Flavonols and Flavones in Foods and their Relation with Cancer and Coronary Heart Disease Risk

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Michaël Gerard Leo Hertog

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Collection State Museum
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Stellingen

1. De berekening van Kühnau in 1976 dat de gemiddelde consumptie van flavonolen, flavonen en flavanonen ongeveer 100 milligram bedraagt is een overschatting als gevolg van gebrekkige gegevens over flavonoïdgehaltes van voedingsmiddelen.

Dit proefschrift

2. De term "non-nutritieve stof" (stof zonder voedingswaarde voor de mens) voor flavonoïden gaat voorbij aan het feit dat flavonoïden een beschermende werking in het menselijk lichaam hebben.

Dit proefschrift

3. Naast vitamine C, vitamine E en carotenoïden leveren ook flavonoïden een belangrijke bijdrage aan het antioxidantgehalte van onze voeding.

Dit proefschrift

4. Behalve alcohol dragen ook flavonoïden in rode wijn bij aan het verlaagd risico voor coronaire hartziekten van rode-wijndrinkers ("The French Paradox").

Frankel EN, et al. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. Lancet 1993;43:454-57

Dit proefschrift

5. De lage sterfte aan coronaire hartziekten in Japan, ondanks het hoge percentage rokers, is niet alleen door een lage gemiddelde consumptie van verzadigd vet te verklaren, maar ook door de Japanse gewoonte om veel (groene) thee te drinken.

Dit proefschrift

6. The possibility - no, probability - of risk factors common to all [non-infectious chronic diseases] must be considered, rather than merely examining factors which might represent risks for one particular disease.

Dennis P. Burkitt. Lessons for health from our paleolithic ancestors. Cardiovascular Risk Factors 1991;1:253-258

7. Das Ethos einer zukünftigen Naturwissenschaft verlangt, daß nicht nur Unehrlichkeit, sondern auch Einäugigkeit als unmoralisch zu gelten hat.

Hans Primas. Umdenken in der Naturwissenschat. GAIA 1992;1:1-15

8. In de meeste Nederlandse kantines is het nog steeds niet mogelijk om een gezonde en smakelijke lunch samen te stellen. Daarom dient het huidige assortiment aangevuld te worden met een uitgebreide keuze aan verse salades (salad bars), zoals het in menig buurland al lang praktijk is.

9. De matige kwaliteit van Nederlandse literaire vertalingen blijkt uit de roman "De Slinger van Foucault" van Umberto Eco waarin de vluchtroute van Casaubon door het nachtelijk Parijs met behulp van de Nederlandse vertaling *niet* na te lopen is, terwijl dat met behulp van de Franse en Duitse vertaling en het Italiaanse origineel wèl mogelijk is.

10. De invoering van het AIO-stelsel met al haar controversen heeft in ieder geval voor AIO's en OIO's het verzinnen van één van de maatschappijkritische stellingen makkelijker gemaakt.

11. Advertenties voor AIO/OIO banen dienen de volgende waarschuwingstext te bevatten: *Promotieonderzoek verrichten is schadelijk voor uw welzijn. Het kan tot slapeloosheid, buikklachten en tot verlies van sociale contacten leiden.*

12. De grootste moeilijkheden ondervinden beginnende basisschoolleerkrachten niet in de omgang met leerlingen, maar vooral in het contact met hun ouders. De PABO's dienen daarom veel meer aandacht te besteden aan het trainen en ontwikkelen van sociale en communicatieve vaardigheden in de omgang met ouders.

13. De grootste energie- en geduldvreters op autowegen zijn de anderen !
(vrij naar J-P Sartre: "L'enfer, c'est les autres...")

14. De wereld gaat aan begrip ten onder.

15. Het publiceren van artikelen en het afronden van een proefschrift zijn de krenten in de promotieonderzoekspap.

Stellingen behorend bij het proefschrift:

Flavonols and Flavones in Foods
and their Relation with Cancer
and Coronary Heart Disease Risk

van Michaël G.L. Hertog

Wageningen, 8 april 1994

Abstract

Flavonols and flavones in foods and their relation with cancer and coronary heart disease risk

Ph.D. thesis, Agricultural University Wageningen, State Institute for Quality Control of Agricultural Products, Wageningen, the Netherlands, and National Institute of Public Health and Environmental Protection, Bilthoven, the Netherlands

Michaël G.L. Hertog

Flavonoids are polyphenolic antioxidants occurring ubiquitously in vegetable foods. Flavonols and flavones inhibit chemically induced tumors in rodents. The flavonol quercetin also inhibits LDL oxidation and platelet aggregation *in vitro*. We therefore decided to investigate the relation between flavonoid intake and cancer and coronary heart disease risk in humans. The three flavonols quercetin, kaempferol, myricetin, and the two flavones luteolin and apigenin were selected because of their anticarcinogenic and antioxidant activities and because of their ubiquitous occurrence in foods. We first developed and validated a HPLC method for the quantitative determination of these flavonoids in foods. We then determined the flavonol and flavone content of 28 types of vegetables, 12 types of fruits and 9 types of beverages commonly consumed in The Netherlands. Quercetin was the main flavonoid and occurred in most fruits, beverages and in some vegetables. Mean intake of flavonols and flavones combined among Dutch adults was 23 mg/day, and main dietary sources of these flavonoids were tea (48 %), onions (38%), and apples (8 %). Mean flavonol and flavone intake of 805 men aged 65-84 years participating in the Zutphen Elderly Study 1985 was 26 mg/day. During five year follow-up period 75 men had a first diagnosis of cancer, of which 28 men had lung cancer. Thirty-four men died from all-cause cancer. Intake of flavonols and flavones in 1985 was not related to subsequent (lung) cancer morbidity (*P* trend 0.54) and mortality (*P* trend 0.51). Between 1985 and 1990 43 men died from coronary heart disease and 38 men had a first myocardial infarction. Intake of flavonols and flavones, expressed as tertiles of intake, was, independently from known risk- and confounding factors, inversely associated with mortality from coronary heart disease (*P* trend 0.015) and incidence of a first myocardial infarction (*P* trend 0.08). Average intake of flavonols and flavones in 16 cohorts participating in the Seven Countries Study around 1960 was also inversely related to mortality from coronary heart disease after 25 years of follow-up, but it was not related to cancer mortality. In multivariate regression analysis including saturated fat intake, flavonoid intake, and percentage of smokers as independent variables about 90 % of the total variance in coronary heart mortality was explained. Flavonol and flavone intake contributed about 9 % to the explained variance.

We conclude that intake of flavonols and flavones may protect against coronary heart disease in humans, but that it does not seem to be an important determinant of cancer risk. However, more experimental, clinical, and epidemiological evidence is needed before firm conclusions on the health effects of these flavonoids can be drawn.

The investigations described in this thesis were carried out at the DLO State Institute for Quality Control of Agricultural Products (RIKILT-DLO), Department of Micronutrients and Natural Toxins, Wageningen, The Netherlands; Wageningen Agricultural University, Department of Human Nutrition, Wageningen, The Netherlands; and the National Institute of Public Health and Environmental Protection, Department of Chronic Diseases and Environmental Epidemiology, Bilthoven, The Netherlands.

