission of the heterocyclic C-ring.[21] Whether or not the position of the free hydroxyl group in ring B is essential is not known. A flavonoid that has only one free hydroxyl group in ring A at position 7, e.g. 7,4'-dihydroxyflavone, withstands ring fission. Whether two hydroxyls in ring A, or a single hydroxyl group at position 5 are sufficient for ring fission is not known. The presence of *O*-methyl substitution in these essential positions reduces susceptibility to cleavage. Methylation of the 3-hydroxyl group in the C-ring of (+)-catechin also increases resistance to ring scission. The most widespread dietary flavonoids have a 5,7,3',4'-hydroxylation pattern which will enhance ring cleavage by bacteria after hydrolysis of the glycosides in the colon.

Thus, the formation of potentially active metabolites through bacterial degradation in the colon is highly dependent on structural details of the dietary flavonoids involved.

Catechins

After oral administration of labelled catechins to humans some 50% of the radioactivity was recovered in urine, and only 0.5 - 3% of this was in the form of catechin aglycone. Thus, catechins are extensively metabolized.[25,26]

Enzymatic transformations of catechins in body tissues

Unfortunately phase I transformation reactions for catechins have not been described; information on metabolism in liver and other organs is limited to the attachment of various substituents to existing hydroxyl groups, and most of this information is qualitative only. This subject evidently needs closer study.

Sulfates and glucuronides of (+)-catechin were identified after oral administration of (+)-catechin to rodents, monkeys and man (*Table 2*). Intravenous and intraperitoneal administration of this flavonoid to rodents and monkeys also produced these conjugates. Thus, glucuronidation and sulfation in body tissues was demonstrated. Rats and humans excreted sulfates and glucuronides of 3-methoxy-(+)-catechin into urine, plasma and bile after intravenous injection of this compound.[26,51] Because catechins are polyhydroxylated compounds, several sites offer themselves for binding of glucuronic acid or sulfate. In human urine, two glucuronides of 3,3'-dimethoxy-(+)-catechin and of 3-methoxy-(+)-catechin, and a sulfate conjugate of 3-methoxy-(+)-catechin were found.[26] The position of these groups could not be determined. Two different sulfates and a mixed sulfate/glucuronide of (+)-catechin were detected in perfusate and bile of a perfused isolated rat liver.[52] After oral administration of (-)-epigallocatechin-3-gallate and (-)-epicatechin of green tea to human volunteers the major conjugates found in plasma were sulfates, whereas the (-)-epigallocatechin circulated as the glucuronide.[36] Some 20% of the (-)-epigallocatechin-3-gallate was also present as unconjugated compound. In addition to conjugates of (+)-catechin, glucuronides of the main colonic metabolites, the three valerolactones and 3-hydroxyphenylpropionic acid (*Figure 2*), were identified in urine of humans.[47] Sulfates of δ-(3-hydroxyphenyl)-γ-valerolactone and 3-hydroxyphenylpropionic acid were also present.

In vitro incubation of (+)-catechin in liver homogenates produced 3'-methoxy-(+)-catechin, thus showing that O-methylation had occurred.[49] Purified catechol-*O*-methyl transferase (E.C.2.1.1.3) was also able to form the 3'-methoxy compound.[49] Only the 3'-hydroxyl group of (+)-catechin or 3-methoxy-(+)-catechin was methylated, thus suggesting that catechol-*O*-methyl transferase was also involved *in vivo*. Additional evidence for the role of catechol-*O*-methyl transferase in *O*-methylation came from experiments with oral administration of valerolactones, the primary bacterial ring fission products of (+)-catechin, to guinea pigs.[46] Only δ-(3-4-dihydroxyphenyl)-γ-valerolactone was *O*-methylated, whereas δ-(3-hydroxyphenyl)-γ-valerolactone that lacks a catechol group was not *O*-methylated.

In conclusion, catechins were metabolised by liver enzymes to give sulfates, glucuronides, and mixed sulfates/glucuronides which were excreted into bile, urine and plasma. In addition, *O*-methylated catechin conjugates were produced by catechol-*O*-methyl transferase in the liver. Because of the specificity of this enzyme only *ortho*-hydroxy-methoxy metabolites were formed. These phase II reactions occurred in rodents as well in humans. Types of glucuronides depended on species, and preference for sulfation was found in humans.[26]

Bacterial ring cleavage of catechins in the colon

According to Das et al.,[23] the catechin ring is cleaved by microorganisms at the positions indicated by arrows in *Figure 2*. This type of fission is decisive for the basic structures of the successive metabolites: valerolactones (phenyl-C_5: a benzene ring with a side chain of 5 C-atoms), phenylpropionic acids (phenyl-C_3) and benzoic acids (phenyl-C_1). Variations in substituent patterns of these basic structures occurred and were to some extent species dependent (*Table 2*). Identification of the valerolactones was pioneered by Watanabe[53-56] and was the first step in the elucidation of the bacterial metabolism of (+)-catechin in the colon. Catechin labelled with ^{14}C in the A-ring only ([ring A-^{14}C]catechin) and uniformly ^{14}C-labelled catechin ([U-14C]-catechin) were used to further substantiate this general scheme.[23] Oral administration of valerolactones to rats and guinea pigs[23,46] gave rise to the propionic and benzoic acids depicted in *Figure 2*. The free hydroxyl group at the 3 position of catechin was essential for ring fission in the colon by bacteria, as 3-methoxy-(+)-catechin was resistant to ring fission in rat, mouse, marmoset[57] and man.[26]

Animal experiments showed that heterocyclic ring fission of (+)-catechin was wholly mediated by microorganisms in the colon. In the presence of antibiotics which kill the microorganisms, the ring fission products were not produced.[46,50] These metabolites also were formed upon *in vitro* incubation of catechin with intestinal contents and again their formation could be suppressed by addition of antibiotics. Ligation of the bile duct prevents bile that contains conjugated (+)-catechin after intravenous injection to flow into the small intestine. After intravenous injection of (+)-catechin to bile-duct ligated rats no ring fission products were detectable in urine,[45] again showing the crucial role of the gut.

In rats, biliary circulation was an important phenomenon in catechin metabolism. Studies with bile-duct cannulated rats, showed that about 40% of orally administered absorbed (+)-catechin was secreted with bile into the small

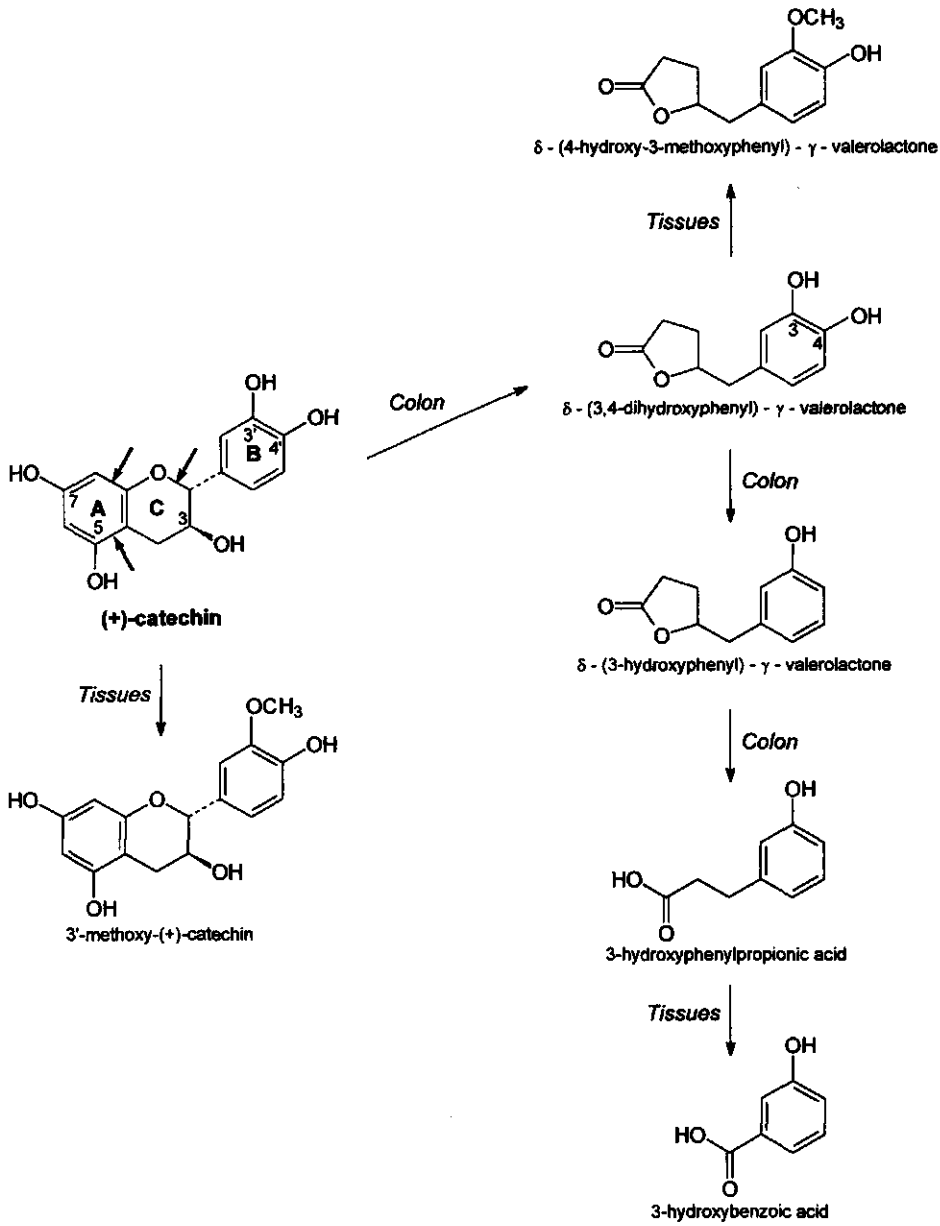

Figure 2. *Metabolic reactions of catechins in body tissues and colon. (+)-Catechin is shown as an example. Conjugation reactions are not shown.*

intestine.[11] Only glucuronide or sulfate conjugates of catechins and 3'-methoxy-(+)-catechin (*Figure 2*), the major hepatic metabolite,[49] were secreted with bile. Catechins secreted with bile were prone to microbial degradation. Subsequently, after hydrolysis of the conjugates, catechin and its phenolic acid and lactone metabolites were reabsorbed.[45,49] About 60% of the metabolites of 3-methoxy-(+)-catechin that were secreted with bile were reabsorbed in the first enterohepatic circulation.[57]

In conclusion, bacteria of the colon cleaved the heterocyclic ring of (+)-catechin to form phenyl-C_5 and phenyl-C_3 metabolites, which were absorbed and excreted into urine both in rodents and in man. In rats secretion of catechin conjugates into bile exposed them anew to bacterial degradation. Presence of a methoxy group at position 3 in (+)-catechin made the molecule resisitant to ring fission in rodents as well in man. Ring fission of other types of catechins was not studied.

Extent of catechin metabolism and species differences

Unconjugated (+)-catechin, valerolactones and phenolic acids excreted into urine represented only 3% of the orally administered [U-^{14}C]-(+)-catechin in rats.[23] As 58% of the dose was excreted in urine (*Table 1*), only 5% of this radioactivity was identified. On oral administration of [U-^{14}C]-(+)-catechin to monkeys a considerably higher percentage, 20% of the dose, was excreted in urine as unconjugated catechin and its phenyl-C_5, phenyl-C_3, and phenyl-C_1 metabolites.[24] Catechin accounted for 3%, and the main metabolite δ-(3-hydroxyphenyl)-γ-valerolactone for 8% of the dose; conjugates of some of these metabolites and of catechin were present, but were not quantified. Ingestion of [U-^{14}C]-(+)-catechin by human volunteers showed that ring fission was only a minor metabolic route;[25] 90% of the urinary radioactivity (50% of the dose) was composed of conjugates of (+)-catechin and 3'-methoxycatechin, and their aglycones accounted for 3% of the dose. Oral administration of 3-[^{14}C]-methoxy-(+)-catechin to humans showed that less than 0.5% of the dose was excreted unchanged in urine; major metabolites were conjugates of the parent compound and of 3,3'-dimethoxycatechin.[26] *O*-methylation of 3-methoxy-(+)-catechin was less important in man than in rodents where *O*-methylation was almost 100%.[26] Thus in man a major part of (+)-catechin is absorbed and subsequently excreted with urine as conjugates.

Only monkeys and humans excreted 3-hydroxyphenylhydracrylic acid in

urine after an oral (+)-catechin dose (*Table 2*).[24,47] The traces of 4-hydroxyphenolic acids in urine reported in one study with rats[45] are puzzling, because only 3-hydroxyphenolic acids are expected based on the scheme depicted in *Figure 2*. Possibly some of these metabolites originate from dietary tyrosine.[50]

Thus, only a few percent of orally administered (+)-catechin and 3-methoxy-(+)-catechin escaped metabolism. The major metabolic reactions were conjugation and *O*-methylation performed by liver cells. In man, ring fission by bacteria in the colon was only of minor importance for these two catechins. Only very limited data were found on the metabolism of an important group of dietary catechins, the epicatechins of tea.

Table 2. *Metabolites of (+)-CATECHIN after oral (p.o.) or intravenous (i.v.) or intraperitoneal (i.p.) administration to various species*

Metabolite	Species	Dose (mg/kg body weight)	Body fluid	Reference
(+)-catechin aglycone	rat	i.v. 100	urine	45
	guinea pig	p.o. 150	urine	46
	monkey	p.o. and i.p. 125	urine	24
	man	p.o. 80	urine	47
	man	p.o. 25	urine, plasma	25
(+)-catechin glucuronide(s)	rat	i.v. 100	urine, bile	45
	guinea pig	p.o. 150	urine	46
	monkey	p.o. and i.p. 125	urine, bile	24
	man	p.o. 80	urine	47
	man	p.o. 25	urine	25
(+)-catechin sulfate(s)	guinea pig	p.o. 150	urine	46
	man	p.o. 80	urine	47
	man	p.o. 25	urine	25
(+)-catechin or its conjugates	man	p.o. 45	serum	48

3'-*O*-methyl-(+)-catechin	rat	p.o. 10	bile	49
	man	p.o. 25	urine, plasma	25
δ-(3-hydroxyphenyl)-γ-valerolactone	rat, guinea pig	p.o. 200	urine, faeces	23
	rat	i.v. 100	urine	45
	guinea pig	p.o. 150	urine	46
	monkey	p.o. and i.p.125	urine	24
	man	p.o. 80	urine	47
δ-(3,4-dihydroxyphenyl)-γ-valerolactone	rat, guinea pig	p.o. 200	urine, faeces	23
	rat	i.v. 100	urine	45
	guinea pig	p.o. 150	urine	46
	monkey	p.o. and i.p. 125	urine	24
	man	p.o. 80	urine	47
δ-(4-hydroxy-3-methoxyphenyl)-γ-valerolactone	rat, guinea pig	p.o. 200	urine	23
	guinea pig	p.o. 150	urine	46
	monkey	p.o. 125	urine	24
	man	p.o. 80	urine	47
3-hydroxyphenylpropionic acid	rat	p.o. 200	urine	23,50
	rat	i.v. 100	urine	45

Table 2 (Contd.). *Metabolites of (+)-CATECHIN after oral (p.o.) or intravenous (i.v.) or intraperitoneal (i.p.) administration*

Metabolite	Species	Dose (mg/kg body weight)	Body fluid	Reference
3-hydroxyphenylpropionic acid	guinea pig	p.o. 150	urine	46
	monkey	p.o. 125	urine	24
	man	p.o. 80	urine	47
	man	p.o. 25	urine	25
4-hydroxyphenylpropionic acid	rat	i.v. 100	urine	45
3-hydroxyphenylhydracrylic acid	monkey	p.o. and i.p. 125	urine	24
	man	p.o. 80	urine	24,47
4-hydroxyphenyllactic acid	rat	i.v. 100	urine	45
4-hydroxyphenylacetic acid	rat	i.v. 100	urine	45
3-hydroxyhippuric acid	rat	p.o. 200	urine	23
	rat	p.o. 200	urine	50
	guinea pig	p.o. 150	urine	46
	monkey	p.o. 125	urine	24
	man	p.o. 25	urine	25

3-hydroxybenzoic acid	guinea pig	p.o. 200	urine	23
	guinea pig	p.o. 150	urine	46
	monkey	p.o. 125	urine	24
	man	p.o. 25	urine	25

Flavonols

Enzymatic transformations of flavonols in body tissues

As for catechins phase I transformation reactions for flavonols have not been described, and this part of their metabolism awaits study. The role of the liver in rats in glucuronidation and O-methylation was demonstrated by intraperitoneal injection of rutin (quercetin-3-rutinoside) and quercetin.[58] 3'-Methoxyquercetin-3-rutinoside and its glucuronide, and conjugates of quercetin, isorhamnetin (3'-methoxyquercetin) and rutin were found in bile. Both 3'-methoxyquercetin and 4'-methoxyquercetin were reported in urine and bile of rats.[28] The presence of the 4'-methoxy isomer was confirmed by NMR and a specific chemical reaction. Again, only *o*-hydroxy-methoxy metabolites were found (*Table 3*), suggesting that catechol-*O*-methyl transferase was involved.[41] Sulfation of quercetin was studied by using perfusion of isolated rat liver.[52] Two double sulfate/glucuronide conjugates constituted 85% of the biliary secreted sulfate-containing conjugates. Sulfation in male rats was twice that in female rats. *In vitro* incubations with unfractionated sulfotransferases of rat liver confirmed these results.

Human data are limited. We found circumstantial evidence for the presence of quercetin conjugates in urine and plasma after oral administration of dietary quercetin to human subjects;[30,62,63] acid hydrolysis of urine and plasma increased the concentration measured. We also found 3'-methoxyquercetin in plasma and urine of these subjects (Hollman et al., unpublished).

Thus, in rats major enzymatic metabolic reactions of flavonols were located in the liver, and they were similar to those of catechins: glucuronidation and sulfation of hydroxyl groups, and *O*-methylation of catechol groups. Conjugation and *O*-methylation of quercetin also occurred in humans.

Bacterial ring cleavage of flavonols in the colon

The proposed flavonol-specific sites of ring fission are depicted by arrows in *Figure 3*. The proposed scheme accounts for the phenylacetic acids (phenyl-C_2) and the phenylpropionic acids (phenyl-C_3) found in various species (*Table 3*). However, direct experimental proof for these types of ring fission in flavonols is not available. The phloroglucinol (1,3,5-trihydroxybenzene) and phloroglucinolcarboxylic acid (2,4,6,-trihydroxybenzoic acid) found in urine of rats after oral administration of

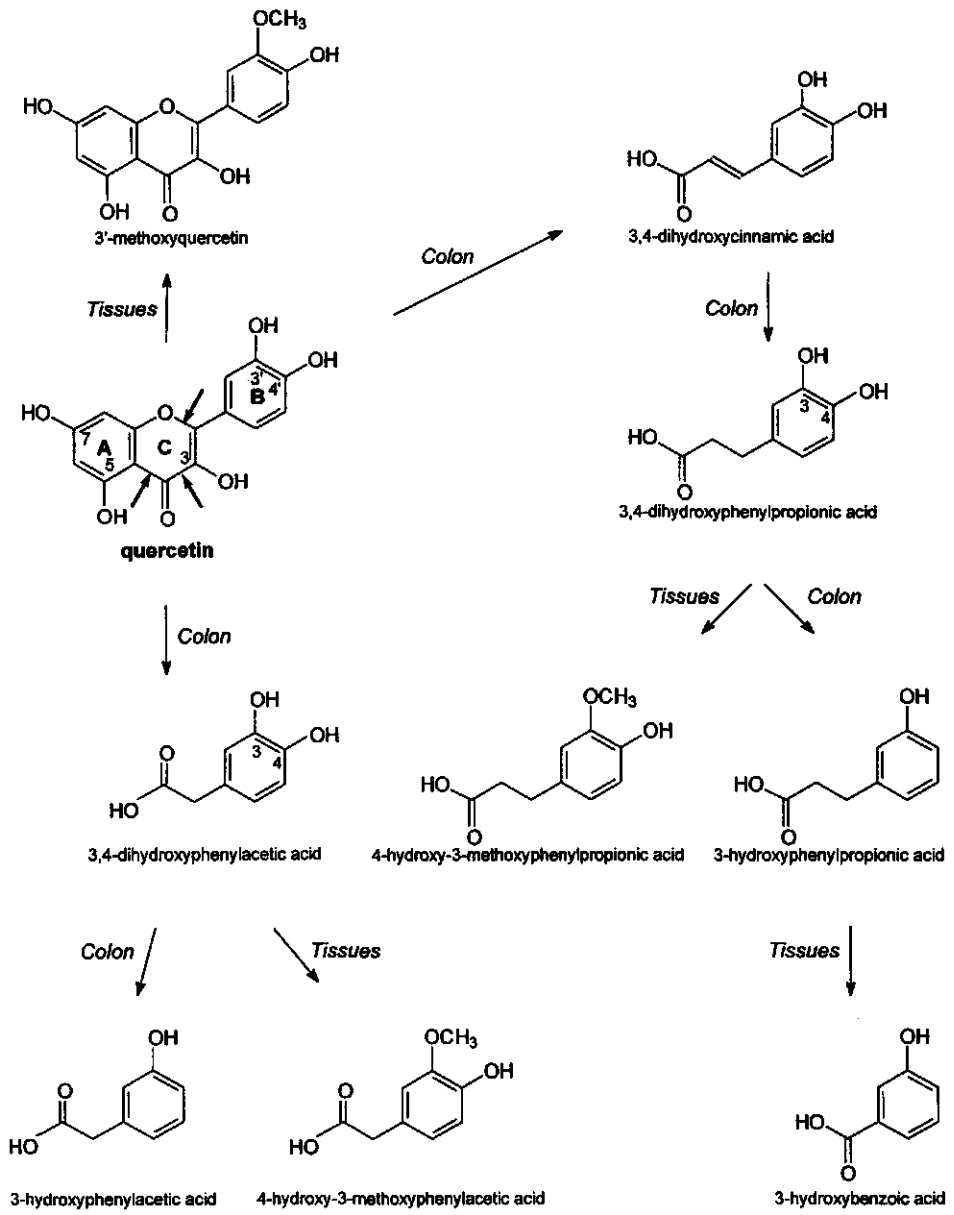

Figure 3. *Metabolic reactions of flavonols in body tissues and colon. Quercetin is shown as an example. Conjugation reactions are not shown.*

quercetin pointed to metabolites with an intact A-ring of quercetin,[64] but they turned out to be analytical artefacts.[27,61] Oral administration of 3,4,5-trihydroxyphenylacetic acid to rats produced 3,4- dihydroxy- and 3-hydroxyphenylacetic acid, which are metabolites of myricetin (3,5,7,3',4',5'-hexahydroxyflavone).[22] Similar to (+)-catechin, these results indicated that the phenolic acids formed only had an intact B-ring (*Figure 3*). Microorganisms mediated these dehydroxylation reactions.

The phenylacetic acids, typical for the proposed ring fission of quercetin, rutin (quercetin-3-*O*-rutinoside) and myricetin were not found in rats treated with an antibiotic to suppress microorganisms.[22,59,65] *In vitro* anaerobic incubation of myricetin, myricitrin (myricetin-3-*O*-rhamnoside)[22] and rutin[65] with rat caecal micro-organisms also produced the metabolites observed in urine after oral administration. Mucosal membranes of the small intestine as well as contents of duodenum, jejunum and ileum were unable to metabolise quercetin in rats,[28] as opposed to the contents of caecum and colon. Thus, microorganisms in the colon cause ring fission of flavonols. The absence of these ring fission products after intraperitoneal injection of rutin in bile duct cannulated rats is an additional indication for the exclusive role of microorganisms in ring fission of flavonols.[65] Biliary circulation of quercetin was indicated by the occurrence of glucuronides and sulfates of quercetin in bile.[28,58] However, no data are available about the extent of biliary secretion and reabsorption.

In plant foods flavonols mainly occur as glycosides. As expected, the metabolites of quercetin and of rutin, the 3-rutinoside of quercetin, were similar (*Table 3 and 4*); microorganisms of the colon probably first hydrolysed rutin to produce quercetin. The rutinose moiety was also removed from quercetin-7-*O*-(ß-hydroxyethyl)rutinoside by microorganisms in the colon of dogs,[67] but, this aglycone was stable against ring fission. The explanation is probably that the ß-hydroxyethyl group is resistant to microbial hydrolysis. This showed that substituents bound to hydroxyls that are resistant to hydrolysis by the gut microorganisms, can have a profound effect on the stability of the ring system. However, the microflora of rats was capable of ring fission of quercetin-7-*O*-(ß-hydroxyethyl)-rutinoside,[68] but failed in the case of quercetin-7,3',4'-tri-*O*- and quercetin-5,7,3',4'-tetra-*O*-(ß-hydroxyethyl)rutinoside. Another interesting observation was made by Griffiths[22] who found that robinetin (3,7,3',4',5'-pentahydroxyflavone) was not degraded to phenolic acids in rats, and also was stable upon incubation with microorganisms. Robinetin lacks the hydroxyl group at position 5 as compared to myricetin which is degraded as expected (*Table 4*).

In summary, bacteria of the colon cleaved the heterocyclic ring of flavonols to form phenyl-C_3 and phenyl-C_2 metabolites, which were absorbed and excreted into urine. These metabolites were found in rodents as well in humans, although most of these studies were performed with rodents. Glycosylation could not stabilize the ring structure as opposed to substituents that formed non-hydrolysable bonds with hydroxyl groups. Secretion into bile of flavonol conjugates may contribute to additional bacterial degradation.

Extent of flavonol metabolism

Quantitave studies are limited, and available only for quercetin. Rats did not excrete the aglycone in urine after oral administration of quercetin aglycone;[28] 1.7% of the dose was excreted as glucuronide and sulfate conjugates of quercetin, while monomethoxylated quercetin conjugates accounted for 3.6%. This accounts for about half of the urinary metabolites in these rats, as 13% of the administered radioactivity was excreted in urine (*Table 1*).

In humans, less than 1% of the orally administered aglycone was estimated to reach the circulation unchanged.[29] We found that humans who were fed quercetin or quercetin glycosides excreted only 0.1% to 0.3% of the dose as unchanged quercetin or its conjugates in urine (*Table 1*), whereas absorption amounted to 20 - 50%.[30] In these human subjects less than 0.5% of the dose was excreted as 3'-methoxyquercetin (Hollman et al., unpublished).

These data indicate that quercetin is extensively metabolised in rats and humans. Only a small part of these metabolites has been quantified. In contrast with (+)-catechin in humans, quercetin is metabolised only to a limited extent via conjugation with sulfate, glucuronic acid or *O*-methylation.

Table 3. *Metabolites of QUERCETIN found after oral (p.o.), intravenous (i.v.), or intraperitoneal (i.p.) administration to various species*

Metabolite	Species	Dose (mg/kg body weight)	Body fluid	Reference
quercetin glucuronide(s) or sulfate(s)	rat	i.p. and p.o.30	bile	58
quercetin glucuronide(s)	rat	p.o. 630 and i.p 315	urine and bile	28
quercetin sulfate(s)	rat	p.o. 630 and i.p 315	urine	28
3'-methoxyquercetin conjugate	rat	i.p. and p.o.30	bile	58
3'-methoxyquercetin and its glucuronide	rat	p.o. 630 and i.p 315	urine and bile	28
4'-methoxyquercetin	rat	p.o. 630 and i.p 315	urine and bile	28
3-hydroxycinnamic acid	rat	p.o.	urine	59
3-hydroxyphenylpropionic acid	rat	p.o.	urine	59
3,4-dihydroxyphenylacetic acid	rat, rabbit, guinea pig, man	p.o.	urine	60
	rat	p.o. 320	urine	61

	rat	p.o.	urine	59
4-hydroxy-3-methoxyphenylacetic acid	rat, rabbit, guinea pig, man	p.o. and i.p.	urine	60
	rat	p.o. 320	urine	61
	rat	p.o. 25	urine	27
	rat	p.o.	urine	59
3-hydroxyphenylacetic acid	rat, rabbit, guinea pig, man	p.o.	urine	60
	rat	p.o. 320	urine	61
	rat	p.o. 25	urine	27
	rat	p.o.	urine	59
3-hydroxybenzoic acid	rat	p.o.	urine	59

Table 4. *Metabolites of RUTIN, KAEMPFEROL and MYRICETIN found after oral (p.o.) or intraperitoneal (i.p.) administration to various species*

Flavonol	Metabolite	Species	Dose (mg/kg body weight)	Body fluid	Reference
rutin (quercetin-3-*O*-rutinoside)	rutin glucuronide	rat	i.p. 30	bile	58
	3,4-dihydroxyphenylacetic acid	rat,rabbit, guinea pig, man	p.o.	urine	60
	3-hydroxyphenylacetic acid	rat,rabbit, guinea pig, man	p.o.	urine	60
	4-hydroxy-3-methoxyphenyl-acetic acid	rat,rabbit, guinea pig, man	p.o.	urine	60
	quercetin-3-*O*-rutinoside glucuronide	rat	i.p. 30	bile	58
	3'-methoxy-3-*O*-rutinoside and its glucuronide	rat	i.p. 30	bile	58
[2',5',6'-H]rutin	3,4-dihydroxyphenylacetic acid	man, rat	man: p.o.10 rat: p.o.100	urine	66 65
	4-hydroxy-3-methoxyphenyl-acetic acid	man, rat	man: p.o.10 rat: p.o.100	urine	66 65
	3-hydroxyphenylacetic acid	man, rat	man: p.o.10 rat: p.o.100	urine	66 65

	3,4-dihydroxytoluene	man, rat	man: p.o.10 rat: p.o.100	urine	66 65
	ß-3-hydroxyphenylhydracrylic acid	man	p.o. 10	urine	66
	3-hydroxyphenylpropionic acid	rat	p.o. 100	urine	65
kaempferol (5,7,3,4'-tetra-hydroxyflavone) and	kaempferol	rat	p.o. 300	urine	21
kaempferol-7-rhamnosido-3-galactorhamnoside	4-hydroxyphenylacetic acid	rat	p.o. 300	urine	21
myricetin and myricetin-3-O-rhamnoside	myricetin	rat	p.o. 300	urine	22
	3,5-dihydroxyphenylacetic acid	rat	p.o. 300	urine	22
	3-hydroxyphenylacetic acid	rat	p.o. 300	urine	22

Flavones and flavanones

Enzymatic transformations of flavones and flavanones in body tissues

Evidence for oxidative phase I reactions of flavones was found in guinea pigs. After intraperitoneal and oral administration of a synthetic flavone lacking hydroxyl groups both 4'-hydroxy- and 3',4'-dihydroxyflavone were excreted in urine.[69] Oral administration of a flavanone lacking hydroxyl groups to rats introduced hydroxyl groups at the 3 or 6 position,[32,70] and these metabolites were excreted in urine. However, proof for the involvement of the liver was not given. Several metabolites formed by reduction of the carbonyl group, for instance flavan-4-α-ol, were identified. However, the corresponding reduction of flavone was never found.[69] Phase II reactions were demonstrated by many workers. Conjugation of baicalein (5,6,7-trihydroxyflavone) and baicalein-6-glucuronide was shown in rats: five conjugates were identified in bile.[71] Baicalein conjugated with two glucuronic acid molecules and the mixed conjugate containing one glucuronic acid and one sulfate predominated.[71] This is in accordance with observations that high molecular weight and high polarity of compounds facilitates their secretion with bile.[40] A study with isolated perfused rat liver[72] showed that diosmin (5,7,3'-trihydroxy-4'-methoxyflavone-7-rutinoside) was secreted with bile as such and as its glucuronide conjugate; diosmetin (5,7,3'-trihydroxy-4'-methoxyflavone) was only secreted as sulfate and glucuronide conjugates. Oral administration of naringin (5,7,4'-trihydroxyflavanone-7-rhamnoglucoside) and hesperidin (5,7,3'-trihydroxy-4'-methoxyflavanone-7-rutinoside) to rats showed that, besides the glucuronides, also the parent glycosides were secreted into bile.[33] On oral administration of diosmetin to rats its glucuronide appeared within minutes in portal venous blood, and no aglycone could be detected.[73] This suggests that the glucuronide was produced on absorption at the level of the intestinal mucosa. It is documented[74] that intestinal mucosa were important for extrahepatic glucuronidation. *In vivo* as well *in vitro* (rat liver microsomes), 5-hydroxyflavone was glucuronidated.[73] This is remarkable, because the 5-hydroxyl group is strongly stabilized by the 4-keto group, or is involved in chelation. Glucuronidation in rats of 7,5-dihydroxyflavone occurred mainly at the 7-position, and of 5,7,3'-trihydroxyflavone at the 7- and 3'-position.[73]

Tangeretin (5,6,7,8,4'-pentamethoxyflavone) was O-demethylated by rat and human liver microsomes;[75] the metabolites formed were not identified separately.

