Bioavailability of flavonoids and cinnamic acids and their effect on plasma homocysteine in humans

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Proefschrift

Ter verkrijging van de graad van doctor
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STELLINGEN

1. Met het oog op de biobeschikbaarheid van fenolen is het mogelijk dat modificatie van de vorm waarin fenolen in voeding vóórkomen meer oplevert dan het verhogen van de hoeveelheid in voeding (n.a.v. dit proefschrift).

2. Indien een hoog homocysteïnegehalte een risico blijkt te zijn voor de gezondheid, dan is de consumptie van fenolen en van voedingsmiddelen die rijk zijn aan fenolen een potentieel risico voor de gezondheid (n.a.v. dit proefschrift).

3. Het is niet ondenkbaar dat in de toekomst op een pak koffie komt te staan: 'geniet, maar drink met mate'.

4. De grote variatie tussen personen in de gevoeligheid voor gewichtstoename bij een overmaat aan energie-inname kan mogelijk deels worden verklaard door verschillen in 'onbewust energieverbruik', zoals spieractiviteit om het lichaam in balans te houden, spontane spiercontracties en zenuwtrekjes (Levine et al., Science 1999;283:212-214).

5. Matig alcoholgebruik beschermt vermoedelijk tegen hart- en vaatziekten. Mensen met hart- en vaatproblemen zouden de waarschuwing 'geniet, maar drink met mate' daarom ook kunnen zien als een voedingsadvies.

6. Voorkómen van ziekte is veelal moeilijker dan genezen.

7. Dat vrijwilligerswerk vrijwel onmisbaar is in de huidige welvarende maatschappij is een vorm van armoede.

8. Reizen door Nederland met de auto of de trein is een goede oefening in 'onthaasting'.

Stellingen behorend bij het proefschrift *'Bioavailability of flavonoids and cinnamic acids and their effect on plasma homocysteine in humans'*
Margreet Olthof, Wageningen, 13 juni 2001

Aan mijn ouders

ABSTRACT

Bioavailability of flavonoids and cinnamic acids and their effect on plasma homocysteine in humans.

PhD thesis by Margreet R. Olthof, Division of Human Nutrition and Epidemiology, Wageningen University, the Netherlands. June 13, 2001

Dietary antioxidants might prevent oxidative damage to tissues and therefore protect against cardiovascular disease and cancer. Dietary phenols are strong antioxidants in vitro but their role in vivo is uncertain. Furthermore, there are only limited data on their bioavailability in humans. The aim of this thesis was to investigate *whether bioavailability data on flavonoids and cinnamic acids support the hypothesis that they can affect health in humans.* Because the group of phenols in foods is huge, we focussed our research on major phenols in foods; the flavonol quercetin, black tea phenols and chlorogenic acid (5-caffeoylquinic acid). We studied their bioavailability and effect on plasma homocysteine in humans, a potential risk factor for cardiovascular disease.

The bioavailability of quercetin and chlorogenic acid depends upon their conjugated moieties. Hollman et al. found that the bioavailability of quercetin-3-rutinoside, a major flavonol in tea, was only 20% of that of quercetin-4'-glucoside. We found that transformation of quercetin-3-rutinoside into quercetin-3-glucoside will improve its bioavailability because the 3-glucoside had the same high bioavailability as the 4'-glucoside. Caffeic acid is a major phenol in coffee, but it is present as a conjugate with quinic acid, called chlorogenic acid. We found that the conjugation of caffeic acid with quinic acid hinders absorption in humans: absorption of chlorogenic acid was only 30% of that of its caffeic acid moiety.

Furthermore, we found that chlorogenic acid, black tea solids and quercetin-3-rutinoside are extensively metabolized in the human body, mainly before they reach the circulation. Their metabolites have no, or less, antioxidant activity in vitro than their parent phenols. Therefore the role of dietary phenols as antioxidants in vivo might be less important than suggested by their in vitro antioxidant activity.

Coffee consumption increases plasma homocysteine, a potential risk factor for cardiovascular disease. Chlorogenic acid from coffee is partly responsible for the homocysteine-raising effect of coffee, because we found that it increased plasma homocysteine. Black tea solids also raised plasma homocysteine, whereas quercetin-3-rutinoside did not. Furthermore, we found that glycination of metabolites of phenols in the body is not involved in the homocysteine-raising effect of phenols.

In conclusion, chlorogenic acid, tea phenols and quercetin are available in the human body, but their effects on health are uncertain. Further research on bioavailability and health effects of dietary phenols is needed.

CONTENTS

1

Introduction

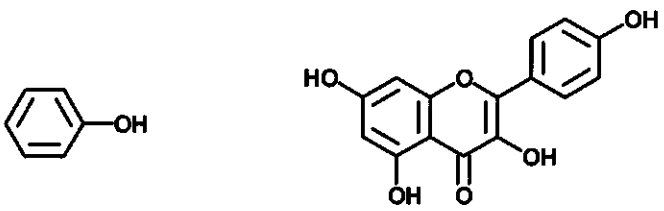
GENERAL INTRODUCTION

Epidemiological studies suggest that consumption of a diet rich in fruits and vegetables reduces the risk of chronic diseases such as cardiovascular disease and cancer (Ness & Powles 1997, World Cancer research Fund/American Institute for Cancer Research 1997), although the epidemiological evidence is not conclusive (Ness et al. 1999).

Dietary antioxidants present in fruits and vegetables might play a role in the prevention of disease because they can prevent oxidative damage to tissues. Oxidative damage is believed to be involved in several diseases (Halliwell 1994, Stohs 1995, Thomas 1995). For example, oxidation of Low Density Lipoprotein (LDL) cholesterol plays a role in the pathogenesis of atherosclerosis (Chisolm & Steinberg 2000). Oxidative damage of tissues and/or DNA might be involved in the cancer process (Halliwell 1994).

Major antioxidants in the diet are antioxidant (pro)vitamins, such as beta-carotene, vitamin C and vitamin E, but their role in disease prevention is still not clear (Buring & Hennekens 1997, Marchioli 1999, Jha et al. 1995). Besides antioxidant vitamins, the interest in the potential role in human health of non-vitamin antioxidants, such as phenols, is increasing. Many phenols have higher antioxidant activities than antioxidant vitamins. For example, the antioxidant activity of the flavonoid quercetin is 4 times higher than that of vitamin E (Rice-Evans et al. 1996).

The term 'phenols' is used for compounds characterized by an aromatic ring structure with one or more hydroxyl groups (Figure 1.1). However a distinction can be made between 'simple phenols' and 'polyphenols'. Simple phenols are compounds containing a single aromatic ring and bearing one or more aromatic hydroxyl groups. Polyphenols are compounds containing at least two aromatic rings each bearing at least one aromatic hydroxyl group (Clifford 1999b). The number and position of hydroxyl groups is related to the antioxidant activity of phenols (Rice-Evans et al. 1996).



(A) (B)

Figure 1.1 Basic chemical structure of 'simple phenols' (A) and an example of 'polyphenols' (B)

In order to study the health effects of phenols, research into the bioavailability of phenols, as well as research into the actual health effects of phenols, is necessary. The studies in this thesis describe the bioavailability, absorption and metabolism of major dietary phenols: the flavonol quercetin, tea phenols, and cinnamic acids. We also studied the effect of these phenols on plasma homocysteine. This introduction provides information on the occurrence of phenols in foods, on the current knowledge with regard to bioavailability of phenols and potential biological effects of phenols. At the end of the introduction the rationale and outline of this thesis are described.

PHENOLS IN PLANTS

Phenols are essential compounds in plants for growth and reproduction, and they protect the plant against UV radiation, pathogens and herbivorous predators (Parr & Bolwell 2000). Plant phenols are synthesized in the plant via the shikimic acid pathway in which the amino acid phenylalanine plays a central role (Figure 1.2) (van Genderen et al. 1996, Parr & Bolwell 2000). The phenylpropanoid unit C_6-C_3:  is the basic structure from which a variety of phenols is synthesized. Phenols can be divided in subgroups based on their backbone carbon structure (Figure 1.2). Within these subgroups various compounds exist, for example more than 4,000 flavonoids have been identified.

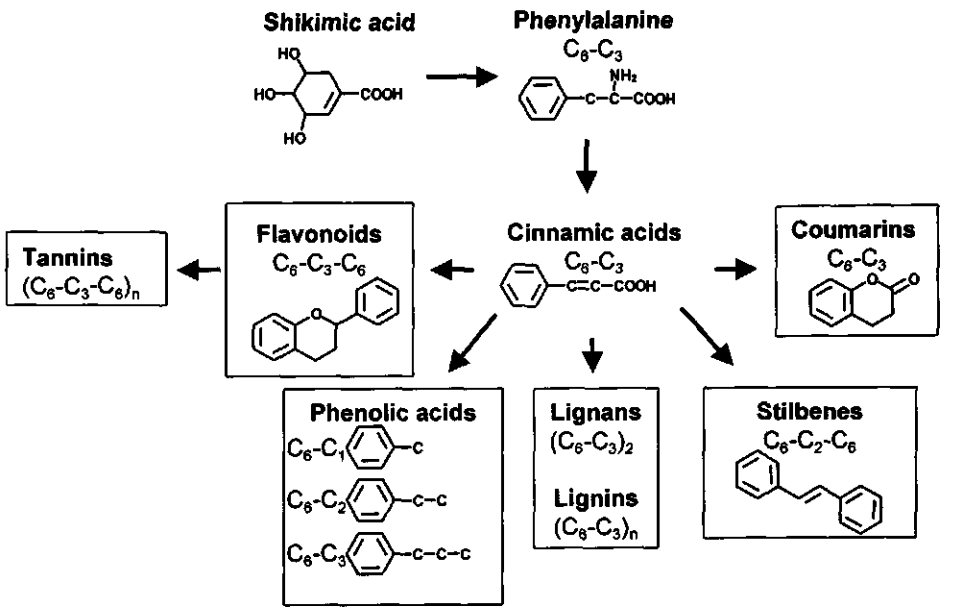


Figure 1.2 Synthesis of plant phenols via the shikimic acid pathway

PHENOLS IN FOODS AND INTAKE IN HUMANS

Phenols are ubiquitous in plants and therefore they are a basic part of the human diet. Furthermore, they are used in the food industry as additives such as colorants, flavors and preservatives. Phenols in foods are generally conjugated with sugars, but links with other compounds such as carboxylic and organic acids, amines, lipids and other phenols also occur (Figure 1.3) (Bravo 1998). Major phenols in the diet are flavonoids and cinnamic acids (Figure 1.2 and Table 1.1). Plant foods are the only dietary sources of flavonoids. Phenolic acids are also derived from plant foods, but they can also be formed in humans out of other phenols.

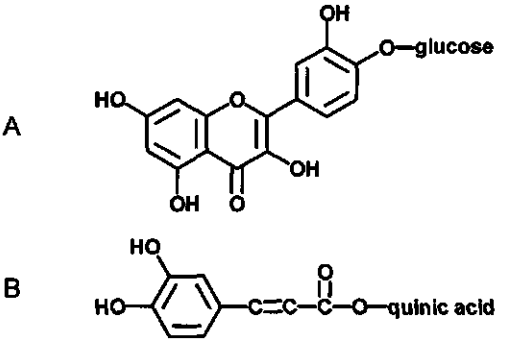


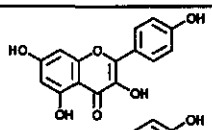
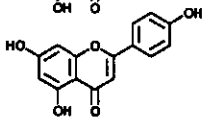
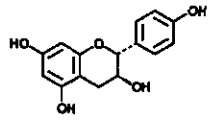
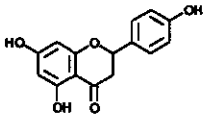
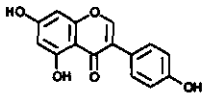
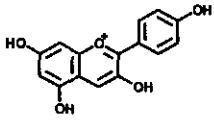
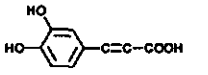
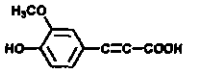
Figure 1.3 Examples of chemical structures of (A) the flavonol quercetin conjugated with glucose called quercetin-4'-glucoside, and of (B) the cinnamic acid caffeic acid conjugated with quinic acid, called chlorogenic acid.

The daily intake of flavonoids and cinnamic acids is difficult to estimate because food composition tables are not available. Kuhnau (Kuhnau 1976) roughly estimated that the daily intake of flavonoids would be approximately 1 g, but later this turned out to be too high (Table 1.1). Flavonoids can be divided into 6 subgroups: flavonols, flavones, catechins, flavanones, isoflavones and anthocyanidins. More than half of the intake of the flavonols and flavones consists of the flavonol quercetin (Hertog et al. 1993a). The estimate of the daily intake of 50 mg of catechins represents the intake of catechins present in foods as single catechin molecules (monomeric catechins) (Table 1.1). However, black tea is a major source of catechins, and during processing of black tea, tea catechins form complex condensation products such as theaflavins and thearubigins, which are difficult to measure (Balentine et al. 1997). Therefore, the actual intake of catechins is probably higher.

Caffeic acid and ferulic acid are major cinnamic acids in foods (Table 1.1). Caffeic acid occurs in food mostly as a conjugate with quinic acid, called chlorogenic acid (Figure 1.3). In coffee drinkers, coffee provides about 90% of the caffeic acid intake (Clifford 1999a, Radtke et al. 1998). Ferulic acid is associated with dietary fiber. The colonic microflora releases ferulic acid from fiber (Kroon et al. 1997). Other sources of

cinnamic acids are fruits such as apples, pears, berries, prunes and peaches, and vegetables such as eggplants and artichokes (Clifford 1999a).

Table 1.1 Chemical structure, major dietary sources, and estimated daily intake of flavonoids and cinnamic acids.

Phenol	Chemical structure	Major sources	Estimates of daily intake	
Flavonoids				
Flavonols		Tea, onions, apples	3-5 mg (Finland and Spain)	(1)
and flavones			10-30 mg (The Netherlands, UK and USA)	(2)
			40-70 mg (Japan and Hawai)	(3)
Catechins		Tea, chocolate	Monomeric catechin: 50 mg (The Netherlands)	(4)
Flavanones		Citrus fruits	No estimate available	(5)
Isoflavones		Legumes (soybeans)	25-50 mg (Japan) 0-10 mg (western world)	(6)
Anthocyanidins		Fruits such as berries and cherries, and wine	180-215 mg	(5)
Cinnamic acids				
Caffeic acid		Coffee	Coffee drinkers; ~500 mg Non-coffee drinkers: <25 mg	(7)
Ferulic acid		Cereal bran, coffee	~ 100 mg	(8)

(1) = (Garcia-Closas et al. 1998, Garcia-Closas et al. 1999, Knekt et al. 2000, Knekt et al. 1997)
(2) = (Hertog et al. 1993a, Rimm et al. 1996, Yochum et al. 1999, Hertog et al. 1997a)
(3) = (Le Marchand et al. 2000, Hertog et al. 1995)
(4) = (Arts et al. 2001, Arts et al. 1999)
(5) = (Pedersen & Dwyer 1998, Kuhnau 1976, Clifford 2000b)
(6) = (Kirk et al. 1999, Nagata et al. 1998, Somekawa et al. 2001)
(7) = (Clifford 1999a, Radtke et al. 1998)
(8) = (Kroon et al. 1997, Clifford 1999a)

Especially beverages such as tea and coffee, seem to be important quantitative sources of flavonoids and cinnamic acids (Table 1.1). However, the estimates of intake of total flavonoids and cinnamic acids are based on the intake of just a few individual flavonoids and cinnamic acids. Thus, in order to determine the total intake of flavonoids and cinnamic acids, and of other subgroups of phenols, more information is needed.

Based on the available estimates, the intake of antioxidants via dietary phenols is comparable with, or even more than that via (pro)vitamins, such as vitamin C, E and carotenoids. Therefore dietary phenols are important as antioxidants in the prevention of diseases.

BIOAVAILABILITY OF PHENOLS

Bioavailability can be defined as 'the fraction of the ingested nutrient that is utilized for physiological functions and storage' (Jackson 1997).

Knowledge about the bioavailability of dietary phenols is very limited. The bioavailability of a compound is mainly determined by its chemical structure. In addition to variations in their basic phenolic structure, plant phenols might differ in degree of glycosylation, conjugation with other phenols, molecular size, degree of polymerization, and solubility. Furthermore, in foods, mixtures of phenols occur. Until now mainly the bioavailability of individual phenols has been studied (Bravo 1998, Hollman 1997a).

Bioavailability of a dietary component is determined by 4 processes (Figure 1.4): A) *absorption* from the small intestine or colon determines whether or not a phenol will become available in the blood circulation and in specific target tissues. In theory it is possible that despite the presence of large amounts of phenols in foods, none of them actually reach the target tissues; B) *metabolism* in the body; C) *distribution* in the body, and D) *excretion* from the body.

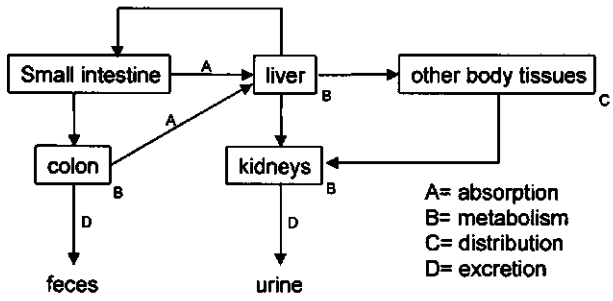


Figure 1.4 Schematic overview of the main bioavailability processes

Absorption of flavonoids and cinnamic acids

For a long time flavonoids from foods were considered to be non-absorbable because they are bound to sugars, the so-called glycosides. These glycosides were supposed not to be liberated into the free flavonoid (aglycone) due to the absence of beta-glucosidase enzymes in the small intestine of mammals (Griffiths & Smith 1972). Therefore oral intake of flavonoids was thought to be of no use (Gugler et al. 1975, Clark & Mackay 1950). However, in contrast to this belief, Hollman et al. showed that flavonoid glycosides could be absorbed from the small intestine. Indeed, the sugar moiety proved to be a very important determinant of absorption of flavonoid glycosides: the flavonol quercetin-4'-glucoside is better absorbed than quercetin-3-rutinoside (Hollman et al. 1995, 1997b and 1999, Felgines et al. 2000). The catechins occur as aglycones in foods. Monomeric catechins are absorbed in humans (Hollman & Arts 2000). The absorption of the complex condensation products of catechins, present in black tea, is uncertain, but they seem to be at least partly absorbable (Hollman et al. 1997c).

Cinnamic acids in foods occur mostly as esters with, among others, quinic acid (Clifford 2000a). The free cinnamic acids are well absorbed in animals and humans (Ader et al. 1996, Jacobson et al. 1983, Camarasa et al. 1988, Fahelbum & James 1977). However, it is unknown whether the esterification of cinnamic acids with other compounds influences their absorption. There are indications that these esters must be hydrolyzed before the cinnamic acids can be absorbed in humans (Booth et al. 1957, Czok et al. 1974, Plumb et al. 1999, Clifford 2000a).

Metabolism, distribution and excretion of phenols

Metabolism, distribution and excretion processes determine the type of circulating metabolites, the sites that these metabolites can reach and the period of time that they are available to target tissues. Metabolism mainly takes place by the colonic microflora and in the liver. However, other organs such as the gut wall and the kidney also play an important role in the metabolism.

Flavonoids are extensively metabolized in the human body as indicated by the low excretion of the original flavonoid structure in urine after ingestion. Only 1-3% of the original quercetin (Hollman et al. 1995) or catechins (Hackett et al. 1983 and 1985) was excreted in urine after volunteers had ingested large amounts. Cinnamic acids are also extensively metabolized because the recoveries in urine were low after ingestion. Of the caffeic acid ester chlorogenic acid none was recovered in urine after ingestion (Booth et al. 1957, Choudhury et al. 1999). Of free caffeic acid and ferulic acid, about 10% was recovered in urine (Camarasa et al. 1988, Choudhury et al. 1999).

Due to extensive metabolism of phenols in humans, it is possible that the metabolites of phenols are the actual compounds that reach the circulation and target tissues.

Therefore, the metabolites of phenols might be more important for potential health effects than the original phenols. Thus, information on the amount and the types of metabolites of dietary phenols, and on their elimination kinetics in humans, is essential.

BIOLOGICAL EFFECTS OF PHENOLS

Beneficial effects of dietary phenols

In the 1930s, flavonoids were thought to have vitamin properties because they seemed to decrease the permeability and fragility of capillary blood vessels. Consequently, they were called 'vitamin P' (derived from permeability) (Bentsáth et al. 1936 and 1937). In later years, the interest in vitamin 'P' weakened due to the belief that flavonoids were not bioavailable (Clark & Mackay 1950). Recently, flavonoids and other phenols have gained interest mainly because of their antioxidant activity and because they were proven to be bioavailable (Hollman 1997a, Rice-Evans et al. 1996).

Oxidation is an essential energy producing process in the body, which generates oxygen free radicals. However, an excess of free radicals might be involved in the pathogenesis of several diseases including cardiovascular disease, cancer and inflammatory diseases (Stohs 1995, Thomas 1995).

Antioxidants can protect against an excess of free radicals by reacting with free radicals, scavenging free radicals, inhibit their formation, or assist with other antioxidant defences (Shahidi & Wanasundara 1992, Pietta 2000, van Acker et al. 2000, Zhu et al. 1999). For example, antioxidant phenols protect against oxidized Low Density Lipoprotein (LDL) cholesterol in vitro (de Whalley et al. 1990, Castelluccio et al. 1995). Oxidized LDL seems to be involved in the development of cardiovascular disease (Chisolm & Steinberg 2000, Steinberg 1997). Phenols might protect against cancer by inhibiting the formation of free radicals and in that way prevent damage of tissues and of DNA (Kasai et al. 2000, Noroozi et al. 1998).

However, the role of antioxidants in humans is still not clear. Epidemiological studies do not show a clear protective effect of the flavonoid subgroups flavonols and flavones on cardiovascular disease and cancer (Table 1.2). However, these flavonols and flavones might not represent the total group of phenols. In addition, inconclusive results from large intervention trials with antioxidant vitamins such as beta-carotene, vitamin C and E also raise doubts about the effects of antioxidants on cardiovascular disease and cancer (Buring & Hennekens 1997, Marchioli 1999, Jha et al. 1995). Moreover, it remains difficult to study the effects of antioxidants in humans, because there are no adequate biomarkers available for measurement of effects of antioxidants in vivo (Witztum 1998).

Adverse effects of dietary phenols

Although phenols are mainly thought to be beneficial to human health, they might also have adverse effects (Skibola & Smith 2000). It is well known that antioxidants can also be pro-oxidants, depending on the circumstances and the dose (Skibola & Smith 2000, Stich 1991, Shahidi & Wanasundara 1992). Therefore, an excess of antioxidants might be harmful.

Traditionally, phenols have been considered to be anti-nutrients. This is due to the fact that they can decrease the digestibility of food as they bind and precipitate macromolecules such as protein, carbohydrates and digestive enzymes (Jung & Fahey 1983, Bravo 1998). Further, phenols form complexes with metals and therefore inhibit the bioavailability of nutrients such as zinc and iron (Brune et al. 1989, Coudray et al. 1998, Layrisse et al. 2000).

A new potential role of phenols in human health is that phenols might raise the plasma concentration of homocysteine in humans. Homocysteine is a sulfur-containing amino acid that is present in human blood. It is produced from methionine through demethylation of S-Adenosylmethionine (SAM) (Figure 1.5) (Finkelstein 1990). A high concentration of homocysteine in blood is a potential risk factor for cardiovascular disease (Nygard et al. 1995, Refsum et al. 1998). However, it is still unsure whether a high plasma homocysteine concentration is a causal risk factor for disease, or whether it merely is a consequence of disease (Brattstrom & Wilcken 2000, Ueland et al. 2000).

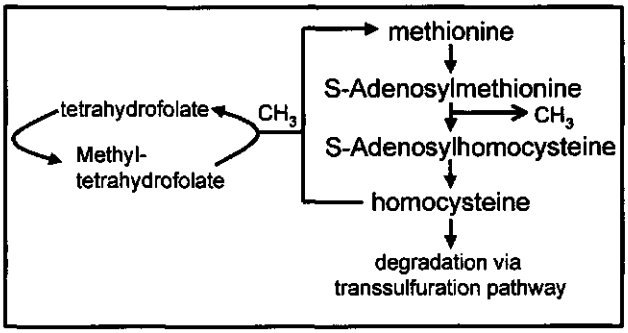


Figure 1.5 Schematic overview of homocysteine metabolism

Nygard et al. (Nygard et al. 1997) found that high coffee consumption is associated with a higher homocysteine concentration in blood. Other epidemiological studies and intervention studies confirmed this (Oshaug et al. 1998, Stolzenberg-Solomon et al. 1999, Grubben et al. 2000, Urgert et al. 2000). The responsible compounds in coffee are unknown. The lipid soluble compounds kahweol and cafestol, that are present in unfiltered coffee and known to raise cholesterol, are not responsible because unfiltered coffee as well as filtered coffee raises plasma homocysteine (Grubben et al.

2000, Urgert et al. 2000). Two other obvious candidates for the homocysteine-raising effects of coffee are caffeine and chlorogenic acid. Caffeine is currently under investigation (personal communication with Dr. P. Verhoef, Wageningen Centre for Food Sciences, Wageningen, The Netherlands). We investigated in *chapter 5* whether the phenol chlorogenic acid might be responsible because it occurs in large amounts in coffee, but in only small amounts in other foods (Clifford 1999a).

Table 1.2 Overview of epidemiological studies of the effect of flavonols and flavones on cardiovascular disease and cancer.

	Outcome	Effect #
Cardiovascular disease		
• (Hertog et al. 1993b, Hertog et al. 1997b)	CHD* mortality	+
	Fatal and non-fatal MI*	0/+
• (Hertog et al. 1995)	CHD* mortality	+
• (Knekt et al. 1996)	Coronary mortality	0/+
• (Keli et al. 1996)	Stroke	+
• (Rimm et al. 1996)	Non-fatal MI*	0
	Total CHD*	0
	CHD* mortality	0
• (Hertog et al. 1997a)	Ischemic heart disease mortality	0/-
	Risk of ischemic heart disease	0
• (Yochum et al. 1999)	CHD* mortality	+
	Stroke mortality	0
• (Knekt et al. 2000)	CVA*	0
• (Hirvonen et al. 2001)	Coronary death	0/+
	Nonfatal MI	+
• (Hirvonen et al. 2000)	Stroke	0
Cancer		
• (Hertog et al. 1994)	Risk of cancer and cancer mortality	0
• (Hertog et al. 1995)	Cancer mortality	0
• (Goldbohm et al. 1995)	Risk of cancer	0
• (Knekt et al. 1997)	Risk of cancer	0/+
• (Le Marchand et al. 2000)	Risk of cancer	0/+
• (Hertog et al. 1997a)	Cancer mortality	0/-
• (Garcia-Closas et al. 1998, Garcia-Closas et al. 1999, Garcia et al. 1999)	Risk of cancer	0/+

0 = no effect;
+ = protective;
- = increased risk;
0/+ = protective, but not statistically significant;
0/- = increased risk, but not statistically significant.

*abbreviations: CHD = coronary heart disease; MI = myocardial infarction; CVA = cerebrovascular disease

RATIONALE AND OUTLINE OF THIS THESIS

The objective of this thesis was to investigate *whether bioavailability data on flavonoids and cinnamic acids support the hypothesis that they can affect health in humans*. Information on the bioavailability processes of dietary phenols will provide valuable insight into the plausibility that they might affect health. Because the group of phenols represents a huge variety of compounds, we focussed our studies on major flavonoids and cinnamic acid in foods: quercetin, black tea phenols, and chlorogenic acid (5-caffeoylquinic acid).

1) Impact of conjugated moieties of quercetin and chlorogenic acid on their bioavailability

Flavonols are important dietary phenols and quercetin is their major representative. In foods quercetin occurs as quercetin glycosides and Hollman et al (Hollman et al. 1995, 1997b and 1999) showed that the sugar moiety determines the bioavailability: the bioavailability of quercetin-3-rutinoside (quercetin attached to a glucose with rhamnose) was only 20% of that of quercetin-4'-glucoside (quercetin attached to a glucose). Quercetin-3-rutinoside is the major quercetin form in tea, and tea is the major source of quercetin. Therefore it would be interesting to see whether the bioavailability of quercetin-3-rutinoside from tea could be improved if quercetin-3-rutinoside is transformed into quercetin-3-glucoside, which in theory is possible (Bokkenheuser et al. 1987, Gunata et al. 1988, Kurosawa et al. 1973). However, it is not known whether the bioavailability of quercetin-3-glucoside is similar to that of the well-bioavailable quercetin-4'-glucoside, i.e. whether the position of glucose plays a role in the bioavailability of quercetin. In *chapter 2* we investigated whether quercetin-3-glucoside had the same high bioavailability as quercetin-4'-glucoside.

Another important group of phenols in foods are the cinnamic acids, of which chlorogenic acid is the major representative (Clifford 1999a). Very little is known about the bioavailability of chlorogenic acid. Therefore we studied the absorption of chlorogenic acid and of its caffeic acid moiety in volunteers without a colon (ileostomy volunteers) in *chapter 3*.

2) Metabolism of phenols in humans

Phenols are extensively metabolized in the human body. Therefore it is important to identify the metabolites of phenols that are actually circulating in the body and may have biological effects. Information on the metabolites of phenols and on the site of metabolism of phenols in humans is essential but scarce. We measured 59 phenolic acids as potential metabolites in the urine of 20 healthy volunteers with a colon after they had ingested chlorogenic acid, phenols from black tea and quercetin-3-rutinoside. Furthermore, to investigate the role of the colonic microflora in the metabolism of phenols we measured potential metabolites of chlorogenic acid, caffeic acid, and

quercetin-3-rutinoside in volunteers without a colon (ileostomy volunteers) in *chapter 4).*

3) Effect of phenols on plasma homocysteine

Coffee increases plasma homocysteine concentrations, a potential risk factor for cardiovascular disease (Nygard et al. 1997, Oshaug et al. 1998, Stolzenberg-Solomon et al. 1999, Grubben et al. 2000, Urgert et al. 2000). Because chlorogenic acid occurs in large amounts in coffee, it might be the responsible compound for the homocysteine-raising effect of coffee. Therefore we investigated whether chlorogenic acid raised plasma homocysteine concentrations. Because metabolism of chlorogenic acid and other phenols is likely to occur via the same pathways, we also determined the effect of black tea solids and quercetin-3-rutinoside on plasma homocysteine *(chapter 5).* We found that chlorogenic acid and black tea solids raised plasma homocysteine, therefore we looked into the mechanism underlying the potential homocysteine-raising effects of phenols in *chapter 6.*

Chapter 7 discusses the implications our findings.