The research in this thesis was supported by grants from the Ministry of Agriculture, Nature Management, and Fisheries; the Dutch Commodity Board of Vegetables and Fruits; and the Netherlands Prevention Foundation.

Contents

Chapter 1

General Introduction

There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable.

There is another which states that this has already happened.

Douglas Adams, The Restaurant at the End of the Universe. Pocket Books, New York, USA 1982

General Introduction

Cancer
- Carcinogenesis

Coronary Heart Disease
- Atherosclerosis and thrombosis

Flavonoids
- Chemical structure
- Function in plants
- Occurrence in foods
- Biologic effects
- Flavonoids as vitamin P
- Flavonoids in carcinogenesis
- Flavonoids in atherosclerosis and thrombosis

Scope of the thesis

References

General Introduction

Cancer and coronary heart disease are the most common causes of premature death in the Western world (WHO, 1990), and are important determinants of the quality of life in the elderly (Fries, 1980). Both cancer and coronary heart disease rates vary greatly between different populations and countries. For instance, in Finland coronary heart disease is very common, whereas Japan has very low incidence rates (Uemura and Pisa, 1988). Stomach cancer on the other hand is a common cause of death in Japan, but it is relatively uncommon in Western Europe and the United States (IARC, 1992). Epidemiological studies have shown that migrants rapidly adapt disease patterns from their new home countries suggesting that in addition to genetic factors (nature) also environmental factors (nurture) including life-style factors play an important role in disease etiology (IARC, 1990). Environmental factors are in principle preventable. There is for instance overwhelming experimental, clinical, and epidemiological evidence that smoking is a major risk factor for several types of cancer (Doll, 1976) as well as for coronary heart disease (Rose, 1990). Another apparent important factor influencing cancer and coronary heart disease risk is diet.

At an ILSI conference on Nutrition and Cancer in Atlanta (USA) in 1991, the epidemiologist Doll estimated that about 20 to 60 % of all cancers could be attributed to dietary factors. This shows that there is a lot of uncertainty. The most consistent associations are observed between a high consumption of fruits and vegetables, particularly raw and fresh green-yellow vegetables and fresh fruits, and a reduced risk of cancers at most sites, but particularly cancer of the stomach, colon, rectum, and lung (Steinmetz and Potter, 1991a; Block et al., 1992). Vegetable foods contain a large number of vitamins such as vitamin C, vitamin E, and ß-carotene which, possibly due to their antioxidant capacities, reduce cancer risk (Steinmetz and Potter, 1991b). Recently the oxidation of low-density lipoproteins has been proposed as a key factor in the onset and development of atherosclerosis (Witzum and Steinberg, 1991). Antioxidant dietary vitamins such as vitamin E, vitamin C, and ß-carotene could therefore also reduce coronary heart disease risk (Gey et al., 1993). Much attention is paid in experimental, clinical, and epidemiological studies to the protective effect of these vitamins.

However, vegetable foods also contain a large number of non-nutritive compounds, i.e. substances without apparent nutritive value for humans, but with pronounced biologic activity (Wattenberg, 1990). An important group of non-nutritive substances are *flavonoids*, polyphenols which occur ubiquitously in foods of vegetable origin (Kühnau, 1976). Evidence for a potentially protective effect of some flavonoids, particularly *flavonols* and *flavones*, on cancer and coronary heart disease risk is provided by *in vitro* and *in vivo* studies but their effects in humans are unknown. Epidemiological studies are needed in which the effect of these flavonoids in humans is investigated. However quantitative data on the flavonoid content of foods, needed for an epidemiological evaluation, are lacking. This thesis describes the development of an analytical method for the determination of flavonols and flavones in foods, their content in relevant foods, and the relation between flavonol and flavone intake and cancer and coronary heart disease risk. First, the mechanisms involved in cancer and coronary heart disease development will be summarized. Subsequently, the structure, the occurrence, and the biologic effects of flavonoids will be reviewed. Finally, a description of the outline of this thesis will be presented.

Cancer

Carcinogenesis

Cancer embraces a group of diseases which have in common that a cell is altered in such a way that it escapes growth control and replicates. In this way a normal functioning of the organ in which the cell proliferates is prohibited (IARC, 1990). There is considerable evidence that carcinogenesis is a multistage process which can be initiated and modulated by chemical agents present in the environment (e.g. diet), but also by viruses and ultraviolet radiation (IARC, 1990). Most experimental evidence on the multistage process of carcinogenesis is derived from the mouse-skin model. In general three distinct steps in chemical carcinogenesis are considered: initiation, promotion, and malignant conversion or progression (Figure 1). In the initiation phase irreversible damage to the DNA of a cell is introduced, which provides the cell with a relative growth advantage, possibly due to the activation of proto-oncogenes (Guyton and Kenseler, 1993). In the promotion phase proliferation of the initiated cell is stimulated, possibly by binding to, and activation of, cellular receptors which are involved in growth control, or by inhibition of intracellular communication (Weisburger JH, 1992). Promotion leads to benign growth that can evoluate

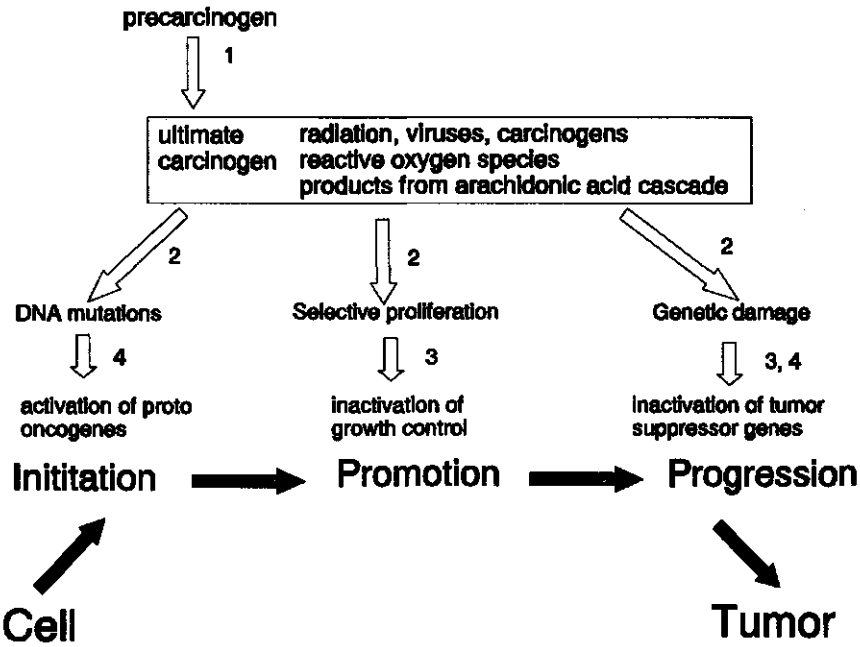

Figure 1. Multistage carcinogenesis. Steps that can be inhibited by anticarcinogens are numbered, see text (adapted from Wattenberg, 1985; Guyton and Kenseler, 1993)

in the third stage, malignant conversion or progression, into cancer. Malignant conversion requires, similarly to initiation, genetic alterations in which growth control is further inhibited, possibly by inhibition of tumor suppression genes (Guyton and Kensler, 1993). It is probable that multiple initiating and promoting events are involved in tumor development and growth. Vogelstein and co-workers presented a multistage genetic model for human colorectal tumor development. In this model at least 5 genetic mutations in affected cells are needed for cancer formation (Vogelstein et al., 1988; Fearon and Vogelstein, 1990). This is roughly consistent with epidemiologic studies which showed that the rate of tumor development is proportional to the fourth to sixth power of the elapsed time (Moolgavkar and Luebeck, 1992). In experimental animal studies it was shown that initiation, promotion, and malignant conversion can be modulated, e.g. stimulated and inhibited, by chemical compounds in the diet. Compounds that inhibit chemically induced carcinogenesis are thus called "anticarcinogens". The mechanisms involved have been extensively reviewed (Wattenberg, 1985; De Flora and Ramel, 1988; Ito and Imaida, 1992) and here only a brief overview will be presented. It should be noted that almost all evidence on the effects of anticarcinogens and the mechanisms involved is provided from experimental (animal) studies and their relevance in humans is unclear.