Anaerobic incubation of [3-^{14}C]hesperetin with rat caecal microorganisms

only produced phenylpropionic acids and no $^{14}CO_2$ nor phenylbenzoic acids (*Figure 4*).This suggested that ß-oxidation of the propyl chain of the phenylpropionic acids was not mediated by bacterial enzymes, but by mammalian enzymes.[31]

In conclusion, flavones and flavanones were metabolised by liver enzymes to give sulfates, glucuronides, and mixed sulfates/glucuronides which were excreted into bile, urine and plasma. Only glucuronides or sulfates were secreted with bile, except for rhamnoglucosides which were secreted as such. Glucuronidation in the intestinal mucosa was observed. These studies were only performed with rats. In addition, *O*-methylation of catechol groups and *O*-demethylation occurred in humans and rats. ß-Oxidation of phenylpropionic acids was found in rats.

Bacterial ring cleavage of flavanones and flavones in the colon

The specific sites of ring fission for flavones and flavanones are shown by arrows in *Figure 4*. The proposed scheme accounts for the phenylpropionic acids (phenyl-C_3) reported in body fluids of various species after various flavones and flavanones (*Table 5,6*). A study with ^{14}C-labelled hesperetin ([3-^{14}C]5,7,3'-trihydroxy-4'-methoxyflavanone) in rats identified the predicted ^{14}C-labelled phenylpropionic acids.[31] Anaerobic incubation of [3-^{14}C]hesperetin with caecal microorganisms did not produce $^{14}CO_2$, which indicated that ß-oxidation of the propyl chain of the phenylpropionic acid was not caused by bacterial enzymes but by mammalian enzymes.

The dose clearly affected the metabolites formed. After a very low oral dose, only ring cleavage products were found in urine,[31] whereas a more than 100 fold increase of the oral dose, the common dose in these experiments, also produced metabolites with an intact ring structure.[33,77]

The synthetic flavone[69] and flavanone[76] both lacking hydroxyl groups were not cleaved by microorganisms, as no phenolic acids were excreted, and metabolites did not change after administration of antibiotics.[69] Experiments with various flavones with 5,7-hydroxylation showed that at least one free hydroxyl group in ring B was required for ring fission.[22]

To summarise, microorganisms in the colon of rats cleaved the heterocyclic ring only of hydroxylated flavones and flavanones to form phenyl-C_3 metabolites, which were absorbed and excreted into urine. Metabolism of flavanones by bacteria in rodents and humans lead to similar metabolites. Human data on metabolism of flavones are not available.

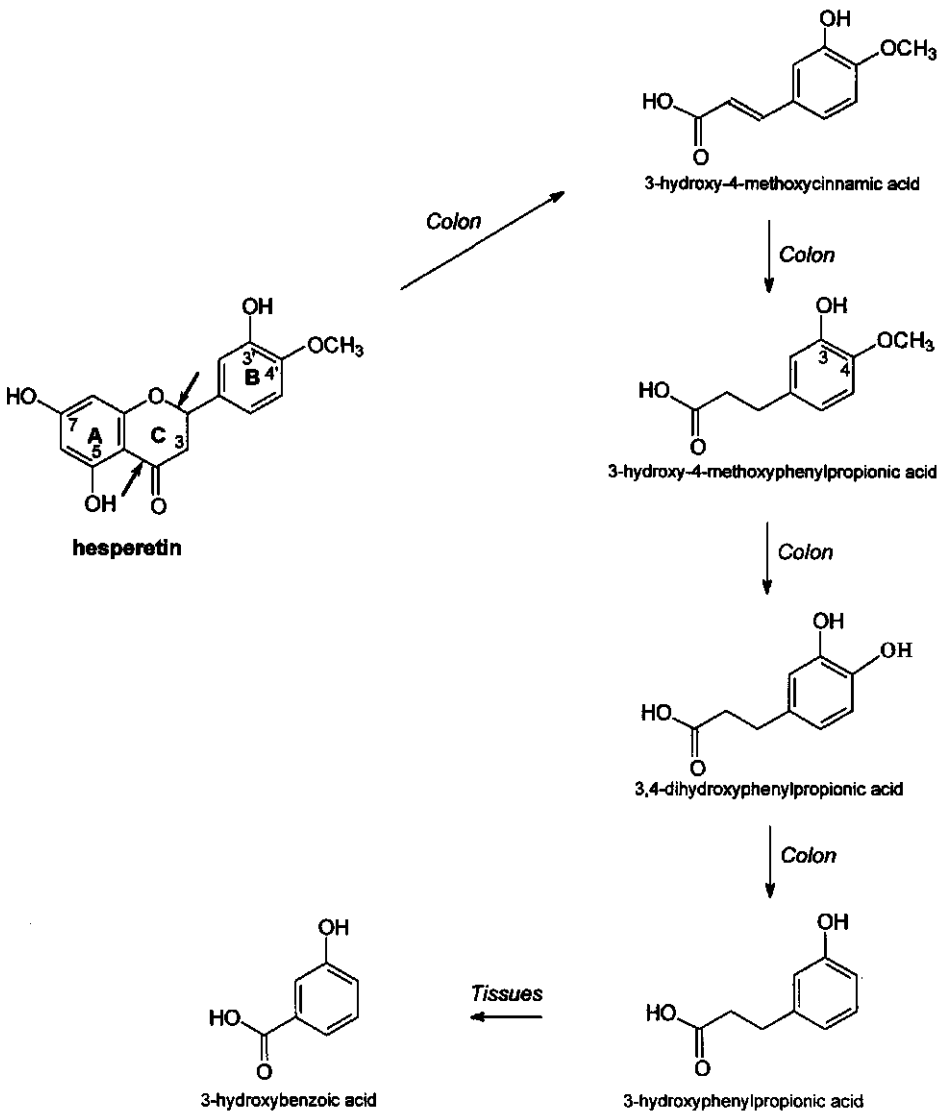

Figure 4. *Metabolic reactions of flavanones in body tissues and colon. Hesperetin is shown as an example. Flavones show similar reactions. Conjugation reactions are not shown.*

Table 5. *Metabolites of FLAVONES found after oral (p.o.) or intraperitoneal (i.p.) administration to rats*

Flavone	Metabolite	Species	Dose (mg/kg body weight)	Body fluid	Reference
flavone	flavone aglycone	rat	p.o. 150, i.p.	urine	76
	flav-3-ene	rat	p.o. 150, i.p.	urine	76
	4'-hydroxyflavone	rat	p.o. and i.p.	urine	69
	3',4'-dihydroxyflavone	rat	p.o. and i.p.	urine	69
tricetin (5,7,3',4',5'-penta-hydroxyflavone)	3,5-dihydroxyphenyl-propionic acid	rat	p.o.	urine	22
tricin (5,7,4'-trihydroxy-3',5'-dimethoxyflavone)	3,5-dihydroxyphenyl-propionic acid	rat	p.o.	urine	22
5,7-dihydroxy-3',4',5'-tri-methoxyflavone	3,5-dihydroxyphenyl-propionic acid	rat	p.o.	urine	22
5,6,7-trihydroxyflavone and its 7-*O*-ß-glucuronide	5,6,7-trihydroxyflavone glucuronides, sulfates, and mixed conjugates	rat	p.o.	bile	71
	6-methoxy-5,7-di-hydroxyflavone	rat	p.o.	bile	71
diosmetin (5,7,3'-trihydroxy-4'-methoxyflavone)	diosmetin-7,3'-diglucu-ronide	rat	p.o. 100	urine, whole blood	73

Table 5 (Contd.). *Metabolites of FLAVONES found after oral (p.o.) or intraperitoneal (i.p.) administration to rats*

Flavone	Metabolite	Species	Dose (mg/kg body weight)	Body fluid	Reference
diosmetin (5,7,3'-trihydroxy-4'-methoxyflavone)	diosmetin-3'-glucuronide	rat	p.o. 100	urine, whole blood	73
	diosmetin glucuronide	rat	p.o. 600	urine	77
	diosmetin aglycone	rat	p.o. 600	urine	77
	3-hydroxyphenylpropionic acid	rat	p.o. 600	urine	77
	3-hydroxycinnamic acid	rat	p.o. 600	urine	77
diosmin (5,3'-dihydroxy-4'-methoxyflavone-7-rutinoside)	diosmetin aglycone	rat	p.o. 1200	urine	77
	3-hydroxyphenylpropionic acid	rat	p.o. 1200	urine	77
	3-hydroxycinnamic acid	rat	p.o. 1200	urine	77
luteolin (5,7,3',4'-tetra-hydroxyflavone)	luteolin aglycone	rat	i.p. 40	urine	78
	monomethoxylated luteolin (2 isomers)	rat	i.p. 40	urine, bile	78

Table 6. *Metabolites of FLAVANONES after oral (p.o.) or intravenous (i.v.) administration to various species*

Flavanone	Metabolite	Species	Dose (mg/kg body weight)	Body fluid	Reference
liquiritigenin (7,4'-dihydroxy-flavanone)	liquiritigenin aglycone	rat	i.v. 5	plasma	79
	liquiritigenin-4'-glucuro-nide, -7-glucuronide, -4',7-disulfate, -4'-glucuronide-7-sulfate, -7-glucuronide-4'-sulfate	rat	i.v. 5	plasma and /or bile	79
eriodictyol (5,7,3',4'-tetra-hydroxyflavanone)	eriodictyol glucuronide	rat	p.o. 900	urine	77
	5,7,4'-trihydroxy-3'-methoxyflavanone	rat	p.o. 900	urine	77
	3-hydroxyphenylpropionic acid	rat	p.o. 900	urine	77
	3-hydroxycinnamic acid	rat	p.o. 900	urine	77
homoeriodictyol (5,7,4'-tri-hydroxy-3'methoxyflavanone)	homoeriodictyol aglycone	rat	p.o. 450	urine	77
	homoeriodictyol glucuronide	rat	p.o. 450	urine	77
	3-hydroxyphenylpropionic acid	rat	p.o. 450	urine	77
	4-hydroxy-3-methoxyphenyl-propionic acid	rat	p.o. 450	urine	77

Table 6 (Contd.). *Metabolites of FLAVANONES after oral (p.o.) or intravenous (i.v.) administration to various species*

Flavanone	Metabolite	Species	Dose (mg/kg body weight)	Body fluid	Reference
homoeriodictyol (5,7,4'-trihydroxy-3'methoxyflavanone)	3-hydroxycinnamic acid	rat	p.o. 450	urine	77
hesperetin (5,7,3'-trihydroxy-4'-methoxyflavanone)	hesperetin aglycone	rat	p.o. 450	urine	77
	hesperetin glucuronides	rat	p.o. 150	bile	33
		rat	p.o. 450	urine	77
		man	p.o. 30	urine	77
	3-hydroxyphenylpropionic acid	rat	p.o. 450	urine	77
		rat	p.o. 1.5	urine	31
	4-hydroxyphenylpropionic acid	rat	p.o. 30-150	urine	33
	3,4-dihydroxyphenyl-propionic acid	rat	p.o. 1.5	urine	31
	3-hydroxy-4-methoxyphenyl-propionic acid	rat	p.o. 1.5	urine	31
	3-hydroxy-4-methoxy-phenylhydracrylic acid	man	p.o. 30	urine	77
	3-hydroxycinnamic acid	rat	p.o. 450	urine	77

hesperidin (5,3'-dihydroxy-4'-methoxyflavanone-7-rutinoside)	hesperidin	rat	p.o 150	bile	33
	hesperetin aglycone	rabbit	p.o. 330	urine	77
		rat	p.o. 450	urine	77
	hesperetin glucuronides	rat	p.o. 150	bile	33
		rabbit	p.o. 330	urine	77
		rat	p.o. 450	urine	77
	3,4-dihydroxyphenyl-propionic acid	rabbit	p.o. 330	urine	77
	4-hydroxy-3-methoxy-phenylpropionic acid	rabbit	p.o. 330	urine	77
	3-hydroxyphenylpropionic acid	rabbit	p.o. 330	urine	77
		rat	p.o. 450	urine	77
	4-hydroxyphenylpropionic acid	rat	p.o. 30-150	urine	33
	3-hydroxy-4-methoxy-phenylpropionic acid	man	p.o. 30	urine	77
	3-hydroxycinnamic acid	rabbit	p.o. 330	urine	77
		rat	p.o. 450	urine	77

Table 6 (Contd.). *Metabolites of FLAVANONES after oral (p.o.) or intravenous (i.v.) administration to various species*

Flavanone	Metabolite	Species	Dose (mg/kg body weight)	Body fluid	Reference
hesperidin (5,3'-dihydroxy-4'-methoxyflavanone-7-rutinoside)	3-hydroxyhippuric acid	rabbit	p.o. 330	urine	77
	3-hydroxybenzoic acid	rabbit	p.o. 330	urine	77
	4-hydroxy-3-methoxybenzoic acid	rabbit	p.o. 330	urine	77
naringenin (5,7,4'-trihydroxyflavanone)	naringenin glucuronides	rat	p.o. 150	bile	33
		rat	p.o. 300	urine	80
	naringenin aglycone	rat	p.o. 300	urine	80
	4-hydroxyphenylpropionic acid	rat	p.o. 30-150	urine	33
		rat	p.o. 300	urine	80
	4-hydroxycinnamic acid	rat	p.o. 300	urine	80
	4-hydroxybenzoic acid sulfate	rat	p.o. 300	urine	80
naringin (5,4'-dihydroxyflavanone-7-neohesperidoside)	naringenin aglycone	rat	p.o. 150	bile	33
		rat	p.o. 600	urine	80

naringin (5,4'-dihydroxy-flavanone-7-neohesperidoside)	naringenin glucuronide	rat	p.o. 150	bile	33
		rat	p.o. 600	urine	80
	4-hydroxyphenylpropionic acid	rat	p.o. 30-150	urine	33
	4-hydroxycinnamic acid	rat	p.o. 600	urine	80
	4-hydroxybenzoic acid sulfate	rat	p.o. 600	urine	80

Anthocyanidins

The limited data available on the metabolism of anthocyanidins indicate that these flavylium flavonoids are metabolised to a much more limited extent than other flavonoids. Cyanidin (3,5,7,3',4'-pentahydroxyflavylium) was not converted to phenolic metabolites when incubated with rat caecal bacteria.[20] Delphinidin (3,5,7,-3',4',5'-hexahydroxyflavilium) and malvin (3,5,7,4'-tetrahydroxy-3',5'-dimethoxy flavylium-3,5-diglucoside) fed to rats or incubated with microorganisms were not metabolized to identifiable compounds, but phenolic compounds could be excluded.[22] After intravenous administration of an extract of *Vaccinium myrtillus* to rats, 20% of the administered dose (based on direct colorimetric measurement) was excreted as such into urine.[81]

Phenolic acids

Ring fission of flavonoids generates phenolic acids that are absorbed and excreted into urine (*Tables 1 - 6*). The primary ring fission products (*Figure 2 - 4*) are susceptible to supplemental metabolism by bacteria in the colon and, after absorption, also by enzymes in body tissues. The major questions are: what are the metabolic reactions acting upon these primary phenolic acids, and what affects these reactions?

Degradation of primary ring fission products of flavonoids by bacteria in the colon

In vitro incubations of cinnamic (phenyl-C_3), phenylpropionic (phenyl-C_3), and phenylacetic (phenyl-C_2) acids with rat caecal bacteria[82] demonstrated that these microorganisms performed the following metabolic reactions. Dehydroxylation of 3,4-dihydroxyphenylpropionic and 3,4-dihydroxyphenylacetic acids to produce 3-hydroxyphenolic acids.[22] Demethylation of *o*-hydroxymethoxyphenolic acids. Reduction of the double bond of cinnamic acids to produce phenylpropionic acids. Decarboxylation of cinnamic and phenylacetic acids, but only when a hydroxyl group at position 4 was present. Decarboxylation of the phenylpropionic acids did not occur. Also after oral administration of 3,4,5-trihydroxyphenylacetic acid, a proposed primary fission product of myricetin, to rats, dehydroxylation was confirmed and produced 3,4-dihydroxyphenylacetic and 3-hydroxyphenylacetic acid which were excreted in urine.[22] Dehydroxylation of δ-(3,4-dihydroxyphenyl)-γ-valero-

lactone, the primary ring fission product of (+)-catechin, was demonstrated in guinea pigs.[46]

Species differences in these metabolic reactions of bacteria were observed. In rabbits no decarboxylation of 3,4-dihydroxyphenylacetic acid occurred.[83] ß-Hydroxylation of phenylpropionic acids which produced phenylhydracrylic acids was only observed in man and monkeys[24,65,66,77] and it was demonstrated that bacteria in the colon carried out this reaction.

Enzymatic transformations of phenolic acids in body tissues

Conjugation of 3-hydroxybenzoic acid with glycine to form 3-hydroxyhippuric acid occurred in rodents and man (*Table 2*), probably in the renal tissues.[84] Conjugation with glucuronic acid or sulfate of valerolactones,[47] of phenylpropionic acids[24,31,46,47] and phenylacetic acids[61] was generally found. *o*-Hydroxymethoxy phenolic acids excreted in urine (*Table 2 - 6*) could have originated from ring fission of *O*-methylated flavonoids secreted with bile. However absorbed ring fission products were also *O*-methylated in guinea pigs,[46] rats,[85-87] and man.[87]
Phenylpropionic acids were converted to benzoic acids by ß-oxidation of the propyl chain in all species.[23-25,31,46,59,77] It was shown that only tissue enzymes could have been involved.

In conclusion, administration of flavonoids *in vivo* yielded a range of substituted phenolic acids in urine (*Table 2 -6*). The presence of these compounds could be explained by the bacterial and tissue enzymatic reactions that act upon phenolic acids. Species differences in these metabolic reactions occurred to some extent.

PHARMACOKINETICS

A quantity of major interest in assessing the biological effects of flavonoids, or indeed of any food component or drug, is the bioavailability. Bioavailability quantitates the exposure of the body (excluding gut and liver) to the substance in question. Bioavailability is often mistakenly equated with absorption. However, bioavailability also includes first-pass metabolism. Bioavailability is defined as the percentage of the ingested flavonoid amount that enters the blood circulation intact

after passage through the liver.[40] It is determined experimentally by giving a flavonoid orally and intravenously and then measuring the ratio of the areas under the plasma flavonoid concentration versus time curves (AUCs). The fate of flavonoids in the body after ingestion is determined by their absorption, distribution and elimination, which each have their own rates and extents. Pharmacokinetic parameters are needed to describe and to predict these processes. Subsequently, predictions of dosage-dependency of plasma levels, achievable plasma levels, and accumulation can be made.

Catechins

The maximum plasma concentration of (+)-catechin and its metabolites was reached after 1 - 3 h, and elimination half-life of the aglycone was about 1 h (*Table 7*). By the time that the maximum concentration in plasma was reached, only about 10% of the catechin was present unchanged.[25] Differences between C_{max}s found by [48] and[88] are probably explained by the inclusion of conjugates in the data of [48] but not of.[88] In plasma, ^{14}C activity was present up to 120 hours, indicating a long persistence of metabolites.[25] A linear relation between the administered dose and the area under the plasma concentration versus time curve (AUC) was found in humans.[88] This indicates that there was no saturable gastro-intestinal absorption and/or no dose dependent first-pass effect of the liver up to an oral dose of 30 mg/kg. The rate of absorption of 3-methoxy-(+)-catechin[26] was similar to that of (+)-catechin (*Table 7*). However, the elimination half-life of total ^{14}C activity after 3-methoxy-(+)-catechin was considerably higher, whereas the elimination of the parent 3-methoxy-(+)-catechin appeared to be very rapid. This could point to storage of 3-methoxy-(+)-catechin or a metabolite in tissues, and subsequent slow release of metabolites. The rate of absorption of various epicatechins of green tea seemed to be dependent on the type of catechin.[36]

Thus, pharmacokinetic data of catechins are scarce, and bioavailability has not been determined. Absorption was moderately rapid, and elimination of the (+)-catechin aglycone was rapid.

Flavonols

After a high oral dose of quercetin aglycone no quercetin aglycone was detected in plasma in man[29] (*Table 7*). Possibly the major fraction of plasma quercetin is conju-

gated to glucuronic acid or sulfate. This could explain why Gugler et al.[29] detected no quercetin in plasma: these authors determined only the aglycone, using a method with a high limit of detection (100 ng/ml). Flavonol glycosides showed moderate to rapid absorption in man.[62,63,90] We[62,63] compared the absorption of quercetin from onions, apples and rutin, and found distinct differences in rates of absorption. Onions contain mainly quercetin-ß-glucosides, whereas apples contain a mixture of quercetin-ß-D-galactosides, and ß-D-xylosides, whereas quercetin is bound to a disaccharide in rutin. We hypothesized that the rapid and better absorption of the quercetin glucoside in onions was caused by the glucose transporter in the small intestine.[30,62,63] Indeed model studies[95] showed that naphthol glucosides were transported by the active Na^+/glucose transporter across the intestinal wall of rats. The elimination of quercetin from plasma was slow in our studies, which implied that quercetin may accumulate in plasma throughout the day with repeated dietary intake. The bioavailability of quercetin in apples and of rutin were both 30% of that in onions. The important role of the sugar moiety in the absorption of
rcetin was also found in a study with ileostomy subjects who lack a colon with
rial flora.[30] The quercetin glucoside in onions was very well absorbed,
tion of the pure quercetin aglycone and quercetin rutinoside was
rinary excretion of total quercetin in these subjects was highest
osides. Thus, the different types of glycosides in these
nd metabolism.
vailability of flavonols has not been determined.
bsorption of quercetin varied between food

,7,3'-trihydroxy-4'-methoxyflavone), was not
nistration to human volunteers.[93] Hydrolysis of
ed, and the elimination of diosmetin from plasma
w. um elimination of tritium was very slow after admini-
strati ats.[92] Absolute bioavailability of 5-methoxyflavone was
ied , and was high: 25% for rats, and 53% for dogs.[94]

Table 7. *Pharmacokinetic parameters of flavonoids. (p.o.: per os; i.v.: intravenous; C_{max}: maximum concentration measured; t_{max}: time to reach C_{max}; $t_{½}$: elimination half-life)*

Flavonoid	Species	Dose (mg/kg body weight)	C_{max} (ng/ml)	t_{max} (h)	$t_{½}$ (h)	Method	Ref.
(+)-catechin	man (n = 3	p.o. 45	15000	1 - 2	1.3	Serum; photometric	48
(+)-catechin	man (n = 6)	p.o. 8	590	1.6	1.3	Serum; aglycone HPLC	88
(+)-catechin	rabbit (n = 8)	i.v. 15	11000	-	0.75	Plasma; aglycone HPLC	89
[U-^{4}C](+)-catechin	man (n = 3)	p.o. 30	12000[a] 500[b]	3[a] 3[b]	- -	Plasma; [a] total radioactivity, [b] aglycone HPLC	25
3-[^{14}C]methoxy-(+)-catechin	rat (n = 3)	i.v. 30	100000[a] 9500[b]	0.1[a] 0.1[b]	6.5[a] < 1[b]	Plasma; [a] total radioactivity, [b] aglycone HPLC	51
3-methoxy-(+)-[U-^{14}C]-catechin	man (n = 3)	p.o. 30	50000[a] 11000[b]	2[a] 2[b]	10[a] «10[b]	Plasma; [a] total radioactivity, [b] aglycone HPLC	26
green tea (-)-epigallocatechin (EGC), EGC-3-gallate (EGCg), (-)-epicatechin (EC), EC-3-gallate (ECg)	man (n = 4)	p.o. 1.3 EGCg 1.2 EGC 0.5 ECg 0.5 EC	 144 140 < 1 60	 4* 1* -* 1*		Plasma; total HPLC * Plasma was measured after 1 h and 4 h.	36
quercetin	man (n = 6)	i.v. 1.5 p.o. 65	3700 < 100	0.1 -	2.4 -	Plasma; aglycone fluorimetric	29

flavonol glycosides of *Ginkgo biloba* extract	man (n = 2)	p.o. ?	28 - 140	2 - 2.5	-	Plasma; total flavonols HPLC	90
quercetin glucosides of onions	man (2) (n = 9)	p.o. 0.9[¶] p.o. 0.9[¶]	200 225	2.9 0.7	17 28	Plasma; total quercetin HPLC [¶] quercetin equivalents	63 62
quercetin glycosides of apples	man (n = 9)	p.o. 1.4[¶]	90	2.5	23	Plasma; total quercetin HPLC	62
quercetin-3-rutinoside	man (n = 9)	p.o. 1.4[¶]	90	9.3	-	Plasma; total quercetin HPLC	62
[^{14}C]7-O-(ß-hydroxyethyl)-quercetine-3-rutinoside	dog (n = 2)	p.o. 22	8750	3-6	-	Plasma; radioactivity	91
[^{3}H]diosmin	rat (n = 5)	p.o.	-	2	-	Serum; radioactivity	92
diosmin	man (n = 2)	p.o. 10	420	1	31.5	Plasma; diosmetin aglycone HPLC	93
5-methoxyflavone	rat (n = 3) dog (n = 2)	i.v. 5 p.o. 10 i.v. 10 p.o. 10	3200 1500 6500 2150	- 0.3 - 1	- - 0.3 -	Plasma; aglycone GC-MS/MS Plasma; aglycone GC-MS/MS	94 94

[a] total radioactivity [b] aglycone HPLC [¶] quercetin equivalents

Tissue distribution of flavonoids

The extended elimination times observed for quercetin and diosmin in humans (*Table 7*) could point to temporary storage of flavonoids or their metabolites in tissues. Studies on tissue distribution were carried out with various labelled flavonoids. After oral administration of [^{3}H]-diosmin to rats the highest concentration of ^{3}H was found in liver.[92] However, ^{3}H started to accumulate in tissue of veins and arteries after 4 hours and still was increasing at the last time (48 h) measured. It was suggested that a metabolite was accumulating in these tissues. The radioactivity associated with 3-[^{14}C]methoxy-(+)-catechin was only recovered with the contents of the alimentary tract; this was caused by enterohepatic circulation of the major metabolite.[57] The distribution of radioactivity in tissues of the rat after oral administration of [4-^{14}C]quercetin[28] showed no evidence for accumulation in any tissue. Six hours after administration the highest radioactivity (0.3% of the administered dose per gram wet organ) was found in the kidney, with liver and blood having somewhat lower concentrations. Because of the position of the label in the quercetin molecule, only quercetin and metabolites with an intact ring structure and phenylpropionic acids would have been detected. Recovery of radioactivity in organs of rats (liver, kidneys, spleen, stomach and gut) was 1.4% of the activity of orally administered [3-^{14}C]hesperetin.[31]

More than 98% of the quercetin in human plasma was bound to proteins.[29] Binding of quercetin to human albumin was 70% - 80%.[96] These observations were confirmed by ultrafiltration;[97] after ultrafiltration to exclude proteins larger than 30 kDa, quercetin was absent in the filtrate of plasma of rats fed quercetin and rutin. Binding of flavonoids to proteins is well-documented.[98] Quercetin and rutin, but not (+)-catechin and 3-methoxy-(+)-catechin were selectively bound to platelets of rabbits *in vitro*.[99]

Thus, the limited data available do not point to storage of intact flavonoids in tissues. However, evidence for accumulation of metabolites was found for diosmin.

SUMMARY AND CONCLUSIONS

Most studies on absorption and metabolism of flavonoids have focused on measuring their urinary metabolites in rodents. Absorption was estimated by measuring urinary excretion using pure aglycones administered at unphysiologically high doses. Absorption as measured this way depended on the type of flavonoid and was between 4 and 58%. The extent of absorption from dietary sources is largely unknown; for instance, data on the catechins of tea, which is a major dietary source are virtually absent. In the diet, most flavonoids except catechins are present not as aglycones but as glycosides. It used to be thought that intact glycosides are not absorbed. However, recently it was found that quercetin glucoside was in fact absorbed much better than the aglycone; this topic thus needs rethinking. Proteins in the diet may theoretically affect flavonoid absorption because they bind polyphenols.[98] Circumstantial evidence for reduced absorption of tea polyphenols by complexation with milk proteins was found in humans;[100] ingestion of tea caused a significant increase of the plasma antioxidant capacity, but not when tea was consumed with milk. However, these authors did not determine polyphenols in plasma. Research on this interaction needs to be done.