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2

Bioavailabilities of quercetin-3-glucoside and quercetin-4'-glucoside do not differ in humans

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The flavonoid quercetin is an antioxidant which occurs in foods mainly as glycosides. The sugar moiety in quercetin glycosides affects their bioavailability in humans. Quercetin-3-rutinoside is an important form of quercetin in foods, but its bioavailability in humans is only 20% of that of quercetin-4'-glucoside. Quercetin-3-rutinoside can be transformed into quercetin-3-glucoside by splitting off a rhamnose molecule. We studied whether this 3-glucoside has the same high bioavailability as the quercetin-4'-glucoside. To that end we fed five healthy men and four healthy women (19–57 y) a single dose of 325 μmol of pure quercetin-3-glucoside and a single dose of 331 μmol of pure quercetin-4'-glucoside and followed the plasma quercetin concentrations. The bioavailability was the same for both quercetin glucosides. The mean peak plasma concentration of quercetin was 5.0 ± 1.0 μmol/L (±SE) after subjects had ingested quercetin-3-glucoside and 4.5 ± 0.7 μmol/L after quercetin-4'-glucoside consumption. Peak concentration was reached 37 ± 12 min after ingestion of quercetin-3-glucoside and 27 ± 5 min after quercetin-4'-glucoside. Half-life of elimination of quercetin from blood was 18.5 ± 0.8 h after ingestion of quercetin-3-glucoside and 17.7 ± 0.9 h after quercetin-4'-glucoside. We conclude that quercetin glucosides are rapidly absorbed in humans, irrespective of the position of the glucose moiety. Conversion of quercetin glycosides into glucosides is a promising strategy to enhance bioavailability of quercetin from foods.

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INTRODUCTION

Flavonoids are polyphenolic compounds that occur in foods of plant origin. The average daily intake of the flavonoid subclasses of flavonols and flavones in The Netherlands is 23 mg (expressed as aglycones) of which quercetin supplies 16 mg (Hertog et al. 1993b). Quercetin is an antioxidant in vitro because it can scavenge radicals, inhibit lipid peroxidation and chelate metals (Rice-Evans et al. 1996). Quercetin was able to inhibit oxidation of LDL in vitro at a concentration as low as 0.25 μmol/L, which is in the physiological range (Manach et al. 1998, de Whalley et al. 1990). Therefore quercetin might contribute to the prevention of cardiovascular disease (Hertog et al. 1993a). However, to induce these health effects in humans, quercetin must enter the systemic circulation. Quercetin in foods is bound to sugars, mainly as β-glycosides, and the bioavailability of these various quercetin glycosides is affected by their sugar moiety (Hollman et al. 1995, 1996a and 1999). Quercetin-3-rutinoside and quercetin-4'-glucoside are important forms of quercetin in foods (Figure 2.1).

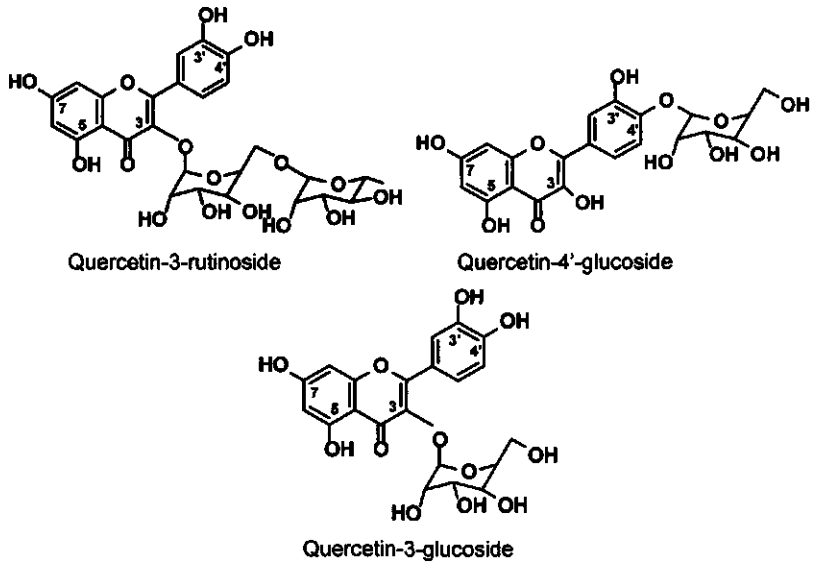

Figure 2.1 Structure of quercetin glycosides

Quercetin-3-rutinoside accounts for ~40% of quercetin in black tea (Engelhardt et al. 1992), and consumption of black tea contributes about 48% to the total flavonol and flavone intake in The Netherlands (Hertog et al. 1993b). Quercetin-4'-glucoside accounts for ~45% of quercetin in onions (Kiviranta et al. 1988), and consumption of onions contributes another 29% to the total flavonol and flavone intake (Hertog et al. 1993b). Although the intake of quercetin-3-rutinoside is twice that of quercetin-4'-

glucoside, the absorption of quercetin-3-rutinoside is only 17% of ingested dose, whereas the absorption of quercetin-4'-glucoside is 52% of ingested dose (Hollman et al. 1995). Furthermore, the bioavailability of quercetin-3-rutinoside is only 20% of that of quercetin-4'-glucoside (Hollman et al. 1999). Therefore it would be interesting to attempt to increase the bioavailability of quercetin-3-rutinoside. Rutinose is a dimer of glucose and rhamnose; therefore quercetin-3-rutinoside can be transformed into quercetin-3-glucoside by splitting of the rhamnose molecule with the enzyme alpha-L-rhamnosidase (Bokkenheuser et al. 1987, Gunata et al. 1988, Kurosawa et al. 1973). The resulting quercetin-3-glucoside differs only from the highly bioavailable quercetin-4'-glucoside in the position of the glucose moiety on the quercetin aglycone. However the bioavailability of quercetin-3-glucoside is unknown. Therefore we tested whether the position of the glucose moiety affected the bioavailability of quercetin glucosides in humans.

MATERIALS AND METHODS

Subjects

The protocol was approved by the Ethical Committee of Nijmegen University Hospital. All subjects were fully informed about the study and signed an informed consent form. Five women and five men started with the study, but one woman was excluded because of problems with blood sampling. Mean age of the remaining nine subjects was 25 y (range 19 - 57 y) and mean body mass index was 21.3 kg/m^2 (range 19.8 - 24.8 kg/m^2). All subjects were healthy based on a medical questionnaire, the absence of protein and glucose in urine and normal values for blood hematocrit, hemoglobin concentration and leukocyte and platelet counts. Subjects were not allowed to use any medicine during the study, except for acetaminophen (paracetamol) and oral contraceptives.

Study design and supplements

The subjects ingested quercetin-3-glucoside or quercetin-4'-glucoside (Figure 2.1) on two different days in random order, and subsequently we measured quercetin in blood over 72 h and in urine over 24 h. Subjects followed a quercetin-low diet from d 3 to 16. To ensure a quercetin-low diet, subjects were given a list of fruits and vegetables which contained more than 15 mg quercetin/kg and of beverages with more than 4 mg quercetin/L (Hertog et al. 1992 and 1993c) and they were instructed not to consume any of them. During the mornings of d 7 and of d 13 the subjects came to the University Hospital Nijmegen after they had fasted overnight. Five of the subjects ingested 325 μmol (151 mg) quercetin-3-glucoside (#011095, Extrasynthese, Genay, France) on d 7 and 331 μmol (154 mg) quercetin-4'-glucoside (#4564, Carl Roth, Amsterdam, The Netherlands) on d 13. The other four subjects received the same supplements in reverse order. Each supplement was dissolved in 10 mL ethanol plus

200 mL of hot water (5% v/v alcohol content). Subjects ingested 2 g of sodium chloride dissolved in 10 mL of water just before they ingested the supplement because the sodium glucose cotransporter might play a role in the absorption of quercetin glucosides and sodium is necessary for the active transport of glucose. During the first 3 h after ingestion of the supplements subjects were allowed to consume water only. We checked compliance with the dietary guidelines with a 24-h recall for d 6 and 12. We calculated intakes with the Dutch food composition table. Average energy intake was 13.4 ± 0.9 (SE) MJ, of which protein provided 14.8 ± 0.5 %, fat 34.7 ± 2.8 %, and carbohydrates 49.8 ± 3.2 %. The mean daily quercetin intake from regular foods during the study was not different between supplement periods and was 7.6 ± 2.3 μmol. Because this was about 2% of the dose of the supplements we conclude that intake of quercetin from regular foods did not affect our results.

Collection of blood and urine samples

We took venous blood samples (10 mL blood per blood sample) into vacuum tubes containing EDTA once before subjects ingested the supplement, and at 15 min, 30 min, 1, 1.5, 2, 4, 6, 8, 12, 24, 36, 48, 60 and 72 h after ingestion. Platelet-poor plasma was prepared by centrifuging the blood for 10 minutes at 2500 x *g* at 4°C. The plasma was stored at -80°C until analysis. On d 7 and 13, subjects collected urine during 24 h in plastic bottles, one for each voiding, with thymol (# 8167; Merck, Amsterdam, the Netherlands) dissolved in isopropanol as preservative. They stored each bottle in dry ice immediately after voiding. At the laboratory we thawed the urine bottles in a water bath of ~40°C, pooled and mixed urine per subject and per supplement day, froze aliquots of urine in liquid nitrogen and stored the urine samples at -80°C until analysis. Subjects took 300 μmol lithium chloride dissolved in 10 mL of water every morning from d 1 until d 14. Urinary recovery of lithium was 94.4 ± 17.2% (mean ± SD), which indicates that collection of urine was complete (Sanchez-Castillo et al. 1987a and 1987b).

Analytical methods

Quercetin, isorhamnetin (3'-methoxyquercetin) and their conjugates with glycosides, glucuronic acid or sulfates in plasma or urine were simultaneously extracted and hydrolyzed to their aglycones with 2 mol/L HCL in aqueous methanol (Hollman et al. 1997). We measured the aglycones by HPLC with fluorescence detection (Hollman et al. 1996b). The limit of detection, i.e. the concentration producing a peak height three times the standard deviation of the baseline noise was 0.007 μmol/L (2 ng/mL) for quercetin in plasma and 0.01 μmol/L (3 ng/mL) for quercetin in urine (Hollman et al. 1997). The limits of detection for isorhamnetin were one-third of those for quercetin (Hollman et al. 1996b). Lithium was measured in undiluted, acidified urine by atomic absorption spectrophotometry (Anonymous 1976).

Data analysis

We used a two-compartment model to describe the pharmacokinetics of quercetin and isorhamnetin. We calculated peak plasma concentration, time to reach peak plasma concentration, elimination half-life and area under the plasma concentration - time curve ($AUC_{0 \rightarrow 72\ h}$) with the MW/Pharm computer package (Proost & Meijer 1992). We calculated the $AUC_{0 \rightarrow 72\ h}$ with the linear trapezoidal rule. Differences between results after ingestion of quercetin-3-glucoside and after quercetin-4'-glucoside were tested for significance by paired t-test with a significance level of $P<0.05$ (SAS Institute Inc., Cary, NC, USA).

RESULTS

The time course of the quercetin (measured as the quercetin aglycone) concentration in blood after ingestion of quercetin-3-glucoside was not different from that after ingestion of quercetin-4'-glucoside (Figure 2.2). The plasma kinetic variables of the two glucosides also did not differ, as did the bioavailability, as indicated by the similar $AUC_{0 \rightarrow 72\ h}$ (Table 2.1).

The concentration of quercetin in plasma rose rapidly after ingestion of quercetin-3-glucoside as well as after ingestion of quercetin-4'-glucoside. The mean peak plasma concentration of quercetin, the time to reach peak concentration, and the elimination half-life of quercetin in plasma did not differ when subjects consumed quercetin-3-glucoside or quercetin-4'-glucoside (Table 2.1).

Table 2.1 Kinetic variables of quercetin absorption and elimination in plasma of nine subjects after one-time ingestion of quercetin-3-glucoside or quercetin-4'-glucoside[1]

Variable		Supplement[2]	
		Quercetin-3-glucoside	Quercetin-4'-glucoside
Plasma total quercetin			
Peak concentration	µmol/L	5.0 ± 1.0	4.5 ± 0.7
	(ng/mL)	1526 ± 315	1345 ± 212
Time to reach peak concentration	(min)	37 ± 12	27 ± 5
Elimination half-life	(h)	18.5 ± 0.8	17.7 ± 0.9
Area under the plasma concentration-time curve ($AUC_{0 \rightarrow 72\ h}$)	(h x µmol/L)	19.1 ± 2.9	17.5 ± 2.4
	(h x ng/mL)	5775 ± 876	5276 ± 730

[1] Values are mean ± SE, n=9. None of the variables differed significantly between supplements.

[2] Subjects ingested 325 µmol (151 mg) of quercetin-3-glucoside or 331 µmol (154 mg) of quercetin-4'-glucoside. Each subject received each supplement in random order at a 6-d interval.

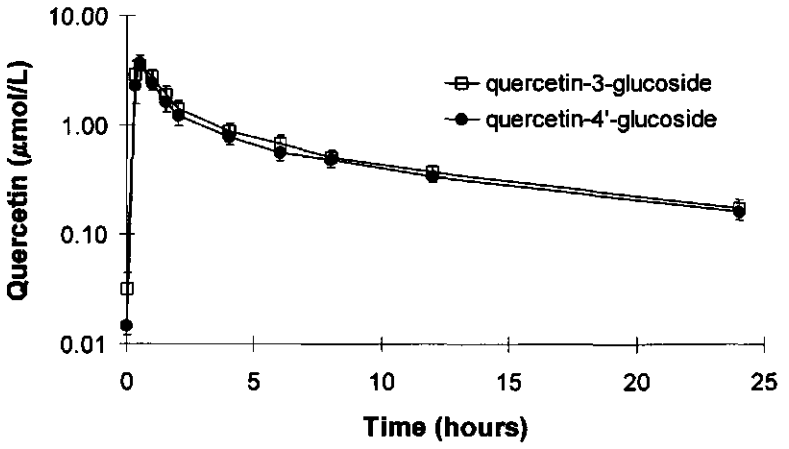

Figure 2.2 Total quercetin concentration in plasma of nine human subjects after ingestion of 325 µmol (151 mg) quercetin-3-glucoside (mean + SE) or 331 µmol (154 mg) quercetin-4'-glucoside (mean - SE). Each subject received each supplement in random order at a 6-d interval.

The amount of quercetin excreted in 24h urine after intake of the 3-glucoside was not different from that after intake of the 4'-glucoside (Table 2.2). Only about 3% of the ingested quercetin was excreted in urine as quercetin aglycone or its conjugates, which indicates that quercetin is extensively metabolized in the human liver and other organs and by the colonic microflora. One of the metabolites of quercetin is isorhamnetin (3'-methoxyquercetin) (Manach et al. 1998, Morand et al. 1998). We indeed found isorhamnetin in plasma and urine after ingestion of both quercetin supplements. Peak concentration of isorhamnetin did not differ between the glucosides and was reached in 51 ±19 min after the quercetin-3-glucoside and in 32 ± 5 min after the quercetin-4'-glucoside. Other kinetic variables of isorhamnetin did not differ between the quercetin glucosides (data not shown). In urine, ~0.6% of the ingested quercetin glucosides was recovered as isorhamnetin (Table 2.2).

Table 2.2 Excretion of quercetin and isorhamnetin in urine of subjects during the first 24 h after one-time ingestion of quercetin-3-glucoside or quercetin-4'-glucoside[1]

		Excretion in urine	
Supplement[2]	Intake	Quercetin	Isorhamnetin
	µmol	% quercetin glucoside intake	
Quercetin-3-glucoside	325	3.0 ± 0.3	0.61 ± 0.08
Quercetin-4'-glucoside	331	2.6 ± 0.4	0.53 ± 0.07

[1] Values are mean ± SE, n=9. None of the variables differed significantly between supplements

[2] Subjects ingested 325 µmol (151 mg) of quercetin-3-glucoside or 331 µmol (154 mg) of quercetin-4'-glucoside. Each subject received each supplement in random order at a 6-d interval

DISCUSSION

The bioavailability of quercetin-3-glucoside is similar to that of quercetin-4'-glucoside. We found that the time to reach peak concentrations was ~30 minutes for both quercetin glucosides and the peak concentration was ~5 μmol/L. This corresponds well with the peak concentration of 3.5 μmol/L for quercetin-4'-glucoside, reported by Hollman et al. (1999), who also found that the bioavailability of quercetin-3-rutinoside was 20% of that of quercetin-4'-glucoside. Therefore our results suggest that enzymatic conversion of quercetin-3-rutinoside into quercetin-3-glucoside will increase bioavailability. Quercetin-3-glucoside itself also occurs commonly in foods such as tea, tomatoes and apples (Engelhardt et al. 1992, Herrmann 1976 and 1988). We may now conclude that this naturally occurring 3-glucoside has the same high bioavailability as the 4'-glucoside.

Quercetin glucosides are absorbed more rapidly than other quercetin glycosides (Hollman et al. 1997 and 1999). The mechanism for quercetin absorption is not known. Hollman et al. (1995 and 1999) speculated that the intestinal sodium-glucose cotransporter is able to transport glucose attached to quercetin through the intestinal cell wall. This idea was supported by the results of Aziz et al. (1998), who found the quercetin-4'-glucoside in human plasma after volunteers had consumed onions. If the sodium-glucose cotransporter plays a role in the absorption of quercetin glucosides, our results would suggest that the absorption of glucose is not affected by its position on the attached quercetin. However, transport of quercetin glucosides by the glucose cotransporter has not been proven yet *in vivo*. For the interpretation of the bioactivity of quercetin from foods in humans, it is important to know in what form quercetin actually circulates in blood. From the results in this study it is unclear in what form quercetin circulates in blood because we measured the concentration of quercetin after hydrolysis to the quercetin aglycone. With regard to bioactivity of various forms of quercetin, quercetin conjugated with glycosides, glucuronic acid, or sulfates also has antioxidant activity *in vitro*, although the antioxidant activity is lower than that of the quercetin aglycone (Manach et al. 1998, Williamson et al. 1996).

In addition to bioavailability data, our study also provided information on the metabolism of quercetin into isorhamnetin (3'-methoxyquercetin). Of the ingested quercetin glucosides ~50% is absorbed in the small intestine and subsequently metabolized, for example into isorhamnetin, in the liver and in other organs. The 50% of ingested quercetin which is not absorbed in the small intestine is metabolized by the colonic microflora into quercetin aglycone and phenolic acids which might be absorbed from the colon (Hollman & Katan 1998, Hollman et al. 1995, Manach et al. 1998). Only 3% of the ingested quercetin is recovered in urine as aglycone or its conjugates. The quercetin in urine might originate from quercetin absorbed in the small intestine and from quercetin absorbed in the colon. Metabolites of quercetin may also be biologically important, because they have antioxidant activity *in vitro*

(Manach et al. 1998, Rice-Evans et al. 1996) and might exert antioxidant effects in humans. In this study we measured isorhamnetin as a metabolite of quercetin. Isorhamnetin concentration in plasma peaked shortly after the quercetin concentration peak. This suggests that both quercetin glucosides are methylated into isorhamnetin immediately after absorption. Methylation of the catechol group of quercetin produces isorhamnetin and it is catalyzed by the enzyme catechol-O-methyltransferase in the liver (Zhu et al. 1994). In quercetin-4'-glucoside the 4' position is occupied by a glucose and thus there is no catechol group available for methylation. Deglucosylation of the 4'-glucoside is needed to release the catechol group. Because the time to reach peak concentrations of isorhamnetin after intake of the 3-glucoside was the same as after intake of the 4'-glucoside this could imply that deglucosylation of the 4'-glucoside is not rate limiting for isorhamnetin formation. Furthermore, isorhamnetin is not an important final metabolite of quercetin because only 0.6% of the ingested quercetin glucosides was excreted in urine as isorhamnetin.

This study shows that it might be possible to increase or decrease bioavailability of quercetin, and maybe of other components in foods and of drugs, by attaching or detaching a glucose molecule. Specifically, treatment of the poorly absorbed quercetin-3-rutinoside from tea with rhamnosidase would transform it into the highly bioavailable quercetin-3-glucoside. Recent research has reinforced the evidence for an inverse association between the intake of flavonoids and death from coronary heart disease (Yochum et al. 1999). If intake of quercetin and related flavonols can indeed be proven to reduce coronary heart disease risk, then production of foods with a more highly bioavailable form of quercetin might become a realistic proposition.

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3

Chlorogenic acid and caffeic acid are absorbed in humans

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Chlorogenic acid, an ester of caffeic acid and quinic acid, is a major phenolic compound in coffee; daily intake in coffee drinkers is 0.5-1 g. Chlorogenic acid and caffeic acid are antioxidants in vitro and might therefore contribute to the prevention of cardiovascular disease. However, data on the absorption of chlorogenic acid and caffeic acid in humans are lacking. We determined the absorption of chlorogenic acid and caffeic acid in a cross-over study with 4 female and 3 male healthy ileostomy subjects. In such subjects, degradation by the colonic microflora is minimal and absorption can be calculated as the amount ingested minus the amount excreted in ileostomy effluent. The ileostomy subjects ingested 2.8 mmol chlorogenic acid and 2.8 mmol caffeic acid on separate days in random order and subsequently collected ileostomy fluid and urine for 24 h. Absorption of chlorogenic acid was 33 ± 17% (mean ± SD) and of caffeic acid 95 ± 4%. Traces of the ingested chlorogenic acid and 11% of the ingested caffeic acid were excreted in urine. Thus, one-third of chlorogenic acid and almost all of the caffeic acid were absorbed in the small intestine of humans. This implies that part of chlorogenic acid from foods will enter into the blood circulation, but most will reach the colon.

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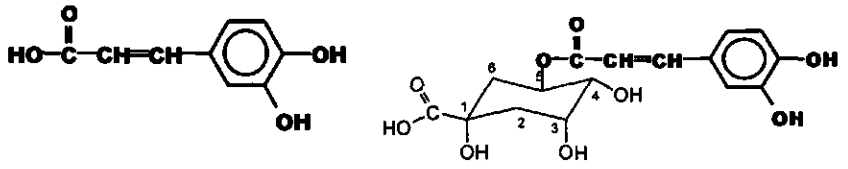
INTRODUCTION

Phenolic compounds form a substantial part of plant foods. Most of these phenolic compounds are antioxidants in vitro (Rice-Evans et al. 1996) and antioxidants might protect against cardiovascular disease.

A major class of phenolic compounds are hydroxycinnamic acids, which are found in almost every plant (Herrmann 1976, Kuhnau 1976). The major representative of hydroxycinnamic acids is caffeic acid, which occurs in foods mainly as an ester with quinic acid called chlorogenic acid (5-caffeoylquinic acid) (Figure 3.1). Coffee is a major source of chlorogenic acid in the human diet; daily intake in coffee drinkers is 0.5-1 g, coffee abstainers will usually ingest <100 mg/d. Other dietary sources of chlorogenic acid include apples, pears, berries, artichoke, eggplant (Clifford 1999). Knowledge concerning the absorption of chlorogenic acid in humans is essential to evaluate possible health effects in vivo because the absorbed fraction of chlorogenic acid will enter into the blood circulation and thus can induce biological effects in the blood circulation. Furthermore, the fraction that is not absorbed will enter into the colon where it might have biological effects. Chlorogenic acid and caffeic acid are antioxidants in vitro (Castelluccio et al. 1995, Rice-Evans et al. 1996), and they might inhibit the formation of mutagenic and carcinogenic N-nitroso compounds because they are inhibitors of the N-nitrosation reaction in vitro (Kono et al. 1995). Further, chlorogenic acid can inhibit DNA damage in vitro (Kasai et al. 2000, Shibata et al. 1999). Therefore, the inverse association between coffee intake and colon cancer in some epidemiological studies (Giovannucci 1998, Baron et al. 1994, Favero et al. 1998, La Vecchia et al. 1989, Tavani et al. 1997) might be explained in part by the chlorogenic acid present in coffee. However, there are no data on absorption of chlorogenic acid or caffeic acid in humans. The major problem in measuring the absorption of chlorogenic acid and caffeic acid in humans is their bacterial degradation in the colon (Scheline 1968). Thus, measurement of fecal excretion of chlorogenic acid and caffeic acid would lead to an overestimation of the amount absorbed. Therefore, we determined the absorption of chlorogenic acid and caffeic acid in healthy ileostomy subjects, who lack a colon. Ileostomy subjects were successfully employed previously to determine the absorption of flavonoids (Hollman et al. 1995), coffee diterpenes (De Roos et al. 1998), and dietary polysaccharides (Englyst & Cummings 1985).



(A) (B)

Figure 3.1 Structure of caffeic acid (A) and chlorogenic acid (B)

MATERIALS AND METHODS

Subjects

The study was approved by the Ethical committee of the Division of Human Nutrition and Epidemiology. We recruited subjects by approaching volunteers who participated in previous studies at our division (Hollman et al. 1995, De Roos et al. 1998). Exclusion criteria were as follows: signs of diseases related to the gastrointestinal tract; resection of >50 cm of the terminal ileum; an ileostomy that did not function properly; use of drugs which influenced gastrointestinal transit; present illness; pregnancy or lactation. Four women and three men, with a mean age of 63 y (range: 46-74 y), and a mean body mass index of 27.1 kg/m^2 (range 23.3 - 34.9 kg/m^2) were admitted to participate and signed an informed consent form. All subject had had a total colectomy between 7 and 27 y ago for ulcerative colitis or polyposis coli. The subjects were healthy, based on a medical questionnaire, normal blood values for hemoglobin, hematocrit and white blood cell counts, and absence of glucose and protein in urine.

Study design and supplements

Subjects followed a diet that was low in chlorogenic acid and quercetin from d 1 to 14. The diet was low in quercetin because one of the supplements we tested was quercetin-3-rutinoside. To ensure adherence to the dietary guidelines, we gave the subjects a list of forbidden foods and beverages. Foods were prohibited if they contained >15 mg/kg of quercetin or chlorogenic acid. Beverages were prohibited if they contained >4 mg/L of quercetin or chlorogenic acid (Clifford 1999, Hertog et al. 1992 and 1993). Because subjects were not allowed to drink coffee and tea during the study, we supplied coffee and tea substitutes. The coffee substitute was an extract made of chicory, rye and barley ('Swiss coffee-like', Tayala AG, Birsfelden, Switzerland) and the tea substitute was an extract of a mixture of herbs ('droommix', Piramide, Veenendaal, the Netherlands). We analyzed the coffee substitute and tea substitute for quercetin and chlorogenic acid and the amounts were within the range of the dietary guidelines (results not shown). Compliance with the dietary guidelines was good. None of the subjects reported consumption of any foods or beverages that were on the list of forbidden foods and beverages during the study. Furthermore, the

low chlorogenic acid and caffeic acid excretion in presupplement ileostomy effluent confirmed that subjects adhered to the dietary guidelines (Table 3.1).
On d 5, all subjects consumed a placebo supplement, which was 200 mL of water. On d 6, 10 and 14 all subjects consumed one of the following supplements in random order: 1000 mg (2.8 mmol) chlorogenic acid (Fluka Chemie, Buchs, Switzerland) or 500 mg (2.8 mmol) caffeic acid (Fluka Chemie) or 220 mg (0.3 mmol) quercetine-3-rutinoside (Rutosidum DAB; BUFA B.V., Uitgeest, The Netherlands). The placebo and the quercetin-3-rutinoside supplements were part of another study; these results will be reported elsewhere. Subjects received the supplements as a powder and were instructed to add 200 mL hot water and to consume the beverage within 5 minutes after preparation. Subjects ingested the supplements between 0700 and 0900 h at home, together with a light breakfast that we provided. The breakfast consisted of wheat bread, cheese, strawberry jam, milk and the coffee and tea substitutes: subjects had a free choice. After this breakfast with supplements, subjects were only allowed to drink water for 3 hours.

Collection of ileostomy effluent and urine

On d 5, 6, 10 and 14 subjects collected one sample of ileostomy effluent and urine just before ingestion of the supplements. After ingestion of the supplements, they collected ileostomy effluent and urine during 24 h. During the daytime, they changed the ileostomy bags every 2 h and immediately stored the bags on dry ice to minimize degradation of the contents by residual bacterial flora. At night, subjects had to change the bags as often as possible.
Subjects collected urine in 0.5 L plastic bottles, with 0.13 g thymol (# 8167; Merck, Amsterdam, The Netherlands) as a preservative and stored the bottles with urine on dry ice immediately after voiding. We checked the completeness of urine collection by assessment of recovery of 270 μmol lithium in urine. It was ingested daily by the subjects as lithium chloride dissolved in 10 mL of tap water from 7 d before the first urine collection. Lithium chloride is completely absorbed and 95% is excreted in urine (Sanchez-Castillo et al. 1987a and 1987b). Urinary recovery of lithium was 94 ± 12% (mean ± SD), indicating good compliance in collecting urine.

Sample preparation

The ileostomy bags were kept frozen with liquid nitrogen during separation of the plastic bags from the contents. The frozen contents were freeze-dried, ground to pass through an 0.5-mm sieve and stored at -20°C until analysis. We thawed the urine bottles in a water bath of ~40°C, pooled and mixed urine per subject and per supplement day, froze aliquots of urine in liquid nitrogen and stored the urine samples at -80°C until analysis. We prepared the samples collected before breakfast (presupplement sample) and the final collection at the end of the 24-h collection period (final sample) separately.