Due to the pioneering work of Wattenberg anticarcinogens have been classified, mainly upon the moment in the process of carcinogenesis in which they are active (Wattenberg, 1985, 1990): inhibitors of carcinogen formation[1]; carcinogen scavengers[2]; agents that inhibit promotion and/or malignant conversion[3]; and agents that stimulate DNA repair mechanisms[4] (see also numbering in Figure 1). Most compounds show several mechanisms of action. Inhibitors of carcinogen formation are substances that stimulate the activity of Phase I and Phase II enzymes, which are important in deactivating potential toxins including carcinogens. Phase I enzymes are microsomal mono-oxygenases such as the P450 family of enzymes. Phase II enzymes conjugate products from Phase I transformation with for instance glutathion, thus making carcinogens more hydrophilic and more easily excreted (Wattenberg, 1985). For instance the activation of the precarcinogen benzo(a)pyrene to its active metabolite (ultimate carcinogen) is inhibited and its excretion is enhanced by induction of especially the Phase II conjugating enzymes (Wattenberg, 1987). Carcinogen scavengers trap ultimate carcinogens by binding and forming inactive complexes. Polycyclic aromatic hydrocarbons are thus inactivated (Wattenberg and Loub, 1978). Antioxidants trap reactive oxygen species which are thought to be involved in promotion and malignant conversion (Guyton and Kensler, 1993). In addition, products from the arachidonic acid cascade such as prostaglandins and leucotriënes stimulate promotion and possibly progression (Fürstenberger et al., 1989). Anti-inflammatory agents which inhibit the arachidonic acid cascade may thus inhibit promotion and malignant conversion (Fischer et al., 1982).

Coronary Heart Disease

Coronary heart disease, also called ischemic heart disease, is a group of disorders characterized by an insufficient flow of blood through the coronary arteries which can result in death of the heart muscle cells. It is caused by a narrowing of the coronary arteries by atherosclerotic plaques or by the formation of a thrombus in an atherosclerotic coronary artery which blocks the lumen (Guyton, 1986).

Atherosclerosis and thrombosis

The development of atherosclerosis (Figure 2) is currently best explained by the response-to-injury hypothesis forwarded by Ross in 1973, modified in 1986, and in 1993 (Ross and Glomset, 1973; Ross, 1986; *idem*, 1993). Injury or inflammation of the endothelial cells in arteries causes the release of growth factors that stimulate platelet aggregation, early thrombosis, and the migration of monocytes and T-lymphocytes to the inner layer of the arterial wall. The monocytes then become macrophages which accumulate cholesteryl esters to form so-called "foam cells". These foam cells build together with the T-lymphocytes and smooth muscle cells, the fatty streak (Ross, 1993). Recently, the oxidative modification of low-density lipoproteins (LDL) has been proposed as a key factor in the initiation and the development of atherosclerosis (Steinberg et al., 1989). Oxidation of LDL is a free radical process known as lipid peroxidation, which causes the oxidation of the unsaturated fatty acids in LDL. In vitro studies show that LDL can be oxidatively modified by endothelial cells, arterial smooth muscle cells, and macrophages (Witzum and Steinberg, 1992). Recent investigations suggest that these modifications may indeed occur *in vivo* (Yla-Herttuala et al.. 1989; Palinski et al., 1989). However the exact nature of the free radical species involved *in vivo* is not known. Chain-breaking antioxidants can inhibit the oxidative modifications of LDL

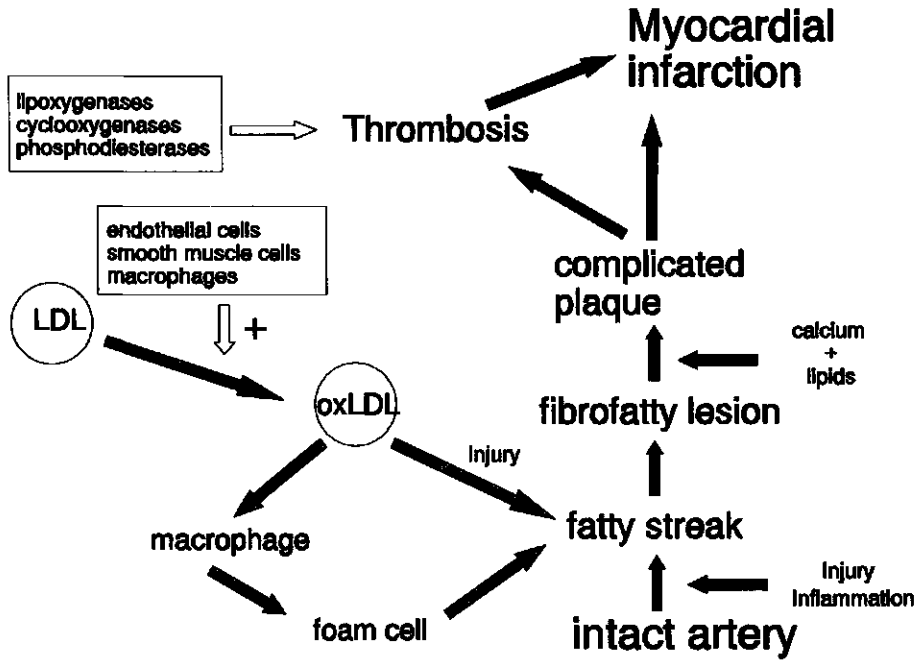

Figure 2. LDL oxidation, atherosclerosis, and thrombosis (adapted from Steinberg et al (1989) and Ross (1993)

by scavenging reactive oxygen species (Esterbauer et al., 1993). Oxidized LDL is, in contrast to native LDL, rapidly taken up by macrophages which convert them into foam cells (Figure 2). Oxidized LDL is also cytotoxic which may cause local endothelial injury, thus favoring the penetration of monocytes and accelerating the formation of the fatty streak (Steinberg, 1989).