The two major sites of flavonoid metabolism are the liver and the colonic flora. Only the liver has been investigated as a metabolic organ. Other tissues such as intestine wall and kidneys may play a role. Phase I biotransformation reactions of liver enzymes have been described only for synthetic flavonoids lacking hydroxyl groups, but evidence for phase II biotrans-formation is abundant. Absorbed flavonoids and their absorbed colonic metabolites are glucuronidated and sulfated by the liver in humans as well in rodents, but the types of glucuronides and the preference for sulfation may vary between species. *O*-methylation of catechol groups is found in humans and rodents. Unabsorbed flavonoids and flavonoid conjugates secreted with bile into the gut are degraded by bacteria in the colon. Hydrolysis of conjugates and glycosides, and ring fission of the aglycones to phenolic acids are the major bacterial reactions. In rats, the metabolites are then absorbed and excreted in urine. However, very few quantitative data on metabolism are available. In metabolism of (+)-catechin ring fission is of minor importance in humans. With other flavonoids, the rather high excretion of CO_2, about one third of the dose, points to notable ring fission in rats.

In humans, conjugation and *O*-methylation of quercetin occurs only to a limited extent, whereas these reactions are of major importance for (+)-catechin

metabolism. One would expect biliary secretion also to occur in man, as glucuronides of flavonoids have molecular weights in excess of 500.[39] However, the significance of biliary secretion and reabsorption in humans is unknown.

Pharmacokinetic data on flavonoids are scarce, probably because selective and sensitive analytical methods to determine flavonoids and their metabolites in plasma, urine and tissues were lacking. Absolute bioavailability of flavonoids has not been determined, but it is becoming evident that relative bioavailability of flavonols differs markedly between foods.

In order to evaluate the impact of dietary flavonoids on human health we need more information on how the nature of the glycoside moiety and of the food matrix affect absorption and metabolism. Such studies should be performed at the low levels of intake which occur naturally, as the high doses used in earlier studies may produce artefacts. Identification of metabolites in body fluids and tissues is also an important goal for further research.

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J Clin Nutr 1996;**50**:28-32.

3

FLUORESCENCE DETECTION of FLAVONOLS in HPLC by POSTCOLUMN CHELATION with ALUMINUM

Hollman PCH, van Trijp JMP, Buysman MNCP
Analytical Chemistry 1996;**68**:3511-3515

Abstract

Flavonols are dietary antioxidants which might prevent coronary heart disease. To be able to study absorption of flavonols in man, we developed a postcolumn derivatization with aluminum for HPLC with fluorescence detection. Variables governing postcolumn chelation, such as water content, buffer, and organic modifier of the eluent, concentration of Al^{3+} and presence of acetic acid in the postcolumn reagent, and temperature were studied and optimized. Of the flavonoids, only flavonols that contain a free 3-hydroxyl and 4-keto oxygen binding site form fluorescent complexes with Al^{3+}. The method has a detection limit of 0.15 ng/mL for quercetin, 0.05 ng/mL for kaempferol, 0.45 ng/mL for myricetin, and 0.05 mg/mL for isorhamnetin, thus improving detectability of quercetin 300 fold as compared to that possible with UV detection. The reproducibility relative standard deviation of the method is 1.4%. This extremely sensitive method enables, for the first time, determination of flavonols in body fluids after consumption of a normal diet.

INTRODUCTION

Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. Flavonoids are categorized into flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids.[1] Potent biological effects have been described in many *in vivo* and *in vitro* studies.[2] These studies point to potentially beneficial health effects of dietary flavonoids.[3] Recently, it was found that the intake of quercetin, the major representative of the flavonol subclass (*Figure 1*), was inversely associated with subsequent coronary heart disease in two prospective cohort studies,[4,5] and a prospective cross-cultural study.[6] The antioxidant properties of flavonoids[7] offer a plausible explanation for the effect found.[8,9] However, the extent of absorption of flavonoids is an important unsolved problem in judging their potential role in the prevention of coronary heart disease.

For human absorption studies, we needed to determine flavonols in blood and urine. An HPLC method with UV detection previously developed[10] did not meet the requirements of sensitivity and specificity in these biological fluids. Thus, extensive sample enrichment and cleanup procedures had to be considered. However, the enrichment attainable would most likely not be sufficient. Consequently, a very sensitive detection technique had to be explored. Fluorometry would be most suited to achieve the detection limit required. In addition, the potential gain in specificity over UV detection could simplify, or even make redundant, sample cleanup.

Flavonols such as quercetin and morin can form fluorescent chelates with a variety of metal ions, and before the advent of atomic absorption spectroscopy at the end of the 1960s, morin was used to quantitate a variety of metal ions by fluorometry.[11] Aluminum chloride as a complexing agent is used routinely in thin-layer chromatography to aid identification of the flavonoid type.[12] Nieder[13] very briefly mentioned the use of aluminum chloride as a postcolumn reagent in HPLC with fluorescence detection to determine flavonols in plasma. However, no details about postcolumn reaction conditions were presented.

The present study was designed to find optimum reaction conditions for postcolumn derivatization of quercetin with aluminum, as quercetin is the major dietary flavonol.[4] In addition, we studied structural properties of flavonols that are required in order to form fluorescent chelates with aluminum. The HPLC conditions for separation of flavonoids previously optimized were used as a starting point.

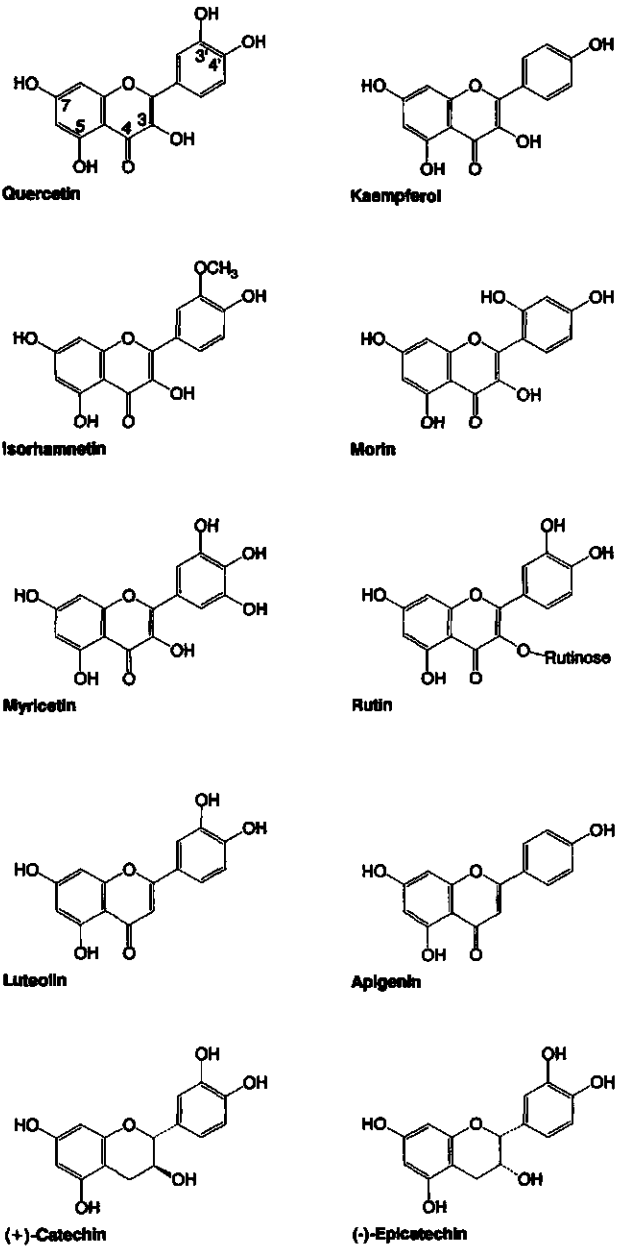

Figure 1. *Structures of flavonols, flavones and catechins.*

EXPERIMENTAL SECTION

Chemicals

All chemicals were reagent grade or HPLC grade.

Fluorescence spectrometer

Fluorescence excitation and emission spectra were recorded with a Perkin Elmer LS 50 B luminescence spectrometer equipped with Fluorescent Data Manager software (Perkin Elmer, Norwalk, CT). Wavelengths of excitation and emission maxima of each flavonoid were determined in 'Prescan mode', varying excitation wavelength from 200 to 450 nm and emission wavelength from 450 to 700 nm, with slits set at 10.0 nm. The final spectra were recorded at 240 nm/min, with slits set at 5.0 nm. Spectra were processed with Slidewrite 6.0 (Advanced Graphics Software Inc., Sunnyvale, CA). Fluorescence spectra of flavonoid standards were recorded both in the eluent (acetonitrile/0.025 M phosphate buffer, pH 2.4, 31:69 v/v), and in the mixture of eluent and aluminum nitrate reagent (proportion, 1 : 0.4) present in the postcolumn reactor when using optimized HPLC conditions. The aluminum nitrate reagent consisted of 1.5 M $Al(NO_3)_3$ in methanol containing 7.2% (v/v) acetic acid. Equimolar solutions (700 nM) of standards were tested in both conditions.

Chromatography

For HPLC analysis we injected 20 μL of the sample onto an Inertsil ODS-2 column (4.6 mm x 150 mm, 5μm; GL Sciences Inc., Tokyo, Japan), protected by an MPLC Newguard RP-18 column (3.2 mm x 15 mm, 7 μm; Brownlee, Applied Biosystems Inc., Foster City, CA) using acetonitrile/0.025 M phosphate buffer pH 2.4 (31:69 v/v) as mobile phase, at a flow rate of 1 mL/min using a Merck Hitachi L-6200 A pump (Hitachi Ltd., Tokyo, Japan). The columns were placed in a column oven set at 30 °C. The column effluent was mixed with 0.4 mL/min 1.5 M $Al(NO_3)_3$ in methanol containing 7.2% (v/v) acetic acid in a postcolumn reactor placed in the column oven. The postcolumn reactor consisted of a 15 m (0.25 mm internal diameter) PTFE tubing (Upchurch Scientific Inc., Oak Harbor, WA) coiled to a diameter of 3 cm and connected to the HPLC column with a low-dead-volume tee (Upchurch Scientific Inc., Oak Harbor, WA). Column effluent and aluminum reagent entered the tee

countercurrently at an angle of 180°. A Merck Hitachi L-6000 A pump was used to generate the aluminum reagent. The fluorescence of the ensuing quercetin-metal complex was measured at 485 nm using a Jasco FP 920 (Jasco Corporation, Tokyo, Japan) fluorescence detector with excitation wavelength set at 422 nm. The detector output was sampled using a Nelson (PE Nelson, Cupertino, CA) series 900 interface and Nelson integrator software (model 2600, rev. 5), and the peak area of the quercetin-metal complex was determined.

RESULTS

Fluorescence spectra

None of the flavonols and flavones (*Figure 1*) showed native fluorescence in the eluent. However, fluorescence of certain flavonols dissolved in the mixture of eluent and aluminum reagent was high, indicating that aluminum had a profound effect (*Figure 2*). The fluorescence was stable within 10 s after mixing. The fluorescence intensities of the various Al^{3+}-flavonol complexes varied considerably (*Table 1*).

Postcolumn reaction conditions

The residence time in the postcolumn reactor had to be at least 40 s to achieve a substantial enhancement of the detector response (*Table 2*). Increase of the length of the reactor coil beyond 20 m only had a marginal effect. Peak broadening caused by the increasing reactor volume was about 1 s (*Table 2*), which can be expected for tubular reactors.[14]

Equimolar solutions of $Al(NO_3)_3$ and $AlCl_3$ in methanol did not differ in detector response and band broadening. Other solvents such as ethanol, acetone and acetonitrile were not suitable, because the solubility of both salts in these solvents was limited. A solution of 0.8 M Al^{3+} could not be obtained.

The detector response increased with the square root of the aluminum concentration (*Figure 3*). Although $Al(NO_3)_3$ concentrations in excess of 1.5 M are expected to increase the detector response, the high viscosity of the reagent at 1.5 M $Al(NO_3)_3$ already required a very high pressure (5000 kPa) in order to reach a reagent flow of 0.4 mL/min. We observed that acetic acid added into the

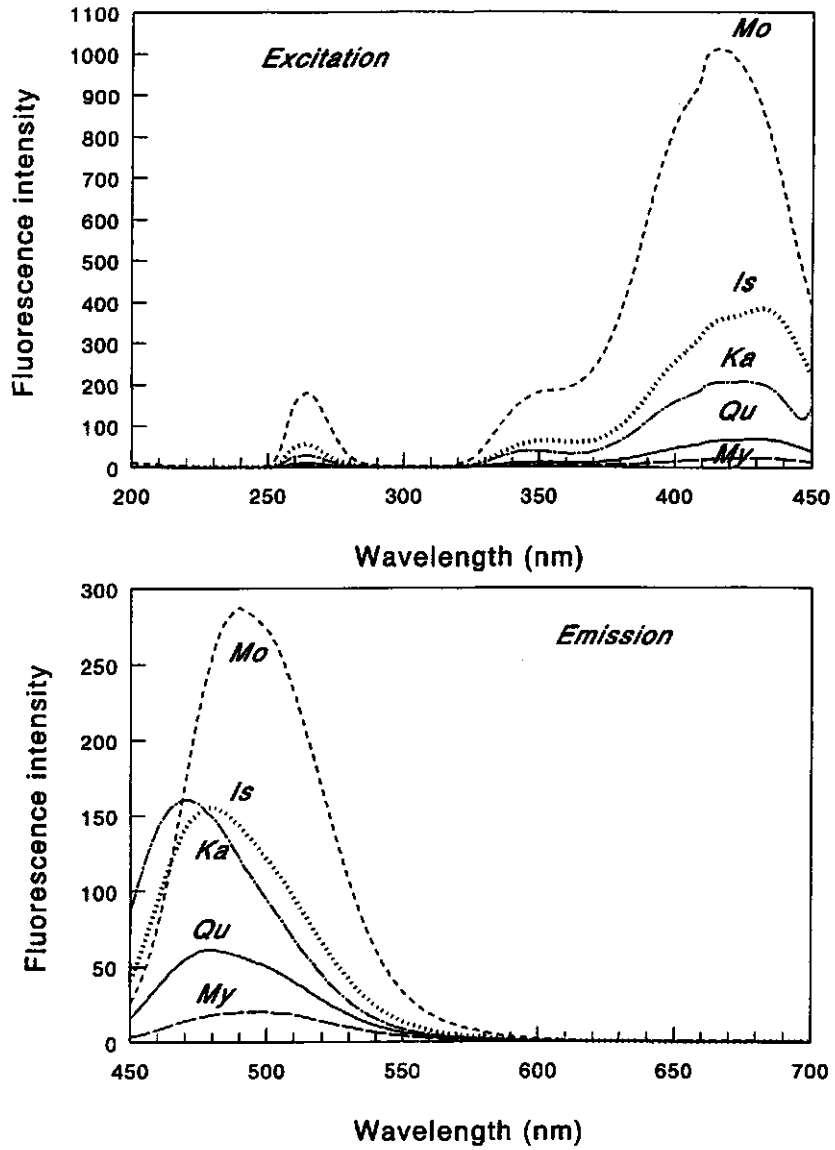

Figure 2. *Fluorescence excitation and emission spectra of flavonols after complexation with aluminum. Flavonols were dissolved in the mixture of eluent and aluminum reagent present in the postcolumn reactor when using optimized conditions for quercetin. Fluorescence intensity is expressed in arbitrary units. Is, isorhamnetin; Ka, kaempferol; Mo, morin; My, myricetin; Qu, quercetin*

aluminum reagent decreased the risk of clogging of the coil. As acetic acid also chelates with Al^{3+}, experiments were carried out with and without acetic acid. A fixed concentration of 7.2% (v/v) was used. No clear effect of acetic acid on the detector response was apparent (*Figure 3*). However, omission of acetic acid increased the frequency of clogging at high $Al(NO_3)_3$ concentrations.

Table 1. *Wavelengths of excitation and emission maxima, and corresponding relative fluorescence intensities of various flavonoids dissolved in the mixture of eluent and aluminum reagent present in the postcolumn reactor when using optimized conditions for quercetin.*

Flavonoid	Maxima $\lambda_{excitation}$ (nm)	Maxima $\lambda_{emission}$ (nm)	Relative fluorescence intensity (%)
Flavonols			
Morin	418	490	100
Kaempferol	420	470	56
Isorhamnetin	430	480	54
Quercetin	430	480	21
Myricetin	428	500	7
Rutin	-	-	0
Flavones			
Luteolin	-	-	0
Apigenin	-	-	0
Catechins			
(+)-catechin	-	-	0
(-)-epicatechin	-	-	0

- no fluorescence could be observed

The highest response was obtained at 34°C, although this maximum was rather broad (*Figure 4*). Thus, a rise in temperature from 24 to 34°C increased the

peak area by 30%. At temperatures higher than 30°C, the detector signal was less stable, as spikes were observed frequently.

Methanol and acetonitrile are commonly used as organic modifiers in reversed-phase HPLC. Increasing water contents in the eluent caused a dramatic drop in detector response for both modifiers (*Figure 5*). Acetic acid added to the $Al_3(NO_3)_3$ reagent reduced this loss of response in the case of methanol. The detrimental effect of water in the acetonitrile-containing eluent was somewhat counteracted by acetic acid, but only at high water concentrations (>45%). To obtain an adequate separation on the reversed-phase column used, the water content of the eluent with acetonitrile has to be about 70%, thus reducing the detector response to only 20% of the maximum achievable value. The maximum detector response was similar for methanol and acetonitrile. The decrease in detector response at <10% water in acetonitrile probably is caused by the moderate solubility of $Al(NO_3)_3$ in acetonitrile.

Table 2. *Length of reaction coil and HPLC detector response as a percentage of the maximum quercetin-Al-complex peak area. Postcolumn reagent: 0.8 M $Al(NO_3)_3$; internal diameter of coil in postcolumn reactor: 0.30 mm; temperature of column and postcolumn reactor: 22°C.*

Length coil (m)	Volume reactor (μL)	Detector response (%)	Residence time♦ (sec)	Peak variance (sec^2)
1.2¶	60	19	348	34
10	710	70	389	39
20	1415	89	428	40
30	2120	100	471	43

¶ internal diameter coil postcolumn reactor = 0.25 mm

♦ total residence time in analytical column plus postcolumn reactor

It was shown previously[10] previously that a phosphate buffer (pH 2.4) improves the peak shape of flavonols in the reversed-phase chromatographic system used. This phosphate buffer enhanced the detector response (*Figure 6*). Highest response was obtained at buffer concentrations between 10 and 30 mM, which coincides with optimum chromatographic conditions for flavonols.[10] Addition of phosphoric acid to the $Al(NO_3)_3$ reagent decreased the detector response only when

when using the eluent with phosphate buffer.

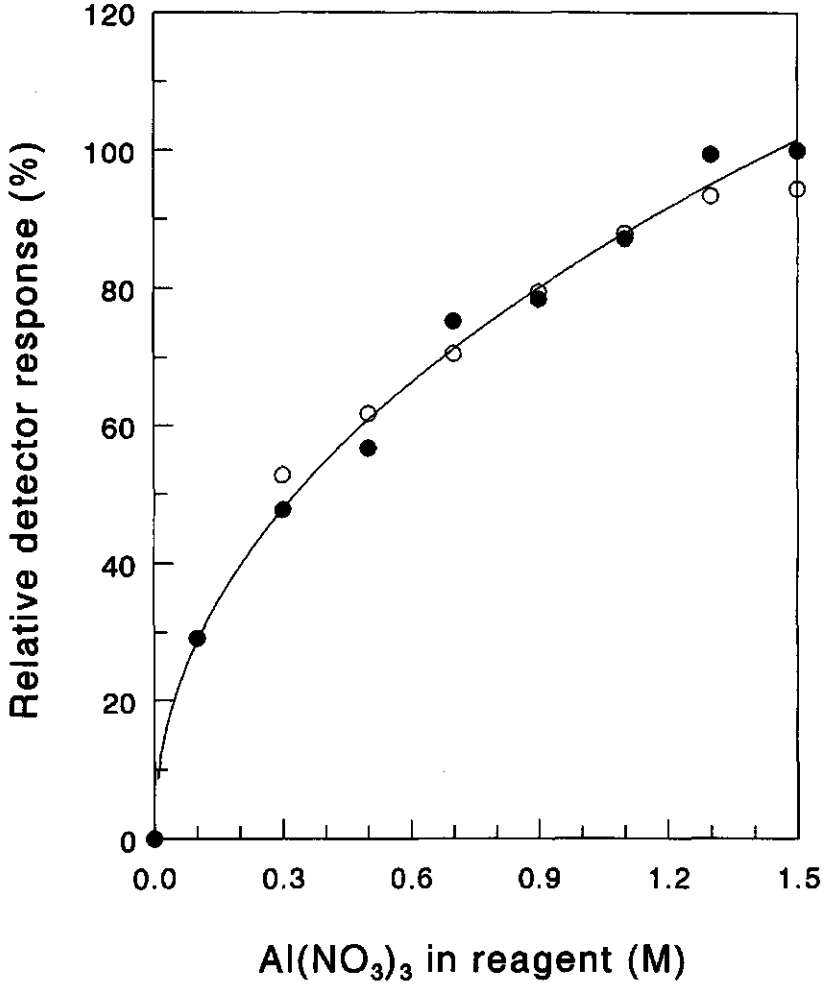

Figure 3. *HPLC detector response to quercetin as a function of the $Al(NO_3)_3$ concentration in the postcolumn reagent. Detector response is expressed as a percentage of the maximum quercetin-Al complex peak area. Each point represents the average detector response of two measurements.*
Eluent: acetonitrile/25 mM phosphate buffer, pH 2.4 (31:69 v/v). Column and postcolumn reactor temperature: 30°C.
●, $Al(NO_3)_3$ with 7.2% acetic acid; ○, $Al(NO_3)_3$ without acetic acid

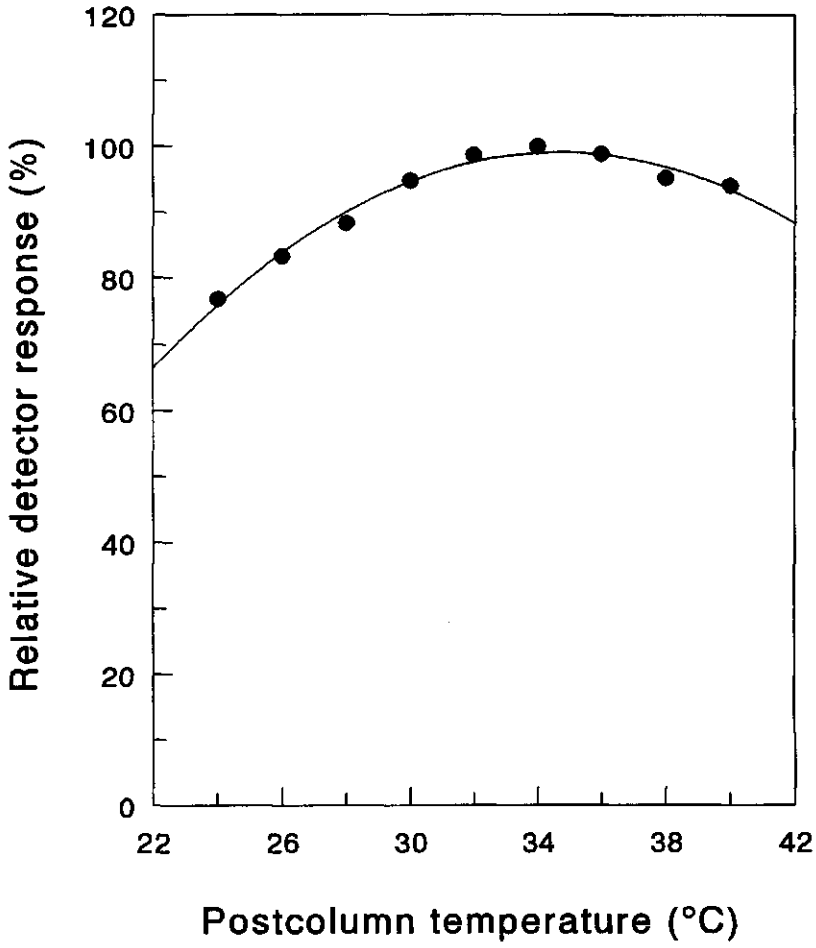

Figure 4. *HPLC detector response to quercetin as a function of the postcolumn temperature. Detector response is expressed as a percentage of the maximum quercetin-Al complex peak area. Each point represents the average detector response of two measurements.*
Eluent: acetonitrile/25 mM phosphate buffer, pH 2.4 (31:69 v/v). Postcolumn reagent: 1.5 M $Al(NO_3)_3$ *with 7.2% acetic acid.*

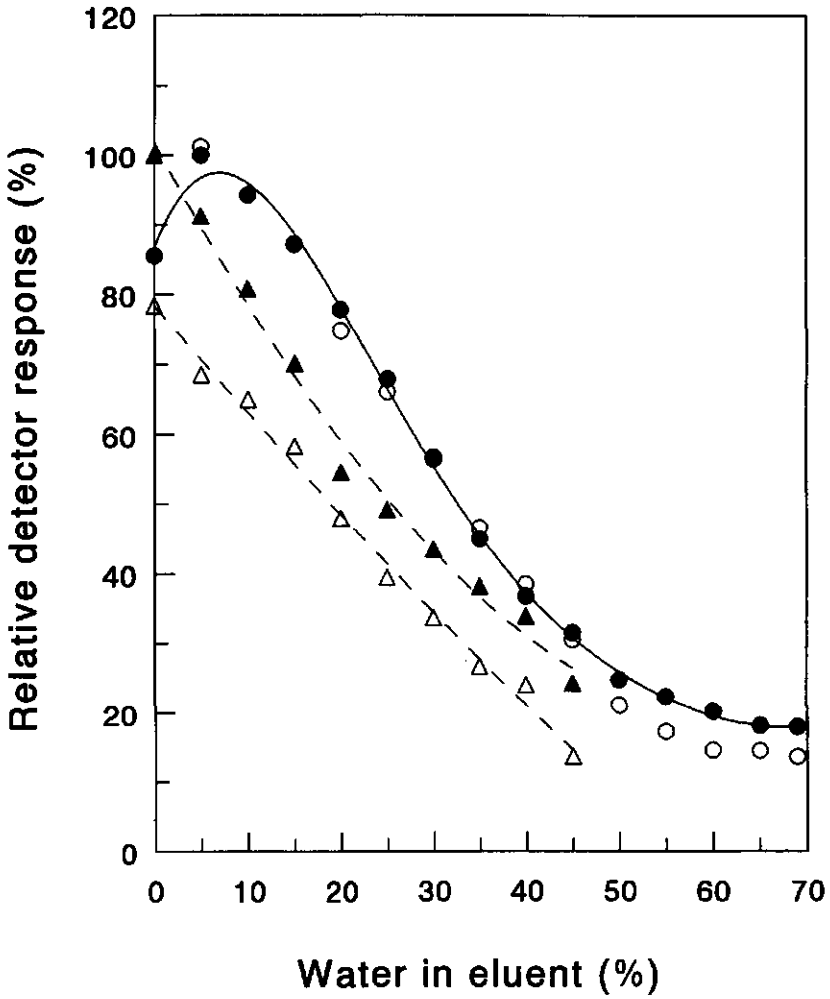

Figure 5. *HPLC detector response to quercetin as a function of the water content in eluents. Detector response is expressed as a percentage of the maximum quercetin-Al complex peak are. Each point represents the average detector response of two measurements.*

Eluent: acetonitrile or methanol without phosphate buffer; postcolumn reagent: 1.5 M $Al(NO_3)_3$; temperature of column and postcolumn reactor: 30°C.

●, acetonitrile, $Al(NO_3)_3$ with 7.2% acetic acid; ○, acetonitrile, $Al(NO_3)_3$ without acetic acid; ▲, methanol, $Al(NO_3)_3$ with 7.2% acetic acid; Δ, methanol, $Al(NO_3)_3$ without acetic acid.

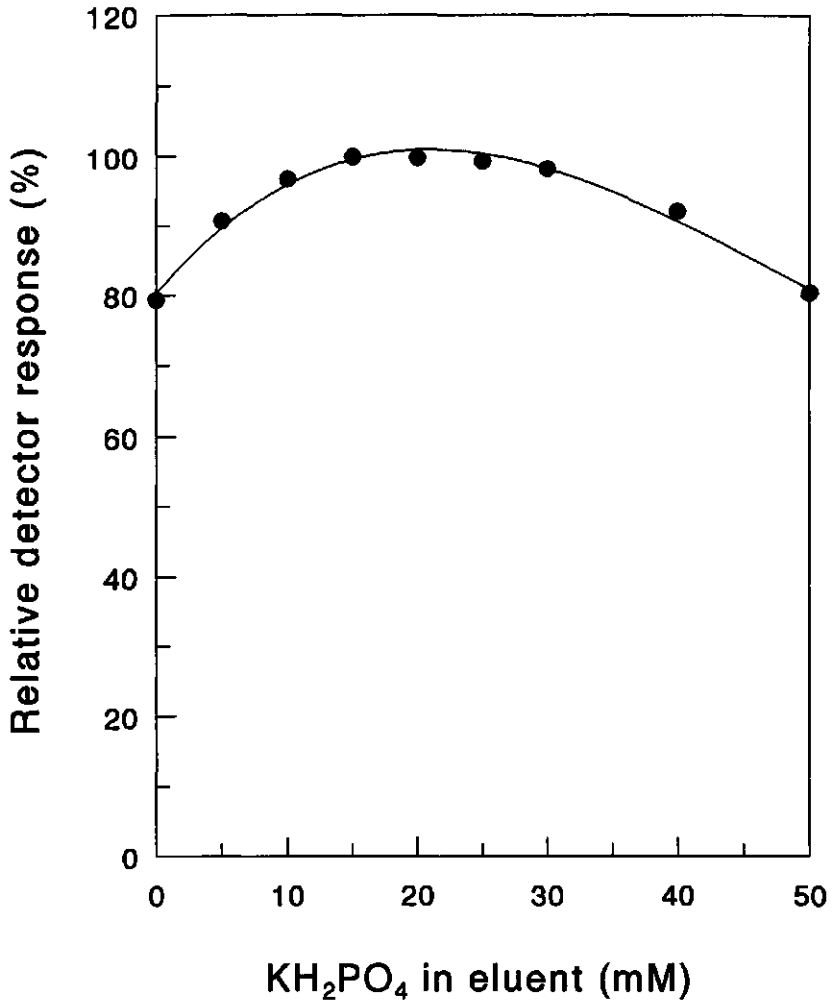

Figure 6. *HPLC detector response to quercetin as a function of the buffer concentration (pH 2.4) in the eluent. Detector response is expressed as a percentage of the maximum quercetin-Al complex peak area. Each point represents the average detector response of two measurements.*
Eluent: acetonitrile/25 mM phosphate buffer, pH = 2.4 (31:69 v/v). Postcolumn reagent: 1.5 M $Al(NO_3)_3$ with 7.2% acetic acid. Temperature of column and postcolumn reactor: 30°C.