Incubation of chlorogenic acid and caffeic acid with gastrointestinal fluids

To check for degradation of chlorogenic acid and caffeic acid in gastrointestinal fluids, we incubated them in vitro in gastric juice and duodenal fluid, and ex vivo in ileostomy fluid. We incubated 30 mg of chlorogenic acid and 15 mg of caffeic acid with 3 mL human gastric juice and 9 mL of water at 37°C for 0.5 and 2 h (Roxburgh et al. 1992, Jebbink et al. 1992). Similar amounts were incubated with 3 mL human duodenal fluid and 9 mL water at 37°C for 1 and 4 h, corresponding to the average and maximal transit time in the small intestine (Layer et al. 1990, Malagelada et al. 1984). Gastric juice and duodenal fluid were obtained from two fasted healthy volunteers with a colon and stored at -20°C.

We also studied the stability of chlorogenic acid and caffeic acid ex vivo during collection of ileostomy fluid and during sample preparation in the laboratory. For this purpose, two ileostomy subjects, who also participated in this study, followed a diet low in chlorogenic acid and quercetin for 4 d. On d 4, they applied three ileostomy bags in total, one bag with 300 mg chlorogenic acid mixed with ~5 g of strawberry jam and one with 150 mg caffeic acid mixed with 5 g of strawberry jam and one bag with 5 g of strawberry jam only. Strawberry jam was used as a vehicle for chlorogenic acid and caffeic acid powder. Strawberry jam itself does not contain chlorogenic acid or caffeic acid. The subjects allowed ileostomy fluid to drain into the bag for ~2 h and kneaded the contents regularly. The ileostomy fluids were stored and analyzed as described.

Analysis of chlorogenic acid and caffeic acid in ileostomy effluent and urine

Chlorogenic acid and caffeic acid in ileostomy effluent were extracted simultaneously by mixing 0.500 g freeze-dried effluent with 25 mL 40% ($^{v}/_{v}$) aqueous methanol containing 2 g *tert*-butylhydroxyquinone/L. The effluent extract was refluxed at 90 °C for 1 h with regular swirling, allowed to cool down and subsequently brought to 50 mL with methanol. The effluent extract was then sonicated for 5 min and filtered through a 0.45 µm filter for organic solvents (Acrodisc CR PTFE; German Sciences, Ann Arbor, MI) before HPLC injection. For HPLC analysis, we injected 20 µL of the effluent extract onto an Inertsil ODS-2 (GL Siences, Tokyo, Japan) column (4.6 x 150 mm, 5 µm 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) using acetonitrile /0.025 mol/L phosphate buffer, pH 2.4 (8:92) as the mobile phase, at a flow rate of 1 mL/min. The columns were placed in a column oven set at 40°C. Ultraviolet absorption was measured at 325 nm. Excretion of chlorogenic acid was calculated as the sum of the excretion of 3-caffeoylquinic acid, 4-caffeoylquinic acid and 5-caffeoylquinic acid in ileostomy effluent (Figure 3.2). Isomers of chlorogenic acid were produced by incubation of a solution of 5-caffeoylquinic acid (pH 8) at 100°C for 30 min (Trugo & Macrae 1984).

The limit of detection, i.e. the concentration producing a peak height three times the standard deviation of the baseline noise, was 3 μg/g freeze-dried ileostomy effluent.
For analysis of caffeic acid in urine, urine was treated with β-glucuronidase/sulfatase before analysis. For analysis of chlorogenic acid in urine, urine was not treated with β-glucuronidase/sulfatase. Urine for measurement of chlorogenic acid and caffeic acid was acidified and brought onto an SPE extraction column. The column was eluted with ethyl acetate. Ethyl acetate was evaporated and acids were derivatized with bis(trimethylsilyl)trifluoroacetamide (BSTFA). Derivates were injected onto a capillary column CP-Sil-5 (Chrompack, Middelburg, The Netherlands) and separated using a temperature gradient. On-line mass spectrometry was used to quantify and identify the acids (Hollman, unpublished results). The detection limit in urine was 0.8 mg/L urine for chlorogenic acid and 0.4 mg/L for caffeic acid.
Lithium was measured in undiluted, acidified urine by atomic absorption spectrophotometry (Anonymous 1976).

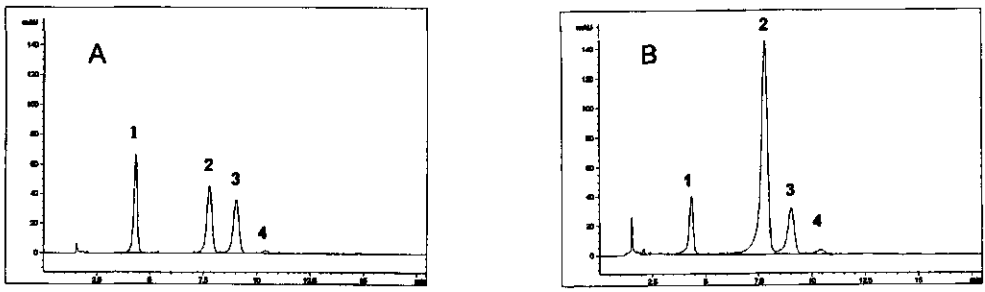


Figure 3.2 Chromatograms of chlorogenic acid isomers and of caffeic acid in standard mixture (A) and ileostomy effluent (B). Peak 1: 3-caffeoylquinic acid or 4-caffeoylquinic acid; Peak 2: 5-caffeoylquinic acid; Peak 3 : 3-caffeoylquinic acid or 4-caffeoylquinic acid; Peak 4: caffeic acid

RESULTS

The HPLC method used for the determination of chlorogenic acid and caffeic acid in ileostomy fluid showed well-resolved isomer peaks. Quantification was not hampered by potential interferences from the sample matrix (Figure 3.2).
Of the ingested chlorogenic acid, 67% was excreted in ileostomy fluid, whereas only 5% of the caffeic acid was excreted (Table 3.1). Traces of the ingested chlorogenic acid and 11% of the ingested caffeic acid were excreted in urine (Table 3.2).
Chlorogenic acid and caffeic acid were recovered almost completely after in vitro incubation in gastric juice and duodenal fluid and after ex vivo incubation in ileostomy fluid (Table 3.3). Thus, the amounts not excreted in ileostomy effluent were likely absorbed rather than degraded in the gut or in the ileostomy bag. The absorption of chlorogenic acid therefore equaled 100% - 67% = 33% and that of caffeic acid 95%.

Table 3.1 Intake of chlorogenic acid and caffeic acid and subsequent excretion in ileostomy effluent over 24 h in 7 subjects[1]

Supplement	Intake	Excretion			Absorption
		Presupplement sample[2]	24-h excretion[3]	Final sample[4]	
	mg				*% of intake*
Chlorogenic acid[5]	1000	2.3 ± 2.5	667 ± 165	2.6 ± 3.8	33 ± 17
Caffeic acid	500	0.3 ± 0.6	27 ± 18	0.1 ± 0.1	95 ± 4

[1] Values are mean ± SD.

[2] Ileostomy effluent sample collected before ingestion of the supplements; amounts represent the mean of 6 subjects because 1 subject did not collect the presupplement ileostomy effluent.

[3] Includes the final but not the presupplement sample.

[4] Ileostomy effluent sample collected at the end of the 24-h collection period.

[5] Excretion of chlorogenic acid was calculated as the sum of the excretion of 3-caffeoylquinic acid, 4-caffeoylquinic acid and 5-caffeoylquinic acid in ileostomy effluent.

Table 3.2 *Intake of chlorogenic acid and caffeic acid and subsequent mean excretion of chlorogenic acid and caffeic acid in urine over 24 h in 7 subjects*[1]

Supplement	intake	24-hour excretion	
		Caffeic acid	Chlorogenic acid[2]
		mg	
Chlorogenic acid	1000	1.7 ± 0.8	2.9 ± 1.5
Caffeic acid	500	53.4 ± 14.1	ND

[1] Values are mean ± SD; ND, not detected.

[2] Excretion of chlorogenic acid was calculated as the excretion of 5-caffeoylquinic acid. 3-caffeoylquinic acid and 4-caffeoylquinic acid were not present in large amounts in urine.

Table 3.3 Stability of chlorogenic acid and caffeic acid expressed as the percentage recovered after incubation with human gastric juice or duodenal fluid in vitro, or with ileostomy effluent ex vivo.

Supplement	Gastric juice[1]		Duodenal fluid[1]		Ileostomy effluent[2]
	Incubation period				
	0.5 h	*2 h*	*1 h*	*4 h*	*2 h*
			%		
Chlorogenic acid	101	99	95	99	98 (97-98)
Caffeic acid	98	101	97	93	97 (96-98)

[1] mean of duplicate analyses

[2] *mean (range) of recoveries in ileostomy bags on the bodies of 2 subjects.*

DISCUSSION

A maximum of 33% of the ingested chlorogenic acid and 95% of the ingested caffeic acid was absorbed from the small intestine in humans. Traces of chlorogenic acid in urine were recovered after ingestion of chlorogenic acid, whereas 11% of caffeic acid in urine was recovered after ingestion of caffeic acid. This indicates that at least some of the chlorogenic acid was absorbed intact. Indications that caffeic acid and chlorogenic acid are absorbed in the small intestine were also found in a rat intestine perfusion model (Spencer et al. 1999). However, because the recovery in urine was not nearly complete, chlorogenic acid and caffeic acid are probably metabolized extensively into other compounds after absorption. Unfortunately, data on metabolism of chlorogenic acid and caffeic acid in the human body are scarce.

Validity of ileostomy model

We measured absorption as the difference between the amount of supplement ingested and the amount excreted in ileostomy fluid in subjects without a colon. Absorption of nutrients in the small intestine of ileostomy subjects is probably not affected by the lack of the colon (Christl & Scheppach 1997), as indicated by their normal serum cholesterol concentrations (Ellegård & Bosaeus 1991), normal absorption of *para*-aminobenzoic acid (Hollman et al. 1995), and of lithium in this study (Sanchez-Castillo et al. 1987a and 1987b).

It is unlikely that appreciable amounts of chlorogenic acid or caffeic acid disappeared through degradation in the gastric juice, duodenal fluid, in the ileostomy bag or during analysis on the laboratory because chlorogenic acid and caffeic acid were recovered completely after in vitro and ex vivo incubations in gastrointestinal fluids (Table 3.3). However, we cannot exclude that part of the supplements were lost somewhere in the gastrointestinal tract; therefore, the absorption values we found in this study should be regarded as maximum absorption values rather than as fixed absorption values. In subjects with a colon absorption of dietary phenolic compounds and their metabolites in the colon is possible. Therefore, in subjects with a colon caffeic acid in urine might originate from dietary chlorogenic acid.

Collection of ileostomy effluent

The amounts of caffeic acid and chlorogenic acid that were not recovered in ileostomy effluent cannot be explained by loss of ileostomy effluent. None of the subjects reported loss of ileostomy effluent during the 24-h collection periods, and the 9-11 ileostomy bags that subjects collected during 24 h also indicated that they had collected all ileostomy effluent. Therefore we conclude that collection of ileostomy effluent was complete.

The subjects in this study collected ileostomy effluent for 24 h, which should be long enough to detect all nonabsorbed supplement in ileostomy effluent because the mean

transit time through the stomach and small intestine is ~8-11h (Fallingborg et al. 1989 and 1990). This was also supported by the fact that the amount of chlorogenic acid and caffeic acid excreted in the final collection of ileostomy effluent at the end of the 24-h period was similar to that in the presupplement collection (Table 3.1). Furthermore, the recovery of 84 ± 19 % (mean ± SD) of quercetin in ileostomy effluent during 24 hours after ingestion of quercetin-3-rutinoside in this study was similar to the recovery during the first 13 h in a previous study (Hollman et al. 1995).

Comparison with previous studies

We found that the absorption of caffeic acid esterified with quinic acid (chlorogenic acid) is three times lower than that of caffeic acid itself. To our knowledge, there are no previous quantitative data on absorption of chlorogenic acid and caffeic acid in humans. The studies that were done on absorption of chlorogenic acid and caffeic acid measured the recoveries of these compounds and their metabolites in urine of rats (Booth et al. 1957, Camarasa et al. 1988).

We recovered 0.3% of chlorogenic acid in urine after ingestion. After ingestion of chlorogenic acid by rats, no chlorogenic acid was found in urine (Choudhury et al. 1999). We recovered 11% of caffeic acid in urine after ingestion, which was comparable to the recovery of 13% found by Camarasa (1988) after ingestion of caffeic acid in rats. After intravenous injection of chlorogenic acid and caffeic acid in rats, only 9% of chlorogenic acid and 26% of caffeic acid were recovered in urine (Choudhury et al. 1999). This indicates that the fraction of chlorogenic acid and of caffeic acid that is absorbed is metabolized extensively in the body and therefore only small amounts are recovered in urine.

Mechanisms of absorption

The absorption of caffeic acid esterified with quinic acid (chlorogenic acid) was less than that of caffeic acid itself. It is possible that chlorogenic acid and caffeic acid are absorbed through different absorption mechanisms. We can envisage two mechanisms for the absorption of chlorogenic acid in humans. The first mechanism might involve absorption of chlorogenic acid as an intact molecule as indicated by the presence of traces of chlorogenic acid in urine after ingestion of chlorogenic acid in our study. We probably only found traces of the absorbed chlorogenic acid in urine because chlorogenic acid is metabolized intensively after absorption (Choudhury et al. 1999). The second mechanism might involve hydrolysis of chlorogenic acid in the stomach and/or small intestine into caffeic acid and quinic acid before absorption. The caffeic acid moiety and the quinic acid moiety are subsequently absorbed (Czok et al. 1974, Westendorf & Czok 1978). If this mechanism plays a role in the absorption of chlorogenic acid, we would expect to find caffeic acid in urine as we found after intake of the caffeic acid supplement. If we assume that the absorption of chlorogenic acid is 33% and the amount of caffeic acid in urine after ingestion of caffeic acid is 11% then

we would expect to recover ~4% of chlorogenic acid in urine as caffeic acid. However, we found only 0.3% of chlorogenic acid as caffeic acid in urine after ingestion of chlorogenic acid, which is ~10 times lower than we expected. This indicates that hydrolysis of chlorogenic acid in the stomach or small intestine is not very important. This is also supported by the fact that we found a large amount of the ingested chlorogenic acid unchanged in ileostomy effluent. Thus, this second mechanism likely does not play an important role in the absorption of chlorogenic acid. Therefore, we propose that in ileostomy subjects most of the absorbed chlorogenic acid is absorbed intact and is metabolized extensively in the liver.

Caffeic acid is probably absorbed through different absorption mechanisms than chlorogenic acid. We can envisage two mechanisms for the absorption of caffeic acid in humans. The first mechanism for absorption of caffeic acid might involve passive absorption of caffeic acid in the stomach. This is supported by the fact that caffeic acid and the structurally related compound cinnamic acid were rapidly absorbed in rats (Fahelbum & James 1977). Further, in the acid environment of the stomach, caffeic acid will be primarily in the nonionic form, which can be absorbed by passive nonionic diffusion. Passive absorption in the small intestine is not very likely because at a pH of ~7 in the small intestine, caffeic acid will be mainly in the ionic form, which is difficult to absorb by passive diffusion (Wolffram et al. 1995). The second mechanism for absorption of caffeic acid might involve absorption by an active transport mechanism in the small intestine. Results from in vitro studies indicate that in the small intestine, an active Na+-dependent transport mechanism might be involved in the absorption of cinnamic acids such as caffeic acid (Ader et al. 1996, Wolffram et al. 1995). Both mechanisms, passive absorption in the stomach and active absorption in the small intestine, might play a role in the absorption of caffeic acid in humans.

Chlorogenic acid, caffeic acid and health

The absorbed fraction of chlorogenic acid and caffeic acid and its metabolites might induce biological effects in the blood circulation. Chlorogenic acid and caffeic acid inhibit oxidation of Low Density Lipoproteins in vitro (Laranjinha et al. 1994, Nardini et al. 1995) and might therefore protect against cardiovascular disease. There are no in vivo data available that show that chlorogenic acid is present in the blood circulation after ingestion, but caffeic acid is present in blood after ingestion by rats (Camarasa et al. 1988). In our human study we did find chlorogenic acid and caffeic acid in urine, which also suggests that a small part will be present as such in blood. We did not measure chlorogenic acid and caffeic acid in blood because we did not have an available method of analysis.

The fraction of chlorogenic acid that escapes absorption is present throughout the whole gastrointestinal tract, where it might induce biological effects. Chlorogenic acid and caffeic acid are antioxidants in vitro (Castelluccio et al. 1995, Rice-Evans et al.

1996), and they might inhibit the formation of mutagenic and carcinogenic N-nitroso compounds (Kono et al. 1995). Further, chlorogenic acid can inhibit DNA damage in vitro (Kasai et al. 2000, Shibata et al. 1999). Therefore, chlorogenic acid, the major phenolic compound in coffee, might be involved in the inverse association between coffee consumption and colon cancer that was found by some epidemiological studies (Baron et al. 1994, Favero et al. 1998, Giovannucci 1998, La Vecchia et al. 1989, Tavani et al. 1997), but not all (Baron et al. 1997). Thus the one-third of ingested chlorogenic acid that is absorbed could have biological effects in the blood circulation, and the fraction of chlorogenic acid that is not absorbed could have biological effects in the colon in humans.

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4

Metabolism of chlorogenic acid, quercetin-3-rutinoside and black tea phenols in humans

Margreet R. Olthof, Peter C. Hollman, Johan M.M. van Amelsvoort, Martijn B. Katan

Dietary phenols are antioxidants and their consumption might contribute to the prevention of cardiovascular disease. Coffee and tea are major dietary sources of phenols. Dietary phenols are extensively metabolized in the body. Lack of quantitative data on their metabolites hinders a proper evaluation of the potential biological effects of dietary phenols in vivo. We identified and quantified phenolic acid metabolites in urine of twenty healthy humans with an intact colon after they had ingested chlorogenic acid (major phenol in coffee), quercetin-3-rutinoside (major flavonol in tea) and black tea phenols. Half of the ingested chlorogenic acid and 43% of the tea phenols were metabolized into hippuric acid, but quercetin-3-rutinoside was mainly metabolized into phenylacetic acids: 3-hydroxyphenylacetic acid (40%), 3-methoxy-4-hydroxyphenylacetic acid (9%) and 3,4-dihydroxyphenylacetic acid (6%). In contrast, in seven humans without a colon we found only traces of phenolic acid metabolites after they had ingested chlorogenic acid and quercetin-3-rutinoside. This implies that the colonic microflora converts most of these dietary phenols into metabolites which then reach the circulation. Metabolites of phenols have lower antioxidant activity than their parent compounds, therefore the contribution of dietary phenols to the antioxidant activity in vivo is probably lower than expected from in vitro tests.

Submitted for publication

INTRODUCTION

Dietary phenols are antioxidants *in vitro*, and might therefore contribute to the prevention of cardiovascular disease (Hollman et al. 1999a, Halliwell 1994). Two important groups of phenols in foods are flavonoids and cinnamic acids. Major flavonoids in foods are flavonols, flavones and catechins. The intake of flavonols and flavones from foods in The Netherlands is about 23 mg/day, of which 16 mg/day is quercetin (Hertog et al. 1993a) and the intake of monomeric catechins is 50 mg/day (Figure 4.1) (Arts et al. 2001). Tea is an important dietary source of flavonoids: 1 L of strong tea can provide about 0.5 g of phenols, of which a large part consists of polymerized flavonoids (mainly catechins) such as theaflavins and thearubigins (Figure 4.1) (Balentine et al. 1997). In The Netherlands, tea provides almost 50% of the daily intake of flavonols and flavones (Hertog et al. 1993a) and 55% of the daily intake of monomeric catechins (Arts et al. 1999).

The major representative of dietary cinnamic acids is caffeic acid. In foods caffeic acid is mainly conjugated with quinic acid, which yields chlorogenic acid (5-caffeoylquinic acid) (Figure 4.1). Coffee is the major source of dietary chlorogenic acid: 1 L of coffee provides 500-800 mg of chlorogenic acid, which corresponds with about 250-400 mg of caffeic acid (Clifford 1999). Coffee consumption provides up to 90% of the daily intake of cinnamic acids, mainly caffeic acid: coffee drinkers ingest about 0.5 - 1 g cinnamic acids/day, whereas coffee abstainers ingest not more than 100 mg/day (Radtke et al. 1998, Clifford 1999).

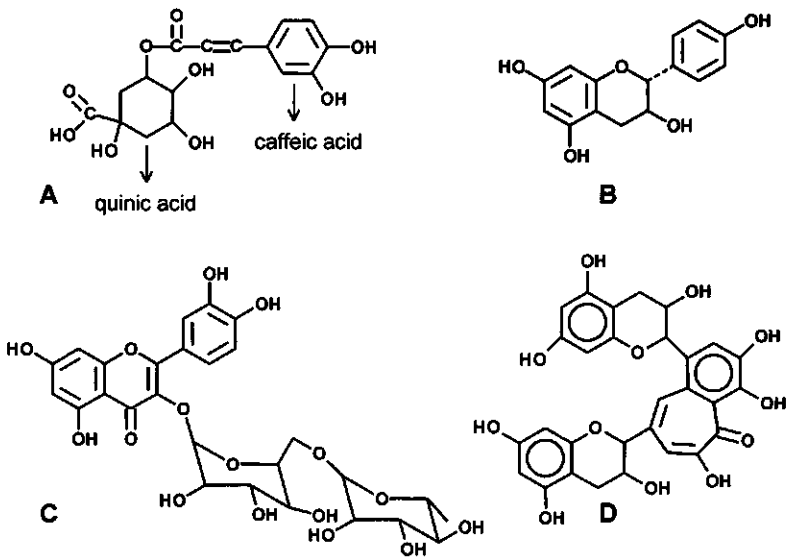

Figure 4.1 Chemical structures of chlorogenic acid (A), monomeric catechin (B), quercetin-3-rutinoside (C), theaflavin (D)

Dietary phenols are antioxidants and they can protect Low Density Lipoprotein (LDL) cholesterol from oxidation in vitro (de Whalley et al. 1990, Meyer et al. 1998, Nardini et al. 1995, Natella et al. 1999). Oxidized LDL might play a role in the pathogenesis of atherosclerosis and it might therefore be associated with an increased risk for cardiovascular disease (Heinecke 1998, Witztum 1998, Steinberg 1997). However, the relationship between intake of flavonoids and cardiovascular disease in epidemiological studies is inconclusive (Geleijnse et al. 1999, Hertog et al. 1993b, 1995 and 1997, Yochum et al. 1999, Keli et al. 1996, Knekt et al. 1996 and 2000, Rimm et al. 1996).

Thus, dietary phenols are strong antioxidants in vitro (Rice-Evans et al. 1996), but their contribution to the antioxidant defense in vivo is uncertain. One reason for this uncertainty is that dietary phenols are extensively metabolized in the body into unknown compounds. As an example, only 1-2% of ingested phenols is excreted intact in urine (Hollman & Katan 1998). If metabolism of phenols occurs before they can reach the circulation and act as antioxidants, then in vitro tests of antioxidant activity might be less relevant to the in vivo situation. Therefore it is important to identify the circulating metabolites of phenols and the site where they are produced. These metabolites might actually have antioxidant activity in vivo, although most of the metabolic reactions reduce the antioxidant activity of the parent phenol (Pietta et al. 2000, Rice-Evans et al. 1996, Manach et al. 1998, Morand et al. 1998). In the liver and kidney the hydroxyl groups of phenols are conjugated with glucuronic acid or sulfate, or a carboxyl group is esterified with glycine to increase water solubility. In addition, hydroxyl groups of phenols can be O-methylated, and oxidation of the side chain may occur. The colon bacteria can deconjugate phenols, cleave the rings, and remove hydroxyl and methyl groups (Scheline 1978b). Knowledge about the metabolites of dietary phenols is thus crucial, because the in vivo biological activity of the circulating metabolites of dietary phenols might be different from that of the dietary phenols themselves.

There are only few data on the metabolism of dietary phenols in humans. Together with data from animal and in vitro studies all these studies indicate that dietary phenols are metabolized mainly into phenolic acids, or into even smaller compounds (Hollman & Katan 1998). However, no controlled dietary trial in humans has been done to identify and quantify the profile of phenolic acid metabolites after ingestion of important dietary phenols. Furthermore, data on the site of metabolism in humans are also scarce.

Therefore, we studied in humans the potential phenolic acid metabolites of 3 major dietary phenols: chlorogenic acid, quercetin-3-rutinoside, and black tea phenols which consist of mainly catechins (Figure 4.1). These phenols are important dietary phenols and are stronger antioxidants in vitro than antioxidant vitamins (Rice-Evans et al. 1996, Williamson et al. 1999). For this purpose Hollman et al. (P.C.H. Hollman et al,

manuscript in preparation) developed a method to measure 59 phenolic acids in urine. Furthermore we investigated the site of metabolism of dietary phenols by comparing the metabolites formed in volunteers with an intact colon with those formed in volunteers who lack a colon (ileostomy subjects).

SUBJECTS AND METHODS

Subjects

Subjects with a colon

Ten women and ten men with a mean (±SD) age of 24 ± 8 y and a mean body mass index of 22.2 ± 2.5 kg/m^2 participated. They were healthy as judged by a medical questionnaire; normal blood values for hemoglobin, hematocrit and white blood cell counts; and absence of glucose and protein in urine. They were not allowed to take any drugs or other supplements during the study, except for acetaminophen (paracetamol) and oral contraceptives. The study was fully explained to the subjects and they gave their written informed consent.

Subjects without a colon (ileostomy subjects)

Four women and three men, with a mean (± SD) age of 63 ± 10 y, and a mean body mass index of 27.1 ± 3.8 kg/m^2 were admitted to participate (Olthof et al. 2001a). All subject had had a total colectomy between 7 and 27 years ago for ulcerative colitis or polyposis coli. Exclusion criteria were as follows: signs of diseases related to the gastrointestinal tract; resection of more than 50 cm of the terminal ileum; an ileostomy that did not function properly; use of drugs which influenced gastrointestinal transit; present illness; pregnancy or lactation. They were healthy as judged by a medical questionnaire; normal blood values for hemoglobin, hematocrit and white blood cell counts; and absence of glucose and protein in urine. The study was fully explained to the subjects and they gave their written informed consent.

Methods

Subjects with a colon

The study in subjects with a colon was approved by the Medical Ethical Committee (Wageningen University). Throughout the 4-weeks cross-over study subjects consumed a controlled diet low in phenols which was largely provided by us. In addition to the diet subjects ingested 4 supplements, each for 1 week in random order. We had a menu cycle of 7 days, so that the menu on the day that blood and urine were collected in each supplement week (i.e. day 7) was always the same, as were the other days (i.e. days 1-6) in each of the four supplement weeks. To achieve the diet low in phenols, we supplied the subjects daily with foods low in phenols, which provided 90% of the energy required to maintain body weight. The remaining 10% of energy was chosen by the subjects from a list of food items low in phenols. Foods

were considered low in phenols if they contained <15 mg quercetin or chlorogenic acid/kg, and beverages if they contained <4 mg quercetin or chlorogenic acid/L (Clifford 1999, Hertog et al. 1992 and 1993c). Because coffee and tea consumption were not allowed we provided the volunteers with the following substitutes: for coffee, an extract made of chicory, rye and barley (Swiss coffee-like; Tayala AG, Birsfelden, Switzerland) and for tea, tea bags containing a mix of herbs (droommix; Piramide, Veenendaal, The Netherlands) or tea bags containing stinging nettle (Jacob Hooy, Limmen, The Netherlands). Chemical analyses of these coffee and tea substitutes showed that the amounts of chlorogenic acid and flavonols such as quercetin were within the allowed ranges, as described before.

In addition to the diet subjects ingested each day one of the following supplements (Figure 4.1): 2 g (5.5 mmol) of chlorogenic acid (Fluka Chemie AG, Buchs, Switzerland), or 4 g of black tea solids (LN-0173-02, kindly provided by Unilever Research Vlaardingen, The Netherlands), or 440 mg (0.7 mmol) of quercetin-3-rutinoside (Rutosidum DAB, BUFA B.V., Uitgeest, The Netherlands), or 0.5 g of citric acid as placebo (AC Citricum; Fagron, Nieuwerkerk a/d IJssel, The Netherlands). The 2 g of chlorogenic acid corresponds with the amount of chlorogenic acid present in ~1.5 L of strong coffee. The 4 g of black tea solids corresponds with ~2 L of strong black tea. Black tea solids contain 30-40 g of polyphenols per 100 g, thus 4 g of black tea solids provided ~4.3 mmol polyphenols, mainly catechins (Balentine et al. 1997). The amount of quercetin in 440 mg quercetin-3-rutinoside corresponds with the amount in ~13 L of black tea (Hertog et al. 1993c) and is 13 times higher than the daily intake of quercetin (Hertog et al. 1993a). The chlorogenic acid, quercetin-3-rutinoside and citric acid, and half of the black tea extract (2g) were dissolved in hot water before ingestion. Subjects took the supplements under our supervision, just before the hot meal at noon. The other one-half of the black tea solids was used for tea preparation and consumption at home: 1 g between 0800 and 1000 (on Saturdays and Sundays: between 0800 and 1100), and 1 g between 1800 and 2000. The volunteers were urged to maintain their usual pattern of physical activity during the study.