The fatty streak can progress into an intermediate fibrofatty lesion by increased migration of monocytes and proliferation of smooth muscle cells. The fibrofatty lesion consists mainly of smooth muscle cells, elastic fibre proteins, collagen, and proteoglycans (Ross, 1993). The fibro-fatty lesions develop further into fibrous plaques, which often become calcified by precipitation of calcium with lipids. The atheromous plaque which is thus produced narrows by itself the lumen of the artery, but is also prone to rupture with subsequent exposure of its content to the flowing blood. The rupturing or fissuring of plaques leads to platelet aggregation which can lead to occlusive thrombosis, the so-called complicated plaque (Ross, 1993). Occasionally, the thrombotic clot breaks away and flows (embolus) to a more peripheral branch of the coronary arterial tree where it blocks the artery (Fuster et al., 1992). Coronary thrombotic occlusions of sufficient size cause an acute myocardial infarction. Thrombosis is a process that is closely controlled by a large number of enzymes, including phosphodiesterases, lipoxygenases, and cyclooxygenases, but the controlling mechanisms are only poorly understood. The activity of these enzymes can be modulated by several (dietary) compounds, which leads to altered platelet aggregation and thrombotic tendencies (Badimon et al., 1993).

Flavonoids

Chemical structure

Flavonoids share the common skeleton of diphenylpyrans (C6-C3-C6), e.g. two benzene rings (A and B) linked through a heterocylic pyran or pyrone ring (C) (Figure 3) (Kühnau, 1976). Flavonoids comprise one of the large groups of secondary plant metabolites occurring widely throughout the plant kingdom, including food plants. Flavonoids consist of flavonols, flavones, flavanones, anthocyanidins, catechins, and biflavans (Table I). Over 4000 different types of flavonoids have been described and this number is today still increasing (Markham, 1989).

Of particular importance in this review are flavonols and flavones because of their potentially protective role in carcinogenesis, atherosclerosis, and thrombosis. In common with other flavonoids the most frequently found flavonols and flavones are those with B-ring hydroxylation in the 3'- and 4'-positions (Herrmann, 1988). Flavones lack the hydroxygroup at C3 that characterizes the flavonols. Quercetin and kaempferol are typical flavonols, the corresponding flavones being luteolin and apigenin respectively (Figure 3). Flavonols and flavones occur in foods usually as *O*-glycosides, with D-glucose as the most frequent sugar residue. Other sugar residues are D-galactose, L-rhamnose, L-arabinose, D-xylose, as well as D-glucuronic acid. In general the sugars with D-series occur as the ß-glycoside, whereas the L-series occur in the α-configuration. The preferred binding site of the sugar residues are C3 and less frequently the C7-position (Herrmann, 1976, 1988). The sugar-free part of the flavonoid molecule is called the aglycone.

Table 1. Different types of flavonoids (see also Figure 3) and their determinants in the diet (adapted from Kühnau, 1976)

Compound	Basic Structure	Typical food	Representative (aglycon)	Estimated intake in the US
Flavonols	3-hydroxyflavonoids	vegetables, fruits	quercetin	160 mg = flavonols, flavones, and flavanones
Flavones	3-desoxyflavonoids	vegetables, citrus fuits	apigenin	
Flavanones	3-desoxyflavonoids (no C2-C3 double bound)	citrus fruits	hesperitin	
Anthocyanidins	4-desoxyflavonoids (no C2-C3 double bound)	berries, colored fruits	cyanidin	180 mg
Catechins	3-hydroxyflavonoids (no C2-C3 double bound)	tea, wines	epigallocatechin	220 mg
Biflavans	catechin and flavandiol dimers	fruits	proanthocyanidin	460 mg

Due to the variation in hydroxylation and glycosidation patterns more than 1000 different flavonols and flavone glycosides have been described. These flavonoids share the common structure of a few hundred types of flavonoid aglycones. Most of these flavonoids occur only specifically in individual plant species (Markham, 1982).

Function in plants

Several functions of flavonoids in plants have been either demonstrated or proposed. These include: protection of plants from UV light, insects, fungi, viruses, and bacteria; pollinator attractors; plant hormone controllers; and enzyme inhibitors (Markham, 1989). The ubiquitous occurrence of flavonoids in higher plants and their rapid turn-over suggest that flavonoids are indeed important to plants. In foods flavonoids may play important roles as natural colorants, flavoring compounds (bitterness), and protection against oxidation (Singleton, 1981). Because of their specific occurrence in (plant) foods, flavonoids are often used for taxonomic purposes and for the tracking down of adulterations (Markham, 1989).

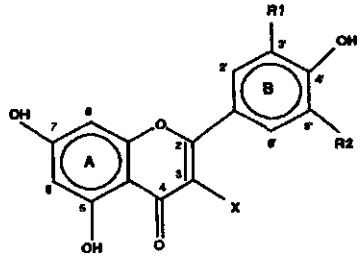

Figure 3. Flavonols: X=OH; Quercetin: R1=OH, R2=H; Kaempferol: R1=H,, R2=H; Myricetin: R1=OH, R2=OH. Flavones: X=H; Apigenin: R1=H, R2=H; Luteolin: R1=OH, R2=H

Occurrence in foods

The occurrence of flavonols and flavones in foods has been reviewed by Herrmann (1976, 1988). Flavonols and flavones occur mainly in the leaves and in the outer parts of the plants, while only trace amounts are found below the soil surface. An exception are onions which contain a large amount of quercetin 4'-D-glucosides. In vegetables quercetin glycosides predominate, but glycosides of kaempferol, luteolin, and apigenin are also present. Fruits almost exclusively contain quercetin glycosides (Herrmann, 1988). Kühnau estimated in 1976 that daily flavonoid intake in the US was approximately 1 gram of which about 100 mg (expressed as aglycons) consisted of flavonols and flavones (see also Table I) (Kühnau, 1976). However, so far little attention has been paid to quantitative aspects of the determination of flavonols and flavones in foods, e.g. completeness of extraction and hydrolysis, and analyses involved mostly whole foods including edible and non-edible parts. Furthermore the quantitative data published were mainly obtained with thin-layer chromatography followed by a spectrophotometric measurement. This analytical method is today considered obsolete and it may have resulted in an over- or underestimation of the flavonoid content of foods.

Biologic effects

The history of flavonoids has been characterized by controversies regarding their biological effects and significance. Around 1940 flavonoids were thought to have vitamin properties. In the seventies flavonoids were suspected mutagens and carcinogens, whereas in the eighties much attention was paid to their antimutagenic and anticarcinogenic activities. Finally, in recent years the antioxidant capacities of flavonoids and their potential role in both, inhibition of LDL oxidation and platelet aggregation was reported. In the following a short review of the biologic effects of flavonoids will be presented. Other effects of flavonoids have been reported such as immune-stimulating effects, anti-allergic effects, anti-viral effects, estrogenic activity, and antidiarrhetic activity (Singleton, 1981; Mabry et al., 1984; Middleton and Kandaswami, 1992). However, it is beyond the scope of this overview to summarize all effects of flavonoids that have been reported.