Linearity, precision, reproducibility and detection limit

At optimized conditions (see *Experimental section*), the calibration graph was linear up to 5000 ng/mL, showing an extended dynamic range. The within-series relative standard deviation of the peak area of a 50 ng/mL standard quercetin was 1.2% (n = 12). Over a period of 1 month, the reproducibility relative standard deviation of the area of a 50 ng/mL standard quercetin was 3.2% (n = 12). Using a calibration graph for each series of analyses to convert this area into a concentration, as is done in quantitative analyses, improved the reproducibility of this 50 ng/mL standard quercetin to 1.4%.

The limit of detection of standard solutions, i.e., the concentration of the flavonol resulting in a peak height of 3 times the standard deviation of the baseline noise, was 0.15 ng/mL for quercetin, 0.05 ng/mL for kaempferol, 0.45 ng/mL for myricetin, and 0.05 ng/mL for isorhamnetin.

DISCUSSION

We have found that flavonols can be determined by fluorescence detection with high sensitivity after chelation with Al^{3+}. We have set up a postcolumn HPLC system and optimized reaction conditions for chelation. A limit of detection of 0.15 ng/mL can be achieved for quercetin without sample enrichment, thus improving detection considerably as compared to the detection limit of 50 ng/mL with ultraviolet detection.[10]

Reaction conditions

A high concentration of aluminum nitrate and an extended residence time in the postcolumn reactor were necessary to obtain sufficient fluorescence intensity. This indicates that the reaction conditions for formation of the complex are rather unfavourable. As a consequence, application in a postcolumn reactor needs careful optimization. The water content of the eluent required for adequate separation in reversed-phase chromatography had a disastrous effect on the detector response. Szabó and Beck[15] found that the absorbance of Al-morin in ethanol decreased sharply with increasing water content of the reagent, and they speculated that the increasing dielectric constant enhanced dissociation of the Al-morin complex.

Alternatively, solvatation of Al^{3+} with water could lead to a less reactive compound compared to the aluminum ion complexes with methanol and acetonitrile. The beneficial effect of acetic acid could be explained on the basis of formation of a more reactive complex of Al^{3+} and acetic acid. The temperature of the postcolumn reactor was an important variable and showed an optimum, as also was observed by White and Lowe[16] for the Al-morin complex. Addition of phosphate buffer to the eluent for improvement of the chromatographic process also enhanced the fluorescence response, possibly by decreasing the pH of the medium.[17] Increasing amounts of phosphate buffer or phosphoric acid had only an inhibitory effect. Phosphates are known to remove metals from flavonols, and fluorometric methods for the determination of trace quantities of phosphates based on this principle have been described.[18,19]

Structural requirements for complexation

Metal ions may bind to the 3-hydroxyl and 4-keto oxygen and to the 5-hydroxyl and 4-keto oxygen (*Figure 1*).[12,20] Two adjacent hydroxyls, such as the 3'- and 4'-hydroxyls in quercetin, may also be involved in chelation, but in acid solution this complex is labile.[12,20] Plant flavonols always contain both a 3- and a 5-hydroxyl group, but complexation occurs only at the 3-hydroxyl-4-keto-oxygen site.[20] This study shows that the 3-hydroxyl-4-keto-oxygen site is essential for fluorescence. Flavonoids without a 3-hydroxyl group, viz., flavones and catechins, escape fluorescence detection. A free 3-hydroxyl group is essential, as rutin, containing a sugar bound to the 3-hydroxyl group, does not form a fluorescent chelate either.

Morin, kaempferol and isorhamnetin show the highest fluorescence intensities (*Figure 2, Table 1*). Apparently, the adjacent hydroxyl groups at positions 3',4' or 5' in quercetin and myricetin inhibit fluorescence. The tendency to form an additional Al^{3+} chelate involving these *o*-hydroxyl groups might inhibit its fluorescence. Thus, the increase in fluorescence caused by acetic acid and phosphate buffer could be explained by disrupture of the Al-quercetin chelate at position 3'-4' at low pH.[12,20] The high fluorescence of Al-morin may be caused by formation of an internal hydrogen bond between the 2'-OH and the heterocyclic oxygen at position 1. This confers molecular rigidity to the molecule, which increases fluorescence efficiency.

Application in sample analysis

We have used this postcolumn HPLC system for the routine determination of quercetin in body fluids. Because of the high sensitivity and selectivity of this fluorescence detection technique, minimal sample preparation is required, and 40 samples/day can be easily analyzed.[21,22]

Acknowledgments

We thank Prof. Martijn B. Katan for valuable comments on the manuscript. This work was supported by grants from the Foundation for Nutrition and Health Research and the Netherlands Heart Foundation (94.128).

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1282. (**Chapter 4**)

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4

ABSORPTION of DIETARY QUERCETIN GLYCOSIDES and QUERCETIN in HEALTHY ILEOSTOMY VOLUNTEERS

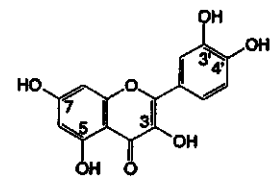
Hollman PCH, de Vries JHM, van Leeuwen SD, Mengelers MJB, Katan MB
American Journal of Clinical Nutrition 1995;**62**:1276-1282

Abstract

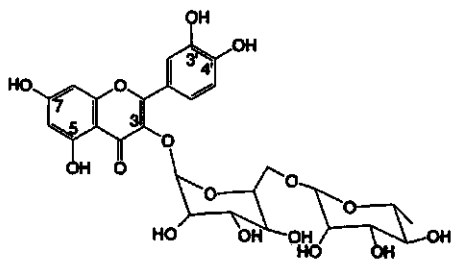
Quercetin is a dietary antioxidant that prevents oxidation of low density lipoproteins *in vitro*. Intake of quercetin was inversely associated with coronary heart disease mortality in elderly Dutch men. However, the extent of absorption of quercetin in humans is unclear. The aim of this study was to quantify absorption of various forms of quercetin. Nine healthy ileostomy subjects were studied, to avoid losses caused by colonic bacteria. They followed a quercetin-free diet for 12 days; on days 4, 8 and 12 they received a supplement of fried onions at breakfast (rich in quercetin glucosides) equivalent to 89 mg aglycone, pure quercetin rutinoside (the major quercetin compound in tea) equivalent to 100 mg aglycone, or 100 mg pure quercetin aglycone, in random order. Subsequently, participants collected ileostomy effluent and urine for 13 hours. *In vitro* incubations of quercetin or its glycosides with gastrointestinal fluids showed minimal degradation. Absorption of quercetin, defined as oral intake minus ileostomy excretion and corrected for 14% degradation within the ileostomy bag, was 52 ± 15% for quercetin glucosides from onions, 17 ± 15% for quercetin rutinoside, and 24 ± 9% for quercetin aglycone. Mean excretion of quercetin or its conjugates in urine was 0.5% of the amount absorbed; quercetin excretion in urine was negatively correlated with excretion in ileostomy effluent ($r = -0.78$, $n = 27$). We conclude that humans absorb appreciable amounts of quercetin and that absorption is enhanced by conjugation with glucose.

INTRODUCTION

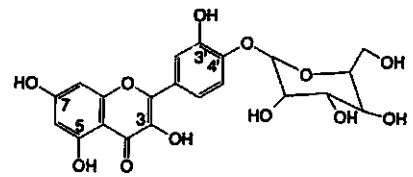
Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. Flavonoids are categorized into flavonols, flavones, catechins, flavanones and anthocyanidins.[1] Quercetin (*Figure 1*), the major representative of the flavonol subclass, is a strong antioxidant,[2] and prevents oxidation of low-density lipoproteins *in vitro*.[3] Oxidised low-density lipoproteins are atherogenic and are considered to be a crucial intermediate in the formation of atherosclerotic plaques.[4] This agrees with our observation that the intake of flavonols and flavones was inversely associated with subsequent coronary heart disease in both the Zutphen Elderly Study,[5] a prospective cohort study, and in the Seven Countries Study,[6] a cross-cultural study.



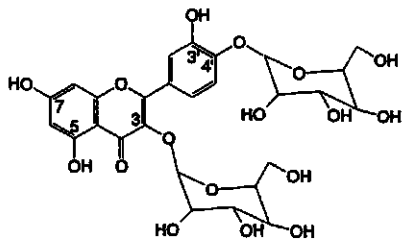
Quercetin aglycone (I)



Quercetin-3-*O*-ß-rutinoside (IV)



Quercetin-4'-*O*-ß-D-glucoside (II)



Quercetin-3,4'-*O*-bis-ß-D-glucoside (III)

Figure 1. *Structure of quercetin aglycone (**I**) and quercetin glycosides; **II** and **III** are the major species in onions,[16,17] and **IV** is the major species in tea.[18]*

The average dietary intake of quercetin in the Netherlands is 16 mg/day,[7] which is similar to that of vitamin E (7 - 10 mg/day), ß-carotene (2 - 3 mg/day), and vitamin C (70 - 100 mg/day).[8] However, the extent of absorption of flavonoids is an important unsolved problem in judging their many alleged health effects.[9] Indeed, it is often stated that flavonoids present in foods cannot be absorbed from the intestine because they are bound to sugars as glycosides.[1] Only free flavonoids without a sugar molecule, the so-called aglycones, are considered to be able to pass through the gut wall, and no enzymes that can split these predominantly ß-glycosidic bonds are secreted into the gut or present in the intestinal wall.[1,10] Hydrolysis only occurs in the colon by microorganisms, which at the same time degrade flavonoids.[1] On the other hand, it was shown in one human study that the aglycone quercetin was not absorbed either.[11] Nieder[12] suggested that flavonol glycosides from *Ginkgo biloba* were absorbed in human subjects, but no information on the extent of absorption was given.

A major problem in studying the absorption of quercetin in humans is its degradation by microorganisms in the colon. For that reason measurement of faecal excretion in normal human subjects would lead to an overestimate of the amount absorbed. We therefore studied quercetin absorption in healthy ileostomy subjects with complete small intestines. Ileostomy subjects with minimal ileal resection were successfully employed previously to determine absorption of minerals and trace elements,[13] dietary starch and nonstarch polysaccharides,[14] and cholesterol.[15]

The present study was designed to determine absorption of quercetin from onions and of a major glycoside from tea, because tea and onions are the main dietary sources besides wine.[7] Onions contain mainly quercetin glucosides, [16,17]whereas quercetin rutinoside predominates in tea[18] (*Figure 1*). Quercetin aglycone, i.e. free quercetin with no sugar attached, was included as a model compound.

SUBJECTS AND METHODS

Subjects

Ileostomy subjects have had their large intestine completely removed and the terminal ileum brought out onto the anterior abdominal wall as a fistula. We recruited nine subjects (five females, four males), mean age 45 (range 22-62) years, mean body mass index 26 (range 21-33) kg/m^2. All had had total colectomies for

ulcerative colitis or polyposis coli on average 13 (range 1 - 26) years ago. In the operation an average of 8 (range 0 - 40) cm of the terminal ileum had been removed. None of the subjects had evidence of Crohn's disease or malabsorption, and all had well-functioning ileostomies. Subjects were not hospitalized, and were leading normal active lives. One subject was a psychologist, one was a clerk, one was a fruit farmer, one was a cleaner, one was a student, one was a retired civil engineer, and the other three were housewives. They were all judged healthy by a gastroenterologist on the basis of a medical questionnaire and had no record of gastric surgery. All subjects were unmedicated.

The protocol was approved by the Nijmegen University Hospital Ethical Committee and was fully explained to the participants, who gave their written informed consent.

Study design, foods, and supplements

Subjects followed a quercetin-free diet for 12 days. On days 4, 8, and 12 we fed them three different quercetin-containing supplements in random order, at breakfast at the Department between 7:45 and 9:30 am. After the quercetin-rich breakfast, participants collected ileostomy effluent and urine for 13 hours. Absorption was calculated as the difference between the amount of quercetin in the supplements and in the subsequent ileostomy effluent.

To ensure a quercetin-free diet, participants were given a list of vegetables and fruits containing more than 15 mg quercetin/kg and of beverages with more than 4 mg quercetin/L,[19,20] and were instructed not to consume any of them. Because proteins are known to bind polyphenols,[21] the quercetin-supplemented breakfasts were low in protein; they consisted of protein-free bread, margarine, jams made from quercetin-free fruits and other sweets such as chocolate sprinkles, coffee without milk, quercetin-free soft drinks, and mineral water. We fried 333 g yellow onions with 20 g margarine, 15 g tomato ketchup and 1 g Italian herbs; 150 g of this dish, corresponding to 215 g raw yellow onions, constituted the onion supplement. It contained 89 $\pm$ 14 mg quercetin (n = 9) as determined by HPLC.[22] For the two other breakfasts 220 mg quercetin-3-*O*-ß-rutinoside (Rutosidum DAB, #339994; OPG Farma, Utrecht, The Netherlands), equivalent to 100 mg aglycone, or 112 mg quercetin-dihydrate (#Q-0125; Sigma, St Louis), equivalent to 100 mg aglycone, were administered as capsules. The capsules also contained 80 mg *para*-aminobenzoic acid (#361334; OPG Farma). *Para*-aminobenzoic acid is completely

absorbed and excreted with urine in humans.[23] The onion breakfast was supplemented with a capsule containing 80 mg *para*-aminobenzoic acid. Subjects also ingested 25 radioopaque barium-salt-impregnated plastic ringlets (outer diameter 3 mm) as a recovery marker. Subjects were instructed not to eat anything and to drink only water or coffee without milk after the experimental breakfasts until lunch.

Energy and nutrient intakes were calculated using the Dutch food composition table.[24] The breakfasts provided 1.52 ± 0.61 MJ (362 ± 145 kcal), with protein accounting for 2.0 ± 1.6% of energy, fat for 41.2 ± 12.6%, and carbohydrates for 56.4 ± 13.2%. The onion breakfast provided 3.8 ± 1.3% of energy from protein and the other breakfasts provided 1.0 ± 0.5%; no differences were found for fat and carbohydrates between the three breakfasts supplied. Average energy intake on days 3, 7, and 11, according to 24-h dietary recalls, was 11.26 ± 2.72 MJ, of which protein provided 14.6 ± 3.7% of energy, fat 37.7 ± 5.9%, and carbohydrates 47.2 ± 5.3%, with no differences between breakfast periods.

Collection of samples

After the quercetin-rich breakfast, subjects returned home or went to work and collected urine and stoma effluent until they went to bed between 10:00 and 11:45 pm. On average, effluent and urine were collected for 13.4 ± 0.7 hours. Subjects changed the ileostomy bags every 2 to 5 hours (on average, 3.5 ± 1.5 hours) according to their normal routine and immediately stored the bags in a polystyrene box containing dry ice. They collected urine in plastic bottles containing 0.1 g thimerosal (#T-5125; Sigma) and stored each bottle in dry ice immediately after voiding. Three of the subjects (#1, #2, and #8) collected urine every two hours, which allowed us to study the rate of excretion of quercetin.

Sample preparation

The filled plastic ileostomy bags were kept frozen with liquid nitrogen, the bags were removed, and the frozen contents were freeze-dried, ground to pass through an 0.5 mm sieve, and stored at -20 °C until analyzed less than 21 weeks later. Urine samples were thawed in a water bath at 40 °C and mixed, and aliquots were taken within 30 min, frozen with liquid nitrogen, and stored at -40 °C until analyzed less than 7 weeks later.

Samples collected before breakfast (prebreakfast sample) and the final collection at the end of the day (final sample) were prepared separately, as were all samples from the three subjects who collected urine every two hours. The other samples were pooled by subject and treatment day and thoroughly homogenised.

Incubation of quercetin supplements with gastrointestinal fluids *in vitro*

Amounts of raw onions, quercetin-3-rutinoside, and quercetin corresponding to 3 mg quercetin aglycone were incubated with 3 mL human gastric juice[25] and 9 mL water at 37 °C for 0.5 and 2 hours. This mimicked stomach contents after the experimental breakfasts.[26]

Similar amounts were also incubated with 1.5 mL human duodenal fluid[27] and 9 mL water at 37 °C for 1 and 4 hours, corresponding to the average and maximal transit time in the small intestine, respectively.[28]

The stability of quercetin in ileostomy fluid was studied as follows. About 6 months after the experiments, three of the volunteers followed a quercetin-free diet for two days. At noon of the second day, they applied an ileostomy bag containing either 30 mg quercetin aglycone emulsified with 6.7 g margarine or 50 g finely ground fried onions prepared as described. Subjects allowed ileostomy fluid to drain into the bag for 3 - 4 hours and kneaded the contents occasionally. The contents were then stored and studied as described above.

Analytical methods

Quercetin glycosides and glucuronides were simultaneously extracted and hydrolysed to the aglycone by using 2 M HCl in aqueous methanol. By varying acid concentration and the duration of extraction and hydrolysis the following procedure was found to be optimal for urine: 12.5 mL methanol containing 2 g *tert*-butyl hydroxyquinone/L and 5 mL 10 M HCl were added to 7.5 g urine followed by mixing, the extract was refluxed at 90 °C for two hours with regular swirling, allowed to cool and subsequently brought to 50 mL with methanol. For ileostomy effluent, 40 mL 62.5% (%) aqueous methanol containing 2 g *tert*-butyl hydroxyquinone/L and 10 mL 10 M HCl were added to 0.500 g freeze-dried effluent and then mixed. The extract was refluxed at 90 °C for two hours with regular swirling, allowed to cool and subsequently brought to 100 mL with methanol. Urine and effluent extracts were sonicated for 5 min and filtered through a 0.45 μm filter for

organic solvents (Acrodisc CR PTFE; Gelman Sciences, Ann Arbor, MI) before HPLC analysis. We injected 10 μL onto an Inertsil ODS-2 (GL Sciences Inc, Tokyo) column (4.6 x 150 mm, 5μm particle size) protected by an MPLC Newguard RP-18 (Brownlee; Applied Biosystems Inc, San Jose, CA) column (3.2 x 15 mm, 7 μm particle size) by using acetonitrile:0.025 M phosphate buffer pH 2.4 (31:69 (%$_v$)) as the mobile phase, at a flow rate of 1 mL/min. The columns were placed in a column oven set at 30 °C. The eluent was mixed with 0.4 mL/min 1.5 M $Al(NO_3)_3$ in methanol containing 7.5% (%$_v$) acetic acid in a postcolumn stainless steel reaction coil (0.25 mm x 15 m) placed in the column oven. The fluorescence of the ensuing quercetin-metal complex was measured at 490 nm with a Merck Hitachi F-1000 (Tokyo) fluorescence detector with the excitation wavelength set at 400 nm. Further details were described elsewhere.[22]

The limit of detection, i.e. the concentration producing a peak height three times the standard deviation of the baseline noise, was 5 ng/g for urine and 2 μg/g for ileostomy effluent. Recovery of quercetin in onion extract, of pure quercetin-3-rutinoside, and of quercetin aglycone added at a quercetin concentration of 800 μg/g to freeze-dried ileostomy effluent free of quercetin was 91.6 $\pm$ 3.0%, 91.6 $\pm$ 0.3%, and 90.0 $\pm$ 0.1% respectively (n = 2). Addition of 0.5 μg quercetin aglycone per gram of urine yielded a recovery of 98.7 $\pm$ 8% (n = 3).

All determinations were carried out in duplicate. We included a control sample of freeze-dried effluent in each series of analyses; all values were within 868 $\pm$ 103 μg/g (mean $\pm$ 2 SD, n = 18). For urine analyses a urine sample of the previous series was always included. The between-series coefficient of variation was 4% (n = 11).

Quercetin absorption was calculated as the difference between the amount in the supplements and in the ileostomy effluent corrected for 9% analytical losses plus 5% degradation within the ileostomy bag (*see Results*).

Para-aminobenzoic acid was determined photometrically by using fluorescamine (#F-9015; Sigma) after hydrolysis with 0.1 M HCl for 40 min in a boiling water bath.[29] Addition of 0.15 mg *para*-aminobenzoic acid per gram of urine yielded a recovery of 94.2 $\pm$ 2% (n = 6). A urine control sample was included in each series of analysis; values were within 66.8 $\pm$ 3.1 mg (mean $\pm$ 2 SD, n = 14).

Statistical analysis

Because the amounts of quercetin excreted were expected to follow a log-normal distribution, values as proportions of intake were first converted to $\log_{10}$ values. The Shapiro-Wilk test for normality did not give evidence for nonnormality. Differences between treatments were tested by analysis of variance using the Statistical Package for Social Sciences, SPSS/PC+ (SPSS Inc, Chicago) with subject, type of breakfast and previous type of breakfast as independent variables. The significance of differences was determined by paired *t* test.

RESULTS

Stability of quercetin and glycosides in gastrointestinal fluids

Quercetin aglycone and glycosides were stable *in vitro* in gastric juice for at least 2 hours and in duodenal fluid for at least 4 hours (*Table 1*).

Incubation with ileostomy fluid for 3.25 hours yielded a recovery of 86% (*Table 1*). The analytical recovery of quercetin added to freeze-dried ileostomy fluid was 91% (*see Methods*); therefore, the loss through degradation in an ileostomy bag carried on the body for 3.25 hours was about 5%.

Compliance with the quercetin-free diet

The average quercetin intake from regular foods on days 3, 7 and 11 according to 24-h dietary recalls was 1.1 $\pm$ 1.4 mg. No difference in quercetin intake was observed between the three 4-day periods. Quercetin excretion in prebreakfast effluent samples was on average 3% of the total daily amount (*Table 2*) and on average 2% in prebreakfast urine (*Table 3*).

Excretion of quercetin

The total amount of quercetin excreted in ileostomy effluent (*Figure 2*) was highly dependent on the type of supplement ($P < 0.01$). After correction for 14% analytical losses plus degradation during time in the ileostomy bag, average absorption was 52% for quercetin from onions, 17% for quercetin-3-rutinoside, and

24% for the pure aglycone (*Table 2*). No significant relation with the subject or with the supplement given in the previous period was found. Excretion of quercetin in urine was also significantly higher for quercetin from onions than for the aglycone, which was again higher than that for the rutinoside (*Table 3*). Again, no statistically significant relation with the subject or the supplement of the previous period was found. Still, one subject, depicted by ● in *Figure 2*, did excrete markedly less quercetin in ileostomy effluent after consumption of the quercetin-3-rutinoside and much more in urine than did the other subjects.

Table 1. *Stability of quercetin recovered from various sources during incubation with human gastric juice or duodenal fluid in vitro, or ileostomy effluent ex vivo.*

	Gastric juice[¶]		Duodenal fluid[¶]		Ileostomy effluent[§]
Source	0.5 h	2 h	1 h	4 h	3.25 ± 0.6 h
	(%)	*(%)*	*(%)*	*(%)*	*(%)*
Onions	95.8	95.2	98.2	97.4	86.8 ± 4.5
Quercetin-3-rutinoside	91.9	92.7	95.7	94.7	-
Quercetin aglycone	94.9	88.7	98.8	108.4	85.9 ± 2.1

[¶] mean of duplicate determinations.

[§] mean ± SD of incubations in ileostomy bags on the bodies of three volunteers. Analytical recovery after additions to freeze-dried effluent averaged 91.1 ± 2.1% (n = 6).

- not determined.

Collection of effluent and urine

Out of 25 radioopaque ringlets swallowed together with each supplement, on average 21 ± 8 were found after consumption of the onions, 17 ± 9 after the quercetin rutinoside, and 24 ± 2 after the quercetin aglycone supplements. On 7 of 27 person-days fewer than 22 of the 25 radioopaque ringlets ingested were recovered in the effluent. In the effluent of one subject no ringlets were found at all after two of the breakfasts but quercetin excretion in this subject was similar to that

in the other subjects, indicating that all of the effluent was probably collected. A mechanical barrier in the connection between ileum and ileostomy bag may have caused the ringlets to be lost.

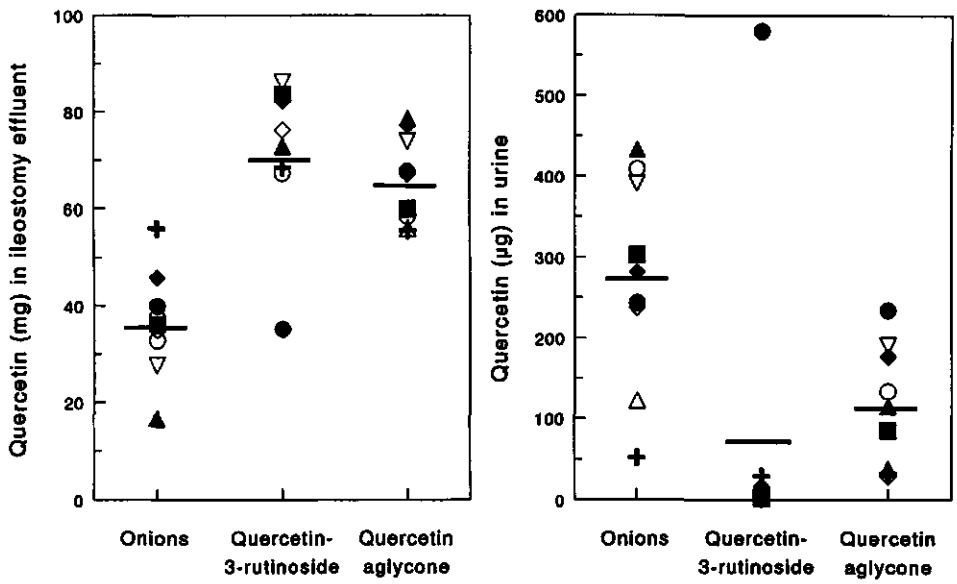


Figure 2. *Total amount of quercetin (mg) excreted in daytime ileostomy effluent, and total amount of quercetin (µg) excreted in daytime urine of nine subjects after consumption of various supplements. Each subject is depicted by a different symbol. Horizontal bars depict means.*

The final samples of effluent, collected just before bedtime, were analyzed separately. They contained on average 6% of the total amount of quercetin excreted after the onions and the aglycone breakfast and 15% after the quercetin-3-rutinoside breakfast (*Table 2*). This mean of 15% was caused by one subject, who excreted 88% of the total amount in this final sample. His ringlet recovery was only 7 of 25, which also indicated a long transit time. Thus the total amount of quercetin excreted in effluent after rutinoside by this subject may have been even higher than the 86 mg recovered in 13 hours, and absorption correspondingly lower.

Urinary recovery of *para*-aminobenzoic acid was 85.5 ± 11.6%. Two

volunteers showed recoveries of *para*-aminobenzoic acid between 64% and 82% for all treatments, but their urinary quercetin output was above average, which speaks against lack of compliance in collecting urine. The final sample of urine, collected just before bedtime, contributed 3% on average to the total daily output of quercetin (*Table 3*), which indicated that the peak of urinary quercetin excretion lay well within the 13-hours period.

Table 2. *Intake of quercetin at breakfast and subsequent mean cumulative excretion in ileostomy effluent over 13 hours*[1]

Supplement (to breakfast)	Intake in terms of aglycone	Excretion in ileostomy effluent			Absorption[§]
		Pre-breakfast sample	Total[♦]	Final (prebedtime) sample	
	(mg)	*(mg)*	*(mg)*	*(mg)*	*(%)*
Onions (n = 9)	89 ± 14	1.8 ± 1.1	37 ± 11	2.9 ± 3.3	52 ± 15
Quercetin-3-rutinoside (n = 9)	100 ± 5	1.3 ± 0.9	72 ± 15	11 ± 25	17 ± 15
Quercetin aglycone (n = 9)	100 ± 5	1.7 ± 1.3	66 ± 9	3.4 ± 5.5	24 ± 9

[1] Mean ± SD. Total excretion as proportion of intake differed significantly ($P < 0.02$) among all three supplements after rejection of the outlying quercetin rutinoside results of subject #4 (● in *Figure 2*).

[♦] Includes the final but not the prebreakfast sample.

[§] Corrected for 9% analytical loss plus 5% degradation within the ileostomy bag.

Rate of urinary quercetin excretion

The three subjects (#1, #2, and #8) who had collected urine every two hours (*Figure 3*) reached 90% of their cumulative excretion within 5.6 ± 0.3 hours after the onion supplement, and within 7.8 ± 1.3 hours after administration of quercetin aglycone. The rate of urinary quercetin excretion was significantly higher ($P < 0.05$) after the onion supplement. Administration of quercetin-3-rutinoside did not yield measurable amounts of quercetin in urine in these three subjects.

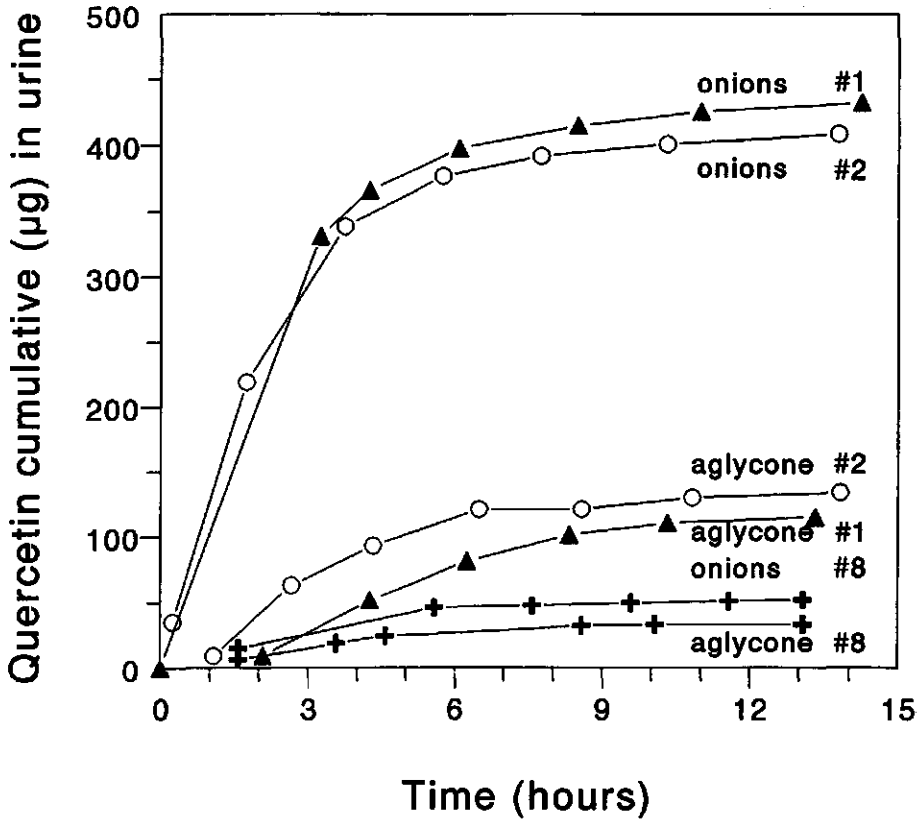


Figure 3. *Cumulative amount of quercetin (µg) excreted in urine of subjects #1, #2, and #8 after consumption of onions and quercetin aglycone. Each subject is depicted by a different symbol. Time course of quercetin excretion after quercetin rutinoside is not shown because no measurable amounts were found.*

Table 3. *Intake of quercetin at breakfast and subsequent mean cumulative excretion of quercetin in urine over 13 hours*[1].