Subjects without a colon (ileostomy subjects)

The study in volunteers without a colon was approved by the Medical Ethical committee (Wageningen University). The design is described in detail in Olthof et al. (2001a). In summary, in this 2-weeks cross-over study subjects followed a diet which was low in chlorogenic acid and quercetin from day 1 to day 14. To achieve this, subjects had to follow dietary guidelines. To ensure adherence to the dietary guidelines we gave the subjects a list of prohibited foods and beverages, which were high in phenols. Foods were considered high in phenols if they contained >15 mg quercetin or chlorogenic acid/kg, and beverages if they contained >4 mg quercetin or chlorogenic acid/L (Clifford 1999, Hertog et al. 1992 and 1993c). Because subjects

were not allowed to drink coffee and tea during the study we supplied the coffee and tea substitutes. Compliance with the dietary guidelines was good. None of the subjects reported that he or she consumed any foods or beverages that were on the list of prohibited foods and beverages during the study.
On day 5 all subjects consumed a placebo supplement, which was 200 mL of water. On day 6, 10 and 14 all subjects consumed one each of the following supplements in random order: 1000 mg (2.8 mmol) of chlorogenic acid (Fluka Chemie AG, Buchs, Switzerland) or 500 mg (2.8 mmol) of caffeic acid (Fluka Chemie AG, Buchs, Switzerland) or 220 mg (0.3 mmol) of quercetine-3-rutinoside (Rutosidum DAB; BUFA B.V., Uitgeest, The Netherlands). Subjects received the supplements as a powder and were instructed to add 200 mL hot water and to consume the beverage within 5 minutes after preparation. Subjects ingested the supplements between 0700 and 0900 at home, together with a light breakfast that we provided.

Collection of urine and blood

Subjects with a colon

On day 7 of each of the four supplement weeks subjects collected urine during 24 hours. Thus each subject collected urine 4 times. Subjects collected urine in 0.5 L plastic bottles with 0.13 g thymol (# 8167; Merck, Amsterdam, The Netherlands) as a preservative and stored the bottles with urine on dry ice immediately after voiding. We measured the recovery of 277 μmol lithium in the 24 h urine of day 7 to check the completeness of urine collection. Lithium was ingested daily during the study by the subjects as lithium chloride dissolved in 10 mL of tap water. Lithium chloride is completely absorbed and 95% is excreted in urine (Sanchez-Castillo et al. 1987a, and 1987b). Lithium was measured in undiluted, acidified urine by atomic absorption spectrophotometry (Anonymous 1976). Urinary recovery of lithium was 105.0 ± 9.0% (mean ± SD), which indicates good compliance in collecting urine.
On day 7 of each supplement period we collected two blood samples for measurement of plasma homocysteine. These results are published elsewhere (Olthof et al. 2001b).

Subjects without a colon

On days 5, 6, 10 and 14 subjects collected 24 h urine as described above. We took no blood from these subjects.

Analyses of phenolic acids in urine

Subjects with a colon

We analyzed the urine samples twice: 1) without deconjugation of the phenolic acid metabolites and 2) after deconjugation with β-glucuronidase/arylsulfatase. Chlorogenic acid was measured without prior deconjugation, and it was not measured after deconjugation. After that, urine was acidified and brought onto a Solid Phase

Extraction (SPE) column. The column was eluted with ethyl acetate. Ethyl acetate was evaporated and acids were derivatized with bis(trimethylsilyl)trifluoroacetamide (BSTFA). Derivates were injected onto a capillary gas chromatography column and separated using a temperature gradient. On-line Mass Spectrometry was used to quantify and identify phenolic acids (Table 4.1). The method will be described in full elsewhere (P.C.H. Hollman et al, manuscript in preparation). This method for phenolic acid metabolites in urine was optimized to measure the following classes of metabolites: phenylpropionic and cinnamic acids (phenyl-C_3: a benzene ring with a side chain of 3 C-atoms), phenylacetic acids (phenyl-C_2) and benzoic acids (phenyl-C_1). We could purchase standards of 59 different phenolic acids and we detected 27 of them in the urine samples of our volunteers. Two phenolic acids were excluded because more than half of the samples had concentrations of these acids that were below the detection limit in all supplement periods, thus 25 phenolic acids were regarded as potential metabolites of the supplements (Table 4.1). The detection limit in urine was 4 mg/L urine for chlorogenic acid, 5 mg/L for hippuric acid and 0.4 mg/L for other phenolic acids.

Subjects without a colon

We analyzed the urine samples of subjects without a colon only after deconjugation with β-glucuronidase/arylsulfatase as described above (Table 4.2), except for chlorogenic acid which was measured without prior deconjugation. Of the 27 phenolic acids that were detected in urine of subjects without a colon 10 phenolic acids were excluded because more than half of the samples had concentrations of these acids that were below the detection limit in all supplement periods. Thus 17 phenolic acids were regarded as potential metabolites of the supplements.

Statistical analyses

For each subject, we calculated the excretion of each of the phenolic acids after each of the three treatments minus its excretion after the placebo. Statistical significance and 95% confidence intervals of the mean differences were calculated using Student's *t* test. We chose to use a one-sided significance level because we reasoned that rises in the excretion of phenolic acids on placebo relative to the phenol supplements must be spurious. Further, because we tested several phenolic acids as potential metabolites (multiple testing) we decided to use an alpha of 0.001 for statistical testing in order to distinguish genuine metabolites of dietary phenols from compounds that were present by accident. To indicate possible other (quantitatively less important) metabolites we reported p-values between 0.01 and 0.001 separately (one-sided) (Tables 4.1 and 4.2). Statistical analyses were done with SAS (SAS Institute Inc, Cary, USA).

RESULTS

Volunteers with a colon

Hippuric acid (N-benzoylglycine) was the major metabolite of chlorogenic acid and of phenols from tea (Table 4.1 and 4.3). Additional phenyl-C_1 and phenyl-C_3 metabolites were formed after chlorogenic acid and after black tea supplementation, but their amounts were small. No hippuric acid or other phenolic acids of the phenyl-C_1 group and the phenyl-C_3 group were formed after quercetin-3-rutinoside. Phenylacetic acids (phenyl-C_2 metabolites) were the major metabolites of quercetin-3-rutinoside (Tables 4.1 and 4.3).

Almost all of the chlorogenic acid ingested was recovered in urine as hippuric acid if we assume that only the caffeic acid moiety of chlorogenic acid yields hippuric acid. However, the quinic acid moiety of chlorogenic acid might also be metabolized into hippuric acid in humans (Quick 1931, Adamson et al. 1970, Scheline 1978a). If we assume that each molecule of chlorogenic acid yields 2 molecules of hippuric acid, then 49.5 mol% of the ingested chlorogenic acid was recovered in urine as hippuric acid (Table 4.3). Of the ingested chlorogenic acid 1.7% was recovered unchanged in urine. We did not assess quercetin-3-rutinoside or tea phenols in urine. However, in other human studies less than 0.5% of ingested quercetin-3-rutinoside was recovered in urine in the form of as quercetin or its conjugates (Hollman et al. 1995 and 1997a), and about 1-6% of monomeric catechins were recovered in urine of humans after ingestion of green tea (Yang et al. 1998, Li et al. 2000).

The absolute excretion of 4-hydroxyphenylacetic acid in urine was in general higher than that of other phenolic acids after all treatments including placebo, but excretion was not influenced by the supplements ingested (Table 4.1). 4-hydroxyphenylacetic acid is probably a metabolite of dietary phenylalanine and tyrosine (Teuchy & Van Sumere 1971, Booth et al. 1960). These amino acids were present to an equal extent in the background diet of all supplement periods.

Treatment of the urine with β-glucuronidase and arylsulfatase enzymes before GC-MS analysis markedly increased the number and amounts of phenolic acids detected in urine (Table 4.4). This indicates that most phenolic acids were conjugated with glucuronic acid or sulfates.

Volunteers without a colon

We did not recover hippuric acid as a metabolite of chlorogenic acid in volunteers without a colon and we found only trace amounts of caffeic acid (3,4-dihydroxycinnamic acid) and ferulic acid (3-methoxy-4-hydroxycinnamic acid) as metabolites of chlorogenic acid (Tables 4.2 and 4.3). This contrasts with the results in volunteers with a colon. After volunteers without a colon had ingested caffeic acid we recovered caffeic acid and ferulic acid as main metabolites in urine plus small amounts of phenyl-C_1 and phenyl-C_3 metabolites (Tables 4.2 and 4.3). After subjects

had ingested quercetin-3-rutinoside we did not find any metabolites in urine (Tables 4.2 and 4.3).

Table 4.1 Amount (μmol/24h; mean ± SE) of phenolic acids excreted in urine of subjects with a colon. Twenty subject ingested placebo, 2 g (5.5 mmol) chlorogenic acid, 440 mg (0.7 mmol) quercetin-3-rutinoside and 4 g black tea solids (~4.3 mmol phenols) each for 7 days each. Supplements were ingested in random order.

Phenolic acids in urine[$]	Supplements			
	Placebo	Chlorogenic acid	Quercetin-3-rutinoside	Black tea solids
Total phenolic acids	3300 ± 200	9300 ± 800	3800 ± 200	5300 ± 400
Phenyl-C_1 metabolites				
Benzoic acid	26 ± 2	33 ± 3 **	27 ± 2	32 ± 4
3-OH-benzoic acid	ND	6.3 ± 0.8 #**	ND	6.6 ± 1.3 #**
4-OH-benzoic acid	51 ± 4 ‡	54 ± 4	51 ± 4	56 ± 6
2,4-diOH-benzoic acid	15 ± 3	13 ± 2 ‡	16 ± 2	15 ± 3 ‡
3,4-diOH-benzoic acid (protocatechuic acid)	25 ± 2	44 ± 5 ‡**	27 ± 2	31 ± 3
3,5-diOH-benzoic acid	45 ± 5	42 ± 4 ‡	47 ± 4	48 ± 5
3,4,5-triOH-benzoic acid (gallic acid)	ND	ND	ND	15 ± 1 ‡**
3-OCH_3-4-OH-benzoic acid (vanillic acid)	96 ± 16	114 ± 16	91 ± 11	83 ± 7
3,5-diOCH_3-4-OH-benzoic acid (syringic acid)	6.7 ± 0.6	13.6 ± 2.2 ‡*	8.3 ± 1.9	6.2 ± 0.7 ‡
N-benzoylglycine (hippuric acid)	2500 ± 200	8100 ± 800 **	2700 ± 200	4400 ± 400 **
N(2-OH-benzoyl)glycine (salicyluric acid)	5.5 ± 0.6 ‡	9.2 ± 1.5 ‡*	6.2 ± 1.2 ‡	9.7 ± 1.4 ‡*
Phenyl-C_2 metabolites				
Phenylacetic acid	125 ± 7	143 ± 10	131 ± 7	162 ± 17
2-OH-phenylacetic acid	8.4 ± 0.5 ‡	8.0 ± 0.6 ‡	8.2 ± 0.6	6.5 ± 0.6 #
3-OH-phenylacetic acid	21 ± 3	18 ± 2	259 ± 51 **	68 ± 6 **
4-OH-phenylacetic acid	180 ± 20 ‡	180 ± 20	170 ± 20 ‡	190 ± 20
3,4-diOH-phenylacetic acid	19 ± 1	24 ± 2 ‡*	52 ± 6 **	26 ± 2 **
3-OCH_3-4-OH-phenylacetic acid (homovanillic acid)	59 ± 3	49 ± 3	110 ± 15 **	63 ± 5
Phenylhydroxyacetic acid (mandelic acid)	37 ± 2	41 ± 3	38 ± 2	48 ± 6

Table 4.1 continued

Phenolic acids in urine[$]	Supplements			
	Placebo	Chlorogenic acid	Quercetin-3-rutinoside	Black tea solids
Phenyl-C_3 metabolites				
3-OH-cinnamic acid	ND	20 ± 3 ‡**	ND	ND
4-OH-cinnamic acid	3.5 ± 0.3 #	4.2 ± 0.5 #	ND	ND
3,4-diOH-cinnamic acid (caffeic acid)	ND	56 ± 8 **	ND	ND
3-OCH_3-4-OH-cinnamic acid (ferulic acid)	30 ± 3	75 ± 6 **	30 ± 3	38 ± 4
3,5-diOCH_3-4-OH-cinnamic acid (sinapinic acid)	2.8 ± 0.5 #	ND	2.6 ± 0.5 #	ND
3-(3,4-diOH-phenyl)propionic acid	ND	97 ± 16 ‡**	ND	5 ± 1 #*
5-caffeoylquinic acid (chlorogenic acid)	ND	100 ± 20 ‡**	ND	ND

[$] The following phenolic acids were absent in all subjects during all supplements: 2,3-diOH-benzoic acid; 2,6-diOH-benzoic acid; 2,3,4-triOH-benzoic acid; 2,4,6-triOH-benzoic acid; 2-OCH_3-benzoic acid; 3-OCH_3-benzoic acid; 4-OCH_3-benzoic acid; 2,3-diOCH_3-benzoic acid; 2,4-diOCH_3-benzoic acid; 2,6-diOCH_3-benzoic acid; 3,4-diOCH_3-benzoic acid; 3,5-diOCH_3-benzoic acid; 3,4,5-triOCH_3-benzoic acid; 2,5-diOH-phenylacetic acid (homogentisic acid); 2-OCH_3-phenylacetic acid; 3-OCH_3-phenylacetic acid; 4-OCH_3-phenylacetic acid; 3,4,5-triOCH_3-phenylacetic acid; cinnamic acid; 2-OH-cinnamic acid; 2-OCH_3-cinnamic acid; 3-OCH_3-cinnamic acid; 4-OCH_3-cinnamic acid; 3-(phenyl)propionic acid; 3-(2-OH-phenyl)propionic acid; 3-(4-OH-phenyl)propionic acid; 3-(2,4-diOHphenyl)propionic acid; 3-(2-OCH_3-phenyl)propionic acid; 3-(4-OCH3-phenyl)propionic acid; 3-(3,4-diOCH_3-phenyl)propionic acid; 3-(3,4,5-triOCH_3-phenyl)propionic acid; phenylaceturic acid

The following phenolic acids were above the detection limit in less than half of the subjects after each supplement period and therefore they are not shown in the table: 2-OH-benzoic acid and 2,5-OH-benzoic acid.

ND = non-detectable; peaks had to be above the detection limit in at least half of the subjects, otherwise the phenolic acid is not shown in the table.

‡ = peaks of 1 - 5 subjects were below detection limit or missing

\# = peaks of 6 - 10 subjects were below detection limit or missing

* = $0.001 \leq p \leq 0.01$, one-sided, significantly different from placebo

** = $p \leq 0.001$, one-sided, significantly different from placebo

Table 4.2 Amount (μmol/24h; mean ± SE) of phenolic acids excreted in urine in subject without a colon. Seven subject ingested placebo, 1 g (2.8 mmol) chlorogenic acid, 220 mg (0.3 mmol) quercetin-3-rutinoside and 500 mg (2.8 mmol) caffeic acid on 1 day each. Supplements were ingested in random order.

Phenolic acids in urine[$]	Supplements			
	Placebo	Chlorogenic acid	Quercetin-3-rutinoside	Caffeic acid
Total phenolic acids	1000 ± 200	1100 ± 200	1100 ± 300	2100 ± 300
Phenyl-C_1 metabolites				
4-OH-benzoic acid	7.5 ± 2.5 ‡	6.9 ± 0.7 ‡	7.9 ± 1.6 ‡	6.0 ± 1.2 ‡
3,4-diOH-benzoic acid (protocatechuic acid)	7 ± 1 ‡	9 ± 1	9 ± 2	68 ± 10 **
3,5-diOH-benzoic acid	9 ± 1 ‡	10 ± 2 ‡	10 ± 2 ‡	20 ± 8 ‡
3-OCH_3-4-OH-benzoic acid (vanillic acid)	33 ± 12	24 ± 5	46 ± 17	141 ± 25 *
N-benzoylglycine (hippuric acid)	510 ± 70	490 ± 30	460 ± 40	590 ± 70
N(2-OH-benzoyl)glycine (salicyluric acid)	40 ± 30 ‡	ND	30 ± 30 ‡	ND
Phenyl-C_2 metabolites				
Phenylacetic acid	18 ± 7 ‡	ND	32 ± 13 ‡	31 ± 6 ‡
2-OH-phenylacetic acid	6.1 ± 0.6	5.8 ± 0.6 ‡	5.9 ± 0.4	5.6 ± 0.3 ‡
3-OH-phenylacetic acid	8.6 ± 0.8	7.5 ± 0.6 ‡	8.6 ± 0.5	8.4 ± 0.5 ‡
4-OH-phenylacetic acid	300 ± 200 ‡	400 ± 200	400 ± 200	400 ± 300
3,4-diOH-phenylacetic acid	6.0 ± 0.8 ‡	7.2 ± 0.8 ‡	7.3 ± 1.1	7.0 ± 0.9 ‡
3-OCH_3-4-OH-phenylacetic acid (homovanillic acid)	32 ± 2	33 ± 3	33 ± 3	35 ± 2
Phenylhydroxyacetic acid (mandelic acid)	8 ± 2 ‡	7 ± 2 ‡	10 ± 3 ‡	14 ± 2 ‡
Phenyl-C_3 metabolites				
3,4-diOH-cinnamic acid (caffeic acid)	ND	9 ± 2 *	ND	300 ± 30 **
3-OCH_3-4-OH-cinnamic acid (ferulic acid)	8 ± 1	21 ± 3 *	9 ± 1	361 ± 24 **
3-(3,4-diOH-phenyl)propionic acid	ND	ND	ND	20 ± 3 **
5-caffeoylquinic acid (chlorogenic acid)	not measured	9.3 ± 1.4 ***	not measured	ND

$ The following phenolic acids were absent in all subjects during all supplements: 2,3-diOH-benzoic acid; 2,6-diOH-benzoic acid; 2,3,4-triOH-benzoic acid; 2,4,6-triOH-benzoic acid; 2-OCH_3-benzoic acid; 3-OCH_3-benzoic acid; 4-OCH_3-benzoic acid; 2,3-diOCH_3-benzoic acid;

2,4-diOCH_3-benzoic acid; 2,6-diOCH_3-benzoic acid; 3,4-diOCH_3-benzoic acid; 3,5-diOCH_3-benzoic acid; 3,4,5-triOCH_3-benzoic acid; 2,5-diOH-phenylacetic acid (homogentisic acid); 2-OCH_3-phenylacetic acid; 3-OCH_3-phenylacetic acid; 4-OCH_3-phenylacetic acid; 3,4,5-triOCH_3-phenylacetic acid; cinnamic acid; 2-OH-cinnamic acid; 2-OCH_3-cinnamic acid; 3-OCH_3-cinnamic acid; 4-OCH_3-cinnamic acid; 3-(phenyl)propionic acid; 3-(2-OH-phenyl)propionic acid; 3-(4-OH-phenyl)propionic acid; 3-(2,4-diOH-phenyl)propionic acid; 3-(2-OCH_3-phenyl)propionic acid; 3-(4-OCH3-phenyl)propionic acid; 3-(3,4-diOCH_3-phenyl)propionic acid; 3-(3,4,5-triOCH_3-phenyl)propionic acid; phenylaceturic acid

The following phenolic acids were above the detection limit in less than half of the subjects after each supplement period and therefore they are not shown in the table: benzoic acid; 2-OH-benzoic acid; 3-OH-benzoic acid; 2,4-diOH-benzoic acid; 2,5-diOH-benzoic acid, 3,4,5-triOH-benzoic acid; 3,5-diOCH_3-4-OH-benzoic acid; 3-OH-cinnamic acid; 4-OH-cinnamic acid; 3,5-diOCH_3-4-OH-cinnamic acid.

ND = non-detectable; peaks had to be above the detection limit in at least half of the subjects, otherwise the phenolic acid is not shown in the table.

‡ = peaks of 1 - 3 subjects were below detection limit or missing

* = $0.001 \leq p \leq 0.01$, one-sided, significantly different from placebo

** = $p \leq 0.001$, one-sided, significantly different from placebo

*** = $0.001 \leq p \leq 0.01$, one-sided, significantly different from caffeic acid

Table 4.3 Amounts of metabolites of phenols in urine as percentage (calculated on a molar basis) of the amount of the parent compound ingested. 20 healthy volunteers with a colon ingested chlorogenic acid (5.5 mmol), quercetin-3-rutinoside (0.7 mmol), black tea solids (~4.3 mmol phenols) and placebo on 7 days each, in random order. 7 healthy volunteers without a colon ingested chlorogenic acid (2.8 mmol), caffeic acid (2.8 mmol) and quercetin-3-rutinoside (0.3 mmol) and placebo on 1 day each, in random order.

	Volunteers **with** colon		Volunteers **without** colon	
	Mean	95% CI	Mean	95% CI
Chlorogenic acid supplement				
▪ 5-caffeoylquinic acid (chlorogenic acid)	1.7	1.0-2.4	0.2	0.1-0.4
▪ N-benzoylglycine (hippuric acid)	49.5*	37.1-61.8		
▪ 3(3,4-dihydroxyphenyl)propionic acid	1.7	1.1-2.3		
▪ 3,4-dihydroxycinnamic acid (caffeic acid)	1	0.6-1.3	0.3	0.1-0.4
▪ 3-methoxy-4-hydroxycinnamic acid (ferulic acid)	0.8	0.6-1.0	0.5	0.2-0.7
▪ 3,4-dihydroxybenzoic acid (protocatechuic acid)	0.3	0.2-0.5		
▪ 3-hydroxycinnamic acid	0.3	0.2-0.4		
▪ Benzoic acid	0.1	0.1-0.2		
▪ 3-hydroxybenzoic acid	0.1	0.1-0.1		
▪ 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid)	0.1	0.0-0.2		
▪ 3,4-dihydroxyphenylacetic acid	0.1	0.0-0.2		
▪ N(2-OH-benzoyl)glycine (salicyluric acid)	0.1	0.0-0.1		
*Caffeic acid supplement***				
▪ 3-methoxy-4-hydroxycinnamic acid (ferulic acid)			12.7	10.7-14.8
▪ 3,4-dihydroxycinnamic acid (caffeic acid)			10.6	8.0-13.2
▪ 3-methoxy-4-hydroxybenzoic acid (vanillic acid)			3.9	1.9-5.9
▪ 3,4-dihydroxybenzoic acid (protocatechuic acid)			2.2	1.3-3.1
▪ 3-(3,4-dihydroxyphenyl)propionic acid			0.7	0.4-1.0
Quercetin-3-rutinoside supplement				
▪ 3-hydroxyphenylacetic acid	39.7	21.9-57.4	no metabolites were found	
▪ 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid)	8.5	4.0-13.1		
▪ 3,4-dihydroxyphenylacetic acid	5.5	3.4-7.6		
*Black tea supplement****				
▪ N-benzoylglycine (hippuric acid)	42.8	29.6-55.9		
▪ 3-hydroxyphenylacetic acid	1.1	0.8-1.4		
▪ 3,4,5-trihydroxybenzoic acid (gallic acid)	0.3	0.3-0.4		
▪ 3,4-dihydroxyphenylacetic acid	0.2	0.1-0.3		
▪ 3-hydroxybenzoic acid	0.1	0.1-0.2		
▪ N(2-OH-benzoyl)glycine (salicyluric acid)	0.1	0.1-0.2		
▪ 3-(3,4-dihydroxyphenyl)propionic acid	0.1	0.0-0.1		

* Based on the assumption that each molecule of chlorogenic acid yields 2 molecules of hippuric acid. For the other phenolic acids we assumed that each molecule of chlorogenic acid yields one molecule of the phenolic acid

** this supplement was not ingested by volunteers with a colon

*** this supplement was not ingested by volunteers without a colon

Table 4.4 Amount of conjugated phenolic acids as proportion of the total amount of phenolic acids. The total amount of phenolic acids includes the unconjugated phenolic acids and the phenolic acids conjugated with sulfates and/or glucuronic acid. Twenty volunteers with a colon ingested chlorogenic acid, quercetin-3-rutinoside, black tea solids and placebo on 7 days each, in random order.

Position on the aromatic ring		Phenyl-C_1	Phenyl-C_2	Phenyl-C_3
		(benzoic acids)	(phenylacetic acids)	(cinnamic acids)
-OH	-OCH_3	% conjugated phenolic acid		
2		*	100	*
3		100	13	100
4		74	13	100
4	3	79	48	72
4	3,5	100	*	*
2,4		52	*	*
3,4		100	74	73
3,5		45	*	100
3,4,5		25	*	*
Phenylhydroxyacetic acid (mandelic acid)			90	

* We did not measure these compounds in this study

DISCUSSION

We found that phenols from foods are extensively metabolized in the human body. Further, a large part of the ingested phenols will probably never enter the peripheral circulation as such, because of prior metabolism. Evidently the dietary phenols that reach the colon are degraded there into metabolites. These are then absorbed from the colon and after circulation and further metabolism in the liver and kidneys they are excreted into urine. It is also possible that part of the ingested phenols is absorbed in the jejuno-ileum and returned to the gut with the bile via the enterohepatic circulation. Thus the colon plays an important role in the metabolism of phenols that reach the colon. In subjects with a colon we recovered in urine about half of ingested chlorogenic acid and tea phenols as hippuric acid, and about half of the quercetin-3-rutinoside as phenylacetic acids. In contrast, in subjects without a colon we recovered only minor amounts of metabolites after they had ingested chlorogenic acid or quercetin-3-rutinoside. The fraction that we did not recover in urine was probably metabolized into compounds that we could not measure, or were excreted with feces. The difference between subjects with a colon and subjects without a colon can be

explained by the fact that chlorogenic acid as well as quercetin-3-rutinoside are not well absorbed in the small intestine and thus will become available for metabolism by the colonic microflora, which is absent in subjects without a colon. Indeed we found that 67% of the ingested chlorogenic acid was excreted unchanged with the stoma effluent and 84% of the ingested quercetin-3-rutinoside was excreted in the form of quercetin or its conjugates (Olthof et al. 2001a, Hollman et al. 1995). To our knowledge this is the first controlled trial that identified and quantified the main metabolites of 3 major dietary phenols in humans.

Metabolism of chlorogenic acid

The most pronounced difference in the metabolic profile of chlorogenic acid between volunteers with and without a colon was the absence of hippuric acid in volunteers without a colon (Table 4.3). After caffeic acid, again no hippuric acid was found in volunteers without a colon. Unfortunately we have no data on caffeic acid metabolism in volunteers with a colon.

We previously found in subjects without a colon that almost all caffeic acid and about one-third of ingested chlorogenic acid appeared to be absorbed from the small intestine (Olthof et al. 2001a). Our results therefore suggest that hippuric acid arises from the fraction of chlorogenic acid that reaches the colon. This would mean that the colonic microflora is required for the metabolism of chlorogenic acid and caffeic acid into hippuric acid. Dehydroxylation is a main metabolic reaction that is carried out by the colonic microflora (Scheline 1978b, Dayman & Jepson 1969). Therefore this might be a limiting step in the formation of hippuric acid from chlorogenic acid and caffeic acid in subjects lacking the colon.

In volunteers with a colon, two-thirds of the ingested chlorogenic acid reaches the colon, where the colonic microflora probably first hydrolyses chlorogenic acid into caffeic acid and quinic acid (Olthof et al. 2001a, Scheline 1978a, Plumb et al. 1999). Subsequently, the caffeic acid moiety is dehydroxylated by bacteria in the colon, and then to a large extent beta-oxidized into benzoic acid after absorption (Figure 4.2). The quinic acid moiety is dehydroxylated into cyclohexane carboxylic acid, and then aromatized into benzoic acid by the colonic microflora (Adamson et al. 1969 and 1970, Cotran et al. 1960, Indahl & Scheline 1973) or, after absorption in body tissues (Beer et al. 1951, Svardal & Scheline 1985). The benzoic acid formed is conjugated with glycine and excreted in urine as hippuric acid (Figure 4.2, Table 4.3).

In volunteers without a colon, one-third of the ingested chlorogenic acid and almost all caffeic acid is absorbed from the small intestine (Olthof et al. 2001a). After chlorogenic acid is hydrolyzed into caffeic acid and quinic acid, the caffeic acid moiety of chlorogenic acid is probably beta-oxidized into benzoic acids, without prior dehydroxylation (Scheline 1978a, Booth et al. 1957, Azuma et al. 2000). This is supported by the presence of small amounts of 3,4-dihydroxybenzoic acid and 3-methoxy-4-hydroxybenzoic acid as metabolites of caffeic acid in volunteers without a

colon (Table 4.3). Part of these benzoic acids might also be conjugated with glycine and excreted in urine as hydroxylated hippuric acids. Unfortunately, we could not measure the hydroxylated hippuric acid derivatives because we did not have their standards, except for 2-hydroxyhippuric acid that was not present (Table 4.2). The quinic acid moiety of chlorogenic acid is probably not aromatized into benzoic acid without prior dehydroxylation (Beer et al. 1951, Scheline 1978a).

Another explanation for the fact that we did not find hippuric acid as a metabolite of chlorogenic acid and caffeic acid in subjects without a colon might be that subjects without a colon received a single dose of the phenols while subjects with a colon received the phenols for 7 days. It is possible that a single dose was not enough to produce hippuric acid. However, others (Jacobson et al. 1983, Booth et al. 1957) did find metabolites of a single dose of chlorogenic acid and caffeic acid which makes this explanation less likely.