Flavonoids as vitamin P

Already in 1936 Szent-Györgyi and co-workers reported that flavonoid preparations from citrus peel and paprika could heal scorbutic pigs where vitamin C alone did not (Bentsáth et al., 1936). Flavonoids had especially strong inhibitory effects on the permeability of capillaries and Szent-Györgyi and coworkers coined the term vitamin P (for permeability) for flavonoids (Bentsáth et al., 1937). However, due to subsequent contradictory results obtained by other scientists, the claims that flavonoids were indispensable like vitamins were not substantiated and the term vitamin P was dropped in 1950. As it was clear that flavonoids showed biologic activities the term "bioflavonoid" was introduced (Singleton, 1981). This term is today still in use although not universally. In subsequent years the studies on the effects of flavonoids, mainly citrus flavonoids, were directed to the vascular system, particularly on the capillary fragility and increase of the resistance of normal capillaries to trauma's (Singleton, 1981). Much attention was also paid to the vitamin C sparing activity

of flavonoids (Kühnau, 1976). However no clear clinical evidence on the effects of flavonoids in humans was presented.

Flavonoids in carcinogenesis

Renewed attention was paid to flavonoids in 1965 when the potentially carcinogenic effects of bracken fern (*Pteridium aquilinium*) was reported (Evans and Mason, 1965). Two major flavonoids, e.g. quercetin and kaempferol present in bracken fern were suspected to be the major carcinogens in this plant. Quercetin was mutagenic in several *in vitro* short term tests (Nagao et al., 1991). However quercetin did not show genetic toxicity in several *in vivo* tests (Yoshida et al., 1980; Aeschbacher et al., 1982). Some evidence for carcinogenicity of quercetin was shown in two studies from the same laboratory (Pamucku et al., 1981; Ertürk et al., 1981), in which quercetin at 0.1 % in the diet induced bladder and liver tumors in rats. Between 1981 and 1983 three large long-term experimental animal studies were carried out in which quercetin or its glycoside rutin were administered in levels between 0.1 % and 10 % of the diet to several strains of mice, rats, and hamsters. All of these studies failed to find any carcinogenic activity of these flavonoids (Saito et al. 1980; Hirono et al., 1981; Morino et al. 1982). Quercetin and other flavonoids are now generally considered to be not carcinogenic. This is supported from findings in 1984 that a compound isolated in bracken fern named Ptaquilosid, is probably responsible for the carcinogenic activity of bracken fern (Hirono et al., 1984).

Equally around 1985 Wattenberg suggested that flavonoids, particularly flavonols and flavones, could have important antimutagenic and anticarcinogenic effects (Wattenberg, 1985). Quercetin and other hydroxylated flavonoids inhibited the metabolic activation of carcinogens by modulation of the activity of detoxifying enzymes; formed inactive complexes with ultimate carcinogens; acted as scavenger of reactive oxygen species; and inhibited arachidonic acid metabolism (see also Figure 1). The anticarcinogenic and antimutagenic effects of flavonoids have been reviewed in 1992 by Huang and Ferraro, therefore only a short summary will be presented here. Topical application of quercetin and other flavonoids inhibited rat skin tumor promotion induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Kato et al., 1983) (Wei et al., 1990), possibly by inhibition of epidermal ornithine decarboxylase activity. Quercetin and other flavonoids also inhibited 7,12-dimethylbenz(a)anthracene-, benzo(a)pyrene-, 3-methylcholanthrene-, and *N*-methyl-*N*-nitrosourea-induced skin tumorigenesis in mice (Mukhtar et al., 1988). Of particular interest are two studies in which the effect of dietary administered flavonols were investigated. Verma and co-workers reported that dietary quercetin inhibited tumor initiation by 7,12-dimethylbenz(a)anthracene (DMBA) and tumor promotion with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in the mouse skin (Verma et al., 1988). By using an experimental model of colon cancer, Deschner and co-workers showed that under low fat intake, dietary quercetin and rutin suppressed hyperproliferation of colonic epithelial cells and ultimately colon tumor incidence (Deschner et al., 1991). The results of these animal studies thus suggest that flavonols and flavones may inhibit tumor development in human beings. However, the anticarcinogenic effects of these flavonoids in humans have not been studied.

Flavonoids in atherosclerosis and thrombosis

Flavonoids are strong antioxidants and oxidant damage is thought to play an important role in atherogenesis. The antioxidant effects of flavonoids have been repeatedly investigated and reviewed (Takahama, 1985; Bors and Saran, 1987; Cotelle et al., 1992; Limasset et al., 1993). In general optimum antioxidant activity of flavonoids is associated with multiple phenolic groups (especially 3' and 4' hydroxygroups), a carbonylgroup at C-4; and free C3 and C5 hydroxygroups (Robak et al., 1988). Flavonols such as quercetin which combines these features scavenged superoxide anions (Robak and Gryglewski, 1988), hydroxyl radicals (Husain et al., 1987), lipid peroxy radicals (Sorata et al., 1982), and they formed ligands with metal ions (Takahama, 1985). However, quercetin and myricetin did also show pro-oxidant actions *in vitro* in the presence of Fe^{3+} (Laughton et al., 1989). Flavonoids inhibited LDL oxidation by macrophages *in vitro*, probably by protecting α-tocopherol in LDL from being oxidized by free radicals, by reducing the formation of free radicals in the macrophages or by regenerating oxidized α-tocopherol (De Whalley et al., 1990). Quercetin also reduced the cytotoxicity of oxidized LDL, whereas flavones such as apigenin were completely ineffective (Negre-Salvagyre et al., 1992). Although the precise mechanisms are unknown, quercetin probably blocks the generation of the intracellular cytotoxic signals, possibly by inhibition of enzymes that are involved in signal transduction. Frankel and co-workers showed that phenolics, including quercetin, extracted from red wine inhibited copper-catalyzed oxidation of human LDL *ex vivo* (Frankel et al. 1993).

Flavonoids also affect the activity of enzymes that are involved in inflammatory processes, blood platelet aggregation, and platelet adheration to vascular walls. However, the exact mechanisms involved in the inhibition of inflammatory responses and their relation with platelet aggregation are only poorly understood. The flavonols quercetin and rutin showed for instance to be modest inhibitors of platelet aggregation in platelet-rich plasma *in vitro*, but they are powerful anti-platelet agents *in vivo* (Gryglewski et al., 1987). Flavonols are in general relatively strong inhibitors of lipoxygenases, whereas they are only modest inhibitors of cyclooxygenases (Moroney et al., 1988; Laughton et al., 1991). Flavonols were also reported to stimulate cyclooxygenases when arachidonic acid was used a substrate (Robak et al., 1988). Possibly flavonols inhibit phosphodiesterases resulting in a rise of cyclic AMP. Cyclic AMP potentiates the antiaggregating effects of prostacyclin (Landolfi et al., 1984; Mower et al., 1984). It was also reported that quercetin reduces the availability of free intracellular CA^{2+}, probably by inhibiting CA^{2+} -dependent ATPase (Beretz et al., 1982). The authors speculate that the reduced availability of CA^{2+} could prevent the activation of actomyosin of the cytoskeleton which is needed for platelet aggregation. Flavonols bind *in vitro* and *in vivo* to platelet membranes and might inhibit the interaction of activated platelets with vascular endothelium (Gryglewski et al., 1987). In addition flavonols, due to their antioxidant activity, inhibit locally destruction of endothelial prostacyclin and endothelium-derived relaxing factor (EDRF) by lipid peroxides which are generated by activated platelets. Prostacyclins and EDRF both inhibit platelet aggregation and have vasodilatory activity (Gryglewski et al., 1987). Quercetin showed indeed vasodilatory effect on the isolated rat aorta (Duarte et al., 1993). In summary, the antithrombotic effects of these flavonoids could be mediated through at least two or three different mechanisms. Evidence on the effects of flavonoids on atherosclerotic complications and thrombotic tendencies in humans is lacking.