Supplement (to breakfast)	Intake in terms of aglycone	Excretion in urine: Pre-breakfast sample	Excretion in urine: Total[♦]	Excretion in urine: Final (pre-bedtime) sample	Total excretion as proportion of Intake
	(mg)	*(μg)*	*(μg)*	*(μg)*	*(%)*
Onions (n = 9)	89 ± 14	1.2 ± 1.9	275 ± 129	5.7 ± 3.5	0.31 ± 0.14
Quercetin-3-rutinoside (n = 9)	100 ± 5	3.6 ± 5.1	73 ± 190	1.7 ± 2.5	0.07 ± 0.19
Quercetin aglycone (n = 9)	100 ± 5	1.0 ± 1.1	115 ± 75	4.4 ± 6.0	0.12 ± 0.08

[1] Mean ± SD. Total excretion as proportion of intake was significantly different for all three supplements ($P < 0.02$).

[♦] Includes the final but not the prebreakfast sample.

DISCUSSION

We found that significant amounts of the quercetin glucosides present in onions and, to a lesser extent, of pure quercetin aglycone are absorbed by the human small intestine. This contradicts the widely held view that dietary flavonoids are poorly absorbed in humans and that the glycosides present in foods are especially poorly absorbed.[1,10] Absorption amounted to 52% for onions, 17% for quercetin-3-rutinoside, and 24% for quercetin aglycone. True absorption could be even higher if absorbed flavonoids are reexcreted with bile as was found in rats.[10,30] However, no data on reexcretion of flavonoids in human studies are available.

Validity of the ileostomy model

We measured absorption as the difference between ingestion and excretion in healthy volunteers who lacked a colon. Jejunoileal absorption in such subjects is

probably equivalent to that in normal subjects with an intact colon, as indicated by their normal serum cholesterol concentrations[15] and absorption of *para*-aminobenzoic acid. Excluding the data of two subjects in whom recovery of *para*-aminobenzoic acid was low did not alter the results or conclusions.

It is unlikely that any quercetin disappeared through degradation in the stomach or duodenum because *in vitro* incubations with gastric juice or duodenal fluid mimicking normal conditions showed no loss of quercetin. Incubation of onions and quercetin in ileostomy fluid for 3 h produced an apparent loss of 14%. Some 9% of this was in fact due to analytical losses as shown by the *in vitro* recovery experiments (*Methods*). Thus breakdown in the ileostomy effluent itself was only some 5%. Degradation of sterols by microorganisms in ileostomy fluid was previously also reported to be small.[15]

Incomplete collection of ileostomy effluent by the volunteers is also unlikely in view of the high recoveries of the nonabsorbable marker and of the quercetin when fed as quercetin rutinoside.

Quercetin excretion in urine was strongly and negatively correlated with excretion in ileostomy effluent (*Figure 4*). This again suggests that low output of quercetin in stoma effluent was truly due to high absorption.

Comparison with previous studies

Our results show that quercetin glucosides of onions are better absorbed than is the aglycone. Absorption of glycosides was also suggested by Nieder.[12] However, no information about the nature of the glycosides was available.

Gugler et al.[11] found no quercetin in urine or plasma after oral administration of 4 g quercetin aglycone to humans and concluded that less than 1% could have been absorbed. The high limit of detection and the high dose could account for the difference in absorption (1% versus 24%) with the present study. Ueno et al.[30] found that at least 20% of orally administered ^{14}C labelled quercetin aglycone was absorbed in rats. The present study agrees with those results.

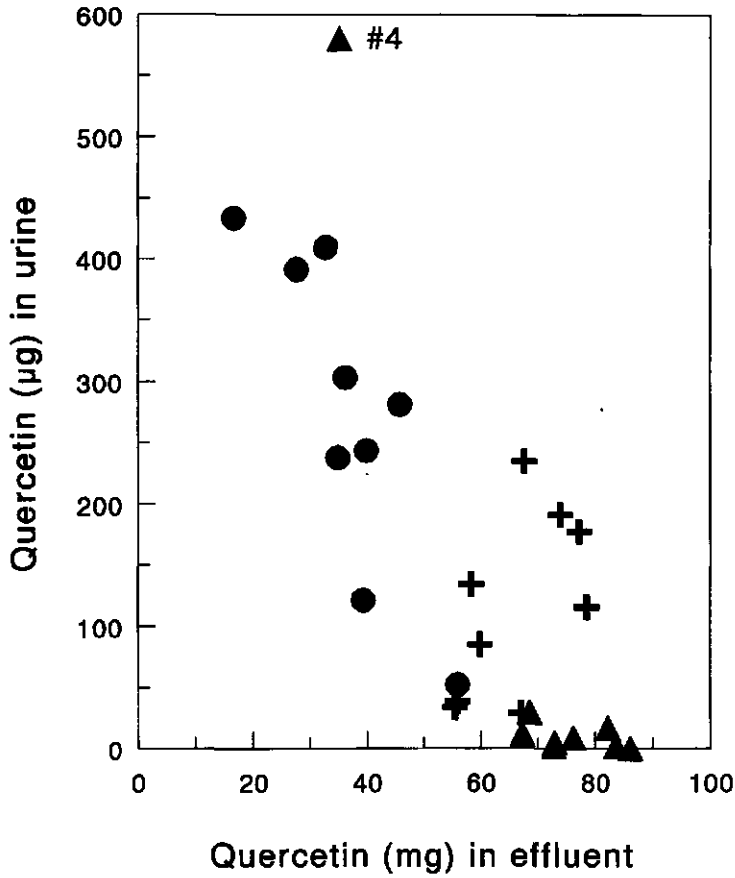


Figure 4. *Correlation between total quercetin excreted in urine and in ileostomy effluent in nine subjects who consumed three supplements in random order; r = -0.78, n = 27*

●, *onions;* ▲, *quercetin-3-rutinoside;* +, *quercetin aglycone.*

Metabolism of quercetin

Figure 3 suggests that the rate of urinary excretion of quercetin was higher for the glucoside from onions than for quercetin aglycone, because the time to reach 90%

of the cumulative excretion was about 2 hours shorter for the glucoside. This could be explained by more rapid absorption of the glucoside, assuming the rate of elimination of the glucoside and aglycone are the same.

After the onion breakfast 41.1% was recovered in ileostomy fluid and 0.3% in urine. Thus, 58.6% went undetected, 2.5% of which may have been degraded in the ileostomy bag and another 3.3% was lost during sample preparation. Like many other compounds,[31] absorbed quercetin is probably extensively modified before being excreted by the kidneys. Our assay would pick up quercetin glucuronides and similar conjugates, but O-methylated quercetin, a hepatic metabolite in rats,[10,30] would escape detection and so would metabolites in which the ring structure itself is altered. In addition to the formation of such undetectable metabolites an acute high dose such as was given here might also be partly stored and released slowly over subsequent days.[32]

Mechanisms of absorption

Quercetin glucosides from onions need to be liberated from the food matrix before being absorbed, whereas absorption of quercetin aglycone and quercetin rutinoside from the gut would probably be greater because these were administered as powders. Poor solubility of quercetin rutinoside does not seem to be a major factor because it was well absorbed in subject #4. Thus there is a predominant effect of the sugar moiety on the absorption of quercetin. We speculate that intestinal sugar carriers may play a role in flavonoid absorption. Model studies by Mizuma et al.[33] on the absorption of naphthol glycosides in everted small intestines of rats support such a mechanism. Absorption was higher for naphthol glucoside than for the galactoside, and higher for the ß-anomer than for the α-anomer; also the absorption of these glycosides was inhibited by the absence of Na^+ which is needed for active Na^+/glucose cotransport, and by the inhibitor of glucose transport phloridzin. Such active transport of ß-glucosides of foreign compounds by the glucose transporter offers a possible explanation for the high absorption of quercetin from onions, in which it is present as ß-D-glucosides. The quercetin group might thus be drawn into the enterocyte by its glucose moiety, which is transported by the glucose carrier. The aglycone (i.e. free quercetin) would then fail to be absorbed because it lacks a sugar. However, experiments are needed to study the role of the active Na+/glucose cotransporter in the absorption of quercetin glucosides. The poor absorption of quercetin rutinoside is puzzling, especially in view of indications that

diosmin, the rutinoside of the flavone diosmetin, is absorbed in humans after oral administration.[34] Studies of the absorption of rutinose itself and of various rutinosides are required to solve this discrepancy.

In contrast with the other eight subjects, subject #4 showed low ileostomy and high urinary excretion after consumption of the quercetin-3-rutinoside (*Figure 4*). This may be due to a variant type of intestinal physiology; further studies of such subjects might yield clues to the mechanism of absorption of flavonoids. Rutinose is a disaccharide consisting of glucose and rhamnose (*Figure 1*). Possibly subject #4 has a ß-glycosidase in the small intestine that splits off rhamnose and transforms the rutinoside into a well-absorbable glucoside.

Thus quercetin glucoside as present in onions is absorbed efficiently in humans. If the glucose transporter is involved in this then quercetin will enter the blood stream as the glucoside; this might affect its distribution, metabolism and excretion. Quantitative data for separate quercetin glycosides in foods are needed for evaluation of the extent of absorption of quercetin from other foods such as tea.

Acknowledgments

The authors thank the volunteers for their willingness, enthusiasm and effort; JGAJ Hautvast for his suggestion to study ileostomy subjects; F Nagengast, GP van Berge Henegouwen, R Mees, and N van Vliet for recruitment and selection of subjects; S van Gend, E te Brinke, J Kanis, P v.d. Bovenkamp, J Bos, J van Trijp, D Venema, B van de Putte, M Sins, and J Lenting for technical assistance; JBMJ Jansen and M Maas, for gastric juice and duodenal fluid; and H van der Voet for assistance in the statistical evaluation.

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5

ABSORPTION and DISPOSITION KINETICS of the DIETARY ANTIOXIDANT QUERCETIN in MAN

Hollman PCH, van der Gaag MS, Mengelers MJ, van Trijp JM, de Vries JHM, Katan MB

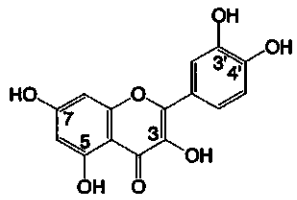
Free Radical Biology & Medicine 1996;**21**:703-707

Abstract

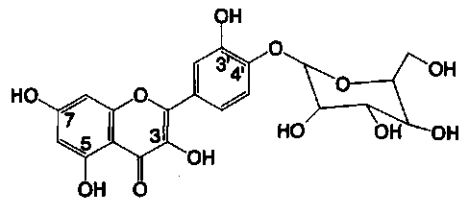
Quercetin is a dietary antioxidant that prevents oxidation of low density lipoproteins *in vitro* by scavenging of free oxygen radicals. Its intake was inversely associated with coronary heart disease mortality in Dutch elderly men. However, data on absorption of quercetin in man are scarce and contradictory. We studied the time course of the plasma quercetin concentration in two subjects after ingestion of fried onions containing quercetin glucosides equivalent to 64 mg of quercetin aglycone. Peak plasma levels of 196 ng/ml were reached after 2.9 h, with a half-life of absorption of 0.87 h. The half-life of the distribution phase was 3.8 h, and of the subsequent elimination phase 16.8 h. After 48 h the plasma concentration was about 10 ng/ml. We conclude that quercetin glucosides from onions are absorbed and are eliminated slowly throughout the day. Thus, the dietary antioxidant quercetin could increase the antioxidant capacity of blood plasma.

INTRODUCTION

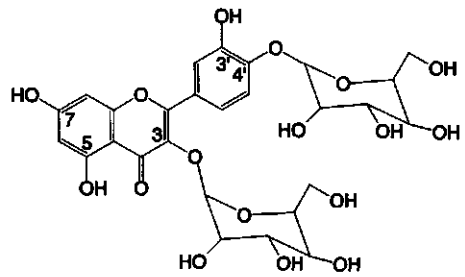
Flavonoids (*Figure 1*) are polyphenolic compounds that occur ubiquitously in foods of plant origin. Flavonoids have a variety of biological effects in numerous mammalian cell systems, *in vitro* as well *in vivo*.[1] Recently much attention has been paid to their antioxidant properties that affect oxygen free radicals and lipid peroxidation. Oxygen free radicals and lipid peroxidation might be involved in several pathological conditions such as atherosclerosis, cancer, and chronic inflammation.[2] The antioxidative and lipid peroxidation inhibiting potential of flavonoids predominantly resides in their radical-scavenging capacity.[3,4] Most flavonoids are effective radical scavengers.[5-7]



Quercetin aglycone (I)



Quercetin-4'-O-ß-D-glucoside (II)



Quercetin-3',4'-O-bis-ß-D-glucoside (III)

Figure 1. *Structure of quercetin aglycone (**I**) and quercetin glucosides: **II** and **III** are the major species in onions*[15,16]

Quercetin, the major representative of the flavonol subclass of flavonoids, is a strong antioxidant.[8] It prevents oxidation of low density lipoproteins *in vitro*,[9] and may therefore contribute to the prevention of atherosclerosis.[10] Indeed, we found that the intake of flavonols and flavones was inversely associated with subsequent coronary heart disease both in the Zutphen Elderly Study,[11] a prospective cohort study, and in the Seven Countries Study,[12] a cross-cultural study. The average dietary intake of quercetin in the Netherlands is 16 mg/day,[13] mainly originating from tea, onions and apples. In US men,[14] onions, together with tea, are the major sources. Onions contain mainly quercetin-4'-*O*-ß-D-glucoside and quercetin-3,4'-*O*-bis-ß-D-glucoside[15,16] (*Figure 1*).

In judging a potential health effect of quercetin, its absorption is an important unsolved problem. Because flavonoids in foods are only present bound to sugars - mainly as ß-glycosides - they are thought to be non-absorbable from the intestine.[17] In one human study quercetin without its sugar moiety (the aglycone) was not absorbed either.[18] Nieder[19] found that flavonol glycosides from *Ginkgo biloba* were absorbed in human subjects, but no information on the administered amount and type of glycosides was given. We now report results of a pilot study on the absorption of quercetin from onions in humans.

MATERIALS AND METHODS

Subjects

We recruited two subjects (1 female, 1 male), ages 22 and 43 years, and Body Mass Index 20.9 and 23.7 kg/m^2, respectively. They were both healthy based on a medical questionnaire. Their values for blood haematocrit and for leucocyte, platelet, and erythrocyte counts, mean erythrocyte cell volume, haemoglobin, and mean corpuscular haemoglobin concentration were normal. Subjects did not use any medication. The protocol was approved by the Wageningen University Ethical Committee, and was fully explained to the participants, who gave their written informed consent.

Study design, foods, and supplements

Subjects followed a quercetin-free diet for 5 days. They were given a list of those

vegetables and fruits that contain more than 15 mg/kg of quercetin and of beverages with more than 4 mg/l of quercetin,[20,21] and were instructed not to consume any of these products. On day 4 at 8:00 am, a breakfast supplemented with freshly fried onions was served. As proteins are known to bind polyphenols,[22] the breakfast was low in protein; we provided protein-free bread, margarine, jams made from quercetin-free fruits, other sweets such as chocolate sprinkles, coffee without milk, quercetin-free soft drinks, and mineral water. We fried 333 g of yellow onions with 20 g of margarine, 15 g of tomato ketchup, and 1 g of Italian herbs; 150 g of this dish, corresponding to 215 g of raw yellow onions, was added to the breakfast. Analysis of duplicate portions[23] showed that the onions provided 64.2 mg of quercetin expressed as aglycone. Subjects were instructed not to eat anything and to drink only water or coffee without milk after the experimental breakfasts up until lunch.

Collection of plasma samples

Venous blood samples were taken into vacuum tubes containing EDTA before breakfast (0 h), and at the time intervals depicted in *Figure 3*; zero time was 10 min after the start of the breakfast. Plasma was prepared within 15 min by centrifugation at 20 °C for 10 minutes at 2000 g; it was stored at -80°C until analysis less than 5 weeks later.

Determination of quercetin in plasma

Quercetin, quercetin glycosides, glucuronides, and sulfates were simultaneously extracted and hydrolysed to the aglycone using 2 M HCl in aqueous methanol.[23] In a 4 ml vial (#WAT022468, Waters, Milford, MA, USA), 1000 µl of methanol containing 2 g/l *tert*-butyl hydroxyquinone (TBHQ) and 400 µl of 10 M HCl were added to 600 µl of plasma and mixed. The vial was sealed tightly with a cap (#72711, Waters, Milford, MA, USA) and septum (#73008, Waters, Milford, MA, USA), inserted into a preheated aluminum block (Reacti-Block C-1, Pierce Europe, Oud-Beijerland, The Netherlands), heated in an oven at 90 °C for two hours, allowed to cool, and centrifuged at 1000 g for 15 min after addition of 2000 µl of methanol containing 2 g/l TBHQ. For HPLC analysis we injected 20 µl of the upper layer onto an Inertsil ODS-2 column (4.6 x 150 mm, 5µm; GL Sciences Inc., Tokyo, Japan) protected by an MPLC Newguard RP-18 column (3.2 x 15 mm, 7 µm; Brownlee,

Applied Biosystems Inc., Foster City, CA, USA) using acetonitrile/methanol/0.025 M phosphate buffer pH 2.4, 10:38:52 (v/v/v) as mobile phase, at a flow rate of 1 ml/min. The columns were placed in a column oven set at 30 °C. The column effluent was mixed with 0.4 ml/min 1.5 M $Al(NO_3)_3$ in methanol containing 7.5% (v/v) acetic acid in a post-column stainless steel reaction coil (0.25 mm x 15 m) placed in the column oven. The fluorescence of the ensuing quercetin-metal complex was measured at 485 nm using a Jasco FP 920 (Jasco Corporation, Japan) fluorescence detector with excitation wavelength set at 422 nm.

Adequate resolution was achieved (*Figure 2*). Addition of 100 ng of quercetin aglycone per ml plasma yielded a recovery of 88 ± 11% (n = 5). All determinations were carried out in duplicate in one series of analyses. The standard deviation of duplicates was 12 ng/ml. The limit of detection, defined as

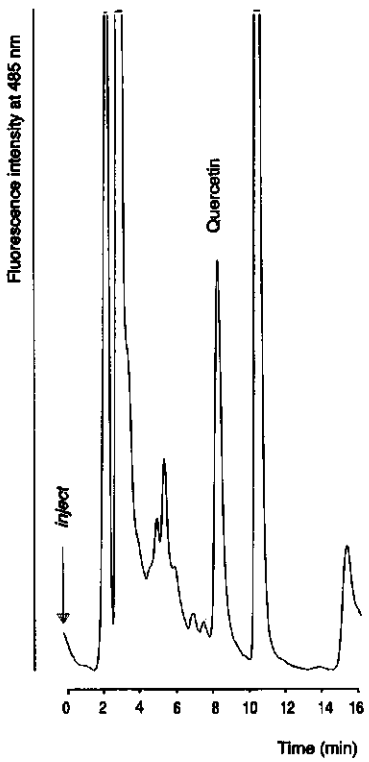


Figure 2. *Chromatogram of quercetin in plasma of subject 1, four hours after ingestion of the onion supplemented breakfast.*

the concentration of quercetin in the sample resulting in a peak height of 3 x the SD of the baseline noise, was 5 ng/ml.

Data analysis

We used the two-compartment open model $C(t) = -C e^{-kt} + A e^{-\alpha t} + B e^{-\beta t}$ to describe the absorption and disposition of quercetin,[24] and calculated the parameters of this model with nonlinear regression analysis using PCNONLIN version 4.0 (SCI Software, ClinTrials Inc., Lexington, KY, USA).

RESULTS AND DISCUSSION

The mean peak plasma level of quercetin was 196 ng/ml. Peak levels were reached 2.9 h after ingestion of the onions (*Figure 3*), with an average half-life of absorption of 0.87 h (*Table 1*). Disposition of quercetin in plasma was biphasic. The half-life of the first phase, the distribution phase, was 3.8 h, and that of the subsequent elimination phase 16.8 h. We could still detect quercetin 48 h after ingestion of the onions; at that time the plasma concentration was about 10 ng/ml.

Nieder[19] found a similar absorption profile after administration of flavonol glycosides from *Ginkgo biloba*, but elimination was more rapid with a half-life of 2 - 4 h. After oral administration of 4 g of quercetin aglycone, Gugler et al.[18] could not detect any quercetin aglycone in plasma, using a limit of detection of 100 ng/ml. Neither quercetin aglycone nor quercetin conjugates were detectable in urine. After intravenous administration of 100 mg of quercetin aglycone Gugler et al.[18] found an elimination half-life of 2.4 h. However, in both studies the plasma concentration was only measured regularly up until 9 - 12 hours after administration. Most likely, the distribution phase was erroneously identified as the elimination phase. After oral administration of diosmin, a glycoside of the flavone diosmetin,[25] the elimination half-life of the aglycone diosmetin was between 26 to 43 hours in human subjects. It was suggested that this relatively slow elimination of diosmetin was caused by enterohepatic recirculation. Biliary excretion of quercetin has also been described in rats.[26] However, no data are available on biliary excretion of quercetin glucuronides in man and their potential subsequent reabsorption from the gut.

The elimination half-life of 17 hours implies that repeated intake of quercetin

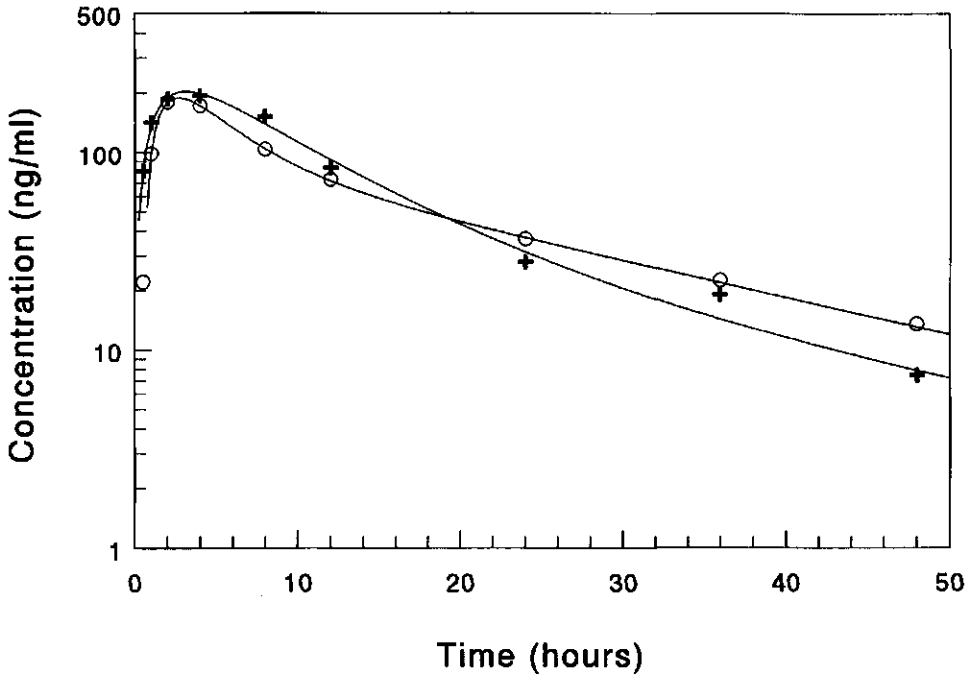


Figure 3. *Quercetin concentration in plasma of two subjects after ingestion of 150 g of fried onions containing quercetin glucosides equivalent to 64 mg of aglycone;* o, *subject 1;* +, *subject 2*

glucosides would lead to a buildup of the concentration in plasma. We have measured here a peak plasma concentration of 200 ng/ml or 0.6 μM after administration of a single high dose of dietary quercetin, equivalent to four times the average Dutch daily intake.[13] Concentrations of the dietary antioxidant ß-carotene in human plasma are similar to this value.[27] Thus, plasma quercetin levels in subjects who regularly eat onions may approach those of ß-carotene.

We have found that quercetin glucosides present in onions are absorbed moderately rapidly, and are eliminated slowly throughout the day. Possibly intestinal sugar carriers play a role.[28] Quercetin could, thus, contribute significantly to the antioxidant defences present in blood plasma. The present data, together with our findings on quercetin excretion in ileostomy volunteers[29] should put to rest any doubts as to whether the dietary antioxidant quercetin is absorbed in man.

Table 1. *Kinetic parameters of quercetin absorption and disposition in two subjects after ingestion of 150 g of fried onions containing quercetin glucosides equivalent to 64 mg of aglycone.* $C(t) = -C e^{-k t} + A e^{-\alpha t} + B e^{-\beta t}$ *was used as a model, where t equals time and C(t) plasma quercetin concentration.*

Parameter		Subject 1	Subject 2	Mean
Absorption				
absorption rate constant, k	(h^{-1})	0.97	0.68	0.83
half-life, $t_{½}$(abs)	(h)	0.71	1.02	0.87
peak level	(ng/ml)	190	204	196
time to reach peak level	(h)	2.7	3.1	2.9
Distribution				
slope, α	(h^{-1})	0.28	0.14	0.21
half-life, $t_{½}$(α)	(h)	2.5	5.1	3.8
Elimination				
slope, ß	(h^{-1})	0.043	0.040	0.042
half-life, $t_{½}$(ß)	(h)	16.3	17.2	16.8

Acknowledgments

This work was supported by grants from the Foundation for Nutrition and Health Research and the Netherlands Heart Foundation (94.128). We thank Joke Barendse for blood sampling.

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6

RELATIVE BIOAVAILABILITY of the ANTIOXIDANT QUERCETIN from VARIOUS FOODS in MAN

Hollman PCH, van Trijp JMP, Buysman MNCP, van der Gaag MS, Mengelers MJB, de Vries JHM, Katan MB

Submitted

Abstract

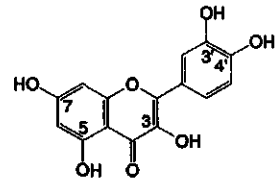
The time course of the plasma quercetin concentration was studied in nine subjects after ingestion of major dietary sources, viz. fried onions containing 225 μmol glucose conjugates of quercetin, equivalent to 68 mg of quercetin aglycone, apples containing 325 μmol of both glucose- and non-glucose quercetin glycosides, and of 331 μmol quercetin-3-rutinoside. Peak plasma levels were 0.75 μmol/L (225 ng/mL) less than 0.7 h after ingestion of the onions, 0.30 μmol/L (90 ng/mL) 2.5 h after the apples, and 0.30 μmol/L (90 ng/mL) 9 h after the rutinoside. Half-lives of elimination were 28 h for onions and 23 h for apples. Bioavailability of both quercetin from apples and of pure quercetin rutinoside was 30% relative to onions. We propose that the sugar moiety of the glycoside is an important determinant of absorption. Repeated consumption of quercetin-containing foods may lead to accumulation of quercetin in blood and increase the antioxidant capacity of blood plasma.

INTRODUCTION

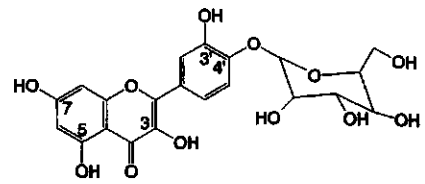
Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin.[1] Flavonoids can function as antioxidants because they scavenge radicals, inhibit lipid peroxidation, and chelate metals.[2] Oxygen free radicals and lipid peroxidation are probably involved in the formation of atherosclerotic plaques, and have also been put forward as intermediates in cancer and chronic inflammation.[3]

Quercetin, the major representative of the flavonoid subclass of flavonols, is a strong antioxidant[4] and prevents oxidation of low density lipoproteins (LDL) in vitro.[5]Oxidised LDL has been found in atherosclerotic lesions of humans,[6] and increased plasma concentrations of autoantibodies against oxidized LDL occur in patients with atherosclerosis.[7,8] Quercetin might therefore contribute to the prevention of atherosclerosis.[9] Indeed, the intake of flavonols and flavones was inversely associated with subsequent coronary heart disease in several prospective studies,[10-13] but not all studies.[14,15] The average dietary intake of quercetin in the Netherlands is 16 mg/day (53 μmol/day),[16] mainly originating from tea, onions and apples.

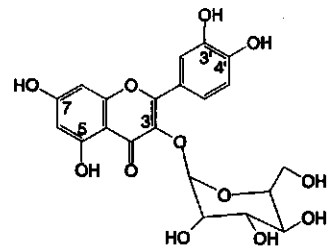
In order to act as an antioxidant, quercetin first has to be absorbed from the diet, and subsequently reach plasma and tissue levels that can contribute to the antioxidant defenses of the human body. Formerly, flavonols were thought to be poorly absorbed from the intestine[1] because in foods they are present bound to sugars, mainly as ß-glycosides which cannot be hydrolysed by pancreatic enzymes. However, we found that human absorption of quercetin-ß- glucosides from onions was 52%, whereas absorption of quercetin without its sugar moiety, the so-called aglycone, was 24%, and of quercetin-ß-rutinoside, also called rutin, was only 17%.[17] In a subsequent pilot study we showed that when subjects consumed fried onions, their peak plasma quercetin concentration was reached within 3 h and then decreased slowly throughout the day.[18] These data suggest that the sugar moiety of quercetin glycosides is an important determinant of their absorption. Onions contain mainly glucose glycosides of quercetin, i.e. glucosides,[19,20] apples a variety of quercetin glycosides, e.g. galactosides, arabinosides, rhamnosides, xylosides, and glucosides,[21-23] and quercetin rutinoside is a major glycoside in tea [24](*Figure 1*). The present study was designed to determine the pharmacokinetics of these various quercetin glycosides from important dietary sources of quercetin.