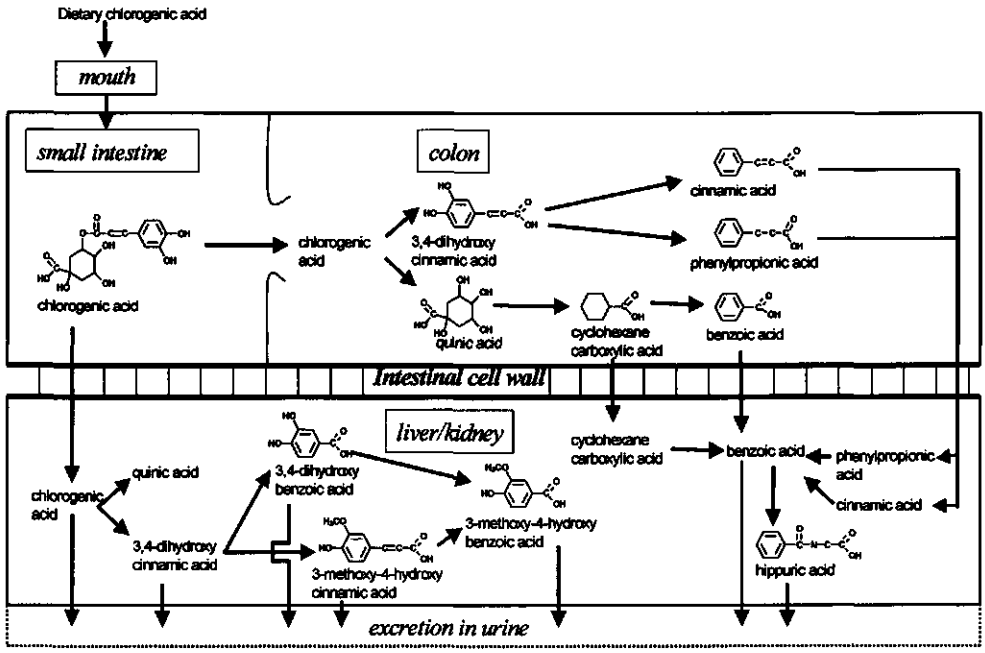

Figure 4.2 Proposed metabolic pathway of chlorogenic acid in humans (based on the results in this paper and (Hollman & Katan 1998))

Metabolism of tea phenols

The phenols in black tea were mainly metabolized into hippuric acid (Tables 4.1 and 4.3) which agrees with results of Clifford et al. (2000). Hippuric acid probably originates from catechins in tea, which are the major phenols in tea (Balentine et al. 1997). Part of the catechins are absorbed and excreted in urine as catechin conjugates and as 3'-methoxycatechin (Hollman et al. 1997b, Hackett et al. 1983 and 1985, Yang et al. 1998). Catechins that reach the colon will undergo cleavage of the

catechin ring into valerolactones by microorganisms in the colon (Figure 4.3). Valerolactones are then metabolized into phenylpropionic acids in the colon, which are further metabolized into benzoic acids and excreted in urine as hippuric acid (Das 1969a, 1969b and 1971, Groenewoud & Hundt 1986, Scheline 1970). We did not measure the valerolactones in urine but others did find them in urine (Hollman et al. 1997b, Li et al. 2000).

Gallic acid (3,4,5-trihydroxybenzoic acid) was present in urine only in the tea period. It probably originates from gallic acid present in tea, or from its esters with catechins (epigallocatechin gallate, epicatechin gallate) (Aucamp et al. 2000, Hodgson et al. 2000). We also found a small amount of 3-hydroxyphenylacetic acid as a metabolite in urine after intake of tea. This could be a metabolite of quercetin-3-rutinoside, a flavonoid which is present in small amounts in tea (Table 4.3) (Hertog et al. 1993c).

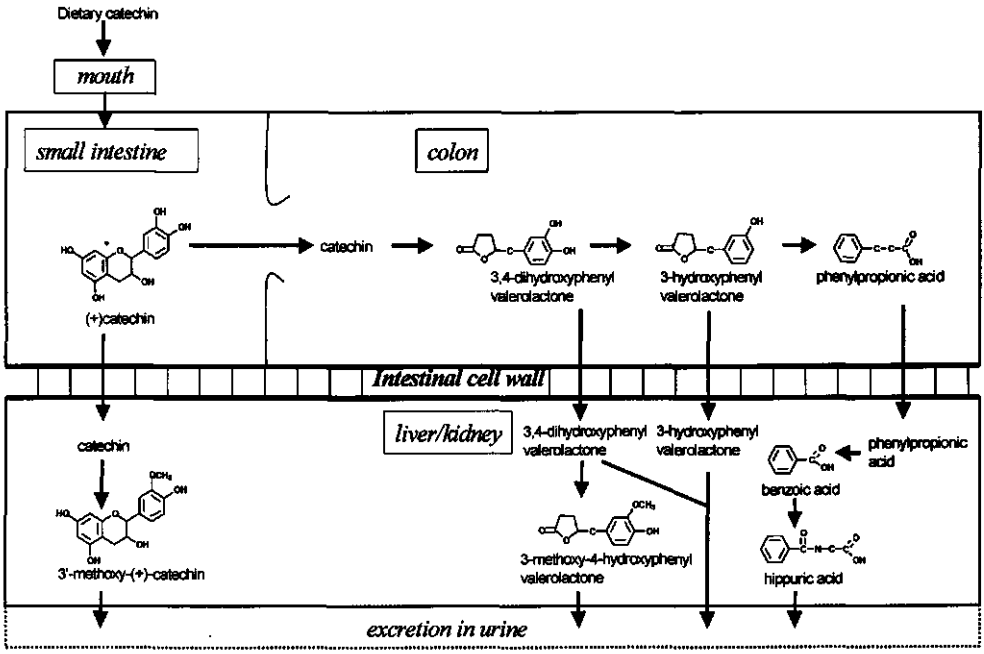

Figure 4.3 Proposed metabolic pathway of catechin in humans (based on the results in this paper and (Hollman & Katan 1998))

Metabolism of quercetin-3-rutinoside

Quercetin-3-rutinoside is not well absorbed in the small intestine of humans and about 83% will be transported into the colon and metabolized there (Hollman et al. 1995, Olthof et al. 2001a). Therefore it was not surprising that we did not find metabolites of quercetin-3-rutinoside in humans without a colon. In volunteers with a colon we recovered more than half of the ingested quercetin-3-rutinoside in humans as phenylacetic acids (phenyl-C_2 acids), which agrees with results of other studies (Baba et al. 1981 and 1983, Sawai et al. 1987, Booth et al. 1956). Our results indicate that

the quercetin-3-rutinoside is probably first deglycosylated to quercetin aglycone (Bokkenheuser et al. 1987) and then ring cleavage of the quercetin moiety into phenolic acids occurs by the colonic microflora (Figure 4.4). In contrast to humans, in rats the metabolites of quercetin-3-rutinoside also include phenylpropionic acid (phenyl-C_3 acids) in addition to phenylacetic acids (Baba et al. 1983, Nakagawa et al. 1965). This might be explained by differences in the colonic microflora of rats versus that of humans and thereby in the sites of ring cleavage. A microorganism which is present in human feces that produces phenylacetic acid from quercetin is *Eubacterium Ramulus* (Schneider & Blaut 2000, Schneider et al. 1999). Our data suggest that phenylacetic acids are subsequently absorbed from the colon and further metabolized in the liver and kidneys. The main metabolic reactions that occur after absorption is methylation in the liver, as indicated by the presence of 3-methoxy-4-hydroxyphenylacetic acid in urine (Tables 4.1 and 4.3) (Baba et al. 1983, Booth et al. 1956).

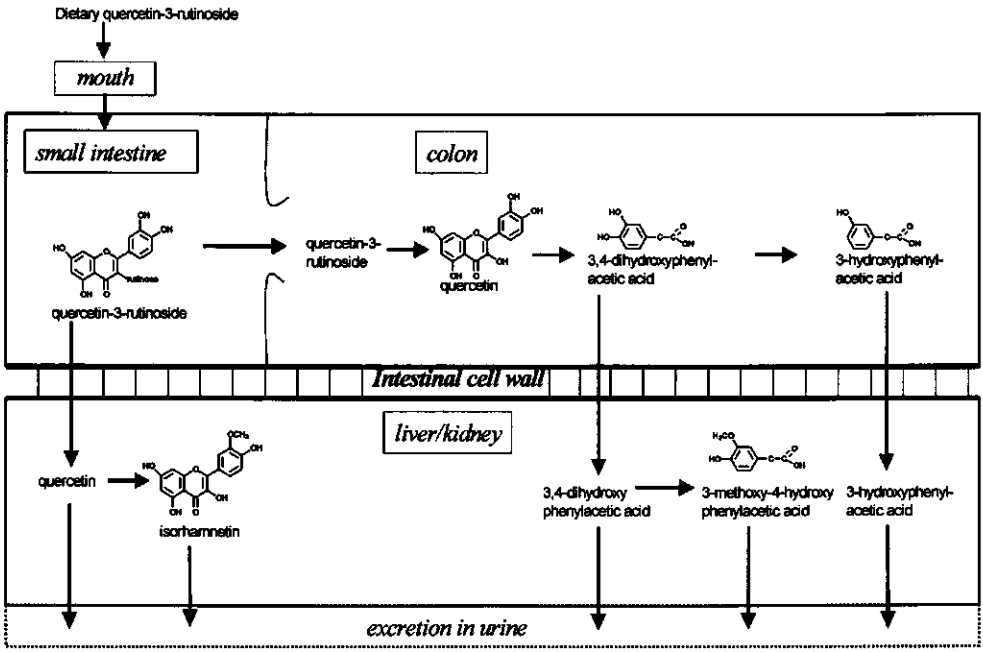

Figure 4.4 Proposed metabolic pathway of quercetin in humans (based on the results in this paper and (Hollman & Katan 1998))

Because the colonic microflora plays an important role in the metabolism of quercetin the metabolites of quercetin could differ between quercetin forms that are well absorbed in the small intestine and those that are less well absorbed. However, 50% of the well-absorbed quercetin forms will still reach the colon and is thus available for metabolism there (Hollman et al. 1995). Therefore, we expect that the amount of each metabolite excreted in urine might differ between various quercetin forms, but not the

type of metabolites. Further, more of the original quercetin reaches the circulation after ingestion of well-absorbed quercetin forms than after less-absorbed quercetin forms (Hollman et al. 1999b). However, data on the metabolites of well-absorbed quercetin forms, such as quercetin glucosides are as yet incomplete.

Antioxidant activity

Dietary phenols are strong antioxidants in vitro (Rice-Evans et al. 1996), but our data show that their antioxidant action in vivo is uncertain because they are extensively metabolized. In general, the metabolites of dietary phenols that we found have much lower antioxidant activity than their parent compounds, or have no antioxidant activity at all. Hippuric acid, the most important metabolite of chlorogenic acid and of tea phenols has no antioxidant activity, because it has lost its hydroxyl groups. Phenylacetic acids, the major metabolites of quercetin-3-rutinoside, have antioxidant activity in vitro that is similar to that of vitamin E, but lower than that of the parent compound quercetin (Rice-Evans et al. 1996, Pietta et al. 2000).

However, because we measured the metabolites of dietary phenols in urine it is possible that intact phenols or intermediary metabolites actually circulate in blood and that these might act as antioxidants in vivo. Unfortunately we did not measure metabolites in blood, because we did not have an analysis method available. However, we hypothesize that the metabolic reactions that lower antioxidant activity of the parent compound occur mainly before they can reach the circulation (Figures 4.2 - 4.4). The breakdown of flavonoids and phenolic acids into smaller molecules through ring cleavage and beta-oxidation in the colon and the liver drastically lowers their antioxidant activity (Rice-Evans et al. 1996, Natella et al. 1999). Subsequently, phenols and their metabolites are conjugated with glucuronic acid, sulphates or glycine, which also lowers their antioxidant activity (Table 4.4) (Morand et al. 1998, Manach et al. 1998).

Conclusion

Dietary phenols are extensively metabolized in humans into compounds with lower antioxidant activity, mainly before they enter the circulation. The antioxidant activity of dietary phenols in vivo might thus be lower than is expected based on their in vitro antioxidant activity.

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5

Consumption of high doses of chlorogenic acid, present in coffee, or of black tea increases plasma total homocysteine concentrations in humans

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Background: In population studies, high intakes of coffee are associated with raised concentrations of plasma homocysteine, a predictor of risk of cardiovascular disease. Chlorogenic acid is a major polyphenol in coffee; coffee drinkers consume up to 1 g chlorogenic acid/d.

Objective: We studied whether chlorogenic acid affects plasma total homocysteine concentrations in humans. For comparison we also studied the effects of black tea rich in polyphenols and of quercetin-3-rutinoside, a major flavonol in tea and apples.

Design: In this crossover study, 20 healthy men and women ingested daily 2 g (5.5 mmol) chlorogenic acid, 4 g black tea solids containing ~4.3 mmol polyphenols and comparable to ~2 L strong black tea, 440 mg (0.7 mmol) quercetin-3-rutinoside, or a placebo daily. Each subject received each of the 4 treatments for 7 d, in random order.

Results: Total homocysteine in plasma collected 4-5 h after supplement intake was 12% (1.2 μmol/L; 95% CI: 0.6, 1.7) higher after chlorogenic acid and 11% (1.1 μmol/L ;95% CI: 0.6, 1.5) higher after black tea than after placebo. Total homocysteine in fasting plasma collected 20 h after supplement intake was 4% (0.4 μmol/L; 95% CI: 0.0, 0.8) higher after chlorogenic acid and 5% (0.5 μmol/L; 95% CI: 0.0, 0.9) higher after black tea than after placebo. Quercetin-3-rutinoside did not significantly affect homocysteine concentrations.

Conclusions: Chlorogenic acid, a compound in coffee, and black tea raise total homocysteine concentrations in plasma. Chlorogenic acid could be partly responsible for the higher homocysteine concentrations observed in coffee drinkers. Whether these effects on homocysteine influence cardiovascular disease risk remains to be established.

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INTRODUCTION

A high homocysteine concentration in blood is a risk factor for cardiovascular disease (Nygard et al. 1995, Refsum et al. 1998). Epidemiologic studies suggest that coffee consumption might be one of the determinants of plasma homocysteine concentrations; plasma homocysteine concentrations in coffee drinkers are up to 2 μmol/L higher than those in coffee abstainers (Nygard et al. 1997, Oshaug et al. 1998, Stolzenberg-Solomon et al. 1999). The results of both an intervention study with unfiltered coffee (Grubben et al. 2000) and an intervention study with filtered coffee (Urgert et al. 2000) support the homocysteine-raising effects of coffee.

The compounds in coffee responsible for this effect are not known. The kahweol and cafestol present in only unfiltered coffee (Urgert et al. 1995) are not responsible because both filtered coffee and unfiltered coffee raise plasma homocysteine concentrations (Grubben et al. 2000, Urgert et al. 2000). One candidate compound is chlorogenic acid, a polyphenol that occurs in large amounts in coffee but in only small amounts in other foods and beverages. Another candidate is caffeine. The amounts of chlorogenic acid and caffeine in coffee are comparable (Clifford 1999, Viani 1988). No studies have been done to investigate the effect of chlorogenic acid or caffeine on plasma homocysteine.

We thus studied the effect of chlorogenic acid on plasma homocysteine concentrations in healthy volunteers. An effect of chlorogenic acid on homocysteine might be mediated by metabolites of chlorogenic acid in the human body. Because metabolism of chlorogenic acid and other polyphenols is likely to occur via the same pathways, we also determined the effect on plasma homocysteine concentrations of polyphenols from black tea and of quercetin-3-rutinoside. Black tea is the tea most commonly consumed worldwide (Balentine et al. 1997) and quercetin-3-rutinoside is a major flavonol in tea and apples (Engelhardt et al. 1992, Herrmann 1976).

SUBJECTS AND METHODS

Subjects

Ten men and 10 women with a mean (±SD) age of 24 ± 8 y and body mass index (in kg/m^2) of 22.2 ± 2.5 participated. The subjects were healthy as judged by a medical questionnaire; normal blood values for hemoglobin, hematocrit and white blood cell counts; and absence of protein and glucose in urine. All subjects were nonsmokers. They were not allowed to take any drugs or other supplements during the study except for acetaminophen (paracetamol) and oral contraceptives. The study protocol was fully explained to the subjects and they gave their written, informed consent. The protocol was approved by the Medical Ethical Committee of the Division of Human Nutrition and Epidemiology.

Methods

Throughout the 4-weeks study, subjects consumed a controlled diet low in polyphenols. To achieve this, we supplied the subjects daily with foods low in polyphenols that provided 90% of the energy required to maintain body weight. The remaining 10% of energy was chosen by the subjects from a list of food items low in polyphenols. Foods were considered low in polyphenols if they contained <15 mg quercetin or chlorogenic acid/kg; beverages were considered low in polyphenols if they contained <4 mg quercetin or chlorogenic acid/L (Clifford 1999, Hertog et al. 1992 and 1993a). Because consumption of coffee and tea was not allowed, we provided the volunteers with the following substitutes: for coffee, an extract made of chicory, rye and barley (Swiss coffee-like; Tayala AG, Birsfelden, Switzerland), and for tea, tea bags containing a mix of herbs (droommix; Piramide, Veenendaal, Netherlands) or tea bags containing stinging nettle (Jacob Hooy, Limmen, Netherlands). Chemical analyses indicated that these substitutes contained only minor amounts of catechins, flavonols or chlorogenic acid. In addition to the controlled diet subjects ingested one of the following supplements each day: *1)* 2 g (5.5 mmol) chlorogenic acid (Fluka Chemie AG, Buchs, Switzerland), *2)* 4 g black tea solids (LN0173-02; kindly provided by Unilever Research Vlaardingen, Vlaardingen, Netherlands) with 30-40% polyphenols by weight (4 g black tea solids contains ~4.3 mmol polyphenols, mainly catechins) (Balentine et al. 1997), *3)* 440 mg (0.7 mmol) quercetin-3-rutinoside (Rutosidum DAB, BUFA BV, Uitgeest, Netherlands), or *4)* 0.5 g citric acid as a placebo (AC Citricum; Fagron, Nieuwerkerk a/d IJssel, Netherlands) (Table 5.1). The 2 g chlorogenic acid is comparable with the amount of chlorogenic acid in ~1.5 L strong coffee, the 4 g black tea solids is comparable with ~2 L strong black tea, and the amount of quercetin in 440 mg quercetin-3-rutinoside is comparable with the amount in ~13 L black tea (Hertog et al. 1993a) and is 13 times higher than the average daily intake of quercetin (Hertog et al. 1993b). Subjects ingested each of these 4 supplements for 1 week in random order. Before ingestion, the chlorogenic acid, quercetin-3-rutinoside, citric acid and one-half of the black tea solids (2 g) were dissolved in hot water. Subjects consumed the supplements under our supervision just before the hot meal at noon. The other one-half of the black tea solids was used for tea preparation and consumption at home: 1 g between 0800 and 1000 (on Saturdays and Sundays, between 0800 and 1100) and 1 g between 1800 and 2000. The volunteers were urged to maintain their usual pattern of physical activity during the study.

On day 7 of each supplement period we collected 2 blood samples: 1 sample in the morning after subjects had fasted overnight, collected ~20 h after the last supplement intake, and 1 postprandial sample, collected 4-5 h after the last supplement intake. We chose to take a fasting blood sample to exclude interference from food consumption shortly before blood sampling. We chose to collect the postprandial

blood sample 4-5 h after supplement intake because the peak concentration of homocysteine after methionine loading, a precursor of homocysteine, occurs at this time point (Andersson et al. 1990, Ueland et al. 1993a). Because the black tea supplement was ingested 3 times daily, the blood sampling times after intake of the black tea supplement were as follows: the fasting sample in the morning was collected ~12 h after the intake of the 1-g dose of black tea solids and ~20 h after the intake of the 2-g dose on the previous day and the postprandial blood sample was collected 4-5 h after intake of 2-g dose of black tea solids at 1200. Blood was collected into vacuum tubes (Venoject II; Terumo Europe NV, Leuven, Belgium) containing EDTA. Blood samples were immediately placed on ice and within 1 h were centrifuged at 2500 x *g* for 10 min at 4°C to obtain plasma. Plasma was separated and stored at -80°C. We also collected urine for the measurement of polyphenolic metabolites; these results will be published elsewhere.

Total homocysteine concentrations were measured by HPLC with fluorometric detection (Ubbink et al. 1991, Ueland et al. 1993b). The interassay CV of the homocysteine assay was <8%. Folate and vitamin B-12 concentrations were measured with ion-capture IMx (Abbott Laboratories, Abott Park, IL) (Wilson et al. 1995, Kuemmerle et al. 1992). The interassay CV of the folate assay was <12% and that of the vitamin B-12 assay was <7%. For the measurement of vitamin B-6 in the chlorogenic acid and the placebo periods, we also collected fasting blood samples into vacuum tubes containing lithium-heparin and immediately stored the tubes at -80°C until analysis. The vitamin B-6 concentration was measured as pyridoxal-5'-phosphate (PLP) in EDTA-treated whole blood by HPLC (Schrijver et al. 1981) after precolumn derivatization with semicarbazide to obtain PLP-semicarbazone (Ubbink et al. 1985). The interassay CV of the vitamin B-6 assay was <7%.

We measured all postprandial blood samples in the same series of analyses. The fasting plasma samples for measurement of total homocysteine, vitamin B-12, and folate concentrations were analyzed in 2 separate series of analyses on separate occasions: in the first series we measured the fasting plasma samples obtained after placebo and chlorogenic acid periods and in the second series we measured the fasting plasma samples obtained after the placebo, black tea, and quercetin-3-rutinoside periods. Thus, a subject's plasma or blood samples obtained after treatment with chlorogenic acid, black tea, or quercetin-3-rutinoside were always analyzed together with the plasma or blood sample of that same volunteer obtained after the placebo period. Therefore, differences between each supplement and the placebo were not affected by analytic variation between series.

Statistical analyses

For each subject, we calculated the differences between values for each of the 3 supplement periods and values for the placebo period. Statistical significance and 95% CIs of the mean differences were calculated by using Student's *t* test. Results were analyzed with use of SAS (version 6.12; SAS Institute, Inc, Cary, NC).

RESULTS

Ingestion of chlorogenic acid raised total homocysteine concentrations by 12% (1.2 μmol/L) in postprandial plasma and by 4% (0.4 μmol/L) in fasting plasma relative to placebo (Table 5.1, Figure 5.1). The rise of total homocysteine in postprandial plasma was 0.8 μmol/L (95% CI: 0.4, 1.2) higher than the rise in fasting plasma. Chlorogenic acid lowered the concentration of folate in fasting plasma by 8% (1.3 nmol/L; 95% CI: 0.6, 2.1) relative to placebo (Table 5.2). Concentrations of vitamins B-6 and B-12 were not significantly affected by chlorogenic acid.

Ingestion of black tea raised total homocysteine concentrations by 11% (1.1 μmol/L) in postprandial plasma and by 5% (0.5 μmol/L) in fasting plasma relative to placebo (Table 5.1, Figure 5.2). The rise in postprandial plasma was 0.6 μmol/L (95% CI: -0.0, 1.2) higher than the rise in fasting plasma. Concentrations of B vitamins were not significantly affected by black tea (Table 5.2).

Ingestion of quercetin-3-rutinoside did not significantly affect total homocysteine concentrations in postprandial and fasting plasma (Table 5.1, Figure 5.3), but lowered the concentration of folate by 11% (2.5 nmol/L, 95% CI: 0.1, 5.0) in fasting plasma (Table 5.2). Concentrations of vitamins B-6 and B-12 were not significantly affected by quercetin-3-rutinoside. It is possible that we did not find an effect of quercetin-3-rutinoside on plasma homocysteine because the dose of quercetin-3-rutinoside used in this study was only 12-16% of the dose of chlorogenic acid and of black tea polyphenols. However, this dose of quercetin-3-rutinoside is ~13 times higher than the average intake of quercetin in the population (Hertog et al. 1993b).

Table 5.1 Change in plasma total homocysteine concentrations compared with placebo in postprandial and fasting plasma samples collected from 20 healthy volunteers after ingestion of chlorogenic acid, black tea, or quercetin-3-rutinoside for 7 d each[1].

Supplement	Intake of polyphenols	Change in total homocysteine concentration compared with placebo[2]	
		Postprandial plasma	Fasting plasma
	mmol/d	μmol/L	
Chlorogenic acid	5.5	1.2 (0.6, 1.7)	0.4 (0.0, 0.8)
Black tea	4.3	1.1 (0.6, 1.5)	0.5 (0.0, 0.9)
Quercetin-3-rutinoside	0.7	0.3 (-0.2, 0.7)	-0.0 (-0.4, 0.3)

[1] Mean (±SD) homocysteine concentrations after each supplement are shown in Figures 5.1-5.3. Postprandial blood samples were collected 4-5 h after the last supplement intake and fasting blood samples were collected ~20 h after the last supplement intake.

[2] Mean (95% CI).

Table 5.2 Concentrations of folate and vitamin B-12 in postprandial and fasting plasma and concentrations of vitamin B-6 in fasting whole blood collected from 20 healthy volunteers after ingestion of placebo, chlorogenic acid, black tea, or quercetin-3-rutinoside for 7 d each[1]

Supplement	Folate		Vitamin B-12		Vitamin B-6:
	Fasting plasma	Postprandial plasma	Fasting plasma	Postprandial plasma	Fasting whole blood
	nmol/L		pmol/L		nmol/L
Placebo	22.6 ± 8.8	19.2 ± 5.0	235 ± 65	229 ± 66	81 ± 17
Chlorogenic acid	15.3 ± 4.8[2,3]	19.6 ± 5.8	247 ± 67[2]	224 ± 57	81 ± 14
Black tea	21.4 ± 6.9	19.5 ± 5.3	238 ± 65	239 ± 69	-[4]
Quercetin-3-rutinoside	20.0 ± 5.6[3]	19.3 ± 5.8	245 ± 82	233 ± 61	-[4]

[1] Mean ± SD. Postprandial blood samples were collected 4-5 h after the last supplement intake and fasting blood samples were collected ~20 h after the last supplement intake.

[2] Folate and vitamin B-12 after chlorogenic acid were measured in another series of analyses. The concentration of folate in plasma after placebo in this series of analyses was 16.7 ± 5.1 nmol/L and that of vitamin B-12 was 242 ± 67 pmol/L

[3] Significantly different from placebo, $P<0.05$.

[4] Not measured.

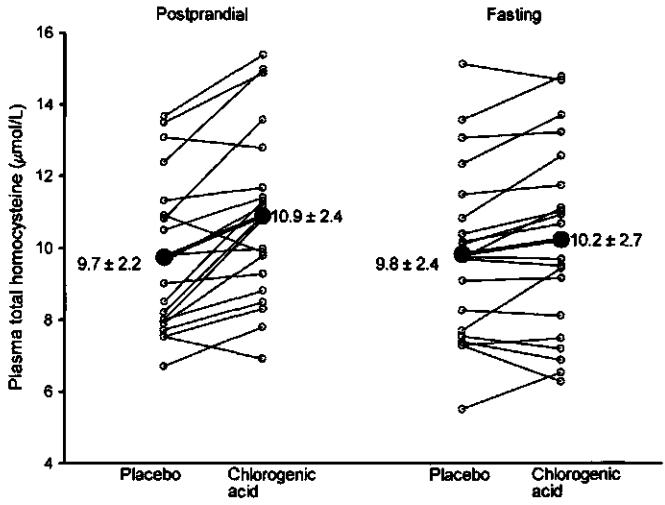

Figure 5.1 Plasma total homocysteine in 20 healthy volunteers after they had consumed 2 g (5.5 mmol) chlorogenic acid or a placebo daily, for 7 d each, in random order. Postprandial blood samples were collected 4-5 h after the last supplement intake and fasting samples were collected ~20 h after the last supplement intake. Shown are the individual (o) and mean ± SD (●) changes.

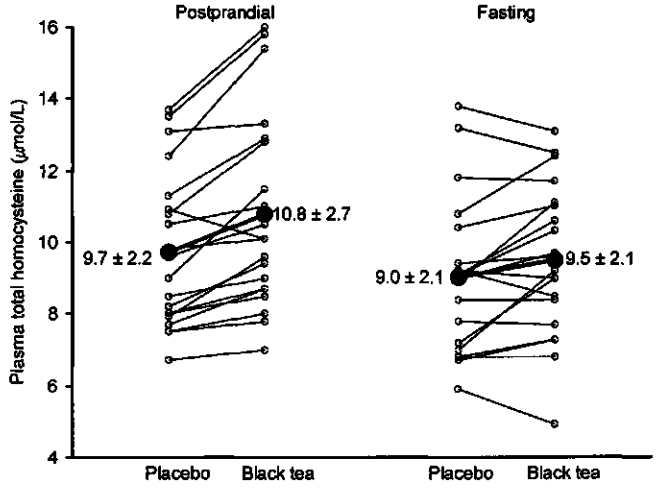

Figure 5.2 Plasma total homocysteine in 20 healthy volunteers after they had consumed 4 g black tea solids (containing ~4.3 mmol polyphenols) or a placebo daily, for 7 d each, in random order. Postprandial blood samples were collected 4-5 h after the last supplement intake and fasting samples were collected ~20 h after the last supplement intake. Shown are the individual (o) and mean ± SD (●) changes.

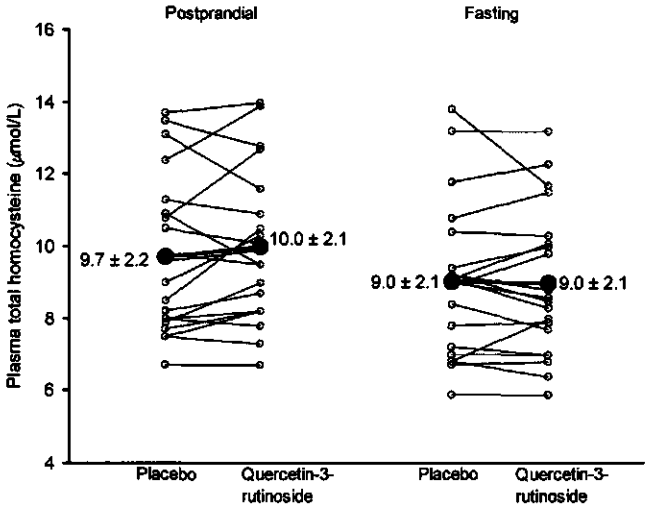

Figure 5.3 Plasma total homocysteine in 20 healthy volunteers after they had consumed 440 mg (0.7 mmol) quercetin-3-rutinoside or a placebo daily, for 7 d each, in random order. Postprandial blood samples were collected 4-5 h after the last supplement intake and fasting samples were collected ~20 h after the last supplement intake. Shown are the individual (o) and mean ± SD (●) changes.

DISCUSSION

We found that consumption of 2 g chlorogenic acid/d by healthy humans raises homocysteine concentrations in postprandial plasma by 12% and in fasting plasma by 4%. Furthermore, we found that consumption of 4 g black tea solids/d also raises homocysteine concentrations in postprandial plasma by 11% and in fasting plasma by 5%. The dose of 2 g chlorogenic acid used in this study is comparable with ~1.5 L strong coffee; the dose of 4 g black tea solids is comparable with 2 L strong black tea. This implies that both black tea and coffee increase plasma homocysteine to the same extent. Thus chlorogenic acid in coffee is at least partly responsible for the higher plasma homocysteine concentrations observed in coffee drinkers (Nygard et al. 1997, Oshaug et al. 1998, Stolzenberg-Solomon et al. 1999, Grubben et al. 2000, Urgert et al. 2000).