Scope of the thesis

In 1989, as this project was designed, its primarily aim was to evaluate the protective effects of non-nutrients on cancer risk. We organized a workshop with experimentalists, epidemiologists, and food chemists in the spring of 1990 in which priorities were set for an epidemiologic evaluation of non-nutritive anticarcinogens (see summary in appendix I). It was concluded that flavonoids were the most promising compounds to study in an epidemiological context. In brief, this choice was based on three previously defined requirements: strong evidence on their anticarcinogenic capacities, their ubiquitous occurrence in vegetables and fruits, and their relatively uncomplicated analytical determination in foods. In the course of 1992 accumulating evidence on the potential role of antioxidants in the inhibition of LDL-oxidation led us to extend the original hypothesis. Some flavonoids, including the ones we investigated are potent antioxidants and inhibit LDL oxidation *in vitro*. Intake of these flavonoids may therefore also reduce coronary heart disease risk.

The two main groups of flavonoids that have been investigated for their biologic effects are flavonols and flavones. Both are present in most vegetables, whereas fruits contain mainly flavonols. Quercetin is the major flavonol and we decided to concentrate specifically on quercetin. Kaempferol and myricetin were added because they are structurally very similar to quercetin in that all three possess a double bond at C2-C3 and a free hydroxygroup at C3. These flavonoids differ only with respect to the number of hydroxygroups (Figure 3). Studies on structure-activity relations for anticarcinogenic and antioxidant activity have indicated that the presence of a free hydroxygroup at C3 was needed although this was not unequivocal (Takahama, 1985; Bors and Saran, 1987; Cotelle et al., 1992; Limasset et al., 1993). We therefore also included the two major food flavones luteolin and apigenin, which lack the C3 hydroxygroup, in our analysis.

As pointed out before no analytical method and no quantitative data on the occurrence of these flavonoids in foods were available. We decided to develop and validate a method for the determination of flavonols and flavones in foods, and to establish a food composition table on these flavonoids which could be used to determine flavonoid intake in humans. Individual intake of flavonols and flavones would then be related to cancer and coronary heart disease risk in a prospective study (The Zutphen Elderly Study). In addition, we investigated the contribution of flavonols and flavones to cross-cultural differences in cancer and coronary heart disease mortality rates (The Seven Countries Study).

A step-by-step procedure was formulated at the start of the study which is summarized in the following aims:

- Develop a validated method for the quantitative determination of quercetin, kaempferol, myricetin, apigenin, and luteolin in foods of vegetable origin (*Chapter 2*)
- Determine the content of these five flavonoids in vegetables, fruits (*Chapter 3*), and beverages (*Chapter 4*) commonly consumed in The Netherlands.
- Calculate mean intake of these flavonoids combined in humans and its determinants (*Chapter 5*)
- Relate flavonoid intake to risk of cancer (*Chapter 6*) and coronary heart disease

(*Chapter 7*) risk in a prospective cohort study (The Zutphen Elderly Study)
- Determine the contribution of flavonoid intake to cross-cultural differences in cancer and coronary heart disease mortality rates in The Seven Countries Study (*Chapter 8*)

A general discussion, conclusions including suggestions for further research, and an epilogue are described in *Chapter 9*.

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Chapter 2

Analytical Method for the Determination of Flavonols and Flavones in Foods

This chapter is based mainly on:

Hertog, M.G.L.; Hollman, P.C.H.; Venema, D.P.
Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. *Journal of Agricultural and Food Chemistry*, 1992, 40, 1591-1598.

ABSTRACT

A rapid method based on RP-HPLC with UV detection is presented for the quantitative determination of five major flavonoid aglycones viz. quercetin, kaempferol, myricetin, luteolin, and apigenin in freeze-dried vegetables and fruits, after acid hydrolysis of the parent glycosides. Completeness of hydrolysis and extraction have been optimized by testing systematically different conditions such as acid concentration, reaction period and methanol concentration in the extraction solution using samples containing various types of flavonoid glycosides. Optimum hydrolysis conditions are presented for flavonol glucuronides, flavonol glucosides and flavone glycosides. Identity of the flavonoids was confirmed with diode-array. Repeatability of the method was good with coefficients of variation (CV) ranging from 2.5-3.1% for quercetin, 4.6-5.6% for kaempferol, 4.6% for myricetin, 3.3% for luteolin and 2.8% for apigenin. CV of the within-laboratory reproducibility was less than two times the CV of repeatability. Recoveries of the flavonols quercetin, kaempferol and myricetin ranged from 77-110 % and recoveries of the flavones apigenin and luteolin ranged from 99-106%. The method presented allows a fast, quantitative and reproducible determination of five flavonoids in freeze-dried foods.

INTRODUCTION*

Flavonoids are polyphenols present in foods of vegetable origin. Quantitative data on the occurrence of flavonoids in foods, which are needed for an epidemiological evaluation of flavonoids is missing (See General Introduction). Furthermore, the data that have been published were mainly based on thin-layer chromatography and spectrophotometric measurements. Recently, Bilyk and co-workers published results of flavonoid analyses in foods based on HPLC and UV-detection (Bilyk *et al.*, 1984; Bilyk and Sapers, 1985, 1986). However, hydrolysis conditions were not optimized in these studies. Quantitative determination of individual flavonoid glycosides in foods is difficult because most reference compounds are not commercially available. Furthermore, more than 50 different glycosides of the most common flavonoids have been described (Herrmann, 1988). Hydrolysis of all glycosides to aglycones offers a practical method for the quantitative determination of flavonoids in foods. Hydrolysis of flavonoids with HCl has been described by Harborne (1965) who performed acid hydrolysis with 2.0 M HCl in boiling 50% aqueous methanol (v/v). Under these conditions flavonol 3-*O*-glucosides are hydrolysed completely within a few minutes whereas complete hydrolysis of flavonol 3,7 and 4'-*O*-glucuronides takes 60-250 minutes. Based on data from the literature (Bilyk and Sapers, 1985; Herrmann, 1988) we selected five food samples viz. lettuce, endive, cranberries, onions and leek in which the three major flavonols quercetin, myricetin and kaempferol occur both as the rapidly hydrolysable glycosides, mainly glucosides and the slowly hydrolysable glucuronides. In lettuce quercetin-3-D-glucuronide predominates and luteolin-3-D-glucuronide is present in trace quantities. In endive kaempferol-3-D-glucuronide predominates. In cranberries high concentrations of quercetin- and myricetin-3-D-glucosides are present. Onions have an exceptionally high content of quercetin-4'-D-glucosides whereas leek contains kaempferol-3-D-glucosides. Celery, containing the flavones apigenin- and luteolin-7-apiosyl-D-glucosides, was used as an additional food sample.

In this paper we present a HPLC method for the determination of the above mentioned flavonoids in freeze-dried vegetables and fruits. Extraction and hydrolysis conditions have been systematically optimized for every food sample by varying acid concentration and reaction period. The effect of flavonoid and glycoside type on hydrolysis conditions could thus be investigated.