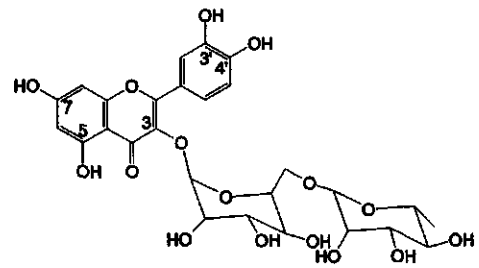
Quercetin aglycone (I)



Quercetin-4'-*O*-ß-D-glucoside (II)



Quercetin-3-*O*-ß-D-galactoside (III)



Quercetin-3-*O*-ß-rutinoside (IV)

Figure 1. *Structure of quercetin aglycone (**I**), quercetin glucoside (**II**) of onions, quercetin galactoside (**III**) of apples and quercetin rutinoside (**IV**).*

MATERIALS AND METHODS

Subjects

We recruited 9 subjects (5 females, 4 males), mean age 24.8 (range 20 - 47) years, with a mean Body Mass Index of 23.2 (range 19.3 - 30.9) kg/m^2. They had no record of gastric or intestinal surgery and were all healthy based on a medical questionnaire, and the absence of glucose or protein in urine. Their values for blood haematocrit, for leucocyte and platelet counts, for haemoglobin concentration, alanine amino transferase, aspartate amino transferase, alkaline phosphatase, and γ-glutamyl transferase were normal. Subjects did not use any medication. The protocol was approved by the Wageningen University Ethical Committee, and was fully explained to the participants, who gave their written informed consent.

Study design, foods, and supplements

Subjects followed a quercetin-free diet during three experimental periods each of 5 days. They were given a list of those vegetables and fruits that contain more than 15 mg/kg (50 μmol/kg) of quercetin and of beverages with more than 4 mg/L (13 μmol/kg) of quercetin;[25,26] they were instructed not to consume any of these products during each of the three 5-day periods. These periods were separated by 9 days without treatment and without a prescribed diet. On day 4 of each experimental period we provided one out of three different quercetin-containing supplements in random order. Supplements were given at breakfast at the Department between 7:40 and 8:20 am. After the quercetin-rich breakfast blood samples were collected periodically over the next 36 hours and urine continuously for 24 hours.

Because proteins are known to bind polyphenols,[27] the quercetin-supplemented breakfasts were low in protein; they consisted of protein-free bread, margarine, jams made from quercetin-free fruits and other sweets such as chocolate sprinkles, coffee without milk, quercetin-free soft drinks, and mineral water. We fried 330 g yellow onions with 20 g margarine, 15 g tomato ketchup and 1 g Italian herbs; 150 g of this dish constituted the onion supplement. We freeze-dried apple peels, variety Jonagold, ground them, and mixed 41.24 g of the powder with 400 g of apple sauce; 434 g of this mixture was supplemented to each subject. Analysis of duplicate portions[28] showed that the onions provided 225 ± 43 μmol (68 ± 13

mg quercetin equivalents) and apples 325 ± 7 μmol (98 ± 2 mg quercetin equivalents) of quercetin. For the third experimental breakfast 220 mg quercetin-3-*O*-ß-rutinoside (Rutosidum DAB, #339994; OPG Farma, Utrecht, The Netherlands), 331 μmol equivalent to 100 mg aglycone was administered in a capsule. The breakfasts were also supplemented with a capsule containing 80 mg *para*-aminobenzoic acid (#361334; OPG Farma) as a recovery marker. *Para*-aminobenzoic acid is completely absorbed and is excreted with urine.[29] Subjects were instructed not to eat anything and to drink only water or coffee without milk from after the experimental breakfasts until lunch. At lunch at the Department, subjects swallowed another capsule with *para*-aminobenzoic acid, and they were instructed to swallow a third capsule with 80 mg *para*-aminobenzoic acid at dinner at home.

Energy and nutrient intakes were calculated using the Dutch food composition table.[30] Average energy intake on day 3 of each period according to 24-h dietary recalls, was 10.4 ± 1.5 MJ, of which protein provided 13.2 ± 1.8% of energy, fat 33.9 ± 5.4%, and carbohydrates 52.5 ± 5.7%, with no differences between treatment periods.

Collection of samples

Venous blood samples were taken into vacuum tubes containing EDTA before breakfast, and at the time intervals depicted in *Figure 2*; zero time was 10 min after the start of the breakfast. Platelet-rich plasma was prepared within 15 min by centrifugation at 20 °C for 10 minutes at 200 *g*; it was stored at -80°C until analysis.

Subjects collected urine in plastic bottles containing 0.13 g thymol (# 8167; Merck) for 24 hours after each supplemented breakfast. They stored each bottle in dry ice immediately after voiding. Three of the subjects, #1, #2, and #4, collected their 24-h urines in 9 to 10 separate portions, which allowed us to study their rates of excretion of quercetin. Upon arrival at the laboratory, urine samples were thawed in a water bath at 40 °C and mixed, and aliquots were taken within 30 min, frozen with liquid nitrogen, and stored at -40 °C until analyzed. Samples from the three subjects who had collected urine every few hours were homogenised per subject per period. The other samples were pooled per subject and per treatment day and thoroughly homogenised.

Analytical methods

Quercetin, quercetin glycosides, glucuronides, and sulfates were extracted from plasma or urine and simultaneously hydrolysed to the aglycone form using 2 M HCl in aqueous methanol.[28] For plasma 1.00 mL of methanol containing 2 g/L *tert*-butyl hydroxyquinone (TBHQ) and 0.40 mL of 10 M HCl were added to 0.60 mL of plasma in a 4 mL vial (#WAT022468, Waters, Milford, MA, USA) and mixed. The vial was sealed tightly with a cap (#72711, Waters, Milford, MA, USA) and septum (#73008, Waters, Milford, MA, USA), inserted into a preheated aluminum block (Reacti-Block C-1, Pierce Europe, Oud-Beijerland, The Netherlands), heated in an oven at 90 °C for 5 hours, allowed to cool, and centrifuged at 1000 g for 15 min after addition of 2.00 mL of methanol containing 2 g/L *tert*-butyl hydroxyquinone (TBHQ). We transferred 1.5 mL of the upper phase into an HPLC vial and added 15 μL 100 g/L ascorbic acid. For urine 15.0 mL methanol containing 2 g *tert*-butyl hydroxyquinone/L and 5 mL 10 M HCl were added to 5 g urine followed by mixing, the extract was refluxed at 90 °C for 8 hours with regular swirling, allowed to cool and subsequently brought to a final volume of 50 mL with methanol containing 2 g/L ascorbic acid. Urine extracts were sonicated for 5 min and filtered through a 0.45 μm filter for organic solvents (Acrodisc CR PTFE; Gelman Sciences, Ann Arbor, MI) before HPLC analysis.

For HPLC analysis we injected 20 μL onto a reversed-phase C18 column connected to a postcolumn reaction coil, where quercetin was transformed into a quercetin-aluminum complex. The fluorescence of the quercetin-metal complex was measured.[31]

The limit of detection, i.e. the concentration producing a peak height three times the standard deviation of the baseline noise, was 0.007 μM (2 ng/mL) for plasma and 0.01 μM (3 ng/mL) for urine. Recovery of 0.33 nmol (100 ng) quercetin aglycone added to 1 mL plasma was 88 ± 3% (n = 6). Addition of 0.83 nmol (250 ng) quercetin aglycone per gram of urine yielded a recovery of 99 ± 7% (n = 3).

Determinations in urine were carried out in duplicate. The relative standard deviation of duplicates was 4 % for plasma and 6 % for urine. We included a control sample of plasma and urine in each series of analyses; all values were within 0.23 ± 0.046 μM (mean ± 2 SD, n = 15) for plasma and within 3.02 ± 0.55 μM (mean ± 2 SD, n = 15) for urine.

Para-aminobenzoic acid in urine was hydrolysed with 0.1 M HCl for 40 min in a boiling water bath and determined photometrically by using fluorescamine

(#F-9015; Sigma).[32] A urine control sample was included in each series of analysis; values were within 66.7 ± 6.1 mg (mean ± 2 SD, n = 7).

Data analysis

We used the two-compartment open model $C(t) = -C e^{-kt} + A e^{-\alpha t} + B e^{-\beta t}$ to describe the absorption and disposition, i.e. distribution, metabolism and elimination, of quercetin.[33] We calculated the parameters of this model with nonlinear regression analysis using PCNONLIN version 4.0 (SCI Software, ClinTrials Inc., Lexington, KY, USA).

Quercetin excreted in urine, the area under the plasma concentration - time curves (AUC), quercetin peak plasma levels as proportion of intake, and times to reach quercetin peak plasma levels were converted to $\log_{10}$ values for statistical testing. Differences between treatments were tested by analysis of variance using the Statistical Package for Social Sciences, SPSS/PC+ (SPSS Inc, Chicago) with subject, type of quercetin source, and type of quercetin source in preceding period as independent variables. The significance of differences was determined by paired t test.

RESULTS

Compliance with the quercetin-free diet

The average quercetin intake from regular foods according to 24-h dietary recalls on the third day of the three diet periods was 1.5 ± 0.9 mg (5 ± 3 μmol). No difference in quercetin intake was observed between the three diet periods. Plasma quercetin concentration in the pre-breakfast plasma samples was on average 0.017 ± 0.013 μM, whereas in the plasma samples 36 h after the supplements it was still 0.040 ± 0.027 μM. Thus, compliance with the quercetin-free diet was excellent.

Plasma concentration of quercetin.

Intake of onions and apples led to a rapid rise, and of the rutinoside (rutin) to a very slow rise of quercetin levels in plasma. The area under the plasma concentration - time curve (AUC), the quercetin peak plasma level, and the time to reach the peak

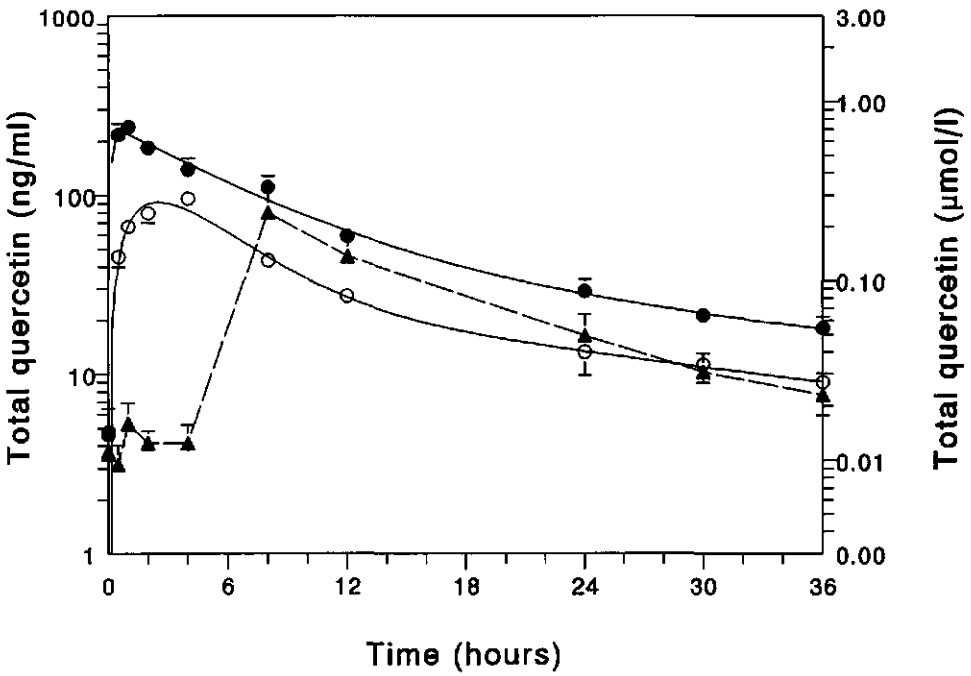


Figure 2. *Total quercetin concentration (mean ± SE) in plasma of nine subjects after ingestion of fried onions conatining 225 µmol quercetin glycosides, apples containing 324 µmol quercetin glycosides and 331 µmol rutin. Each subject received each supplement in random order.*

●, *onions;* ○, *apples;* ▲, *rutinoside*

plasma level were all significantly ($P < 0.001$) dependent on the type of supplement (*Table 1*). No significant relation with individual subject or with supplement given in the preceding period was found.

The mean peak plasma level of quercetin was 0.74 µM (224 ng/mL) after onions, 0.30 µM (92 ng/mL) after apples, and 0.30 µM (90 ng/mL) after quercetin-3-rutinoside. Peak levels were reached 0.7 h after ingestion of the onions, 2.5 h after the apples, and 9 h after the quercetin-3-rutinoside (*Figure 2, Table 1*). In 4 out of 9 subjects the quercetin concentration had reached its maximum observed concentration already at 0.5 h after the onions supplement, the first data point after consumption of the supplement. Therefore, the time needed to reach the peak level may have been overestimated, and the height of the peak underestimated.

To our knowledge no data are available on the tissue distribution of quercetin in man or animals, after absorption. In such a case, compartmental models are useful to describe the time course of the plasma quercetin concentration. A compartment should be interpreted as a collection of tissues that have similar blood flow and quercetin affinity. The change in quercetin concentration in each compartment is described by first-order kinetics.[33] Disposition, i.e. distribution, metabolism and elimination, of quercetin in plasma after the onions and apples supplements was best described by a two-compartment model (*Figure 2*). Regression analysis was only possible for onions and apples; rutin (quercetin-3-rutinoside) escaped the model because the delayed absorption resulted in too few data points to perform a proper regression analysis. The average half-lives of the absorption phase, the distribution phase, and the subsequent elimination phase were not statistically different between onions and apples (*Table 1*). We could still detect quercetin 36 h after ingestion of the supplements; at that time the mean plasma concentration was 0.060 ± 0.027 μM after onions, 0.030 ± 0.030 μM after apples, and 0.027 ± 0.023 μM for quercetin-3-rutinoside (*Figure 2*).

We calculated the relative bioavailability by comparing the areas under the plasma concentration - time curve (AUC) and found that the bioavailabilty of quercetin from both apples and the rutinoside was 30% of that of quercetin from onions (*Table 1*).

Completeness of collection of urine

Urinary recovery of *para*-aminobenzoic acid was 87.3 ± 10.0%, indicating acceptable compliance in collecting urine.[29] On 6 out of 27 person-days subjects reported not to have swallowed the *para*-aminobenzoic acid capsule at dinner, so compliance of these subjects at evening could not be checked. As quercetin was almost completely excreted in urine within the first 10 h after the supplemented breakfasts (*Figure 4*), this uncertainty is of minor importance.

Urinary excretion of quercetin

The total amount of quercetin excreted in urine depended on the type of supplement (*Table 2*). No relation with subject or with supplement given in the previous period was found. Excretion of quercetin and its conjugates in urine was higher for quercetin from onions with 1.39% of the administered dose, than for

Kinetic parameters of quercetin absorption and disposition[1] in nine subjects after one-time ingestion of fried onions containing 225 µmol quercetin glycosides, apples containing 325 µmol quercetin glycosides and 331 µmol rutin. Each subject received each supplement in random order. Data for onions and apples were fitted to a model $C(t) = -C e^{-kt} + A e^{-\alpha t} + B e^{-\beta t}$, *where t equals time and C(t) plasma quercetin concentration. The correlation coefficient r was 0.99 for onions and 0.98 for apples.*

		Supplement		
Parameter		Onions	Apples	Rutinoside
Absorption				
half-life	(h)	0.13 ± 0.09	1.10 ± 0.51	-
peak level	(µM)	0.74 ± 0.05[a]	0.30 ± 0.02[b]	0.30 ± 0.10[b]
	(ng/ml)	224 ± 15[a]	92 ± 6[b]	90 ± 31[b]
time to reach peak level	(h)	0.70 ± 0.36[a]	2.51 ± 0.24[b]	9.3 ± 0.6[c]
Distribution				
half-life	(h)	4.4 ± 1.4	2.4 ± 1.2	-
Elimination				
half-life	(h)	27.8 ± 30.5	22.6 ± 10.6	-
$AUC_{0-\infty}$	(h.ng/mL)	2974 ± 772	1334 ± 155	-
AUC_{0-36h}	(h.ng/mL)	2330 ± 283[a]	1061 ± 125[b]	983 ± 326[b]

[1] mean ± SE

[a,b,c] results with a different superscript letter differ significantly ($P < 0.001$)

- not calculated; regression failed because of too few data points

quercetin from apples with 0.44%, and for the rutinoside with 0.35% (*Table 2*). One subject (#3) excreted much more quercetin in urine after consumption of the quercetin-3-rutinoside than did the other subjects (*Figure 3*). Quercetin excretion of this subject after the rutinoside was only 30% less than that after the onions, whereas all the other subjects excreted at least 80% less after the rutinoside than after the onions.

Subjects #1, #2, and #4, who had collected urine every few hours (*Figure 4*),

reached 90% of their cumulative excretion within 7.9 ± 0.8 hours after administration of the onions supplement, within 8.5 ± 1.1 hours after the apples, and within 12.9 h ± 7.0 hours after the rutinoside.

The area under the plasma concentration - time curve (AUC_{0-36h}) correlated highly with 24-h urinary excretion of total quercetin per subject (*Figure 3*) in these three supplements.

Table 2. *Intake of quercetin at breakfast and subsequent mean cumulative excretion of total quercetin in urine over 24 hours*[1]*. Each subject received each supplement in random order.*

Supplement (to breakfast)	Intake	Excretion in urine	
		Total	Total excretion as proportion of intake
	(μmol)	*(μmol)*	*(%)*
Onions (n = 9)	225 ± 43	3.22 ± 1.60	1.39 ± 0.49[a]
Apples (n = 9)	325 ± 7	1.45 ± 0.71	0.44 ± 0.22[b]
Rutin (n = 9)	331 ± 7	1.17 ± 1.34	0.35 ± 0.41[b]

[1] Mean ± SD.

[a,b] Results with a different superscript letter differ significantly ($P < 0.001$).

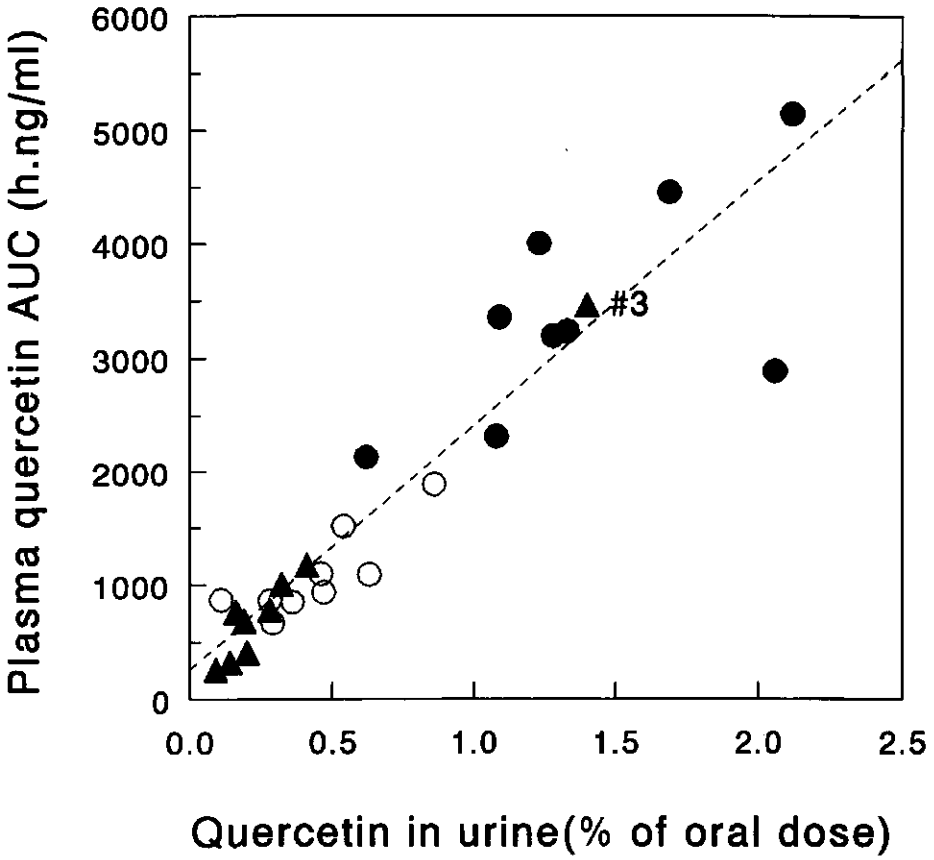


Figure 3. *Correlation between plasma total quercetin AUC_{0-36h}, standardized at a dose of 100 mg quercetin equivalents, and urinary excretion as a percentage of the ingested dose in nine subjects who consumed three supplements in random order; $r = 0.93$, $n = 27$.*

●, *onions;* ○, *apples;* ▲, *rutinoside*

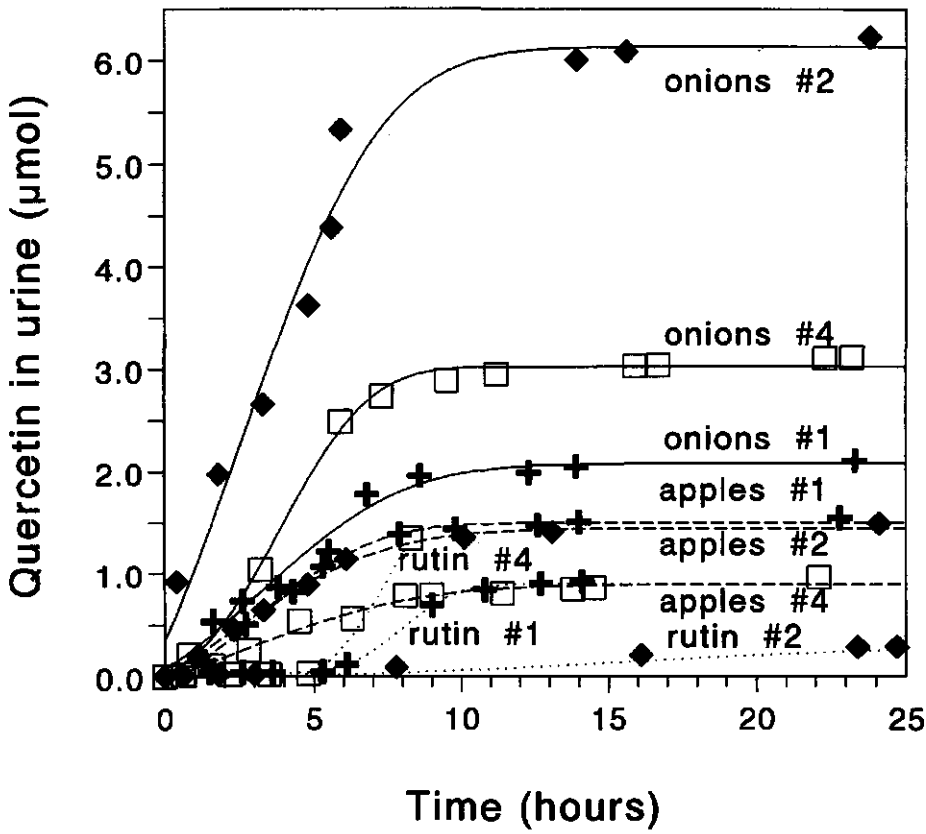


Figure 4. *Cumulative amount of total quercetin (µmol) excreted in urine of subjects #1, #2, and #4 after consumption of onions, apples and rutin (quercetin rutinoside). +, subject #1; ♦, subject #2; □, subject #4.*

DISCUSSION

We found that the dietary antioxidant quercetin can readily enters the circulation after consumption of major dietary sources of quercetin. Absorption kinetics were highly dependent on the type of supplement. A major difference between these supplements is the type of glycoside. Quercetin from onions, which contain only glucosides, was rapidly absorbed, whereas pure quercetin-3-rutinoside, a major species in tea, showed a markedly delayed absorption. The absorption rate from apples, which contain a variety of glycosides, was intermediate. The bioavailabilty of quercetin from apples and of the rutinoside was only one third of that from onions. Thus, these results point to a predominant role of the sugar moiety in the absorption of dietary quercetin in the human body. However, it cannot be ruled out that differences between apples and onions in cell wall structures, location of glycosides in cells or their binding to cell constituents, also affect the liberation of quercetin from these foods in the gastrointestinal tract.

Pharmacokinetics

The biphasic concentration profile in the elimination of quercetin from plasma (*Figure 2*) could point to access of quercetin to poorly perfused tissues, where it may be transiently stored, or tissue binding. However, various conjugates formed upon or after absorption also may cause such a profile.

After consumption of a single portion of onions and apples, a considerable fraction of the absorbed quercetin was present in plasma throughout the day as is indicated by the elimination half-lives of about 24 h. The time course of the plasma quercetin concentration after the rutinoside indicates a similar slow elimination. However, to get a more accurate estimation of these half-lives, the plasma samples should have been taken up to 3 days. The long half-lives implicate that repeated intake of onions, apples, and possibly tea would lead to a build-up of quercetin in plasma. The different rates of absorption of quercetin measured after ingestion of the various supplements will cause quercetin to enter the plasma continuously for a few hours following a mixed meal containing a variety of quercetin sources. We found a peak plasma concentration of 0.75 μM or 225 ng/mL after administration of a single high dose of dietary quercetin equivalent to 4 times the average Dutch daily intake.[16] Concentrations of the dietary antioxidant ß-carotene in human plasma are similar to this value.[34] Thus plasma quercetin levels in subjects who regularly eat

onions may approach those of ß-carotene.

Although urine was only collected for 24 hours, 24-h urinary excretion of quercetin predicted the area under the plasma concentration - time curve (AUC_{0-36h}) excellently in these supplements (*Figure 3*). Thus, the relative bioavailability of quercetin from foods possibly can be predicted by measuring the 24-h quercetin excretion in urine.

Our assay measures total quercetin after hydrolysis of native glycosides and of any glucuronides and sulfates formed by conjugation in the liver or the small intestine.[28] Methylated quercetin formed in the liver[35] is not included, because it is not hydrolysed in our assay. Thus, the concentrations in plasma reflect these conjugates only. It is possible that glucuronides, sulfates and glycosides, if present in plasma, each show a somewhat different distribution and elimination. In addition, for apples each separate glycoside probably is absorbed at its own rate. Thus, the pharmacokinetic parameters calculated reflect the overall absorption and disposition of various conjugates. However, at present no methods are available for the specific determination of these conjugates in plasma.

Comparison with previous studies

Apart from the faster absorption our results for onions are in agreement with those of a pilot study.[18] We also previously showed that quercetin glycosides from onions and quercetin aglycone were absorbed in healthy ileostomy subjects.[17] Absorption profiles of flavonols were determined in human studies by Nieder[36] after administration of flavonol glycosides from *Ginkgo biloba*. However, Nieder[36] only studied 2 subjects, and did not specify the types nor the amounts of flavonol glycosides administered with the extract, whereas urinary excretion was not studied. Gugler et al.[37] could not detect any quercetin aglycone in plasma of human subjects after oral administration of even 4 g of quercetin aglycone, using a limit of detection of 0.33 μM (100 ng/mL). However, their assay did not include quercetin conjugates.

The elimination half-lives of 2 - 4 hours measured by Nieder[36] after oral, and Gugler et al.[37] after intravenous administration were much shorter than those reported here, possibly because in both studies the plasma concentration was only measured regularly up until 9 - 12 hours after administration. Most likely, these authors erroneously identified the distribution phase as the elimination phase. Cova et al.[38] measured a slow elimination with a half-life of 32 hours in human subjects after oral administration of diosmin, a glycoside of the flavone diosmetin.

Disposition of diosmetin also was biphasic, which agrees with our data.

Binding to plasma and urine constituents

We needed a hydrolysis period of 5 to 8 hours to completely liberate quercetin from plasma and urine. This long period came as a surprise. It is to be expected that quercetin in plasma and urine is present either as glucuronide or sulfate.[35] Sulfates are easily hydrolysed,[39] whereas we previously have shown that glucuronides of quercetin are completely hydrolysed within 2 hours.[28] Possibly, other types of conjugates, e.g. glycine conjugates[33] are formed. No data are available on hydrolysis conditions required for these conjugates. In addition, quercetin aglycone added to plasma[18] and urine[17] was completely recovered within two hours of hydrolysis. The extended hydrolysis period suggests that binding of conjugates to plasma and urine constituents differs from that of the aglycone. Alternatively, the active process of absorption as opposed to the *in vitro* addition of the aglycone could lead to a different type of binding. Binding of polyphenols to protein in plasma is to be expected. Manach et al.[40] reported binding to plasma albumin of circulating quercetin and of added aglycone. The nature of the urinary binding constituents or conjugates remains to be solved.

Quercetin concentrations did not differ between platelet-rich and platelet-poor plasma (results not shown). Thus, we could not confirm that quercetin is selectively bound to platelets after absorption from foods as was found by Gryglewski et al.[41] with rabbit platelets *in vitro*.