Chlorogenic acid and plasma total homocysteine

The rise in homocysteine in postprandial plasma of 12% after daily consumption of 2 g chlorogenic acid in this study was lower than the rise of 10-20% in 2 intervention studies after daily consumption of 1 L coffee, which contains ~1 g chlorogenic acid (Grubben et al. 2000, Urgert et al. 2000). The rise in plasma homocysteine concentrations in the present study was also lower than that found in epidemiological studies (Nygard et al. 1997, Oshaug et al. 1998, Stolzenberg-Solomon et al. 1999).

The fact that the rise in plasma homocysteine was lower in our study than in the studies with coffee might indicate that chlorogenic acid is not the only homocysteine-raising factor in coffee.

Beforehand, we did not know how long we had to give the supplements to induce an effect on plasma homocysteine. We expected that a supplementation period of 7 d would be long enough to stabilize plasma homocysteine because the half-life of elimination of plasma homocysteine after a methionine load is ~12 h (Guttormsen et al. 1993). In the present study we indeed showed that a supplementation period of 7 d is long enough to induce an effect on plasma homocysteine. However, we do not know the magnitude of the effect of chlorogenic acid on plasma homocysteine when chlorogenic acid is supplied for >7 d. Furthermore, we found that the effect of chlorogenic acid on homocysteine seems to subside within hours because the rise in homocysteine concentration in postprandial plasma samples, collected ~5 h after ingestion of chlorogenic acid, was significantly higher than that in fasting plasma samples, collected ~20 h after ingestion. We do not know whether the rise in homocysteine might have been even higher at other time points after intake because we collected blood only twice after ingestion of each supplement. We also saw a larger rise of homocysteine in postprandial plasma than in fasting plasma after the intake of black tea, although the fasting blood sample was collected ~12 h after the intake of the last dose (1 g) of the black tea solids. It is possible that the rise in homocysteine was higher in the postprandial blood samples than in the fasting blood samples because the effect of chlorogenic acid and black tea on homocysteine metabolism is fast and short term, like the effect of methionine on plasma homocysteine (Andersson et al. 1990). Other studies are necessary to establish the kinetics of the effect of chlorogenic acid and black tea on plasma homocysteine. We conclude that changes in homocysteine induced by chlorogenic acid or black tea apparently occur within hours rather than days and that the supplementation period of 7 d was adequate.

Black tea and plasma total homocysteine

We found that intake of 4 g black tea solids/d, containing 4.3 mmol polyphenols, raises homocysteine in postprandial plasma by 11% and in fasting plasma by 5%. The magnitude of these rises is similar to that observed after intake of 5.5 mmol chlorogenic acid. However, the dose of black tea used is higher than the mean daily consumption of black tea in the general population (Hulshof et al. 1998) and we do not know the effects of lower doses on plasma homocysteine.

We studied black tea because it is the tea most commonly consumed worldwide and because the only epidemiological study that investigated the association between black tea consumption and homocysteine concentration indicated that black tea might affect plasma homocysteine (Nygard et al. 1997).

In future studies of the effect of tea polyphenols on plasma homocysteine, it might be interesting to study the effect of green tea. Whereas black tea contains mainly polymerized catechins, the major polyphenols in green tea are monomeric catechins (Balentine et al. 1997).

Quercetin-3-rutinoside and plasma total homocysteine

In contrast with chlorogenic acid and black tea, quercetin-3-rutinoside did not significantly affect plasma homocysteine concentrations. This difference might be explained by the relatively low dose of quercetin-3-rutinoside used, which was only 12-16% of the dose of chlorogenic acid and of polyphenols from black tea. Nevertheless, the dose of 0.7 mmol quercetin-3-rutinoside is ~13 times higher than the average quercetin intake in the general population, which is ~0.05 mmol (Hertog et al. 1993b). Therefore, we believe that quercetin intake in the general population does not have a substantial effect on homocysteine concentrations, although we can not exclude the possibility that doses of quercetin higher than used in our study might raise homocysteine. Quercetin-3-rutinoside lowered plasma folate concentrations by 11%. We do not have an explanation for this finding.

Mechanism of the effect of polyphenols on plasma total homocysteine

The mechanisms by which chlorogenic acid and black tea raise plasma homocysteine are not clear. First, changes in plasma homocysteine might be mediated by vitamin B-6, vitamin B-12, and folate, which are involved in the homocysteine pathway. We found that of these vitamins only folate was affected by the supplements. A decrease in plasma folate can lead to an increase in plasma homocysteine as a result of a decrease in remethylation of homocysteine into methionine (Figure 5.4) (Selhub et al. 1993). A decrease in plasma folate, which could explain an increase in plasma homocysteine, was found in fasting plasma only after supplementation with chlorogenic acid and not black tea. In contrast, plasma folate also decreased in fasting plasma after quercetin-3-rutinoside, without a concomitant rise in plasma homocysteine. Furthermore, plasma folate concentrations in the postprandial blood samples were also not significantly affected by the supplements, whereas the largest increase in plasma homocysteine was found in postprandial plasma. Thus, a direct role for folate in the homocysteine-raising effect of chlorogenic acid and black tea polyphenols is unlikely.

We speculate that *O*-methylation reactions that occur in the metabolism of polyphenols are involved in the homocysteine-raising effect of polyphenols. Such methylation reactions transfer a methyl group from *S*-adenosylmethionine to polyphenols and thereby produce homocysteine (Figure 5.4) (Finkelstein 1990). Thus, consumption of a high dose of polyphenols might increase homocysteine production through increased methylation reactions (Zhu et al. 1994). This notion is supported by data from studies on L-dopa (L-3,4-dihydroxyphenylalanine). Like polyphenols, L-dopa

is O-methylated. Indeed, both rats fed L-dopa and Parkinson disease patients treated with L-dopa have higher homocysteine concentrations than do control subjects (Daly et al. 1997, Allain et al. 1995, Kuhn et al. 1998a and 1998b, Muller et al. 1999). Thus, an increase in the O-methylation reactions in the body could result in higher plasma homocysteine concentrations, which might explain why high intakes of polyphenols raise plasma homocysteine.

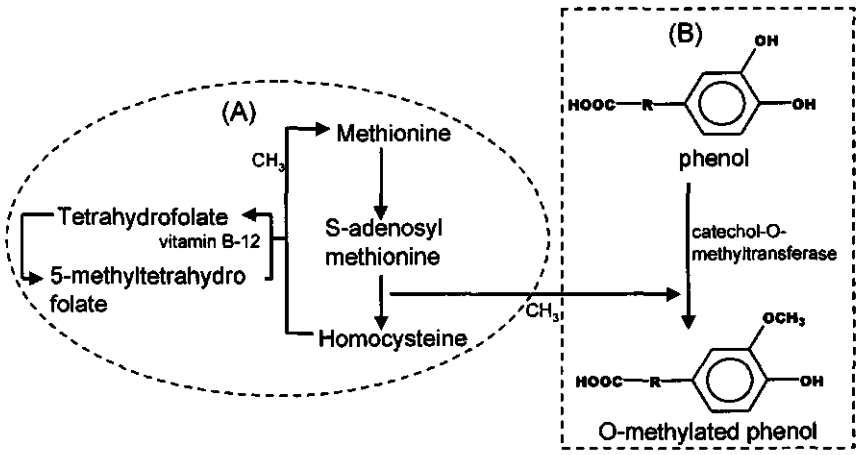

Figure 5.4 Proposed interaction between the hepatic metabolism of homocysteine (A) and the O-methylation of polyphenols (B).

Conclusion

High intakes of chlorogenic acid, which is present in coffee, and of black tea raise plasma homocysteine concentrations. Thus, chlorogenic acid in coffee might be at least partly responsible for the higher plasma homocysteine concentrations of coffee drinkers. A high plasma homocysteine concentration is a predictor of risk of cardiovascular disease (Nygard et al. 1995, Refsum et al. 1998, Ueland et al. 1993a). However, it is still unclear whether a high homocysteine concentration is causally related to cardiovascular disease or is merely an indicator of another process that causes cardiovascular disease.

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6

Effect of chlorogenic acid and its metabolites on plasma total homocysteine concentrations in healthy volunteers

Margreet R. Olthof, Peter C.H. Hollman, Martijn B. Katan

Chlorogenic acid (5-caffeoylquinic acid) is partly responsible for the homocysteine-raising effect of coffee. However the mechanism underlying this effect is unknown. The major metabolite of chlorogenic acid in urine is hippuric acid (N-benzoylglycine). We tested whether exhaustion of endogenous glycine through conversion of chlorogenic acid into hippuric acid increased plasma homocysteine. To that end we fed 11 volunteers chlorogenic acid and benzoic acid, which use glycine for formation of hippuric acid, hippuric acid itself and a placebo. We followed plasma homocysteine concentrations, and excretion of hippuric acid and the supplements in urine. We found a slight increase of 6% in plasma homocysteine in the first 4 hours after ingestion of chlorogenic acid relative to placebo. Benzoic acid and hippuric acid had no effect. After ingestion of chlorogenic acid, benzoic acid and hippuric acid we recovered 72-76 mol% of the dose as hippuric acid in urine. Most of the hippuric acid was rapidly excreted after ingestion of benzoic acid and of hippuric acid, whereas it was excreted between 10-24h after ingestion of chlorogenic acid. Thus glycination of metabolites of phenols does not explain the homocysteine-raising effect of chlorogenic acid, and hippuric acid itself does not either. The mechanism behind the homocysteine-raising effects of chlorogenic acid remains unknown.

In preparation

INTRODUCTION

A high homocysteine concentration in blood is a risk factor for cardiovascular disease (Nygard et al. 1995, Refsum et al. 1998). Coffee consumption is one of the determinants of plasma homocysteine concentration; coffee drinkers have up to 20% (2 μmol/L) higher plasma homocysteine concentration than coffee abstainers (Nygard et al. 1997, Oshaug et al. 1998, Stolzenberg-Solomon et al. 1999, Grubben et al. 2000, Urgert et al. 2000).

Chlorogenic acid (5-caffeoylquinic acid) is a major phenol in coffee (Clifford 1999) and we found in a previous study that 2 g chlorogenic acid (present in ~1.5 L of coffee) raises plasma homocysteine concentrations up to 12%. Black tea solids, which largely consists of phenols, had a similar effect (Olthof et al. 2001b).

The mechanism by which chlorogenic acid and tea solids raise homocysteine concentrations is unknown. B-vitamins probably do not play a role (Olthof et al. 2001b). We speculate that use of glycine for the formation of hippuric acid (N-benzoylglycine) is involved because chlorogenic acid as well as phenols from black tea are metabolized mainly into hippuric acid (Olthof et al. 2001c, Booth et al. 1957, Clifford et al. 2000). This speculation is supported by our finding that quercetin-3-rutinoside was not metabolized into hippuric acid and did not affect plasma homocysteine either. Glycine is involved in several pathways in homocysteine metabolism (Figure 6.1). Glycine is a precursor for serine and both compounds are needed for catabolism of homocysteine into glutathione. Serine is a methyl donor in the folate metabolism. Further, glycine accepts methyl groups from S-adenosylmethionine (SAM) in case an excess of SAM occurs (Finkelstein 1990, Selhub 1999, Wagner 1995). However, we do not know what the effect is on plasma homocysteine of a shortage of endogenous glycine.

We hypothesized that conversion of chlorogenic acid into hippuric acid limits the availability of endogenous glycine which results in an increase in plasma homocysteine. The availability of glycine is indeed a limiting factor in the conjugation of benzoic acid with glycine into hippuric acid (Kubota & Ishizaki 1991, Jackson et al. 1987, Amsel & Levy 1969, Gregus et al. 1992 and 1993, Beliveau & Brusilow 1987).

To test our hypothesis we compared the plasma homocysteine concentrations after ingestion of chlorogenic acid and benzoic acid with that after ingestion of hippuric acid and a placebo in healthy volunteers. Chlorogenic acid and benzoic acid will both use glycine for metabolism into hippuric acid, while hippuric acid will not use glycine (Booth et al. 1957, Quick 1931). This enables us to study the effect of the use of glycine for formation of hippuric acid on plasma homocysteine concentrations. We also studied the kinetics of excretion of several phenolic acid metabolites in urine after intake of the supplements. This provides further insight into the sites where

metabolism takes place. In addition it might give clues to the mechanism behind the homocysteine-raising effect of chlorogenic acid.

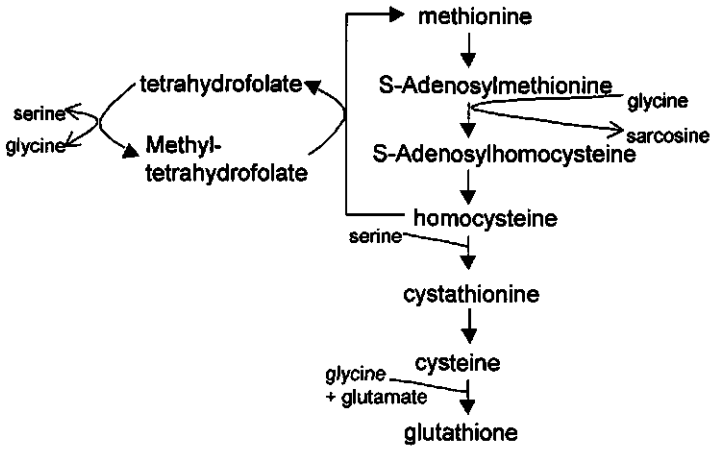

Figure 6.1 Homocysteine metabolism (Finkelstein 1990, Selhub 1999, Wagner 1995)

SUBJECTS AND METHODS

Subjects

Five men and six women with a mean (±SD) age of 29 ± 14 y and a mean body mass index of 22 ± 2 kg/m^2 participated. They were healthy as judged by a medical questionnaire; normal blood values for plasma homocysteine concentrations, liver enzymes, hemoglobin, hematocrit and white blood cell counts; and absence of protein and glucose in urine. All subjects were nonsmokers. They were not allowed to take any drugs or other supplements during the study except for acetaminophen (paracetamol) that was provided by us, and oral contraceptives. The study protocol was fully explained to the subjects and they gave their written informed consent.

Methods

The protocol was approved by the Medical Ethical Committee (Wageningen University). Throughout the 4-weeks randomized cross-over study subjects consumed a controlled diet low in phenols on 2 d per week. To achieve this, we supplied the subjects on these 2 d with foods low in phenols, which provided 100% of the energy required to maintain body weight and subjects were not allowed to eat foods which were not provided by us. Foods were considered low in phenols if they contained <15 mg quercetin or chlorogenic acid/kg, and beverages if they contained <4 mg quercetin or chlorogenic acid/L (Clifford 1999, Hertog et al. 1992 and 1993). Because coffee and tea consumption were not allowed we provided the volunteers with the following substitutes: for coffee, an extract made of chicory, rye and barley (Swiss coffee-like;

Tayala AG, Birsfelden, Switzerland) and for tea, tea bags containing a mix of herbs (droommix; Piramide, Veenendaal, Netherlands) or tea bags containing stinging nettle (Jacob Hooy, Limmen, Netherlands). Chemical analyses indicated that these substitutes contained only minor amounts of catechins, flavonols or chlorogenic acid.
In addition to the controlled diet subjects daily ingested one of 4 supplements on the 2 days per week on which the diet was provided by us. The following supplements were provided by us in random order: either 2.0 g (5.5 mmol) chlorogenic acid (Fluka Chemie AG, Buchs, Switzerland), 1.4 g (8.0 mmol) hippuric acid (Fluka Chemie AG) and 1.2 g (8.0 mmol) sodium benzoate (kindly provided by Flevochemie, Harderwijk, Netherlands) or 0.1 g citric acid as a placebo (AC Citricum; Fagron, Nieuwerkerk a/d IJssel, Netherlands). The 2 g chlorogenic acid is comparable to the amount of chlorogenic acid in ~1.5 L of strong coffee. Before ingestion the supplements were dissolved in hot water. Subjects took the supplements under our supervision, just before breakfast in the morning. The volunteers were urged to maintain their usual pattern of physical activity during the study.
On the second day of each supplement period we collected 8 blood samples: one fasting sample in the morning, and at 1, 2, 4, 6, 8, 10, 24 h after ingestion of the supplement. Just after the blood sample at 4 h, the hot meal was served.
Blood was collected into vacuum tubes (Venoject II; Terumo Europe NV, Leuven, Belgium) containing EDTA. Blood samples were immediately placed on ice, and within 1 hour centrifuged at 2500 x *g* for 10 minutes at 4°C to obtain plasma. Plasma was separated and stored at -80°C. Total homocysteine concentrations were measured by HPLC and fluorometric detection (Ubbink et al. 1991, Ueland et al. 1993). The interassay CV of the homocysteine assay was <8%. Plasma or blood samples a volunteer after ingestion of chlorogenic acid, benzoic acid, hippuric acid and placebo were always analyzed at the same time. Thus differences between each supplement and placebo were not affected by interassay variation.

Urine

On the second day of each supplement period subjects collected urine for 24 h. They started collection just before supplement intake and subsequently collected urine every 2 h after supplement intake, until 10 h after supplement intake. From 10-24 h after intake they collected urine only when needed. Subjects collected urine in 0.5 L plastic bottles with 0.13 g thymol (# 8167; Merck, Amsterdam, Netherlands) as a preservative and stored the bottles with urine on dry ice immediately after voiding. We checked the completeness of urine collection by assessment of recovery of 238 μmol lithium in urine. Lithium was measured in undiluted, acidified urine by atomic absorption spectrophotometry (Anonymous 1976). Subjects ingested lithium chloride, dissolved in 10 ml of tap water, daily during the study from 7 days before the first urine collection. Lithium chloride is completely absorbed and 95% is excreted in urine

(Sanchez-Castillo et al. 1987a and 1987b). Urinary recovery of lithium was 111 ± 14% (mean ± SD), which indicated good compliance in collecting urine.

Analyses of hippuric acid, chlorogenic acid and benzoic acid in urine

We measured hippuric acid, chlorogenic acid and benzoic acid in each urine sample collected every 2 h after ingestion of the supplement of each volunteer, and in a urine sample collected between 10–24 h after ingestion (Figures 6.3-6.5). We measured hippuric acid and benzoic acid in urine after deconjugation with β-glucuronidase/arylsulfatase. Chlorogenic acid was measured in urine without prior deconjungation with β-glucuronidase/arylsulfatase. We measured chlorogenic acid only in urine samples collected after ingestion of the chlorogenic acid supplement and after placebo supplement. Urine was acidified and brought onto an Solid Phase Extraction (SPE) column. The column was eluted with ethyl acetate. Ethyl acetate was evaporated and acids were derivatized with bis(trimethylsilyl)trifluoroacetamide (BSTFA). Derivates were injected onto a capillary column CP-Sil-5B (Chrompack, the Netherlands) and separated using a temperature gradient. On-line MS was used to quantify and identify phenolic acids (Hollman in preparation). The detection limit in urine was 5 mg/L for hippuric acid, 0.8 mg/L for chlorogenic acid, and 0.4 mg/L for benzoic acid. Due to technical problems we could not measure benzoic acid in 4 volunteers and we could not measure chlorogenic acid in 2 volunteers. Furthermore, in 1 volunteer we found unexplained high excretion values for hippuric acid in urine after placebo that were 10-20 times higher than in other volunteers. We calculated the mean excretion of hippuric acid, benzoic acid and chlorogenic acid in urine with and without including this volunteers and reported the results in the results section. For homocysteine measurements we did not exclude the results of this volunteers because they did not differ from that of the other volunteers.

Statistical analyses

For the homocysteine concentrations and for excretion of phenolic acids in urine we calculated at each time point for each subject the differences relative to placebo. The total excretion of a phenolic acid was calculated for each person by summarizing the excretion values at each time point. We reported a missing value in case excretion value at one time point was missing.

Statistical significance and 95% confidence intervals of the mean differences were calculated using Student's *t* test (SAS Institute, Cary, USA).

RESULTS

Four hours after ingestion of chlorogenic acid total homocysteine concentrations were 6 % (0.5 μmol/L; 95%CI: –0.3, 1.3) higher than after placebo (Figure 6.2). Ingestion of benzoic acid and hippuric acid did not affect plasma homocysteine concentrations relative to placebo.

Plasma homocysteine concentrations increased after the hot meal, irrespective of the supplement the subjects ingested at breakfast. At 5-6 h after the hot meal (t=10 h after supplement intake) the plasma homocysteine concentration was 0.5–0.7 μmol/L higher than 1-2 hours after the hot meal (t=6 h) (Figure 6.2). The rise in plasma homocysteine concentrations might have been even higher at later time points, but we do not know this because we did not take blood samples between 10 and 24 h after ingestion of the supplements.

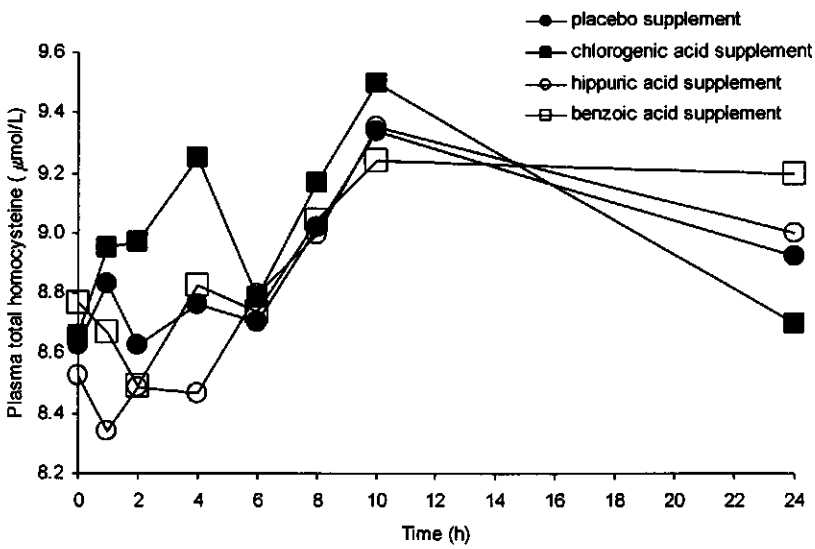

Figure 6.2 Plasma total homocysteine concentrations (mean) after ingestion of 1.2 g (8 mmol) of benzoic acid, 1.4 g (8 mmol) of hippuric acid, 2 g (5.5 mmol) of chlorogenic acid and placebo. Eleven healthy volunteers ingested the supplements, for 2 d each, in random order

Excretion of hippuric acid, chlorogenic acid and benzoic acid in urine

About 72-76 mol% of the ingested chlorogenic acid, benzoic acid and hippuric acid was recovered in urine in the form of hippuric acid (Table 6.1). For chlorogenic acid we calculated the recovery under the assumption that 1 molecule of chlorogenic acid provides 2 molecules of hippuric acid: the caffeic acid moiety as well as the quinic acid moiety of chlorogenic acid can be metabolized into hippuric acid (Adamson et al. 1970, Quick 1931, Scheline 1978).

Hippuric acid was mainly excreted between 10 and 24h after ingestion of chlorogenic acid, whereas it was excreted within the first 4 hours after ingestion of benzoic acid and hippuric acid supplement (Figure 6.3).
Chlorogenic acid and benzoic acid were mainly excreted in urine in the first 4 hours after ingestion of the supplement, but only small amounts were recovered (Figures 6.4 and 6.5).
We excluded one volunteer from the phenolic acid results because of extremely high excretion of hippuric acid after the placebo supplement. When we included the results of this volunteer the recoveries of hippuric acid, chlorogenic acid and benzoic acid in urine were as follows. The mean (95% CI) recoveries of hippuric acid in urine were 74 mol% (44, 104) after chlorogenic acid supplement, 36 mol% (-46, 119) after benzoic acid supplement, and 48 mol% (-15, 112) after hippuric acid supplement. The recovery of chlorogenic acid in urine was 0.4 mol% (0.3, 0.5) after chlorogenic acid supplement. The recoveries of benzoic acid in urine were 0.1 mol% (-0.0, 0.3) after chlorogenic acid supplement, 1.1 mol% (0.6, 1.5) after benzoic acid supplement and 0.3 mol% (0.1, 0.4) after hippuric acid supplement.

Table 6.1 Recovery of hippuric acid, benzoic acid and chlorogenic acid relative to placebo in 24 h urine. Eleven healthy volunteers ingested 1.2 g (8 mmol) of benzoic acid, 1.4 g (8 mmol) of hippuric acid, and 2 g (5.5 mmol) of chlorogenic acid for 2 d each, in random order[1].

	Recovery in urine relative to placebo		
Supplement	Chlorogenic acid	Benzoic acid	Hippuric acid
	mol per 100 mol of supplement		
Chlorogenic acid	0.4 (0.2, 0.5) (n=7)	0.2 (-0.1, 0.4) (n=5)	76 (42, 110) (n=8)
Benzoic acid	Not measured	1.2 (0.9, 1.5) (n=6)	72 (46, 98) (n=10)
Hippuric acid	Not measured	0.2 (0.0, 0.4) (n=6)	75 (52, 99) (n=10)

[1] Mean (95%CI). We excluded one volunteer because of unexplained, extremely high excretion values of hippuric acid after ingestion of the placebo. The results when we included this volunteer are presented in the results section. Data of other volunteers might have been excluded because we had no data on the amount excreted during 24 h. Therefore we indicated in the table on how many volunteers the results were based.

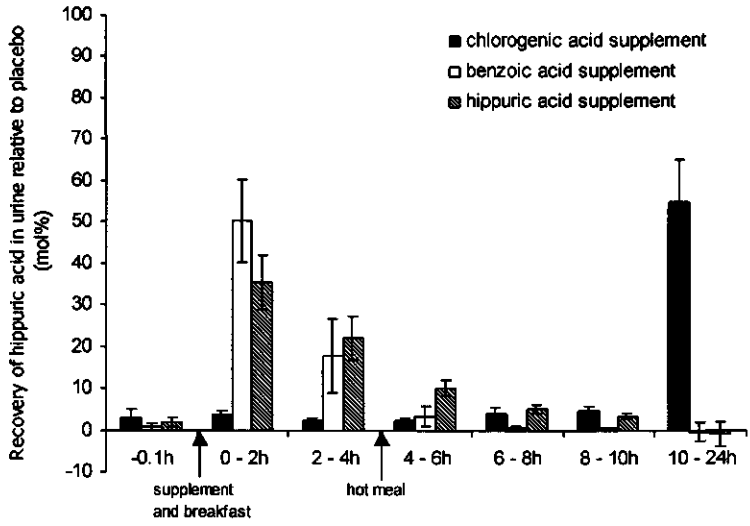

Figure 6.3 Recovery of hippuric acid in urine (mean ±SE) relative to placebo after ingestion of 1.2 g (8 mmol) of benzoic acid, 1.4 g (8 mmol) of hippuric acid and 2 g (5.5 mmol) of chlorogenic acid. Eleven healthy volunteers ingested the supplements for 2 d each, in random order. One volunteer with unexplained high excretion of hippuric acid in the placebo period was excluded in this figure.

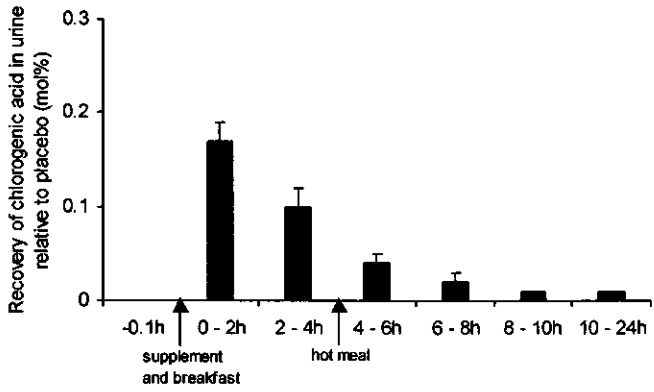

Figure 6.4 Recovery of chlorogenic acid in urine (mean ±SE) relative to placebo after ingestion of 2 g (5.5 mmol) chlorogenic acid. Eleven healthy volunteers ingested the supplements for 2 d each, in random order. One volunteer with unexplained high excretion of hippuric acid in the placebo period was excluded in this figure.

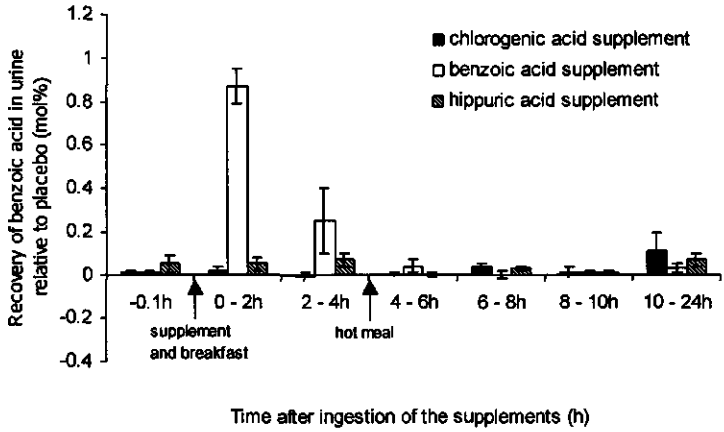

Figure 6.5 Recovery of benzoic acid in urine (mean ±SE) relative to placebo after ingestion of 1.2 (8 mmol) of benzoic acid, 1.4 g (8 mmol) of hippuric acid and 2 g (5.5 mmol) of chlorogenic acid. Eleven healthy volunteers ingested the supplements for 2 d each, in random order. One volunteer with unexplained high excretion of hippuric acid in the placebo period was excluded in this figure.

DISCUSSION

We found in a previous study that the homocysteine-raising effect of coffee might be partly due to chlorogenic acid, a major component in coffee (Olthof et al. 2001b).

In this study we also found that chlorogenic acid raised plasma homocysteine at 4 hours after ingestion although the effect was smaller than in the previous study. The rise in this study was 6% whereas the rise in the previous study was 12% (Olthof et al. 2001b). This difference between the two studies could be due to chance because the 95% confidence interval of the difference between the homocysteine rise in this study and in the previous study included zero. However, other factors could have played a role.