MATERIALS AND METHODS

HPLC

Chromatographic separations were performed on a Nova-Pak C18 (WATERS Associates, Milford, MA) column (3.9 x 150 mm, 4 um) protected by a Perisorb RP-18 (3.9 x 40 mm, 30-40 um) guard column. Both columns were placed in a column oven set at 30°C. The HPLC system consisted of a KRATOS (KRATOS Analytical Systems, Ramsey, NJ) SPECTROFLOW 400 solvent pump controlled by a KRATOS SPECTROFLOW 450 solvent programmer and a LINEAR (LINEAR Inst. Corp. Reno,

* section shortened for publication in this thesis

NV) model 204 UV/VIS detector set at 370 nm. A MARATHON (Spark Holland, Emmen, the Netherlands) autoinjector was used with a fixed 10 uL loop. Two mobile phases were used. The first mobile phase consisted of 25% acetonitrile in 0.025 M KH_2PO_4 (pH 2.4) with a flow-rate of 0.9 mL/min (eluent I). However, since inadequate separation of quercetin and luteolin was achieved, a second eluent consisting of 45% methanol in 0.025 M KH_2PO_4 (pH 2.4) with a flow-rate of 0.9 ml/min was used (eluent II). Detector output was sampled using a NELSON (PE NELSON, Cupertino, CA) series 900 interface and NELSON integrator software (model 2600, rev. 5.0). Quantification was based on peak area as determined by NELSON. Eluent I was used for quantification of the compounds whereas eluent II was used for additional peak identification. However, when both quercetin and luteolin were present eluent II was used for quantification of these flavonoids. A Hewlett-Packard (Palo Alto, CA) Model 1040 A photodiode-array UV-visible detector was used to record UV-spectra of the flavonoids in samples on-line. Spectra were recorded upslope, apex and downslope (220-450 nm, 2 nm steps, sampling interval 1280 ms). The spectra of each peak were superimposed after substraction of the corresponding base line spectrum. Peaks were considered to be pure when there was exact correspondence among the spectra (peak purity match >990). Similarly, peak identity was confirmed by superimposing the spectrum of each peak with the corresponding standard spectrum (peak identity match >990) and by comparison of retention times (time window: 0.5 %) in both eluents. Peaks were only quantitated if they matched the above mentioned criteria.

Sample preparation

Fresh lettuce (*Lactuca sativa* L), leek (*Allium porrum* L), celery (*Apium graveolens* L), onions (*Allium cepa* L), endive (*Chicorium endivia* L) and cranberries (*Vaccinium macrocarpon* Ait.) were purchased in December at a local supermarket and prepared the same day. The whole foods were cleaned, chopped under liquid nitrogen and immediately stored at -20 °C until lyophilized (DELTA, condenser temp -45 °C, pressure 0.01 mbar). The outer dry skin of onions was removed before cleaning. After lyophilisation the freeze-dried tissues were ground to pass a 0.5 mm sieve and allowed to equilibrate in open air. Percentage of moisture was measured (80 °C, vacuo). The food samples were stored at - 20 °C until analyzed.

Standards

Flavonoid standards were purchased from FLUKA (myricetin #70050, quercetin dihydrate #83370, kaempferol #60010 and apigenin #10790) and from ROTH (luteolin #5801). The standards were dissolved in methanol to a concentration of 500 ug/mL and stored at 4 °C. Every week stability of the compounds in methanol was checked spectrophotometrically at 375 nm (flavonols) and 340 nm (flavones) after dilution to 10 ug/mL in methanol. Standard solutions proved to be stable for over three months at 4°C. An exception was myricetin which deteriorated by approximately 10% after one month. Calibration curves of the standards ranging from 0.5 ug/mL to 25 ug/mL were constructed for both eluents. As peak height, peak shape and retention time are dependent on the

composition of the injection solution we decided to match standard and sample solution. Standard stock solutions were diluted in 20 mL 62.5% aqueous methanol to which 2 g/L of antioxidant *tert*-butylhydroquinone (TBHQ) was added. To this solution 5 mL of 6 M HCl was added and subsequently made up to 50 mL with methanol. All compounds had linear calibration curves (peak area vs. concentration) through the origin. R squared values exceeded 0.9995. The limit of detection was defined as the amount of flavonoid which resulted in a peak-height three times the standard deviation of the baseline noise.

Table I. Retention time $t_{(R)}$, capacity factors (k'), and plate numbers of five flavonoids

Peaks	Compound	Eluent I			Eluent II		
		$t_{(R)}$ (min)	k'	plate number	$t_{(R)}$ (min)	k'	plate number
1	myricetin	3.08	2.1	4080	3.45	2.5	1325
2	quercetin	5.78	4.8	5670	6.34	5.3	2080
3	luteolin	5.65	4.7	5330	7.88	6.9	2600
4	apigenin	10.32	9.3	5625	12.9	11.9	3355
5	kaempferol	11.52	10.5	5554	11.53	10.5	3125

Eluent I: 25% acetonitrile in 0.025 M phosphate buffer (pH 2.4). Eluent II: 45% methanol in 0.025 M phosphate buffer (pH 2.4). Detection at 370 nm, 0.01 a.u.f.s. Flow-rate 0.9 mL/min

Extraction and hydrolysis

Unless otherwise stated extracts were prepared as follows: 40 mL of 62.5 % aqueous methanol (2 g/L TBHQ) was added to 0.500 grams of freeze-dried sample material. To this extract 10 mL of 6 M HCl was added and mixed carefully. The extraction solution thus obtained, consisted of 1.2 M HCl in 50% aqueous methanol (v/v). After refluxing at 90 °C for two hours with regular swirling, the extract was allowed to cool and was subsequently made up to 100 mL with methanol and sonicated for 5 minutes. Approximately 2 mL were filtered through a .45 um filter for organic solvents (Acrodisc CR PTFE, Gelman) prior to injection.

RESULTS

Eluent I and eluent II, described under the HPLC section, were both tested for separation efficiency. Standard working solutions were made by diluting the five stock solutions to 5 ug/mL as described before. The working solutions were injected onto the column which was previously equilibrated with the eluent for 60 min.

As can be seen from Table I eluent I yielded, with similar capacity factors, much higher plate numbers compared to eluent II. Interestingly, elution order of the pair luteolin and quercetin and the pair apigenin and kaempferol is reversed in eluent I compared to eluent II indicating a different selectivity of both modifiers for flavonols and flavones.

Analyses of samples revealed that eluent I resulted in less interfering peaks compared to eluent II (Figure 2,6). However, with eluent I quercetin and luteolin could not be separated. Therefore, we decided to apply eluent I for quantification, eluent II for additional peak identification and for quantification only when both, quercetin and luteolin were present. However, as we did not found both quercetin and luteolin in the same product, this proved not to be necessary for the food samples investigated in this study

Wildanger and Herrmann (1973) reported the use of 1% sulphuric acid in methanol for hydrolysis of flavonoid glycosides in plant materials. Accordingly, we investigated the effects of sulphuric acid and hydrochloric acid on hydrolysis of quercetin-3-D-glucuronides in lettuce. Lettuce was prepared and analyzed as described before, taking care that the water/methanol ratio was kept equal for both acids. As can be seen from Table II, higher yields were found with HCl compared to H_2SO_4. All further hydrolysis experiments were thus carried out with hydrochloric acid

Table II. Influence of reaction period, acid type and acid concentration on quercetin yield[a] in lettuce.