Metabolism

Only 0.4 - 1.4% of the administered quercetin was recovered in urine. We have shown previously that human absorption of quercetin glucosides from onions amounted to 52%, whereas absorption of the pure aglycone was 24%, and of rutin 17%.[17] Thus, like many other compounds,[42] absorbed quercetin is probably extensively modified before being excreted by the kidneys. Our assay would pick up quercetin glucuronides and sulfates, but *O*-methylated quercetin, a hepatic metabolite in rats,[35,43] would escape detection and so would metabolites which cannot be converted back into the aglycone by the hydrolysis procedure. Biliary secretion of quercetin glucuronides and sulfates has been described in rats.[35,43] Only a part of these secreted quercetin conjugates will be reabsorbed and detected in

urine. However, no data are available on biliary secretion and reabsorption of quercetin in man. Thus, conjugates of quercetin formed by phase II liver metabolic reactions,[33] except possibly O-methylated quercetin, only make up a small fraction of the metabolites formed. Hitherto, metabolites of quercetin caused by phase I metabolism have not been described.[44] Bacteria of the colon constitute a second metabolic compartment, and transform quercetin into phenolic acids, which can be found in urine.[45,46] Depending on their hydroxylation pattern, these are antioxidants themselves.[47] In addition to the formation of such undetectable metabolites quercetin might also be partly stored and released slowly over subsequent days.[48]

Mechanisms of absorption

The short time to reach peak levels after onion glucosides points to absorption in the stomach or small intestine, whereas the prolonged time needed for the rutinoside to reach its peak, suggests that it transits the small intestine and is absorbed in the colon. We previously showed that intact glucosides are absorbed well.[17] Hydrolysis of the rutinoside may be necessary before absorption can occur, which is in accordance with conventional thinking on flavonoid absorption.[1] Because the rutinoside is a ß-glycoside, only microorganisms in the colon can mediate hydrolysis, but at the same time they also degrade the liberated aglycone.[1] In contrast with the present study, we indeed found practically no urinary excretion of quercetin or its conjugates after administration of the rutinoside to ileostomy subjects who lack a colon.[17] Again this provides evidence for a role of the colon in the absorption of quercetin rutinoside a major species in tea and in the diet of populations where tea is a common beverage. The area under the plasma quercetin concentration - time curve and the urinary quercetin excretion of subject #3 after rutin were both considerably higher than those of the other subjects (*Figure 3*). However, the time to reach a peak level of quercetin in plasma did not differ. Possibly this subject's absorption of rutin in the colon is more efficient. We previously reported another subject with an enhanced absorption of rutin.[17] The reasons for these pronounced interindividual differences deserve further study. These mechanisms explain the limited bioavailability of the rutinoside in comparison with the glucoside. This study suggests that the sugar moiety has a predominant effect on the absorption and plasma levels of quercetin.[17]

We found that quercetin glycosides present in major food sources of flavonols are absorbed, and are eliminated slowly throughout the day. Quercetin could thus

contribute significantly to the antioxidant defences present in blood plasma. Data for separate quercetin conjugates in foods and plasma are needed to fully evaluate the role of the dietary antioxidant quercetin in human health.

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ɛs Nouws for stimulating discussions. This work was supported by grants from the Foundation for Nutrition and Health Research and the Netherlands Heart Foundation (94.128).

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7

A ROLE for the INTESTINAL SODIUM-GLUCOSE COTRANSPORTER in the ABSORPTION of DIETARY FLAVONOIDS in MAN

Hollman PCH, Buysman MNCP, van Trijp JMP, van Gameren Y, Cnossen PJ, de Vries JHM, Katan MB

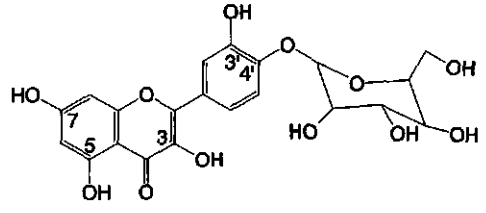
Submitted

Abstract

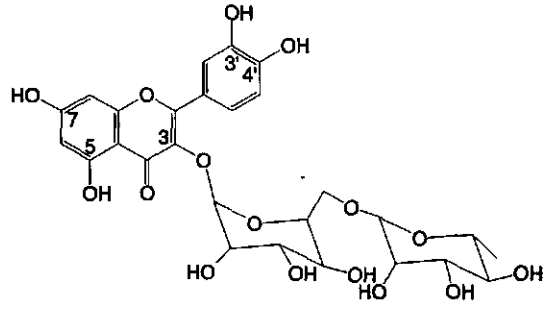
The role of the sugar moiety in the absorption of quercetin glycosides was studied. Nine fasting subjects ingested pure solutions of quercetin-4'- glucoside (311 μmol) and quercetin-3-rutinoside (311 μmol).Intake of quercetin glucoside led to a much more rapid and larger rise of quercetin levels in plasma than intake of the rutinoside. The mean peak plasma level of quercetin was 3.20 μM after the 4'-glucoside and only 0.23 μM after quercetin-3-rutinoside. Peak levels were reached $<$0.6 h after ingestion of the 4'-glucoside, and 5.6 h after the 3-rutinoside. Elimination of quercetin from plasma was similar for the two sources with half-lives of about 24 h. We conclude that the 4'-glucoside was rapidly absorbed from the small intestine, whereas the 3-rutinoside was only absorbed from the colon. We propose that the sodium-glucose cotransporter in the brush border membranes of the small intestine plays a role in the absorption of the 4'-glucoside.

INTRODUCTION

Flavonoids are polyphenolic compounds from plants. Human diets provide several hundred milligrams per day from vegetables, fruits, tea and wine. Flavonoids are antioxidants; they scavenge radicals and inhibit lipid peroxidation.[1] Lipid peroxidation is probably involved in the formation of atherosclerotic plaques.[2] Indeed, the intake of one class of flavonoids, the flavonols, was inversely associated with cardiovascular disease in several[3-6] though not all[7,8] studies in man. However, absorption of flavonoids is thought to be marginal, because most flavonoids are present in foods as ß-glycosides non-hydrolysable by pancreatic enzymes.[9] These are thought to be non-absorbable, but in fact our earlier studies[10] (*Chapter* 6) with foods suggested that the sugar moiety plays an important role in their absorption. We now studied the absorption in man of two pure quercetin glycosides: quercetin-4'-*O*-ß-D-glucoside, a major flavonol in onions,[11] and quercetin-3-*O*-ß-rutinoside (*Figure 1*) occurring in tea, apples and wine,[12,13] the major dietary sources of quercetin.[6]



Quercetin-4'-O-ß-D-glucoside



Quercetin-3-O-ß-rutinoside

Figure 1. *Structure of the quercetin glycosides used.*

METHODS

The study was approved by the Nijmegen University Hospital Ethical Committee, and was fully explained to the participants, who gave their written informed consent. Nine healthy volunteers followed a quercetin-free diet [10] during two periods of 5 days. On the morning of day 3 we fed 311 μmol of quercetin-4'-O-ß-D-glucoside (Spiraeosid 4564, Brunschwig Chemie B.V.) or 311 μmol quercetin-3-O-ß-rutinoside (Rutosidum DAB, OPG Farma) in 10 ml ethanol plus 200 ml water containing 2 g NaCl in random order to fasting subjects. Venous blood samples were taken at the times depicted in *Figure 2*. Quercetin and quercetin conjugates were extracted from plasma and hydrolysed to the aglycone free form using HCl/methanol (*Chapter 6*) and analysed as described.[22]

RESULTS AND DISCUSSION

The absorption kinetics of quercetin-4'-glucoside and quercetin-3-rutinoside differed substantially (*Figure 2*). Intake of the glucoside led to a much more rapid and larger rise of quercetin levels in plasma than intake of the rutinoside. The mean peak plasma level of quercetin was 3.20 ± 0.40 μM (mean ± SE) after the glucoside and only 0.23 ± 0.05 μM after the rutinoside. Peak levels were reached 0.61 ± 0.07 h after ingestion of the glucoside, and 5.6 ± 1.0 h after the rutinoside (*Figure 2*). In 7 out of 9 subjects the quercetin concentration had reached its maximum already at the first time point, 0.5 h after ingestion of the supplement; therefore the actual maximum may have been even higher and reached earlier than suggested by *Figure 2*. Elimination of quercetin from plasma was similar for the two sources with half-lives of 33.7 ± 4.0 h after the 4'-glucoside and 23.3 ± 5.7 h after the rutinoside (*Figure 2*). The bioavailability of the rutinoside, as judged by the area under the plasma concentration - time curve was only 18% of that of the glucoside.

The short time to reach peak levels after the glucoside points to absorption from the small intestine, whereas the rutinoside apparently transits the small intestine without absorption and is only absorbed from the colon. Evidently the sugar moiety has a predominant effect on the absorption of quercetin glycosides. Enzymatic hydrolysis of ß-glycosides in the stomach or small intestine,[9] or hydrolysis by HCl[14] in the stomach is highly improbable. Similar to sugars, diffusion

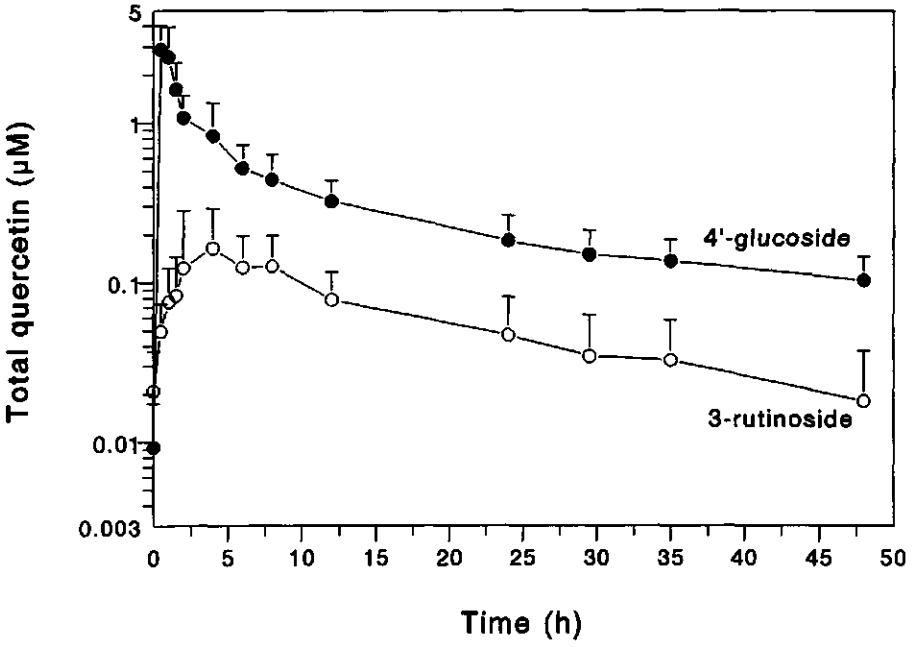


Figure 2. *Total quercetin concentration (mean ± SD) in plasma of nine subjects after ingestion of quercetin-4'-O-ß-D-glucoside (●) and quercetin-3-O-ß-rutinoside (O). Each subject received each supplement in random order.*

of hydrophilic polar glycosides across endothelial membranes is unlikely, thus absorption of the glucoside from the stomach can be excluded. We propose that the intestinal Na^+-glucose cotransporter carries flavonoid glucosides into the enterocyte. The short time to reach peak levels of quercetin is similar to that for D-glucose.[15] Naphthol glycosides were transported across the intestinal wall of rats by the Na^+-glucose cotransporter.[16] Absorption was better for the naphthol glucoside than for the galactoside, and the ß-anomer was absorbed better than the α-anomer,[16] in agreement with the known affinities of the sugar transporter.[17] Studies on the structural requirements of phenyl glucosides for binding to this carrier concluded that transportable ß-D-glucosides have similar three-dimensional structures.[18] Phenyl-α-D-glucosides did not interact with the sugar carrier. Transport by the glucose cotransporter of methylazoxymethanol-ß-D-glucoside[19] and sulphamethazine-D-glucoside was reported.[20] This active transport of ß-D-glucosides offers an explanation for the extensive absorption of quercetin from onions[10] (*Chapter 6*) in

which it is present as a ß-D-glucoside. The intermediate rate of absorption of quercetin from apples (*Chapter 6*) also fits in, because the major glycosides of apples are ß-D-galactosides and ß-D-xylosides.[12] D-xylose and D-galactose are transported by the sugar cotransporter, but xylose less efficiently than glucose and galactose.[17,21] The slow absorption of quercetin-3-rutinoside can be explained because rutinose cannot be transported by Na^+-glucose cotransporter, hence it can only be absorbed after hydrolysis in the colon.

Our data implicate that the glucose carrier in man will efficiently absorb glucose even when attached to a bulky molecule such as quercetin. Absorption of both food components and drugs might therefore be enhanced by attachment of a glucose molecule.

Acknowledgments

This work was supported by grants from the Foundation for Nutrition and Health Research and the Netherlands Heart Foundation (94.128).

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8

GENERAL DISCUSSION

The research described in this thesis focused on the following questions:

- To what extent is dietary quercetin absorbed in humans?
- Which factors affect its absorption from foods?
- How does the concentration of quercetin in plasma change with time after ingestion of various quercetin-rich products?
- What is the bioavailability of quercetin from major dietary sources?

To answer these questions, we performed studies with quercetin-rich supplements or pure quercetin glycosides in healthy ileostomy subjects who lack a colon (*Chapter 4*) or in normal healthy volunteers (*Chapters 5,6,7*). We developed a sensitive analytical method (*Chapter 3*) to be able to measure quercetin in plasma and urine.

We have found that absorption of quercetin glycosides from the diet can be appreciable: up to 52%. The sugar moiety of the glycoside was a major determinant of absorption. The concentration of quercetin in plasma increased quickly after consumption of quercetin-4'-glucoside, and only slowly after the quercetin-3-rutinoside. We propose that the sodium-glucose cotransporter is involved in the absorption of quercetin-4'-glucoside. The elimination half-life of about 24 hours was independent of the type of glycoside. Accumulation of quercetin in plasma will occur because of this long half-life; steady-state levels of 0.2 μM are attainable. The bioavailability of quercetin of apples and of the rutinoside was 30% relative to onions.

Absorption of glycosides versus aglycones

Quercetin glucosides contained in onions were well absorbed, whereas the free aglycone and the rutinoside were less well absorbed (*Chapter 4*). Except for the rutinoside, these results contradict conventional thinking on absorption of ß-glycosides (*Chapter 2*). This traditional way of thinking assumed that ß-glycosides are non-absorbable from the intestine, because they are too hydrophilic for diffusion across intestinal membranes. Following this explanation, the aglycone is expected to have more favourable properties for diffusion. However, model studies on absorption of flavonoid aglycones and glycosides across intestinal membranes to substantiate this aglycone hypothesis are lacking.

As ß-glycosides are only hydrolysable by bacteria in the colon,[1] absorption in ileostomy volunteers would be negligible, because bacteria are practically absent

in these subjects. This provides a way for experimental testing. We indeed found that only a little amount of quercetin was excreted in urine after ingestion of the rutinoside, except in one subject. Absorption of the aglycone was somewhat higher than that of the rutinoside, but the conventional aglycone hypothesis would not predict the high absorption of the glycosides of onions as compared to the aglycone (*Chapter 4*).

However, non-enzymatic hydrolysis of glycosides by HCl in the stomach would be conceivable. Differences in hydrolysis rates of various glycosides and in situ liberation of the aglycone could then possibly favour absorption of the 4'-glucoside.To test this, we mimicked gastric conditions in an *in vitro* incubation experiment with quercetin-4'-glucoside present in onions, and quercetin-3-rutinoside and showed that hydrolysis was completely absent (*Table 1*). This fits with our experience *in vitro* that hydrolysis of glycosides is only complete after boiling with hydrochloric acid at 90°C for at least 2 h.[2]

Thus, these results contradict that ß-glycosides are only absorbable via the aglycone after hydrolysis. Although we only indirectly measured absorption in the study with ileostomy volunteers, plasma quercetin values in normal volunteers (*Chapters 6 and 7*) confirmed that the quercetin glucoside of onions was absorbed far better than the rutinose glycoside.

Table 1. *Resistance of quercetin ß-glycosides against hydrolysis by hydrochloric acid. Quercetin aglycone and glycosides, 100 ng/ml each, were incubated in 0.1 M HCl at 37 °C. The concentration of free quercetin aglycone in the acid solutions was measured after the periods indicated.*

	Free quercetin aglycone (ng/ml)		
Incubation period (min)	Quercetin aglycone	Quercetin-4'-glucoside	Quercetin-3-rutinoside
5	112	<2[1]	<2
10	91	<2	<2
20	101	<2	<2

[1] limit of detection

Mechanisms of absorption

Small intestine

All three studies (*Chapters 4,6,7*) showed that absorption of quercetin contained in onions was markedly more efficient and substantially faster than that of the rutinoside. This suggests different mechanisms of absorption. The time to reach peak levels of quercetin in plasma after consuming onions was less than 0.7 h (*Chapter 6*), and suggests absorption from the stomach or small intestine.[3] A major glycoside of onions is quercetin-4'-glucoside, and the study with a pure solution of this glucoside gave very similar results (*Chapter 7*) to the study with onions (*Chapter 6*). Thus, the matrix of onions did not affect the time to reach peak levels in plasma.On the other hand, absorption of a pure solution of quercetin-4'-glucoside was substantially higher and faster than that of a pure solution of quercetin-3-rutinoside. Consequently, the sugar moiety of the glycoside probably is an important determinant for its absorption. As was shown above, hydrolysis of glycosides in the stomach is highly improbable. Analogous to sugars, diffusion of the hydrophilic polar glycosides across endothelial membranes is unlikely, thus absorption of the glucoside from the stomach can be excluded. We hypothesize that the active sodium-glucose cotransporter, a transport protein complex resident in the brush border membrane of the small intestine, is involved in the absorption of quercetin-4'-glucoside. However, the involvement of this active transporter has to be confirmed in a model system. Brush border membrane vesicles of rabbits or rats would constitute a suitable *in vitro* system[4,5] to do this.

If the sodium-glucose cotransporter is involved in the active absorption of quercetin-4'-glucoside, it can be predicted that other quercetin glycosides also have an efficient absorption. Apart from glucose, galactose has a high affinity for this carrier, whereas xylose also will bind, though with a lower affinity.[4,6,7] In addition, quercetin galactosides and xylosides present in foods also have the favourable ß-anomeric configuration for transport.[8] Results for apples, rich in these glycosides, but also containing substantial amounts of the rutinoside,[9] were consistent with the proposed absorption mechanisms. We observed a broad peak in the plasma quercetin concentration versus time curve, indicating that the various glycosides were absorbed at different rates (*Chapter 6*).

Results of the pilot study with onions (*Chapter 5*) were not entirely consistent with the other onion study (*Chapter 6*). In the pilot study, the rate of absorption was smaller. However, peak levels and elimination half-lives were similar. These studies

used different batches of onions. Onions also contain quercetin-3,4'-diglucoside in addition to the 4'-glucoside.[10] No information on the individual glucosides was available as only total quercetin glycosides were determined. We speculate that the proportion of these two glucosides differed between these studies. This would imply that the 3,4'-diglucoside and the 4'-glucoside were absorbed at different rates. In addition, between-persons variation in absorption also could be an explanation. We found that one of the subjects of the pilot study had a similar slower absorption of the pure 4'-glucoside solution (results not shown).

Colon

The relatively long time needed to reach peak concentrations of quercetin in plasma after ingestion of the 3-rutinoside indicates that the rutinoside was predominantly absorbed from the colon (*Chapter 7*). This time to reach peak levels was at least 9 times longer than that after the 4'-glucoside. We obtained a similar ratio when comparing onions and the rutinoside (*Chapter 6*). The indispensable role of the microflora in the absorption of the rutinoside was apparent by comparing ileostomists (*Chapter 4*) with normal volunteers (*Chapters 6*). When we excluded the high absorber of rutinoside in each study (*see next paragraph*), the average urinary excretion as a percentage of rutinoside intake in the ileostomy study was only 0.010% (95% Confidence Interval:0.002% - 0.018%) as opposed to 0.22% (0.13% - 0.31%) in normal volunteers.

In each study we found a subject with an enhanced absorption of the rutinoside (*Chapters 4,6,7*). As the times to reach peak values were not different from the other subjects, it can be hypothesised that they also absorb the rutinoside from the colon, but more efficiently. This could point to inter-individual variations in gastro-intestinal microflora or an aberrant type of colonic absorption.

Implications for enhancement of absorption of beneficial components

The potential involvement of the sodium-glucose carrier in the absorption of quercetin-4'-glucoside suggests that it has a certain degree of tolerance for substituents in the glucose molecule. Apparently a large substituent at carbon atom C1 of the glucose moiety is possible in the ß-configuration. Previous studies found that smaller substituents, viz. various ß-phenylglucosides, were transported by the sodium-glucose cotransporter.[8,11] It was also found that larger substituents are possible in the ß-configuration at C1 than for the α-configuration.[4,8]. Knowledge on requirements of glycoside structures for transport by the sugar-carrier may be used

to design sugar-conjugated components with improved absorption. Thus, not only the absorption efficiency of a beneficial component might be improved, but also its site of absorption might be chosen.

Bioavailability

We performed single dose studies with onions and apples to compare the bioavailability of quercetin in these foods (*Chapter 6*). Bioavailability of both the quercetin form contained in apples and of pure quercetin rutinoside, was one third relative to onions. However, black tea is the major dietary source of quercetin in the Netherlands.[12] A single dose study with tea was not feasible because of the low quercetin concentration of tea infusion. The major glycosides of quercetin in black tea are quercetin-3-rutinoside, quercetin-3-glucoside and quercetin-3-galactoside.[13] At a rough estimate, the rutinoside accounts for about 50%, the glucoside for 30% and the galactoside for 20% of the quercetin glycosides present in tea leaves. The limited data available indicate that these proportions may vary depending on the origin of the tea.[13] Thus, our choice for the rutinoside to represent tea in these studies was fair. Assuming that both the 3-glucoside and the 3-galactoside are as well absorbed as the 4'-glucoside, which is probably too optimistic, we estimate that the bioavailability of quercetin glycosides of tea is less than two thirds of that of onions.

This estimation of the bioavailability of quercetin contained in tea indicates that the exposure of quercetin from tea was overestimated relative to that of onions in the epidemiological studies (*Chapter 1*). This may be relevant in the Dutch cohort studies, especially when large differences between individuals in tea intake existed. In that case, misclassification of individuals in groups of real quercetin exposure may have occurred.

These differences in bioavailability may also have affected the cross-cultural study, where large differences in dietary quercetin sources between the countries were observed.[14] However, bioavailability from wine, an important dietary source in some of the countries, has not been determined.

Prospects for bioavailability research

We have shown that the type of glycoside is a major determinant of the bioavailability of quercetin in foods. Hence, the amounts and types of quercetin glycosides of a particular food will predict the bioavailability of the quercetin present. To do so, the bioavailability of individual pure quercetin glycosides will have to be determined. Relevant quercetin glycosides have to be chosen, and it can be predicted that less than 10 different types of glycosides will significantly contribute in major dietary sources of quercetin. After determination of the contents and types of glycosides in a particular food, its bioavailability of quercetin can then be calculated using these data on bioavailability of individual glycosides. This 'molecular approach' will make dietary trials with human volunteers superfluous. Without this knowledge, dietary trials with each type of food would be necessary, and these would have little or no predictive value for other foods. In addition, it would be difficult to control all variables in a dietary trial, which would lead to imprecise outcomes.

Accumulation of quercetin in plasma

We measured elimination half-lives for quercetin of about 24 h (*Chapters 5,6,7*). Elimination half-life values for the glucoside and rutinoside of quercetin, as well as those obtained after ingestion of onions and apples were similar. This indicates that, independent of the source, absorbed quercetin is cleared from the body by the same route of elimination. The elimination half-life (24 h) is at least equal, but probably larger than the average consumption interval (3-24h) for quercetin-containing foods. As a consequence, daily habitual consumption of quercetin-rich foods will lead to accumulation of quercetin in plasma, causing a steady-state level in blood. Habitual consumption once a day of quercetin-rich products with an elimination half-life of 24 h, will accumulate the amount of quercetin in the body as compared to the daily dose by a factor 1.4.[15] Assuming a linear dose-response relation, an average steady-state level in plasma of 0.18 μM (55 ng/ml) quercetin can be predicted through habitual consumption once a day of 100 μmol (30 mg) quercetin in the form found in onions. This amount of quercetin is equivalent to 100 g of onions. The minimum plasma concentrations will then be about 0.13 μM (40 ng/ml) and the maximum concentration 0.25 μM (75 ng/ml).[15] However, other quercetin-containing foods,

e.g. tea, will normally be consumed several times during the day, which will decrease the variation in plasma quercetin concentrations.

Analytical aspects

Postcolumn chelation of quercetin enhanced fluorescence intensity considerably (*Chapter 3*) and allowed us to detect quercetin in plasma at levels as low as 7 nM (2 ng/ml). This limit of detection was sufficiently low to adequately monitor the elimination of quercetin from plasma. A drawback of this derivatization procedure is the requirement of a free hydroxyl group in the 3-position of the quercetin molecule. Conjugation at this position blocked chelation and subsequent detection. Therefore, hydrolysis of plasma samples prior to analysis was imperative.

Quercetin proved to be tightly bound to plasma and possibly also to urine constituents. A relatively long hydrolysis period was required (*Chapter 6*). Our attempts to measure quercetin in whole blood were unsuccessful. *In vitro* recovery experiments with quercetin aglycone revealed a high quercetin binding capacity of erythrocytes in our extraction medium. Even prolonged hydrolysis periods were not sufficient to remove quercetin bound to erythrocytes. We could not find any quercetin in whole blood of subjects, whereas we did find normal values in the plasma prepared from these blood samples.

Metabolism

Only a small fraction of the ingested dose of quercetin, 1.4% or less, was excreted in urine, whereas absorption of quercetin of onions was 52% (*Chapters 4 and 6*). These data suggest extensive metabolism of quercetin. Our plasma and urine values included quercetin aglycone and its conjugates that are hydrolysable with hydrochloric acid. In addition to quercetin conjugates we found 3'-methoxyquercetin conjugates in plasma and urine, but not 4'-methoxyquercetin, a metabolite described in rats.[16] However, the concentration of this metabolite also was small. Biliary excretion of absorbed quercetin could be a significant route of excretion (*Chapter 2*). This would expose quercetin conjugates to microbiological degradation. Bacteria can split the heterocyclic oxygen-containing ring of quercetin giving rise to various hydroxyphenolic acids (*see Chapter 2, page 41*). These metabolites

can evidently be absorbed because many researchers have reported their presence in urine (*Chapter 2*).

Thus, we only could detect a small fraction of the orally administered quercetin. Unlike the situation with rodents, the metabolism of quercetin by humans has not been extensively studied. Quantitative data on metabolism of quercetin in rodents are limited but it does point to extensive metabolism (*Chapter 2*).

Plasma concentration and antioxidant activity

A question to be answered by these studies was whether the plasma quercetin concentration after habitual consumption of quercetin-containing foods, may have a potential protective antioxidant effect. As calculated above, average steady-state levels of 0.2 μM quercetin with modest fluctuations throughout the day are attainable. This concentration will be reached after consumption of onions containing 30 mg of quercetin, corresponding to the intake in the highest tertile of the Zutphen Study showing reduction of risk.[17] Average plasma concentrations of antioxidant nutrients are 0.3 - 0.6 μM for ß-carotene, 15 - 40 μM for α-tocopherol, and 30 - 150 μM for ascorbic acid.[18] The antioxidant activity of an individual component has to be taken into account to be able to compare its contribution to the total antioxidant potential of plasma. The Trolox equivalent antioxidant activity (TEAC) of quercetin is 4 fold higher than that of the antioxidant (pro)vitamins.[19] However, quercetin metabolism introduces groups bound to the phenolic hydroxyls (*Chapter 2*). These modifications will decrease the antioxidant activity, because quercetin is a hydrogen-donating antioxidant.[20,21] Quercetin was able to inhibit low density lipoprotein (LDL) oxidation *in vitro*, with IC_{50} values of 2 - 20 μM.[22,23] These data suggest that physiological quercetin concentrations are possibly too low to affect LDL oxidation in plasma. Oxidation of LDL occurs primarily in the arterial wall (intima), into which LDL particles can penetrate.[24,25] It is not known whether quercetin can reach this site of oxidation. Thus, it is not clear whether dietary quercetin can play a role in the protection of LDL particles. However, quercetin protected cells *in vitro* against the cytotoxic effect of previously oxidized LDL with an IC_{50} value of only 0.1 μM.[23]

As was discussed above, bacterial metabolism can cleave the quercetin molecule generating absorbable hydroxyphenolic acids. These molecules also have phenolic hydroxyl groups and show antioxidant activity. Trolox equivalent antioxi-

dant activity (TEAC) values of hydroxyphenolic acids, e.g. hydroxycinnamic acids, are equal or higher than TEACs of antioxidant vitamins.[19,20] Thus, in addition to the parent quercetin molecule, its metabolites may contribute to the antioxidant effect of dietary quercetin in the body.

Apart from its concentration and antioxidant activity, the relative importance of an individual antioxidant in plasma depends on the nature of the oxidant, the type of target molecules to be protected, and its ability to reach the target molecules or cellular and subcellular membranes.[18,26] *In vitro* systems to measure the antioxidant activity should therefore be adapted to its putative biological action.[20] Ultimately, only studies that measure *in vivo* antioxidant effects of a given molecule can be decisive for its potential protective properties.

Do dietary polyphenols contribute to the antioxidant defences of the body?

There are many clinical conditions in which oxygen free radicals are thought to be involved.[27] Consequently, antioxidants in the diet which complement the actions of the antioxidant enzymes could play an important role in the prevention of oxidative damage by these radicals. Much of the research has been focused on coronary heart disease. The role of oxidised LDL proposed in the pathogenesis of atherosclerosis has generated much research. In the laboratory, a whole range of antioxidants has been shown to be able to prevent LDL-oxidation when added to isolated LDL. Not surprisingly, polyphenols[20,22,23,28,29] which by their chemical nature are antioxidants, or food extracts rich in polyphenols such as wine,[30-34] also show this protective action towards *in vitro* LDL-oxidation. However, the clinical relevance of these laboratory findings is still obscure.

Oxygen free radicals can cause structural damage to DNA, activate signal transduction pathways that are related to growth, differentiation, and cell death. They also can modulate the activity of stress proteins and stress genes.[35] Because of these properties oxygen free radicals are carcinogens,[35] which implies that antioxidants might protect against cancer. Indeed, anticarcinogenic properties of tea polyphenols and tea extracts have been demonstrated in many animal models.[36] In contrast to these experimental studies, epidemiological studies on tea consumption are inconclusive.[37,38] No protective effects on stomach or lung cancer were recorded.[37] A protective effect of green tea in colon cancer was suggested.[37] Possibly, beneficial effects from tea are confined to high intakes in high-risk populations.[37]

The family of polyphenols occurring in plant foods is vast, and comprises of galloyl and hexahydrodiphenoyl esters and their derivatives, condensed proanthocyanidins, flavonoids, and hydroxybenzoic and hydroxycinnamic acids.[39] An estimation of the total polyphenol intake is difficult, because only very limited data on food contents of these various polyphenols are available. The often quoted intake of all flavonoids of 1 g/day in the U.S.A.,[40] or 650 mg/day expressed as aglycones, is most certainly too high. We estimate the daily intake of all flavonoids to be a few hundreds of milligram per day expressed as the aglycones (*Chapter 2, page 24*). The flavonol/flavone subgroups accounted for 23 mg/day in the Netherlands.[41] The intake of antioxidant (pro)vitamins is much lower than that of polyphenols. It was recently estimated in the Second Dutch National Food Consumption Survey of 1992, a representative sample of households composed of 6200 Dutch persons aged 1 - 92 years.[42] The average intake of vitamin C was 73 mg/day, of vitamin E 13.7 mg/day and of ß-carotene 1.2 mg/day.