First, in this study subjects ingested the chlorogenic acid before breakfast while in the previous study it was ingested before a hot meal. We saw in this study that the hot meal at noon increased plasma homocysteine, while a breakfast did not. This might be caused by methionine present in a hot meal, because this amino acid is the precursor for homocysteine (Guttormsen et al. 1994, Chambers et al. 1999). We speculate that chlorogenic acid might affect plasma homocysteine more when ingested just before a hot meal, which 'triggers' the homocysteine metabolism, than when ingested before a breakfast.

Second, the length of the supplement periods differed between the two studies: in this study subjects ingested chlorogenic acid for 2 consecutive days while in our previous study it was 7 days. However, we do not think that the length of the supplement period plays an important role because we found in our previous study that the effect of

chlorogenic acid on plasma homocysteine concentration was fast, and almost gone at 20 hours after ingestion of chlorogenic acid (Olthof et al. 2001b).
We conclude that the difference between this study and the previous study is probably due to chance. More studies are needed to determine the effects of chlorogenic acid on plasma homocysteine under various circumstances, such as after ingestion of chlorogenic acid throughout the day, or after ingestion together with various types of foods.

Mechanism behind the homocysteine-raising effect of chlorogenic acid

We speculated that the effect of chlorogenic acid on homocysteine might be due to exhaustion of endogenous glycine which is used for the metabolism of chlorogenic acid into hippuric acid. Therefore we gave volunteers benzoic acid which requires glycine for its conversion into hippuric acid, and hippuric acid itself which does not require glycine. We found that benzoic acid did not affect plasma homocysteine and neither did hippuric acid. One reason for this might be that the dose of benzoic acid in this study was too low to exhaust the glycine pool (Amsel & Levy 1969, Quick 1931). On the other hand, the amount of hippuric acid produced after benzoic acid was comparable to that after chlorogenic acid, and chlorogenic acid did affect plasma homocysteine. Therefore it seems unlikely that the use of glycine in the metabolism of dietary phenols causes their homocysteine-raising effect. Another observation in this study supports this conclusion. We found that hippuric acid is excreted in urine between 10 and 24 h after intake of chlorogenic acid, while plasma homocysteine increased during the first 4 h after intake (Figures 6.2 and 6.3) (Olthof et al. 2001b). Thus the timing of formation of hippuric acid did not seem to coincide with the timing of the rise in homocysteine.
Hippuric acid itself is also not responsible for the homocysteine raising effect of chlorogenic acid, because hippuric acid did not raise plasma homocysteine.
Another mechanism the homocysteine-raising effects of phenols might be that homocysteine is formed through methylation of phenols in the liver. The methyl groups that are needed for methylation of phenols are donated by S-Adenosylmethionine (SAM) which then yields homocysteine. Therefore ingestion of phenols might increase the methylation process which results in an increase in plasma homocysteine concentration. We found only minor amounts of methylated metabolites in urine after chlorogenic acid in the previous study (Olthof et al. 2001c) However, measurements in urine might not represent the situation in the body and therefore this methylation hypothesis should be investigated.

Metabolism of chlorogenic acid and benzoic acid into hippuric acid

Small amounts of chlorogenic acid were recovered in urine, mainly in the first 4 h after ingestion (Figure 6.4). This chlorogenic acid in urine probably originates from the fraction that is absorbed in the small intestine. In contrast, hippuric acid mainly

appeared in urine between 10 and 24 h after ingestion of chlorogenic acid (Figure 6.3). This indicates that the colonic microflora is involved in the formation of hippuric acid from chlorogenic acid (Booth et al. 1957), because transit time through the small intestine is about 10 h (Fallingborg et al. 1989 and 1990). Degradation of chlorogenic acid in the colon rather than endogenously is also supported by our previous findings that two-third of ingested chlorogenic acid is not absorbed in the small intestine but goes to the colon (Olthof et al. 2001a). In addition we found that subjects without a colon did not produce hippuric acid from chlorogenic acid (Olthof et al. 2001c). We recovered 76 mol% of the chlorogenic acid supplement as hippuric acid in urine, this indicates that most of the chlorogenic acid that reaches the colon is metabolized into hippuric acid. In addition, some chlorogenic acid which is absorbed in the small intestine might also reach the colon via the enterohepatic cycle and be metabolized by the colonic microflora. A major part of the degradation products is absorbed from the colon, further metabolized and excreted in urine.

The rapid appearance of hippuric acid in urine within 4 h after ingestion of benzoic acid indicates that benzoic acid is first absorbed and then metabolized into hippuric acid probably in the liver. The recovery of 72% of the ingested benzoic acid dose as hippuric acid as well as the rapid excretion of hippuric acid in urine correlated well with the results of Kubota et al. (1991).

One volunteer had unexplained high excretion of hippuric acid in the placebo period. Although volunteers were instructed to adhere to the controlled diet this volunteer might have eaten something in addition to the controlled diet that lead to the high excretion of hippuric acid in urine. However we have no proof for this. The recoveries of hippuric acid in urine after ingestion of benzoic acid and hippuric acid were drastically affected by the results of this one volunteer. Therefore we showed the results with and without including the results of this volunteer. The kinetics of excretion of hippuric acid after ingestion of the supplements were not affected by the results of this volunteer. We did include the results of this volunteer in our homocysteine analyses because these results were not different from those of the other 10 volunteers. Therefore, we do not believe that the results of this one volunteer affected the conclusions of this study.

Conclusion

Chlorogenic acid in coffee remains a candidate for part of the homocysteine-raising effect of coffee. Exhaustion of endogenous glycine due to the formation of hippuric acid from chlorogenic acid is not involved. Further research is necessary to elucidate the mechanism underlying the homocysteine-raising effects of coffee and of chlorogenic acid.

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7

Discussion

GENERAL DISCUSSION

The objective of this thesis was to investigate *whether bioavailability data on flavonoids and cinnamic acids support the hypothesis that they can affect health in humans.*

We found that the flavonol quercetin, black tea phenols, and cinnamic acids are bioavailable and thus might affect health. However, they are extensively metabolized, mainly before they reach the circulation. Because the metabolites of phenols that we found have lower antioxidant activity in vitro than their parent phenols, the role of dietary phenols as antioxidants in vivo might be less than expected. Therefore, studies on health effects of dietary phenols should include the effects of their metabolites. Furthermore, we found that chlorogenic acid from coffee, and black tea solids might be harmful because they raised concentrations of plasma homocysteine, a potential risk factor for cardiovascular disease. In contrast, the flavonol quercetin did not affect plasma homocysteine.

This chapter discusses the implications of these findings and suggestions for further research.

BIOAVAILABILITY

Why are bioavailability studies important?

Research on bioavailability of dietary phenols is scarce. Nevertheless, it is important for many purposes. Examples of the importance of bioavailability research are:

- Knowledge of the bioavailability of nutrients can clarify discrepancies in epidemiological research. For example, the association between the intake of flavonols, such as quercetin, and health in epidemiological studies is inconclusive (see introduction Table 1.2). This can partly be explained by the fact that these studies did not account for the variation in bioavailability of various quercetin glycosides in foods. Differences in bioavailability of quercetin glycosides might result in different effects in vivo.
- Bioavailability studies will give insight into the concentrations of phenols and their metabolites presented to target tissues, which will help to evaluate their potential biological effects.
- Bioavailability data can be used to improve the design of in vitro studies. For example, the in vitro antioxidant activity of the ingested phenols is often used as an indicator of their activity in vivo. However, we showed that dietary phenols are rapidly and extensively metabolized in humans (*chapters 4 and 6*). Thus, in vitro antioxidant activity of the metabolites of phenols would be more representative for the antioxidant activity of dietary phenols in vivo than that of the ingested phenols.

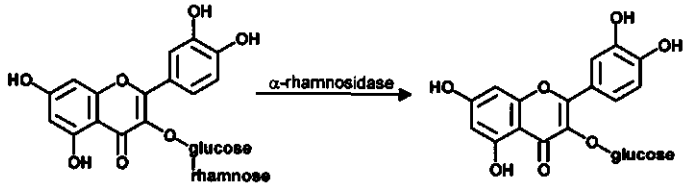
- Knowledge of the bioavailability of food compounds opens ways for targeted modifications of food compounds according to the desired bioavailability characteristics. For example, modification of the conjugated moieties of quercetin or caffeic acid will change the bioavailability (*chapters 2 and 3*). Compounds that are well absorbed will be directed toward the blood circulation. Compounds that are less well absorbed will be directed toward the colon. Therefore the question: "where does this compound go?" might in the future change to "where do we want this compound to go, and where do we want it to stay away?"

Implications of the bioavailability of quercetin, chlorogenic acid and tea phenols

The bioavailability of quercetin and chlorogenic acid depends upon their conjugated moieties. The bioavailability of quercetin-3-rutinoside is much lower than that of quercetin glucosides (*chapter 2*) (Hollman et al. 1999a). The absorption of caffeic acid esterified with quinic acid, yielding chlorogenic acid, is only 30% of that of free caffeic acid (*chapter 3*) (Azuma et al. 2000). Thus differences in the conjugated moieties result in differences in bioavailability of dietary phenols, and in the target tissues that they can reach.

Information regarding bioavailability of phenols will provide insight into which forms are present at which target tissues, and thus into their potential health effects. For example, we found that a large part of chlorogenic acid, present in coffee, will reach the colon after ingestion. This implies that chlorogenic acid, or metabolites formed in the colon might play a role in the potential protective effect of coffee against colon cancer (Tavani & La Vecchia 2000, Giovannucci 1998, Baron et al. 1994, Favero et al. 1998, La Vecchia et al. 1989, Tavani et al. 1997). Furthermore, we found that quercetin-3-rutinoside, chlorogenic acid and tea phenols are extensively metabolized into phenolic acids (*chapter 4*). These phenolic acids, rather than the parent phenols will reach target tissues, and thus might exert biological effects. Therefore, in vitro studies with regard to health effects of phenols should also include the metabolites that reach target tissues.

Insight into the bioavailability of phenols opens up ways for modification of phenols in foods. Increasing the amount of phenols in foods is already possible: the amounts of the flavonols quercetin and kaempferol in tomatoes have each been increased 70-fold by means of genetic modification approaches (personal communication with A. Bovy, Plant Research International, Wageningen, the Netherlands). In addition, our results indicate that it might also be interesting to modify the type of the conjugated moieties of phenols. For example, the poor-bioavailable quercetin-3-rutinoside present in tea can be transformed into the well-bioavailable quercetin-3-glucoside (Bokkenheuser et al. 1987, Gunata et al. 1988, Kurosawa et al. 1973) (Figure 7.1).



Quercetin-3-rutinoside Quercetin-3-glucoside

Figure 7.1 Transformation of quercetin-3-rutinoside into quercetin-3-glucoside

The bioavailability of quercetin from tea would then drastically improve, because the bioavailability of quercetin rutinoside is only 20% of that of the quercetin glucosides (*chapter 2*) (Hollman et al. 1999a). The opposite is also possible: if quercetin glucosides would be transformed into rutinosides, less of the quercetin would be absorbed from the small intestine and more would be directed toward the colon.
Similarly, the bioavailability of caffeic acid can also be changed: the absorption of caffeic acid esterified with quinic acid (chlorogenic acid) is only 30% of that of free caffeic acid (*chapter 3*). Because caffeic acid in foods is mainly present as chlorogenic acid, it will for the most part be directed toward the colon. Hydrolysis of chlorogenic acid into caffeic acid and quinic acid before ingestion will improve the absorption of caffeic acid.
Consequently, in the future we might modify the conjugated moieties of phenols so as to target to which tissues the phenols should go or stay away. However, information on bioavailability and health effects of phenols is as yet too limited to justify such modification.

Phenolic acid metabolites as biomarkers for intake of dietary phenols.

Phenolic acid metabolites in urine can be potential markers for intake of total dietary phenols. Markers of intake that objectively measure intake in individual persons are important tools for epidemiological studies on phenols and health. Currently, intake of individual phenols, such as flavonols and gallic acid, can be estimated by means of measurements of these compounds in blood or urine after ingestion (de Vries 1998, Hodgson et al. 2000). However, no markers are available for the intake of the total group of dietary phenols.
We found approximately half of the ingested chlorogenic acid, black tea phenols, and quercetin-3-rutinoside as phenolic acids in urine, which indicates that these acids could be used as markers of total phenol intake. In addition, the metabolic profile of metabolites in urine might indicate the types of phenols that were ingested. For example, we found that chlorogenic acid and tea phenols are mainly metabolized via

the phenylpropionic acid pathway into hippuric acid (phenyl-C_1). Flavonols, such as quercetin-3-rutinoside, are metabolized via the phenylacetic acid pathway into phenylacetic acids (phenyl-C_2) (*chapter 4*) (Hollman & Katan 1998). One potential problem in using phenolic acids in urine as markers of phenol intake is that not only dietary phenols, but also amino acids such as tyrosine and phenylalanine can be metabolized to phenolic acids and end up in urine (Booth et al. 1960, Teuchy & Van Sumere 1971). This should be taken into consideration when phenolic acids would be used as markers for intake of dietary phenols.

HEALTH

Phenols as antioxidants: important to health?

Phenols are strong antioxidants in vitro, even stronger than antioxidant vitamins such as vitamin E (Rice-Evans et al. 1996). However, antioxidant effects of phenols in the in vivo situation are uncertain.

We found that the dietary phenols, chlorogenic acid, black tea solids and quercetin-3-rutinoside are extensively metabolized into metabolites with no or less in vitro antioxidant activity than their parent phenols (*chapter 4*). This raises doubts as to whether dietary phenols may be important as antioxidants in vivo. Nevertheless, we measured the metabolites of phenols in urine and not in the blood circulation or in specific target tissues. Thus it is possible that other metabolites are present in the blood circulation and are available for antioxidant effects. In intervention studies, the effects of phenols and phenol-rich foods on several markers of antioxidant status are inconsistent. Some studies find that phenols affect antioxidant markers (Wang et al. 2000, Rein et al. 2000, Leenen et al. 2000, Boyle et al. 2000b) but others do not (Boyle et al. 2000a, O'Reilly et al. 2000, Caccetta et al. 2000, Princen et al. 1998).

The uncertainty about efficacy of antioxidants in vivo is also illustrated by the inconsistent results from large intervention trials on antioxidant vitamins and cancer or cardiovascular disease (Marchioli 1999, Buring & Hennekens 1997, McDermott 2000). The effect of vitamin E on cardiovascular disease is not conclusive (Hooper et al. 1999, Yusuf et al. 2000, Collaborative Group of the Primary Prevention Project 2001). Another example is that supplementation of beta-carotene to smokers increased rather than decreased the risk of lung cancer (Anonymous 1994, Omenn et al. 1996).

The lack of reliable markers of in vivo oxidative processes is a major problem in the field of antioxidants, which hinders a proper evaluation of the effects of antioxidants on health (Zock & Katan 1998, Witztum 1998). Isoprostanes as markers for lipid peroxidation in vivo and autoantibodies against oxidized LDL cholesterol might be promising (Roberts & Morrow 2000, Holvoet et al. 1998). More research is necessary before any dietary recommendations can be made with regard to antioxidants.

Coffee and tea: good or bad for health?

Coffee and tea are major sources of dietary phenols. Phenols are believed to be beneficial to health through their antioxidant activity. On the other hand, we found that phenols from coffee and tea might be harmful because they increased homocysteine concentrations in plasma (*chapter 5*), which is a potential risk factor for cardiovascular disease. This apparent inconsistency raises the question whether coffee and tea are good or bad for health.

Unfiltered, boiled coffee increases serum cholesterol concentrations and thereby increases risk of cardiovascular disease (Aro et al. 1987, Urgert & Katan 1997). Although filtered coffee does not raise cholesterol, the data on coffee and cardiovascular disease are inconsistent; case-control studies indicate that coffee is associated with an increased risk, while most prospective studies find no association, or a decreased risk of cardiovascular disease among coffee drinkers (Greenland 1993, Kawachi et al. 1994). Furthermore, coffee might protect against colon cancer, but data are not consistent; case-control studies indicate the coffee protects against colon cancer, whereas the association in prospective studies is less clear (Giovannucci 1998, Tavani & La Vecchia 2000). Nevertheless, chlorogenic acid in coffee could be involved in the potential protective effect of coffee against colon cancer, because two-thirds of the ingested chlorogenic acid are transported into the colon where it might exert biological effects (*chapter 3*).

Tea is a major source of catechins, a subgroup of flavonoids, which are believed to be involved in potential beneficial health effects of tea. However, there is no clear association between intake of tea catechins and risk of cardiovascular disease and cancer in epidemiological studies (Arts 2001). Furthermore, like for coffee, the relation between tea consumption and disease is inconsistent (Tijburg et al. 1997, Hollman et al. 1999b). Epidemiological studies show no clear association between tea consumption and cancer risk (Blot et al. 1996 and 1997, Hollman et al. 1999b, Goldbohm et al. 1996). Epidemiological studies on tea and cardiovascular disease are also inconsistent. Some studies suggest a protective effect of tea (Hertog et al. 1993, Keli et al. 1996, Geleijnse et al. 1999), but others find no association (Stensvold et al. 1992, Klatsky et al. 1990 and 1993). In Wales and Scotland tea drinking is associated with an increased risk of cardiovascular disease (Woodward & Tunstall-Pedoe 1999, Hertog et al. 1997).

Thus, the association between consumption of coffee or tea and disease is not clear. Differences between populations in tea or coffee drinking habits might explain part of the inconsistency, but the most likely explanation is confounding of the associations by other risk factors, or the absence of a true underlying effect. Epidemiological studies cannot completely control for confounding by accompanying lifestyle factors. For example, in most countries tea drinking is associated with a healthy lifestyle, while coffee drinking is associated with a less healthy lifestyle (Schwarz et al. 1994). The

opposite applies to Wales and Scotland: tea drinking is associated with a less healthy lifestyle than coffee drinking (Woodward & Tunstall-Pedoe 1999). This might explain the discrepant finding for tea and cardiovascular disease in these studies. However, the data from all epidemiological studies together are also compatible with no effect of tea and coffee on health.

Thus, it is not yet clear whether tea and coffee are either good or bad for health. Long term intervention trials with tea and coffee can provide definite answers on this issues, but these trials are hardly feasible. However, coffee and tea are common parts of the human diet all over the world. Therefore, it is important to further investigate health effects of these beverages.

Plasma homocysteine

Is homocysteine a causal risk factor for cardiovascular disease?

A high homocysteine concentration is proposed as a biomarker for cardiovascular disease risk. However, the causality of homocysteine in the development of cardiovascular disease is still unclear (Brattstrom & Wilcken 2000, Ueland et al. 2000). The homocysteine-raising effects of coffee, black tea solids and chlorogenic acid raise additional questions about the causal role of plasma homocysteine in cardiovascular disease. If homocysteine is a causal factor we would expect that coffee and tea consumption is related to an increased risk of cardiovascular disease. However, this is not clear from observational studies.

A high coffee consumption is associated with 10-20% rise in plasma homocysteine concentrations in most observational studies (Nygard et al. 1997, Oshaug et al. 1998, Stolzenberg-Solomon et al. 1999), although not in all (Nieto et al. 1997). This homocysteine-raising effect of coffee is confirmed in two intervention studies (Urgert et al. 2000, Grubben et al. 2000). A 10-20% rise in plasma homocysteine would coincide with ~5-10% increased risk of cardiovascular disease in coffee consumers (Ueland et al. 2000). This small increase in risk is probably hard to detect in observational studies which might explain the inconsistent results on the association between coffee consumption and cardiovascular disease (Greenland 1993, Kawachi et al. 1994).

Furthermore, we found that strong black tea raised plasma homocysteine by 11% (*chapter 5*). Only one observational study reported a weak positive association between tea consumption and plasma homocysteine in tea drinkers (Nygard et al. 1997). Other studies do not report on this association. If homocysteine is causal, a homocysteine-raising effect of strong tea might partly explain the positive association between tea and cardiovascular disease risk in the studies in Wales and Scotland, where tea habits differ from those in other populations (Hertog et al. 1997, Woodward & Tunstall-Pedoe 1999). Therefore it would be worthwhile to measure the effects of tea on concentrations of plasma homocysteine in the UK.

Long term, placebo-controlled, homocysteine-lowering intervention trials are necessary to investigate the causality of homocysteine in the development of cardiovascular disease. Only when the results of these intervention studies are known, potential harmful effects of coffee and tea can be discussed.

Possible mechanisms causing the homocysteine-raising effect of dietary phenols.

We found that chlorogenic acid and black tea solids raised plasma homocysteine, while quercetin did not (*chapter 5*). Knowledge of the mechanism causing this homocysteine-raising effect of phenols will give further insight into the effects of dietary compounds on homocysteine metabolism. Furthermore, it helps to predict the effects of other food compounds on plasma homocysteine.

Our study (*chapter 5*) and two intervention studies with coffee (Urgert et al. 2000, Grubben et al. 2000) did not indicate that coffee, black tea solids and chlorogenic acid affect homocysteine metabolism through changes in B-vitamins. Three other possible mechanism that might be involved in the homocysteine-raising effect of phenols are discussed.

- In chapter 6 we tested whether the use of glycine for the metabolism of chlorogenic acid and tea phenols into hippuric acid might be involved in the homocysteine-raising effect (Figure 7.2). Our finding that quercetin was not metabolized into hippuric acid (*chapter 4*), and neither affected plasma homocysteine (*chapter 5*) supports this hypothesis. Glycine is involved in several pathways in the homocysteine metabolism and therefore we hypothesized that possible exhaustion of glycine through the formation of hippuric acid might affect homocysteine metabolism. However, we could not confirm that glycination of metabolites of phenols is involved in the homocysteine-raising effect of phenols.

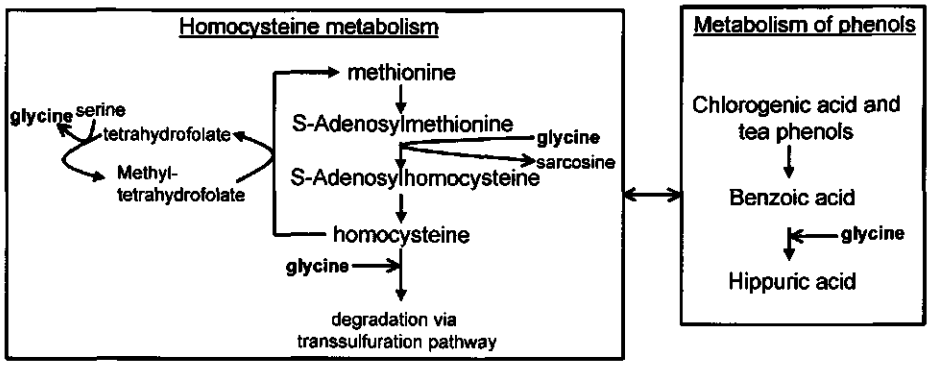


Figure 7.2 Use of glycine in the homocysteine metabolism and in metabolism of phenols.

- The hydroxyl groups of phenols are O-methylated in the liver. The necessary methyl group is derived from S-adenosylmethionine which results in production of homocysteine (Figure 7.3) (Finkelstein 1990). Thus consumption of a high dose of

phenols might increase homocysteine production through increased methylation of phenols (Zhu et al. 1994, Miller et al. 1997). The fact that we did not find many *O*-methylated metabolites of phenols in urine does not support this hypothesis (*chapter 4*). However, measurements in urine might not reflect the situation in the body. Therefore we should test this hypothesis in humans, using substances that are known to undergo *O*-methylation such as L-dopa (L-3,4-dihydroxyphenylalanine). Rats fed L-dopa, and Parkinson disease patients treated with L-dopa have higher homocysteine concentrations than do controls (Daly et al. 1997, Allain et al. 1995, Kuhn et al. 1998a and 1998b, Muller et al. 1999). If this methylation hypothesis is true, then other food compounds or drugs that are *O*-methylated in the liver, might also affect plasma homocysteine.

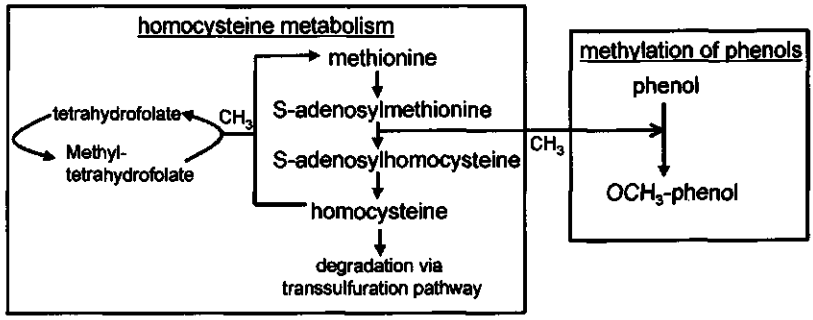


Figure 7.3 Possible relation between methylation of phenols and the homocysteine metabolism

- Another possibility might be that the rise in homocysteine is explained through diuretic effects of coffee, chlorogenic acid and black tea solids: loss of body fluids might increase homocysteine concentrations. However, our results do not support this. Chlorogenic acid and black tea solids increased plasma homocysteine relative to placebo and quercetin-3-rutinoside, but not the amount of urine excreted during 24 hours after intake (*chapter 5*).

FUTURE STUDIES

Metabolites of phenols in blood

We measured metabolites of phenols in urine (*chapter 4*). Measurements of metabolites in blood would have been preferable, but were not possible in the present studies. Future studies should aim at measuring phenols and their metabolites in the blood circulation and target tissues. This will provide ideas about mechanisms behind potential health effects of phenols and it can improve the design of in vitro studies so that they better represent the in vivo situation. Furthermore, this information can be useful in the development of markers for intake of phenols from foods.

Bioavailability of other phenols

Numerous phenols are present in foods, but information with regard to their bioavailability is scarce or lacking. In addition to the current knowledge regarding bioavailability, more research on the bioavailability of other dietary phenols and on factors that influence their bioavailability is necessary.

Role of antioxidants in disease

Currently available biomarkers for measurement of antioxidant activity in vivo are mainly based on in vitro or ex vivo methods that do not necessarily represent the situation in vivo. To assess the importance to health of antioxidants such as phenols, in vivo markers of oxidative damage are needed that both respond to dietary changes and at the same time predict changes in disease risk.

Mechanism underlying the homocysteine-raising effect of phenols

Knowledge about the mechanism underlying the homocysteine-raising effect of dietary phenols will add to the current knowledge about effects of food compounds on the homocysteine metabolism. In addition, it will enable to predict the effects of other food components or drugs on the homocysteine metabolism. This will provide ideas about potential homocysteine-lowering substances.

Causality of plasma homocysteine in cardiovascular disease

Placebo-controlled homocysteine-lowering interventions trials are needed to prove that a decrease in plasma homocysteine lowers the risk of disease. This information is crucial in risk-assessment of food components that increase homocysteine, such as certain phenols.

CONCLUSION

Our studies indicate that dietary phenols could play a role in human health because they are available in the body. We found that the absorption of phenols is dependent upon their conjugated moiety: quercetin glucosides are better absorbed than quercetin-rutinoside; and free caffeic acid is better absorbed than its conjugated derivative chlorogenic acid. This suggests that bioavailability can be modulated by modification of dietary phenols. We also found that phenols are extensively metabolized into phenolic acids, mainly before they reach the circulation. Thus the metabolites of phenols probably circulate in the body and reach target tissues, rather than the ingested phenols. The phenolic acid metabolites have no, or less, antioxidant activity in vitro than their parent phenols. Therefore, the role of dietary phenols as antioxidants in vivo might be less important than suggested by the in vitro antioxidant activity of the parent compounds. We also showed that phenols might have adverse effects on health because some phenols raise plasma homocysteine, a potential risk

factor for cardiovascular disease. Whether beneficial effects of phenols outweigh the adverse effects is as yet unclear.

Our results justify further research with regard to bioavailability and health effects of dietary phenols.

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Summary

Dietary phenols are strong antioxidants in vitro and they are therefore thought to protect against cardiovascular disease and cancer. However, formal proof that phenols can affect health is still lacking. For a proper evaluation of potential health effects of phenols, information on their bioavailability in humans is crucial. The recognition of the need for information on bioavailability of phenols is increasing, but data on bioavailability are still scarce. The aim of this thesis was to investigate *whether bioavailability data on flavonoids and cinnamic acids support the hypothesis that they can affect health in humans.* We studied quantitatively important flavonoids and cinnamic acids from foods: the flavonol quercetin, black tea phenols and chlorogenic acid (5-caffeoylquinic acid).

Impact of conjugated moieties of quercetin and chlorogenic acid on their bioavailability

In *chapter 2* we investigated whether the position of glucose plays a role in the bioavailability of quercetin glucosides. Quercetin is a major representative of the flavonols, a subgroup of flavonoids. In foods, quercetin occurs mainly as quercetin glycosides. Previous studies of Hollman et al. showed that the sugar moiety determines the bioavailability: the bioavailability of quercetin-3-rutinoside (quercetin attached to a rutinose) is only 20% of that of quercetin-4'-glucoside (quercetin attached to a glucose). Tea is the major source of quercetin, but it is present in the poor bioavailable form quercetin-3-rutinoside. Quercetin-3-rutinoside can be transformed into quercetin-3-glucoside by splitting off a rhamnose molecule and it would be interesting to see if this will improve its bioavailability. Therefore, we investigated whether quercetin-3-glucoside had the same high bioavailabilty as the 4'-glucoside. Nine healthy volunteers ingested a single dose of pure quercetin-3-glucoside (325 μmol) and a single dose of pure quercetin-4'-glucoside (331 μmol). Indeed, we found that the quercetin-3-glucoside had the same high bioavailability as the quercetin-4'-glucoside. Thus the position of the glucose moiety did not affect bioavailability of quercetin glucosides. This implies that the bioavailability of quercetin-3-rutinoside will increase considerably when it would be transformed into quercetin-3-glucoside. In general, conversion of quercetin glycosides into glucosides is a promising approach to improve bioavailability of quercetin from foods.

Caffeic acid is a major dietary cinnamic acid, but in foods it is mainly conjugated with quinic acid, called chlorogenic acid. Coffee is its major source. Very little is known about the bioavailability of caffeic acid and chlorogenic acid in humans. In *chapter 3* we investigated the absorption of chlorogenic acid and of caffeic acid in the small intestine of humans. For this purpose we performed our study in ileostomy volunteers. Ileostomy volunteers lack their colon, which provides the opportunity to study the absorption in the small intestine without degradation of the phenols by the colonic microflora. After 7 ileostomy volunteers ingested a single dose of 1 g of chlorogenic acid and a single dose of 0.5 g of caffeic acid we recovered 67% of the ingested chlorogenic acid and 5% of the ingested caffeic acid in ileostomy fluid. This implies

that 33% of the ingested chlorogenic acid and 95% of the caffeic acid is absorbed in the small intestine. Thus, chlorogenic acid from foods can be absorbed but the conjugation of caffeic acid with quinic acid hinders absorption.

Metabolism of phenols in humans

Phenols are extensively metabolized in the human body. Therefore it is important to identify the metabolites of phenols that are actually circulating in the body and may have biological effects. Information on the metabolites of phenols and on the site of metabolism of phenols in humans is scarce. In *chapter 4* we identified and quantified metabolites of chlorogenic acid, quercetin-3-rutinoside and black tea phenols in humans. Twenty healthy volunteers with a colon ingested daily 2 g chlorogenic acid, 4 g black tea solids, 440 mg quercetin-3-rutinoside and a placebo for 7 days each, in random order. We measured 59 phenolic acids in 24-h urine as potential metabolites. Further, we investigated the role of the colonic microflora in the metabolism of dietary phenols by comparing the metabolites formed in volunteers with an intact colon with those formed in 7 volunteers who lack a colon (ileostomy subjects). We found in volunteers with a colon that half of the ingested chlorogenic acid and 43% of the tea phenols was metabolized into hippuric acid (*N*-benzoylglycine). Quercetin-3-rutinoside was mainly metabolized into phenylacetic acids: 3-hydroxyphenylacetic acid (40% of the amount of ingested quercetin-3-rutinoside), 3-methoxy-4-hydroxyphenylacetic acid (9%) and 3,4-dihydroxyphenylacetic acid (6%). In contrast, in volunteers without a colon we found only traces of phenolic acid metabolites after they had ingested chlorogenic acid and quercetin-3-rutinoside. This implies that the colonic microflora converts most of these dietary phenols into metabolites which then reach the circulation. Thus the metabolites of phenols will mainly reach target tissues, rather than the ingested phenols. These metabolites have lower antioxidant activity in vitro than their parent phenols. Therefore the antioxidant activity of dietary phenols in vivo might be lower than suggested by their in vitro antioxidant activity. Furthermore, in vitro studies should include potential health effects of metabolites of phenols and not only those of the phenols ingested.

Effect of phenols on plasma homocysteine

In *chapter 5* we studied the effects of phenols on plasma homocysteine, a potential risk factor for cardiovascular disease. Previous studies showed that coffee consumption increases plasma homocysteine concentrations. Because chlorogenic acid is a major phenol in coffee, we investigated in 20 healthy volunteers whether chlorogenic acid might be responsible for the homocysteine-raising effect of coffee. Homocysteine concentrations in plasma collected 4-5 h after ingestion of 2 g chlorogenic acid (~1.5 L strong coffee) was 12% higher relative to placebo. In plasma collected 20 h after ingestion it was 4% higher. Thus chlorogenic acid is indeed partly responsible for the homocysteine-raising effect of coffee. The homocysteine-raising effect of black tea solids (~2 L strong tea) was similar to that after chlorogenic acid.

Quercetin-3-rutinoside did not affect plasma homocysteine. If homocysteine is proven to be a causal risk factor for cardiovascular disease then consumption of large amounts of coffee or black tea might be a risk for health.

In *chapter 6* we tried to find the mechanism underlying the homocysteine-raising effects of chlorogenic acid and black tea solids. We investigated whether the metabolism of chlorogenic acid and black tea solids into hippuric acid (*chapter 4*) might be related to their homocysteine-raising effect (*chapter 5*). This was supported by the fact that quercetin-3-rutinoside was not metabolized into hippuric acid and did not raise plasma homocysteine either. Hippuric acid is formed through esterification of benzoic acid with glycine. Because glycine is also involved in the homocysteine metabolism, we hypothesized that exhaustion of glycine through formation of hippuric acid might affect homocysteine metabolism. A second objective of this study was to study the kinetics of excretion of several phenolic acid metabolites in urine after intake of the supplements. Eleven healthy volunteers ingested daily 2 g chlorogenic acid and 1.2 g sodium benzoate, which both use glycine for formation of hippuric acid, and 1.4 g hippuric acid and a placebo for 2 days each, in random order. Our results did not indicate that the formation of hippuric acid was involved in the homocysteine-raising effect of phenols. Furthermore, hippuric acid appeared in urine between 10 and 24 h after ingestion of chlorogenic acid. This indicates that the colonic microflora is involved in the formation of hippuric acid from chlorogenic acid, because transit time through the small intestine is about 10 h. This agrees with our findings in *chapter 3* and *chapter 4* that chlorogenic acid is mainly metabolized in the colon.

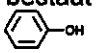
Conclusion

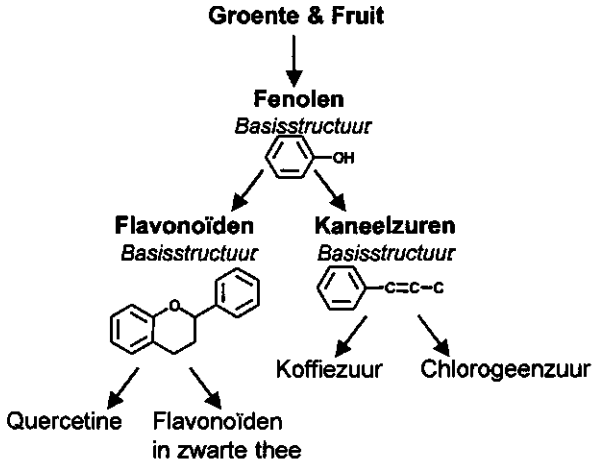
Consumption of phenols from foods will lead to increased concentrations of phenols and their metabolites in the human body. Whether or not this is favorable for health is not yet known. The bioavailability of quercetin and chlorogenic acid depends on their conjugated moieties. Quercetin, chlorogenic acid and black tea phenols are extensively metabolized into phenolic acid metabolites. These metabolites might contribute to the antioxidant activity in vivo, but their antioxidant activity in vitro is less than that of the parent phenols. Therefore, the contribution of dietary phenols to the antioxidant activity in vivo is uncertain. Furthermore, some phenols might also have adverse effects on health. Chlorogenic acid and black tea solids raised plasma homocysteine, a potential risk factor for cardiovascular disease.

In this thesis we studied only a small number of dietary phenols, while the variety in foods is huge. The results of our studies warrant further research on the bioavailability, and on beneficial as well as adverse effects of dietary phenols in humans. Only when we have the complete picture we can decide on the relevance of dietary phenols to human health.

Samenvatting

Bij gezond leven hoort een gezonde voeding. De consumptie van groente en fruit is een onderdeel van een gezonde voeding omdat het de kans op hart- en vaatzieken en kanker mogelijk verkleind. Voedingsstoffen zoals vitamines in groente en fruit zijn hiervoor deels verantwoordelijk. Maar ook andere voedingsstoffen in groenten en fruit, zoals 'fenolen', spelen mogelijk een rol (Figuur 1). Fenolen komen veel voor in groente en fruit en daarnaast in dranken zoals koffie, thee en rode wijn. In dit proefschrift hebben we onderzoek gedaan naar deze fenolen.

De chemische basisstructuur van fenolen bestaat uit een ringstructuur waaraan een hydroxylgroep (OH-groep) gebonden is:  . In de voeding bestaan er veel variaties op deze basisstructuur wat tot gevolg heeft dat er veel verschillende fenolen in de voeding voorkomen.



Figuur 1 Schematisch overzicht van fenolen in de voeding

Op welke manier fenolen de gezondheid zouden kunnen beïnvloeden is niet duidelijk. Mogelijk spelen fenolen een belangrijke rol als 'antioxidanten' en beschermen ze op die manier tegen ziekten zoals hart- en vaatziekten en kanker. Antioxidanten kunnen de vorming van schadelijke vrije radicalen in het lichaam voorkomen of beperken. Vrije radicalen kunnen weefsels beschadigen en daardoor leiden tot ziekte. Voorbeelden van andere antioxidanten in de voeding zijn de vitamines C en E en de voorloper van vitamine A: beta-caroteen.

Echter, er zijn ook aanwijzingen dat fenolen de gezondheid negatief kunnen beïnvloeden. Wij hebben in ons onderzoek onderzocht of fenolen het homocysteïnegehalte in het bloed kunnen verhogen. Een hoog homocysteïnegehalte is een risicofactor voor het ontstaan van hart- en vaatziekten.

In dit proefschrift hebben we onderzocht hoe fenolen zich gedragen in het menselijk lichaam en welk effect fenolen hebben op het homocysteïnegehalte bij mensen. Omdat in de voeding veel soorten fenolen voorkomen hebben we ons beperkt tot 2 groepen fenolen: de flavonoïden en de kaneelzuren (Figuur 1). Binnen deze 2 groepen hebben we weer 4 individuele fenolen gekozen: uit de groep van de flavonoïden hebben we het quercetine en de flavonoïden uit zwarte thee onderzocht; uit de groep van de kaneelzuren hebben we koffiezuur en chlorogeenzuur onderzocht. In de voeding is koffiezuur gebonden met kinazuur en dan heet het chlorogeenzuur.

In het onderzoek in dit proefschrift stonden 3 vragen centraal:

Onderzoeksvraag 1) Worden fenolen opgenomen vanuit de darmen in het bloed en wat is de invloed van de koppeling van fenolen met andere stoffen op de opname?

Onderzoeksvraag 2) Wat gebeurt er met de fenolen in het lichaam? Hiervoor hebben we onderzocht in welke stoffen de fenolen worden omgezet in het lichaam. De stoffen die ontstaan uit fenolen noemen we metabolieten van fenolen.

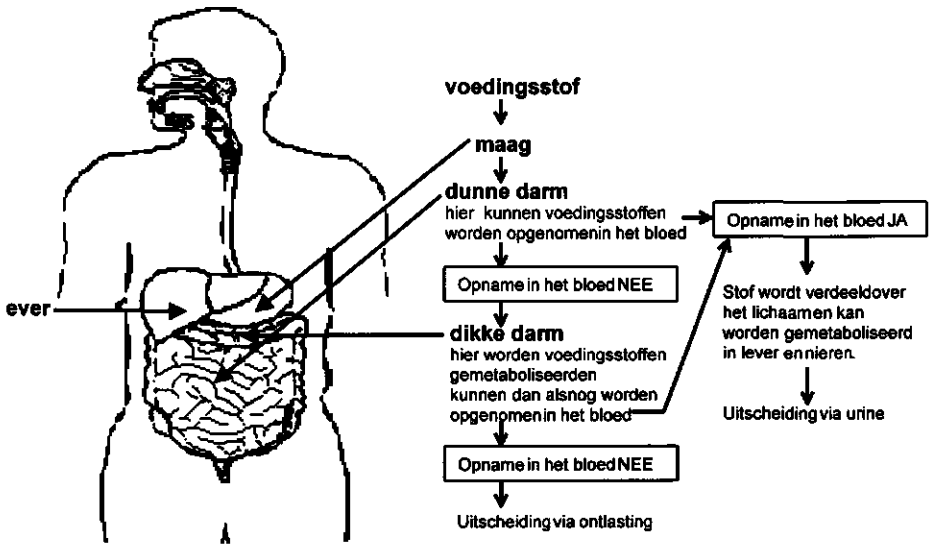
Onderzoeksvraag 3) Wat is het effect van fenolen op het homocysteïnegehalte? Een hoog homocysteïnegehalte zou kunnen leiden tot hart- en vaatziekten.

De onderzoeksvragen 1) en 2) van dit proefschrift kunnen we onderbrengen onder het begrip 'biobeschikbaarheid' van fenolen uit de voeding. Maar wat is biobeschikbaarheid en waarom willen we daar iets over weten? Een belangrijke voorwaarde voor een gezonde werking van voedingsstoffen is dat ze in voldoende mate op de juiste plek in het lichaam terecht moeten komen waar ze hun werking kunnen verrichten. Dit heet 'biobeschikbaarheid'. Biobeschikbaarheid omvat meerdere processen in het lichaam (Figuur 2):

a) Het eerste proces is opname van de stof vanuit de darmen in het bloed. Nadat we een voedingsmiddel of een pil doorslikken komt het via de maag in de dunne darm. In de dunne darm kunnen voedingsstoffen worden opgenomen in het bloed.
b) Het tweede proces is verdeling van de voedingsstof over het lichaam. Via o.a. het bloedvatenstelsel worden de voedingsstoffen verdeeld over het lichaam. Op deze manier kunnen voedingsstoffen verschillende plaatsen in het lichaam bereiken.
c) Een derde proces is metabolisme. Metabolisme in het lichaam houdt in dat stoffen uit de voeding in het lichaam worden afgebroken of worden omgezet in andere stoffen. De stoffen waarin de fenolen worden omgezet noemen we metabolieten

van fenolen. De belangrijkste organen waar metabolisme plaatsvindt zijn de dikke darm, de lever en de nieren.

d) Het vierde proces is de uitscheiding van de voedingsstoffen en/of hun metabolieten in de urine of in de ontlasting. Dit is dus een manier van het lichaam om schadelijke, onbruikbare of overtollige stoffen kwijt te raken.



Figuur 2 Schematisch overzicht van biobeschikbaarheid van voedingsstoffen in het lichaam

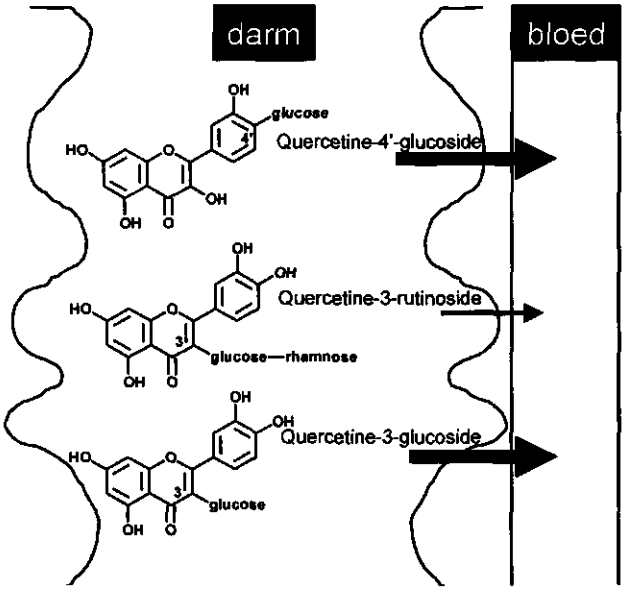
Onderzoeksvraag 1) Worden fenolen opgenomen vanuit de darmen in het bloed en wat is de invloed van de koppeling van fenolen met andere stoffen op de opname?

Fenolen zoals quercetine en koffiezuur komen in de voeding vaak voor gekoppeld met andere stoffen. Omdat deze koppeling invloed kan hebben op de opname van fenolen in het bloed hebben we dit voor quercetine en koffiezuur onderzocht.

Quercetine

Quercetine is in de voeding gekoppeld aan suikers. We weten uit eerder onderzoek dat quercetine wordt opgenomen in het bloed, maar dat het type suiker waaraan quercetine gekoppeld is invloed heeft op de opname. Quercetine waaraan op de 4'-plaats het suiker glucose is gekoppeld (quercetine-4'-glucoside) komt vooral voor in uien en het wordt voor 50% opgenomen in het bloed. Quercetine waaraan op de 3-plaats het suiker rutinose is gekoppeld (quercetine-3-rutinoside) komt vooral voor in thee en wordt maar voor 20% worden opgenomen in het bloed (Figuur 3). Nu is het mogelijk om quercetine-3-rutinoside om te zetten in quercetine-3-glucoside, maar de

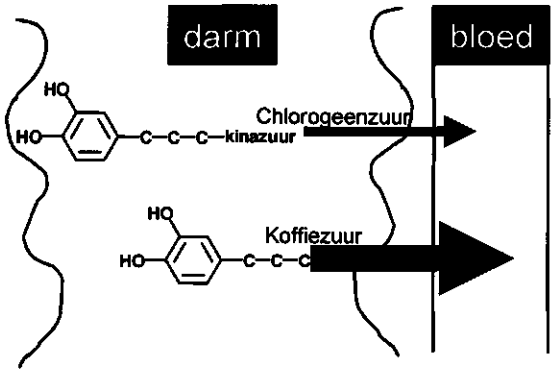
vraag was of dit quercetine-3-glucoside net zo goed opgenomen kon worden als het quercetine-4'-glucoside?
Dit hebben we onderzocht in 9 vrijwilligers. De vrijwilligers namen quercetine-4'-glucoside in op één dag en quercetine-3-glucoside op een andere dag. Na inname hebben we de concentratie quercetine in het bloed en de urine gedurende 24 uur gevolgd. Uit dit onderzoek bleek dat het quercetine-3-glucoside net zo goed opgenomen wordt als het quercetine-4'-glucoside (Figuur 3, *hoofdstuk 2*). Dit betekent dat de beschikbaarheid van quercetine uit thee (quercetine-3-rutinoside) verbeterd wordt als de rutinose wordt omgezet in een glucose. Mocht in de toekomst blijken dat quercetine goed is voor de gezondheid, dan zou het quercetine in onze voeding zo kunnen worden aangepast dat er zoveel mogelijk quercetine vanuit de voeding beschikbaar komt in het lichaam.



Figuur 3 Opname van verschillende vormen van quercetine vanuit de darmen in het bloed: quercetine gebonden aan een glucose wordt beter opgenomen dan quercetine gebonden aan rutinose

Koffiezuur
Koffiezuur is een belangrijke vertegenwoordiger van de kaneelzuren in de voeding. Koffiezuur komt veel voor in koffie, maar in koffie is koffiezuur gebonden aan kinazuur en dan heet het 'chlorogeenzuur'. Wij hebben onderzocht of de koppeling van koffiezuur met kinazuur invloed heeft op de opname vanuit de dunne darm in het bloed in mensen. Dit hebben we onderzocht bij 7 vrijwilligers met een dunne darmstoma. Een stoma is een kunstmatige uitgang voor de ontlasting aan het eind van de dunne darm. Een stoma is nodig als de dikke darm moet worden verwijderd om medische redenen. We hebben voor deze groep vrijwilligers gekozen omdat we

aan het eind van de dunne darm willen meten hoeveel er nog over is van de stoffen die we hebben gegeven. De hoeveelheid stof die is opgenomen vanuit de dunne darm in het bloed kunnen we berekenen uit het verschil tussen de hoeveelheid van de stof die mensen hebben ingenomen en de hoeveelheid stof die we aan het eind van de dunne darm terugvinden.
We vonden dat de opname van chlorogeenzuur (koffiezuur gekoppeld aan kinazuur) 3 keer lager was dan dat van 'vrij' koffiezuur (Figuur 4, *hoofdstuk 3*). Dit betekent dat het kinazuur de opname van koffiezuur remt en dat de opname van chlorogeenzuur uit de voeding zou kunnen worden verbeterd door het kinazuur eraf te halen. Als in de toekomst blijkt dat koffiezuur een gezonde werking heeft, dan zouden voedingsmiddelen zo kunnen worden aangepast dat het koffiezuur wordt losgekoppeld van het kinazuur. Hierdoor wordt het koffiezuur beter beschikbaar.

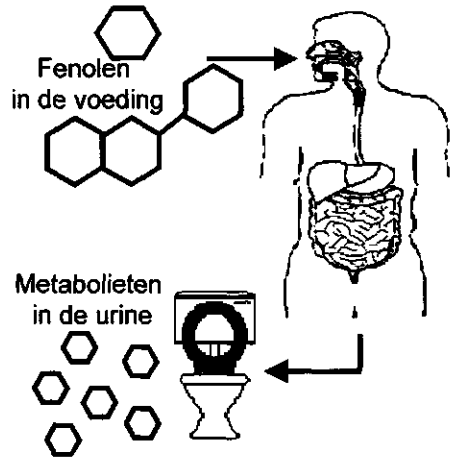


Figuur 4 Opname van chlorogeenzuur en koffiezuur vanuit de darmen in het bloed: koffiezuur wordt beter opgenomen dan chlorogeenzuur.

Onderzoeksvraag 2) Wat gebeurt er met de fenolen in het lichaam? Hiervoor hebben we onderzocht in welke stoffen fenolen worden omgezet in het lichaam. De stoffen die ontstaan uit fenolen noemen we metabolieten van fenolen.

Naast opname van fenolen uit de darmen in het bloed kan het lichaam ook sleutelen aan de fenolen waardoor er andere stoffen ontstaan of waardoor ze worden afgebroken (Figuur 5). De stoffen die ontstaan uit fenolen noemen we metabolieten. De metabolieten van fenolen kunnen ook een fenol-structuur hebben of het zijn stoffen die niet meer op fenolen lijken. Omdat deze metabolieten van fenolen ook in het lichaam circuleren kunnen ze een effect hebben op de gezondheid. Daarom is het belangrijk te weten welke metabolieten er ontstaan uit fenolen. Uit eerder onderzoek is bekend dat fenolen intensief worden omgezet in het lichaam, maar niemand weet precies welke metabolieten er uiteindelijk ontstaan.

In ons onderzoek hebben we onderzocht in welke metabolieten quercetine, chlorogeenzuur en fenolen uit zwarte thee worden omgezet. In ons onderzoek hebben 20 vrijwilligers quercetine, chlorogeenzuur en zwarte thee fenolen ingenomen en daarna hebben wij in de urine van deze vrijwilligers metabolieten van de fenolen gemeten. We vonden dat ongeveer de helft van het chlorogeenzuur en de thee fenolen in het lichaam wordt omgezet in hippuurzuur. Het quercetine werd voor ongeveer de helft omgezet in fenylazijnzuren *(hoofdstuk 4)*.



Figuur 5 Fenolen uit voeding worden in het lichaam omgezet in metabolieten

Tja, en dan....wat zegt dit nu? Om deze vraag te beantwoorden moeten we even terug naar de mogelijke gezondheidseffecten van fenolen. Er wordt gedacht dat fenolen uit voeding gezond zouden kunnen zijn omdat ze sterke antioxidanten zijn. Echter, wij vonden in onze studie dat fenolen die we met de voeding binnenkrijgen door het lichaam worden omgezet in metabolieten die minder goede antioxidanten zijn dan de fenolen uit voeding. Daarom zou de antioxidantwerking van fenolen in het lichaam minder kunnen zijn dan tot nu toe werd gedacht. Helaas kunnen we de antioxidantwerking van voedingsstoffen niet meten in het lichaam, maar in de toekomst wordt dit misschien wel mogelijk.

Onderzoeksvraag 3) Wat is het effect van fenolen op het homocysteïnegehalte? Een hoog homocysteïnegehalte zou kunnen leiden tot hart- en vaatziekten.

Het effect van fenolen op de gezondheid van mensen is nog onbekend. De ideale manier om dit te onderzoeken is om te kijken of in een grote groep mensen die veel fenolen eet minder mensen ziek worden dan in een groep mensen die weinig fenolen eet. Helaas is dit soort onderzoek in de praktijk moeilijk uit te voeren.
Een oplossing hiervoor is het meten van de effecten van fenolen op markers in het lichaam die het risico op ziekte kunnen voorspellen. Een marker is bijvoorbeeld een stof die we kunnen meten in het lichaam en waarvan de concentratie in het lichaam iets zegt over het risico op ziekte. Een voorbeeld van zo'n marker is het homocysteïnegehalte. Homocysteïne is een eiwitdeeltje dat bij iedereen in het bloed aanwezig is. Mensen met hart- en vaatziekten hebben vaker een verhoogd homocysteïnegehalte dan gezonde mensen en daarom wordt gedacht dat een verhoogd homocysteïnegehalte kan leiden tot hart- en vaatziekten. Het homocysteïnegehalte als marker voor hart- en vaatziekten is dus te vergelijken met het cholesterolgehalte, omdat een verhoogd cholesterolgehalte ook kan leiden tot hart- en vaatziekten.
Ons vermoeden dat fenolen een effect hebben op het homocysteïnegehalte, is ontstaan omdat in eerder onderzoek is gevonden dat koffieconsumptie het homocysteïnegehalte verhoogd. Chlorogeenzuur (koffiezuur gekoppeld aan kinazuur) is een belangrijk fenol in koffie en zou daarom dus de oorzaak kunnen zijn van het homocysteïne-verhogende effect van koffie. Wij hebben het effect van chlorogeenzuur op het homocysteïnegehalte gemeten bij 20 gezonde vrijwilligers. Na inname van chlorogeenzuur werd in het bloed van de vrijwilligers het homocysteïnegehalte gemeten. Daarnaast hebben we ook het effect van quercetine en van fenolen uit zwarte thee op het homocysteïnegehalte gemeten bij deze vrijwilligers. We vonden dat chlorogeenzuur en fenolen uit zwarte thee het homocysteïnegehalte verhogen, maar quercetine niet *(hoofdstuk 5)*. Dit betekent dat het chlorogeenzuur in koffie dus deels verantwoordelijk is voor het homocysteïne-verhogende effect van koffie. Naast chlorogeenzuur kunnen nog andere stoffen in koffie aanwezig zijn die het homocysteïnegehalte kunnen verhogen.
Naar aanleiding van het homocysteïne-verhogende effect van chlorogeenzuur en thee fenolen hebben we onderzocht of de omzetting van chlorogeenzuur en thee fenolen in hippuurzuur (*hoofdstuk 4*), misschien kon verklaren op welke manier fenolen het homocysteïnegehalte kunnen beïnvloeden. Namelijk, als blijkt dat de omzetting van chlorogeenzuur en thee fenolen in hippuurzuur de stijging van het homocysteïnegehalte veroorzaakt, dan zouden we dit ook kunnen verwachten van andere fenolen in de voeding die worden omgezet in hippuurzuur. Echter, in ons

onderzoek hebben we gevonden dat de omzetting van fenolen in hippuurzuur waarschijnlijk niets te maken heeft met de stijging in homocysteïne *(hoofdstuk 6)*. Op welke manier fenolen het homocysteïnegehalte wel kunnen beïnvloeden blijft vooralsnog onbekend.
Uit ons onderzoek blijkt dat chlorogeenzuur uit koffie en fenolen uit zwarte thee het homocysteïnegehalte kunnen verhogen. Wat dit betekent voor de gezondheid is nog onduidelijk. Als een verhoogd homocysteïnegehalte leidt tot hart- en vaatziekten, dan zou dit kunnen betekenen dat koffie en thee dus ongezond zijn. Echter, uit grote studies die het verband tussen koffie en thee consumptie en hart- en vaatziekten hebben onderzocht komt niet naar voren dat koffie en thee ongezond zijn. Daarnaast is het ook nog niet zeker of mensen met een verhoogd homocysteïnegehalte inderdaad meer risico hebben op hart- en vaatziekten dan mensen met een normaal gehalte. Een verhoogd homocysteïnegehalte zou ook nog een gevolg kunnen zijn van de ziekte. Momenteel wordt veel onderzoek gedaan naar de vraag of een verhoogd homocysteïnegehalte een risico is voor de gezondheid en over een paar jaar zullen we hierover meer weten. Dus voor nu geldt: geniet van een heerlijk kopje koffie of thee!

Conclusie

In ons onderzoek hebben we gevonden dat fenolen uit de voeding wel worden opgenomen in het bloed, maar dat de hoeveelheid die wordt opgenomen afhankelijk is van de koppeling van de fenolen met andere stoffen.
In het lichaam worden fenolen omgezet in metabolieten en deze metabolieten zijn minder goede antioxidanten dan de fenolen uit voeding zelf. Daarom is de werking van fenolen als antioxidanten in het lichaam waarschijnlijk minder belangrijk dan tot nu toe werd gedacht.
Tenslotte hebben we gevonden dat chlorogeenzuur uit koffie en fenolen uit zwarte thee het homocysteïnegehalte kunnen verhogen. Wat dit betekent voor de gezondheid is nog niet duidelijk.

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Vele handen hebben het werk wat in dit proefschrift beschreven staat licht(er) en plezierig gemaakt!

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Margreet

Curriculum vitae

Margreet Renate Olthof was born on March 19, 1972 in Hardenberg, the Netherlands. In 1990 she passed secondary school, Atheneum, at the 'Jan van Arkel' in Hardenberg. In the same year she started the study 'Human Nutrition' at the former Wageningen Agricultural University. As part of that study she conducted research projects on Human Nutrition (at the former Department of Human Nutrition, Wageningen Agricultural University in collaboration with the University Hospital Nijmegen, Jan - Jul 1994; and at TNO-Nutrition, Sep 1995 – Jan 1996), on Animal Nutrition (at the University of Missouri, USA, Sep 1994 – Feb 1995), and on Epidemiology (at the former Department of Epidemiology and Public Health, Wageningen Agricultural University, Apr – Aug 1995). In 1996 she received the MSc degree (cum laude) and started working as a research assistant at the former department of Human Nutrition of the Wageningen Agricultural University. In February 1997 she started as a PhD-fellow on a collaborative project of the division of Human Nutrition and Epidemiology of the Wageningen University and the State Institute for Quality Control of Agricultural Products (RIKILT), Wageningen, the Netherlands. She joined the education program of the Graduate School VLAG (advanced courses in Food Technology, Agrobiotechnology, Nutrition and Health Sciences). In June 1997 she attended the Annual New England Epidemiology Summer Program at Tufts University, Boston, USA. She was the chair of the PhD-board of the division of Human Nutrition and Epidemiology from 1997 through 1999. She was a member of the PhD-Study Tour committee that organized a two-week study tour to South Africa in 1999. She was selected to participate in the sixth European Nutrition leadership Program (ENLP), Luxembourg.

She is currently working as a research associate on the project 'Diet, homocysteine and cardiovascular disease' at the Wageningen Centre for Food Sciences (WCFS), Wageningen, the Netherlands.

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