In vitro data on the antioxidant activity of polyphenols show that they are better antioxidants than the classic antioxidant vitamins.[19,20] Thus, if the antioxidant activity is an important biological property, the potential role of dietary polyphenols in disease prevention is very promising. However, different from the antioxidant vitamins, only little is known on the human physiology of polyphenols. This thesis shows that absorption of quercetin can be appreciable, and that plasma concentrations are comparable to ß-carotene. But nothing whatsoever is known about tissue distribution and target tissues for quercetin. Data on absorption of other polyphenols in humans are not known, except for catechins. However, the data available show that absorption is more than marginal, thus exposure of body tissues to dietary flavonoids seems feasible at least.

To be able to evaluate the role of dietary antioxidants in disease prevention, there is a need to develop assays for assessing *in vivo* free radical damage and protection against it. Isoprostanes as markers for lipid peroxidation *in vivo* look favourable.[43] Autoantibodies against oxidised LDL[44] seem a more promising tool than the old Esterbauer test.[45] Important questions to be addressed are: what biomolecule is the dietary antioxidant supposed to protect, and will the compound be present *in vivo* at or near the biomolecule in sufficient concentration? Research on these questions has only very recently been started. Ultimately a benefit to cardiovascular disease or cancer has to be proved in randomised clinical trials. Even the supposed benefits of the antioxidant vitamins to cardiovascular disease remain to be demonstrated in clinical trials.[46]

CONCLUSIONS

Absorption

- Absorption of quercetin glycosides can be appreciable: at least 50%.
- The sugar moiety of quercetin glycosides is a major determinant of absorption: conjugation with glucose enhances absorption.
- Quercetin-4'-glucoside is absorbed from the small intestine; the active sodium-glucose cotransporter is probably involved.
- Quercetin-3-galactoside and quercetin-3-xyloside may also be transported by the sodium-glucose carrier.
- Glucosylation of a beneficial compound potentially offers opportunities for enhancement of its absorption.
- Quercetin-3-rutinoside is absorbed from the colon: hydrolysis of the sugar moiety by bacteria is required.

Metabolism

- Quercetin is extensively metabolised: less than 1.5% of the ingested quercetin excreted in urine had an intact flavonoid structure.

Bioavailability

- Bioavailability of both the quercetin form found in apples and of pure quercetin rutinoside was 30% relative to onions.
- Bioavailability of the quercetin form found in tea is estimated at less than 66% relative to onions based on the composition of quercetin glycosides of tea.
- Quantitative data for separate glycosides of a food can predict the bioavailability of quercetin contained in it.

Plasma concentration

- Steady-state levels of quercetin of about 0.2 μM are attainable, due to accumulation.
- Plasma concentration is comparable to ß-carotene, but smaller than that of antioxidant vitamins.
- The concentration of quercetin in plasma is probably too low to prevent LDL-oxidation.
- Metabolites of quercetin potentially may play an antioxidant role.

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Summary

Flavonoids are secondary plant metabolites belonging to the large and diverse group of polyphenols. The ubiquitous occurrence of flavonoids in plant foods is of interest because it may help to explain the inverse association between consumption of vegetables and fruits and risk of chronic diseases. Flavonoids are antioxidants, because of their phenolic hydroxyl groups. Oxidative damage to tissues is hypothesised to play an important role in cancer and cardiovascular disease. Quercetin, a flavonoid occurring in tea, onions, apples and red wine, is a strong antioxidant and prevents oxidation of low density lipoproteins (LDL) *in vitro*. Oxidised LDL has been found in atherosclerotic lesions of humans, and increased plasma concentrations of autoantibodies against oxidized LDL occur in patients with atherosclerosis. Indeed, the intake of quercetin was inversely associated with subsequent cardiovascular disease in some but not all studies (*Chapter 1*). Sufficient absorption from the diet is a prerequisite for its potentially protective role in cardiovascular disease. This thesis describes studies on the human absorption and elimination kinetics of dietary quercetin glycosides, the predominant form in foods.

In *Chapter 2* we reviewed results of studies on the absorption, metabolism and pharmacokinetics of flavonoids reported in the literature. Most studies have been carried out with free flavonoid aglycones in rodents using unphysiologically high doses. Absorption depended on the type of flavonoid and was between 4 and 58%. The absorption from dietary sources, containing flavonoid glycosides, is largely unknown. In the metabolism of flavonoids the liver and the colon are important. Evidence for phase II biotransformation reactions in the liver is abundant. Colonic bacteria are involved in hydrolysis of glycosides and conjugates and in ring fission producing phenolic acids which are absorbed. Three types of ring fission depending on the structure of the flavonoid C-ring have been described. The susceptibility to ring cleavage is determined by the pattern of free hydroxyl groups in the flavonoid molecule. Very few quantitative data on metabolism are available. Pharmacokinetic data on flavonoids are scarce and absolute bioavailability of dietary flavonoids has not been determined.

To be able to study the absorption of flavonols in man, we developed a postcolumn derivatization technique for flavonols in HPLC with fluorescence detection using aluminum (*Chapter 3*). Variables governing postcolumn chelation

of quercetin, such as water content, buffer and organic modifier of the eluent, concentration of Al^{3+} and presence of acetic acid in the postcolumn reagent, and temperature were studied and optimized. Of the flavonoids, only flavonols that contain a free 3-hydroxyl and 4-keto oxygen binding site form fluorescent complexes with Al^{3+}. The method has a detection limit of 0.50 nM (0.15 ng/mL) for quercetin, 0.17 nM (0.05 ng/mL) for kaempferol, 1.5 nM (0.45 ng/mL) for myricetin, and 0.17 nM (0.05 mg/mL) for isorhamnetin, thus improving detectability of quercetin 300 fold as compared to that possible with UV detection. The reproducibility relative standard deviation of the method was 1.4%.

In *Chapter 4*, we studied the absorption of various forms of quercetin. Nine healthy ileostomy subjects were studied, to avoid losses of quercetin caused by colonic bacteria. They followed a quercetin-free diet for 12 days; on days 4, 8 and 12 they received a supplement of fried onions, rich in quercetin glucosides (295 μmol), at breakfast, pure quercetin rutinoside (331 μmol), a major quercetin compound in tea or 331 μmol (100 mg) pure quercetin aglycone, in random order. Subsequently, participants collected ileostomy effluent and urine for 13 hours. *In vitro* incubations of quercetin or its glycosides with gastrointestinal fluids showed minimal degradation. Absorption of quercetin, defined as oral intake minus ileostomy excretion and corrected for 14% degradation within the ileostomy bag, was 52 $\pm$ 15% for quercetin glucosides from onions, 17 $\pm$ 15% for quercetin rutinoside, and 24 $\pm$ 9% for quercetin aglycone. Mean excretion of quercetin or its conjugates in urine was 0.5% of the amount absorbed; quercetin excretion in urine was negatively correlated with excretion in ileostomy effluent (r = -0.78, n = 27). Thus, humans absorb appreciable amounts of quercetin and absorption is enhanced by conjugation of quercetin with glucose.

We studied the time course of the plasma quercetin concentration in two subjects after ingestion of fried onions containing 212 μmol quercetin glucosides (*Chapter 5*). Peak plasma levels of 0.65 μM (196 ng/ml) were reached after 2.9 h, with a half-life of absorption of 0.87 h. The half-life of the distribution phase was 3.8 h, and of the subsequent elimination phase 16.8 h. After 48 h the plasma concentration was about 0.033 μM (10 ng/ml). These data showed that quercetin glucosides contained in onions are absorbed and are eliminated slowly throughout the day. Thus, the dietary antioxidant quercetine could increase the antioxidant capacity of blood plasma.

In *Chapter 6* we studied the absorption and elimination kinetics of quercetin contained in major dietary sources, viz. fried onions containing 225 μmol glucose

compounds of quercetin, apples containing 325 μmol of both glucose- and non-glucose quercetin glycosides, and of 330 μmol quercetin-3-rutinoside. Peak plasma levels were 0.75 μM (225 ng/ml) 0.7 h after ingestion of the onions, 0.30 μM (90 ng/ml) 2.5 h after the apples, and 0.30 μM (90 ng/ml) 9 h after the rutinoside. Half-lives of elimination were 28 h for onions and 23 h for apples. Bioavailability of both quercetin from apples and of pure quercetin rutinoside was 30% relative to onions. Urinary excretion of quercetin was 1.39% of the ingested dose for onions, 0.44% for apples, and 0.35% for quercetin-3-rutinoside. We concluded that quercetin glucosides contained in onions are absorbed rapidly, that the rutinoside is absorbed slowly, and that the overall absorption rate of quercetin glycosides contained in apples is intermediate. We propose that the sugar moiety of the glycoside is an important determinant of absorption. Absorbed quercetin was eliminated only slowly from the blood. Repeated consumption of quercetin-containing foods may therefore lead to accumulation of quercetin in blood and increase the antioxidant capacity of blood plasma.

The role of the sugar moiety in the absorption of quercetin glycosides was studied in *Chapter 7*. Nine fasting subjects ingested pure solutions of quercetin-4'-glucoside (311 μmol) and quercetin-3-rutinoside (311 μmol). Intake of quercetin glucoside led to a much more rapid and larger rise of quercetin levels in plasma than intake of the rutinoside. The mean peak plasma level of quercetin was 3.20 μM after the glucoside and only 0.23 μM after the rutinoside. Peak levels were reached <0.6 h after ingestion of the glucoside, and 5.6 h after the rutinoside. Elimination of quercetin from plasma was similar for the two sources with half-lives of about 24 h. We concluded that the glucoside is rapidly absorbed from the small intestine, whereas the rutinoside is only absorbed from the colon. We propose that the sodium-glucose cotransporter in the brush border membranes of the small intestine plays a role in the absorption of the glucoside.

In conclusion (*Chapter 8*), absorption of quercetin glycosides present in foods can be appreciable. The sugar moiety of the glycoside is an important determinant of its absorption, and absorption of glycosides is possible without prior hydrolysis of the sugar bond. We propose that quercetin-4'-glucoside is drawn into the enterocyte by its glucose moiety, which is carried by the sodium-glucose cotransporter. The composition of a food in terms of quercetin glycosides will predict the bioavailability of the quercetin contained in it. After absorption, quercetin is only slowly eliminated from plasma. This will lead to accumulation of quercetin in plasma after regular consumption of quercetin-containing foods.The

plasma concentration of quercetin can be comparable to ß-carotene, but is smaller than that of the antioxidant vitamins. Antioxidant metabolites of quercetin formed after ingestion may contribute to the potential antioxidant protection by dietary quercetin.

Samenvatting

Flavonoïden zijn secundaire plantenmetabolieten die tot de grote en diverse groep van polyfenolen behoren. Het wijdverspreid voorkomen van flavonoïden in plantaardige voedingsmiddelen is interessant omdat dit mogelijk een verklaring biedt voor het inverse verband tussen groente- en fruitconsumptie en het risico voor chronische ziekten. Flavonoïden zijn antioxidanten doordat ze fenolische hydroxylgroepen bevatten. Er wordt verondersteld dat oxidatieve schade aan weefsels een belangrijke rol speelt in hart- en vaatziekten en kanker. Quercetine, een flavonoïd dat voorkomt in thee, uien, appels en rode wijn, is een krachtige antioxidant en voorkomt oxidatie van lage dichtheids lipoproteïnen (LDL) in het laboratorium (*in vitro*). In een aantal, maar niet alle, epidemiologische studies werd dan ook een invers verband gevonden tussen de inneming van quercetine en hart- en vaatziekten (*Hoofdstuk 1*). Een voorwaarde voor deze mogelijk beschermende rol in hart- en vaatziekten is dat quercetine voldoende geabsorbeerd wordt uit voedingsmiddelen. Dit proefschrift beschrijft onderzoek naar de kinetiek van absorptie en eliminatie bij de mens van quercetine glycosiden, de voornaamste vorm in levensmiddelen.

In *Hoofdstuk 2* gaven we een overzicht van studies over absorptie, metabolisme en farmacokinetiek gerapporteerd in de literatuur. De meeste van deze studies werden uitgevoerd met vrije flavonoïd aglyconen in knaagdieren, waarbij niet-fysiologische hoge doses werden gebruikt. De absorptie hing af van het type flavonoïd en bleek tussen 4 en 58% te liggen. De absorptie uit de voeding, waarin glycosiden van flavonoïden voorkomen, is grotendeels onbekend. De lever en het colon zijn belangrijk voor het metabolisme van flavonoïden. Er is overvloedig bewijs voor phase II biotransformatie reacties in de lever. Bacteria in het colon zijn betrokken bij de hydrolyse van glycosiden en conjugaten en bij ringsplitsing, waarbij fenolische zuren ontstaan die geabsorbeerd kunnen worden. Er zijn drie soorten ringsplitsing beschreven, die afhankelijk zijn van de structuur van de C-ring van het flavonoïd. De gevoeligheid voor ringsplitsing wordt bepaald door het patroon van vrije hydroxylgroepen in het flavonoïdmolecuul. Er zijn slechts weinig kwantitatieve gegevens over metabolisme beschikbaar. Farmacokinetische gegevens zijn er nauwelijks, en de absolute biobeschikbaarheid van flavonoïden die aanwezig zijn in voedingsmiddelen is niet bepaald.

Om de absorptie van flavonolen bij de mens te kunnen bepalen, ontwikkelden we voor flavonolen een postcolumn derivatiseringstechniek met aluminium, toe

te passen in HPLC met fluorescentiedetectie (*Hoofdstuk 3*). Variabelen die de postcolumn complexering van quercetine beïnvloeden, nl. het watergehalte, de buffer en de organische modifier van het eluens, de concentratie van Al^{3+} en de aanwezigheid van azijnzuur in het reagens voor de postcolumnreactie, en de temperatuur werden bekeken en geoptimaliseerd. Alleen flavonolen die als bindingsplaatsen een vrije 3-hydroxyl en een 4-keto zuurstof hebben, konden fluorescerende complexen met Al^{3+} vormen. De methode had een detectiegrens van 0.50 nM (0.15 ng/ml) voor quercetine, 0.17 nM (0.05 ng/ml) voor kaempferol, 1.5 nM (0.45 ng/ml) voor myricetine en 0.17 nM (0.05 ng/ml) voor isorhamnetine. Dit betekent dat de detecteerbaarheid van quercetine met een factor 300 vergroot wordt in vergelijking to UV-detectie. De reproduceerbaarheids relatieve standaardafwijking van de methode was 1.4%.

In *Hoofdstuk 4* bestudeerden we de absorptie van verschillende vormen van quercetine. Negen gezonde proefpersonen met een ileostoma namen deel. Deze proefpersonen werden gekozen om verliezen van quercetine door bacteriële afbraak in het colon te voorkomen. Ze volgden een quercetinevrij dieet gedurende 12 dagen; op de dagen 4, 8 en 12 kregen ze bij het ontbijt in willekeurige volgorde een supplement van gebakken uien, die quercetine glucosiden (295 μmol) bevatten, of zuiver quercetine rutinoside (331 μmol), een belangrijk glycoside in thee, of 331 μmol (100 mg) zuiver aglycon. Vervolgens verzamelden de proefpersonen hun ileostoma vloeistof en urine gedurende 13 uur. *In vitro* incubatie van quercetine en quercetine glycosiden met gastro-intestinale vloeistoffen liet slechts minimale afbraak zien. De absorptie van quercetine, gedefinieerd als orale inneming minus uitscheiding met ileostoma vloeistof en gecorrigeerd voor 14% afbraak in de ileostoma zak, was 52 $\pm$ 15% voor de quercetin glucosiden uit uien, 17 $\pm$ 15% voor het quercetine rutinoside, en 24 $\pm$ 9% voor het quercetine aglycon. De gemiddelde uitscheiding van quercetine of quercetine conjugaten met de urine was slechts 0.5% van de geabsorbeerde hoeveelheid; de uitscheiding van quercetine in de urine was negatief gecorreleerd met de uitscheiding in ileostoma effluent (r = -0.78, n = 27). Absorptie van quercetine bij de mens is dus aanzienlijk en wordt versterkt door conjugatie van quercetine met glucose.

We bepaalden het tijdsverloop van de concentratie van quercetine in plasma bij twee proefpersonen na inneming van gebakken uien die 212 μmol quercetine glucosiden bevatten (*Hoofdstuk 5*). Piek plasmawaarden van 0.65 μM (196 ng/ml) werden bereikt na 2.9 uur; de halfwaardetijd voor de absorptie was 0.87 uur. De halfwaardetijd voor de distributiefase was 3.8 uur, en die voor de eliminatiefase

16.8 uur. Na 48 uur was de concentratie in plasma ongeveer 33 nM (10 ng/ml). Uit deze gegevens volgt dat quercetine glucosiden uit uien geabsorbeerd worden en slechts langzaam gedurende de dag worden geëlimineerd. De antioxidant quercetine aanwezig in de voeding zou dus de antioxidantcapaciteit van bloed plasma kunnen vergroten.

In *Hoofdstuk 6* werd de kinetiek van absorptie en eliminatie van quercetine uit belangrijke bronnen in de voeding bepaald, nl. uit gebakken uien die 225 μmol glucoseverbindingen van quercetine bevatten, uit appels die zowel glucose als niet-glucose glycosiden van quercetine bevatten (325 μmol), en van 330 μmol quercetine-3-rutinoside. Piek plasmawaarden waren 0.75 μM (225 ng/ml) 0.7 uur na consumptie van de uien , 0.30 μM (90 ng/ml) 2.5 uur na de appels, en 0.30 μM (90 ng/ml) 9 uur na het rutinoside. De eliminatie halfwaardetijd was 28 uur voor uien en 23 uur voor appels. De biobeschikbaarheid van zowel quercetine uit appels als van het quercetine rutinoside was 30% vergeleken met uien. De uitscheiding van quercetine in urine was 1.39% van de geconsumeerde hoeveelheid met de uien. Dit was 0.44% voor de appels en 0.35% voor het quercetine-3-rutinoside. We concludeerden dat de quercetine glucosiden uit uien snel worden geabsorbeerd, dat het rutinoside langzaam wordt geabsorbeerd, en dat de overall absorptiesnelheid van quercetine glycosiden uit appels er tussenin ligt. Volgens ons is de suikergroep van het glycoside een belangrijke bepalende factor voor absorptie. Het geabsorbeerde quercetine werd slechts langzaam uit het bloed verwijderd. Herhaalde consumptie van quercetine bevattende voedingsmiddelen zal daarom leiden tot accumulatie van quercetine in het bloed en derhalve de antioxidant capaciteit van het bloed verhogen.

De rol van de suikergroep bij de absorptie van quercetine glycosiden werd bekeken in *Hoofdstuk 7*. Negen nuchtere proefpersonen kregen oplossingen van quercetine-4'-glucoside (311 μmol) en quercetine-3-rutinoside (311 μmol) te drinken. Inneming van het quercetine glucoside leidde tot een veel snellere en grotere stijging in quercetine spiegels in plasma dan inneming van het rutinoside. De gemiddelde piekwaarde van quercetine in plasma was 3.20 μM na het glucoside en slechts 0.23 μM na het rutinoside. De piekwaarden werden < 0.6 uur na de inneming van het 4'-glucoside bereikt en 5.6 uur na het rutinoside. De eliminatie van quercetine uit plasma was vergelijkbaar voor beide bronnen: de halwaardetijden waren ongeveer 24 uur. We conludeerden dat het glucoside snel wordt geabsorbeerd in de dunne darm, terwijl het rutinoside slechts langzaam in het colon wordt geabsorbeerd. We veronderstellen dat de natrium-glucose cotransporter in

de 'brush border' membranen van de dunne darm een rol speelt bij de absorptie van het glucoside.

Geconcludeerd wordt (*Hoofdstuk 8*) dat de absorptie van quercetine glycosiden aanwezig in voedingsmiddelen aanzienlijk kan zijn. De suikergroep van het glycoside is een belangrijke bepalende factor voor absorptie, en absorptie van glycosiden is mogelijk zonder voorafgaande hydrolyse van de suikerbinding. We veronderstellen dat quercetine-4'-glucoside de enterocyt ingetrokken wordt via de suikergroep, die vervoerd wordt door de natrium-glucose cotransporter. Kennis van de samenstelling van een voedingsmiddel voor wat betreft de quercetine glycosiden maakt het mogelijk de biobeschikbaarheid van quercetine te voorspellen. Na absorptie wordt quercetine slechts langzaam verwijderd uit het plasma. Dit veroorzaakt accumulatie van quercetine in plasma bij geregelde consumptie van quercetine bevattende voedingsmiddelen. De concentratie van quercetine in plasma kan vergelijkbaar zijn met die van ß-caroteen, maar is kleiner dan die van de antioxidantvitamines. Metabolieten met antioxidantwerking gevormd na consumptie dragen mogelijk bij aan de potentiële antioxidantbescherming door quercetine bevattende produkten.

Dankwoord

Blijkbaar is na mijn afstuderen, nu al weer heel wat jaren geleden, het onderzoeksvuurtje altijd blijven smeulen. Martijn, jij hebt in de afgelopen jaren dit vuurtje vakkundig opgestookt door voor de benodigde brandstof en zuurstof ('de brainstorms', discussies, ideeën, opmerkingen, kritische vragen, kontakten, E-mailtjes) te zorgen. Jouw aanstekelijke enthousiasme om uit te zoeken hoe iets nu precies in elkaar steekt ("...maar worden die stoffen ook geabsorbeerd?"...) maakte mij duidelijk dat onderzoek heel spannend kan zijn. En dat was dit onderzoek zeker! En als ons onderzoek dan opgeschreven moest worden, dan was er altijd dat onverbiddelijke potlood. Het potlood dat geen wollig taalgebruik duldt, duidelijke grafieken (groter!) vraagt, vage beweringen schrapt, en heldere conclusies eist.

Als ik me goed herinner begonnen mijn eerste kontakten met de Vakgroep Humane Voeding zo rond 1984. Twijfels waren er toen over de vergelijkbaarheid van de doodgewone analyses voor eiwit, koolhydraten, vet enz. in voedingsmiddelen. Dat moest maar eens 'geringtest' worden in internationaal verband. Toevallig hadden wij daar bij het RIKILT enige ervaring mee, en het samenwerkingsverband was gauw gelegd. Dit project liep blijkbaar tot beider tevredenheid, want in de loop der jaren nam het aantal projecten waarin we samenwerkten gestaag toe. Een intensivering van de samenwerking ontstond toen het RIKILT besloot zich sterker te profileren als onderzoeksinstituut, en daar de consequentie uit trok dat er AIO-projekten moesten komen. Professor Hautvast, voor u was het een vanzelfsprekende zaak dat er een Voedings-AIO zou moeten komen; en uiteraard kwam hij er ook: Michaël Hertog. Uw inspanningen om het voedingsonderzoek binnen de landbouwkundige onderzoeksinstituten de plaats te geven die het toekomt, werden hiermee beloond. Ik heb altijd met erg veel plezier op uw vakgroep rondgelopen en heb hier veel inspirerende kontakten kunnen leggen. Hierdoor is in de loop der jaren bij mij het 'thuisgevoel' gestadig gegroeid.

Het leek zo simpel Jeanne. We geven een aantal proefpersonen flink wat uien en appels te eten en kijken dan wat we in het bloed zien verschijnen. Van jou heb ik geleerd dat een gecontroleerde voedingsproef heel wat meer inhoudt. Door jouw efficiënte manier van werken liepen de proeven met vrijwilligers steeds op rolletjes.

Zonder mijn achterland, de analytische chemie, zo ruim vertegenwoordigd op het RIKILT-DLO, was dit proefschrift nooit tot stand gekomen. Wim de Wit, jij gaf me de mogelijkheid dit promotie-onderzoek te starten. Bert-Jan, je had er

gelukkig begrip voor dat een promovendus niet al te veel andere dingen aan zijn hoofd wil hebben: je hebt me efficiënt afgeschermd van een aantal taken.

De vele uren die ik vooral in het begin van dit onderzoek in het laboratorium heb doorgebracht hebben mij er opnieuw van overtuigd dat analytische chemie een ontzettend leuk vak is. John, Dini, Michel en Betty, ik heb jullie analytische vakkennis altijd hard nodig gehad. John, jouw aandacht en interesse voor automatisering en GLP (je labjournaals zijn wat mij betreft de 'gouden' standaard) zijn onmisbaar geweest voor het succesvol lopen van de toch wel heel precaire postcolumn-methode. Dini, ofschoon je slechts voor een beperkt deel van je tijd aan dit project deelnam, leverde jouw rijke ervaring met flavonoïdenonderzoek goede ideeën op. Bovendien kon ik met een gerust hart andere zaken aan jou overlaten. Michel, je kwam er pas later bij toen we dreigden te verdrinken in grote aantallen monsters. Die monster-stromen verdwenen als sneeuw voor de zon omdat jij je handen stevig liet wapperen en er geen been in zag weken achter elkaar tot na zevenen 's avonds door te werken. Daarnaast wist je te zorgen voor een prima werksfeer. Betty, jouw interesse in en betrokkenheid bij ons flavonoïdenonderzoek heb ik steeds erg weten te waarderen.

Jan en Matti, jullie degelijk maalwerk stond aan de basis van veel van onze analyses. Marcel, jij verhoogde mijn absorptiesnelheid van de farmacokinetiek aanzienlijk, waardoor je o.a. voorkwam dat ik met een verkeerd beeld over bioavailability opgezadeld werd. Jacques, met plezier denk ik terug aan onze gesprekken, aan de koffietafel of op ons zonovergoten terras, die over meer dan alleen plasmawaarden of renale klaring gingen. En dan was er natuurlijk Jacob, die altijd wel in was voor een goed gesprek dat meestal al vlot vooral niet over het onderzoek ging. Tini, jouw belangstelling en enthousiasme voor 'het onderzoek' was verfrissend en zeer stimulerend.

Onno Korver, Lilian en Karin, jullie bijstand in woord maar vooral ook in daad was onmisbaar voor het onderzoek. Ik heb het steeds erg plezierig gevonden met jullie ideeën uit te wisselen; dat moesten we maar lang instandhouden.

Zonder proefpersonen geen voedings- of supplementenproef, en daarom vanaf deze plaats bedankt voor jullie bereidwilligheid, uithoudingsvermogen, trouw, humor, gezelligheid .....En dan waren er gelukkig ook studenten die hun afstudeeropdracht Voeding op het RIKILT wilden uitvoeren. Jullie leverden kleurrijke, originele, en altijd enthousiaste bijdragen, en zorgden daarbij voor heel wat afwisseling op het lab: Henriëtte (pionier), Coby en Lotje (kruidig), Sonja (Murphy kreeg jou niet klein) en Saskia (groente- en fruitkoningin), Martijn (talloze

prikmomenten en pompoensoep), Else en Yvonne (day and night shifts), Hanneke (chips,.... kilo's uien), Thomas (...van krantenjongen tot..).

Onze proefpersonen lieten zich steeds zeer gewillig bloed afnemen, en dat kwam zeker niet op de laatse plaats door de vakkundige prikkers Joke, Jan, Robert, en Marga. Monique, je hebt door je prikwerk een essentiële proef mogelijk gemaakt: geen moeite was je teveel zodat alles gladjes verliep. Het was daarnaast ook heel gezellig daar in dat verre vroege Nijmegen.

Onderzoek doen is leuk, maar het wordt dubbel zo leuk als je er ook nog over kunt praten met promovendi-lotgenoten. Rob, Martine, Karin, Annet, Saskia, Marjolein, Ingeborg, Peter, Kees, Nynke, Gerda, Marijke, en Reggy, onze PhD-tour in de V.S. staat in mijn geheugen gegrift. Behalve wetenschappelijk interessant, was het voor mij onvergetelijk in Atlanta samen te genieten van 'Grease', dé film uit jullie jeugd. Overigens was daar ook de niet te versmaden 'Beach Club'.

Shelagh and Mike, thank you very much for polishing my English. And Mike, I hope we will continue to meet anywhere in Europe.

Pa, ik ben natuurlijk apetrots op de prachtige pastel die mijn boekje opsiert. Dat flavonoïden kleur geven aan de wereld om ons heen weet jij natuurlijk al lang gezien de prachtige impressies van de natuur die je in de loop der jaren op papier en linnen hebt gemaakt. Ik vind het erg leuk dat 'mijn' uien je geïnsprireerd hebben tot een prachtige serie pastels en aquarellen.

Onderzoek houdt zich niet aan de kantoortijden en vreet daarnaast veel vrije tijd. Paula, Pascal en Danielle, dat tijd een schaars artikel is hebben jullie aan den lijve moeten ondervinden. Jullie waren de allerbeste supporters die ik me wensen kon. Zonder jullie was dit proefschrift misschien wel nooit geschreven, en vooral, er zou helemaal niks aan geweest zijn!

Over de auteur

Peter Hollman werd in 1951 geboren in Maastricht en behaalde het diploma HBS-B in Den Bosch. Op de middelbare school werd zijn belangstelling voor de chemie gewekt en ging hij vervolgens aan de toenmalige Technische Hogeschool in Eindhoven studeren. In 1974 studeerde hij hier af bij de vakgroep Instrumentele Analyse van de afdeling Scheikundige Technologie. Nadat hij de niet te ontlopen en zeer knellende wapenrok afgelegd had, volgde een korte periode als leraar natuurkunde aan de VWO/HAVO-afdeling van een middelbare school. Begin 1977 trad hij in dienst van het Rijkszuivelstation Leiden. Eind 1977 volgde zijn aanstelling bij het Rijkskwaliteitsinstituut voor Land- en Tuinbouwprodukten (i.o.). Bij dit instituut heeft hij analytisch chemisch onderzoek gedaan aan een breed scala aan producten van de land- en tuinbouw. Zijn interesse evolueerde naar (micro)-nutriënten in voedingsmiddelen en de kwaliteit van analytische gegevens op dit gebied. Eind jaren 80 initieerde hij onderzoek naar bioactieve stoffen (flavonoïden) in plantaardige voedingsmiddelen die mogelijk een rol spelen bij het voorkómen van chronisch ziekten. Sindsdien is hij projectleider van het onderzoek naar gezondheidsbeschermende stoffen in de voeding. Het onderzoek beschreven in dit proefschrift werd in september 1993 gestart en werd uitgevoerd bij het RIKILT-DLO en de Vakgroep Humane Voeding van de Landbouwuniversiteit Wageningen.

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