Reaction period	H_2SO_4		HCl	
	0.3 M	0.6 M	0.6 M	1.2 M
1 hour	70 mg/kg 27%	141 mg/kg 55%	174 mg/kg 68%	216 mg/kg 85%
2 hours	108 mg/kg 42%	198 mg/kg 78%	185 mg/kg 73%	230 mg/kg 90%
4 hours	134 mg/kg 53%	209 mg/kg 82%	204 mg/kg 80%	245 mg/kg 96%
6 hours	181 mg/kg 71%	231 mg/kg 90%	209 mg/kg 82%	255 mg/kg 100%

Results are expressed in mg/kg dry weight and as percentage of maximum yield found in this sample.
[a] Mean for duplicate determination

Table III. Influence of percentage methanol and a reaction period of 2 or 4 hours in extraction medium on flavonoid yield (mg/kg dry weight) in three food samples.

Food	methanol	20 %		50 %		80 %	
	reaction period	2 hr	4 hr	2 hr	4 hr	2 hr	4 hr
				mg/kg dry weight			
onion	quercetin	5009	4746	5238	4775	4443	3091
endive	kaempferol	--	--	170	222	133	1161
celery	luteolin	--	--	153	296	154	256
	apigenin	--	--	756	1583	768	1300

-- not determined

Optimization of extraction and hydrolysis

The six food samples were prepared and analyzed as described before. Three hydrochloric acid concentrations were tested (1.2 M, 1.6 M and 2.0 M) and the reaction period was varied (0.5, 1, 2, 4 and 6 hours) in the procedure described under the Extraction and Hydrolysis section. The lowest acid concentration and the shortest reaction period were omitted in the analysis of lettuce, endive and celery. Determinations at each HCl concentration and reaction period were carried out in duplicate for each food sample.

Henning (1980) reports that flavonoid glycosides are more soluble in water and flavonoid aglycones more soluble in methanol. Extraction efficiency could thus depend on the water/methanol ratio. In a preliminary experiment, two additional extraction solutions with methanol concentrations of 20 % and 80 % were tested in the analysis of onions, endive and celery. Care was taken to keep acid concentration in all extraction solutions equal. It appeared that extraction was most efficient with 50 % aqueous methanol. Flavonoid levels using 50% methanol were up to 30 % higher compared to 20 % and 80 % aqueous methanol (Table III). All further experiments were thus carried out with 50% aqueous methanol (v/v).

Stability of the flavonoid standards under hydrolysis conditions was tested. It appeared that all flavonoids (5 ug/mL) were stable in a solution consisting of 2.0 M HCl in 50% aqueous methanol (v/v) (2 g/L TBHQ) and boiling for up to 6 hours. Losses were less than 5% (results not shown). The influence of acid concentration and reaction period on flavonoid yield in the six food samples is presented in Figures 1,3-5. All graphpoints are the mean of duplicate determination. Results are expressed as percentage of the highest yield found in that food sample and as milligrams per kilogram of dry weight (right y axis). Highest amounts found are summarized in Table IV together with the corresponding hydrolysis condition.

Onions, leek, and cranberries: glucosides of quercetin, kaempferol and myricetin

In onions, we found a high amount of quercetin (5076 mg/kg dry weight) but no kaempferol could be detected. The highest yield was found using 1.2 M HCl and a reaction period of two hours (Figure 1). Increasing acid concentration and reaction time led to a significant degradation of quercetin. After six hours, quercetin had been degraded up to 70% using 2.0 M HCl. Chromatograms of the onion sample analyzed with both eluents are shown in Figure 2b. A small unknown peak with a retention time corresponding to luteolin ($t_{(R)}$= 8.25 min) was detected in eluent II (Figure 2a). However, the spectrum of this peak did not match the spectrum of luteolin.

Kaempferol (295 mg/kg dry weight) was detected in leek. Results are summarized in Figure 1. Highest levels of kaempferol were found using 1.6 M HCl and a reaction period of four hours. We also detected trace quantities of quercetin (20 mg/kg dry weight) when both 1.2 M HCl and a reaction period of two hours and 1.6 M HCl and a reaction period of one hour were used. Quercetin was only detected with eluent I and due to the low level, identification was only tentative. At more severe hydrolysis conditions the quercetin peak disappeared.

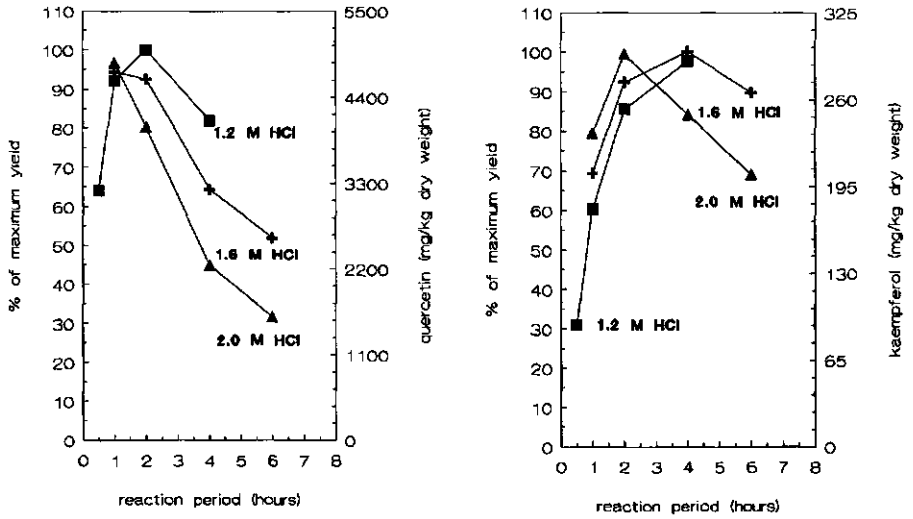

Figure 1. Influence of acic concentration and reaction period on quercetin yield in ***onion*** *and kaempferol yield in* ***leek.***

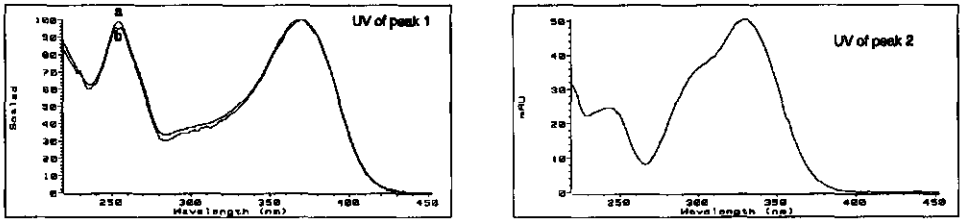

Figure 2a: Superimposed diode-array spectrum of quercetin standard (a) and quercetin in onion (b) both recorded in eluent I. Spectrum of unknown peak (2) at $t_{(R)}$ *= 8.25 min recorded with eluent II.*

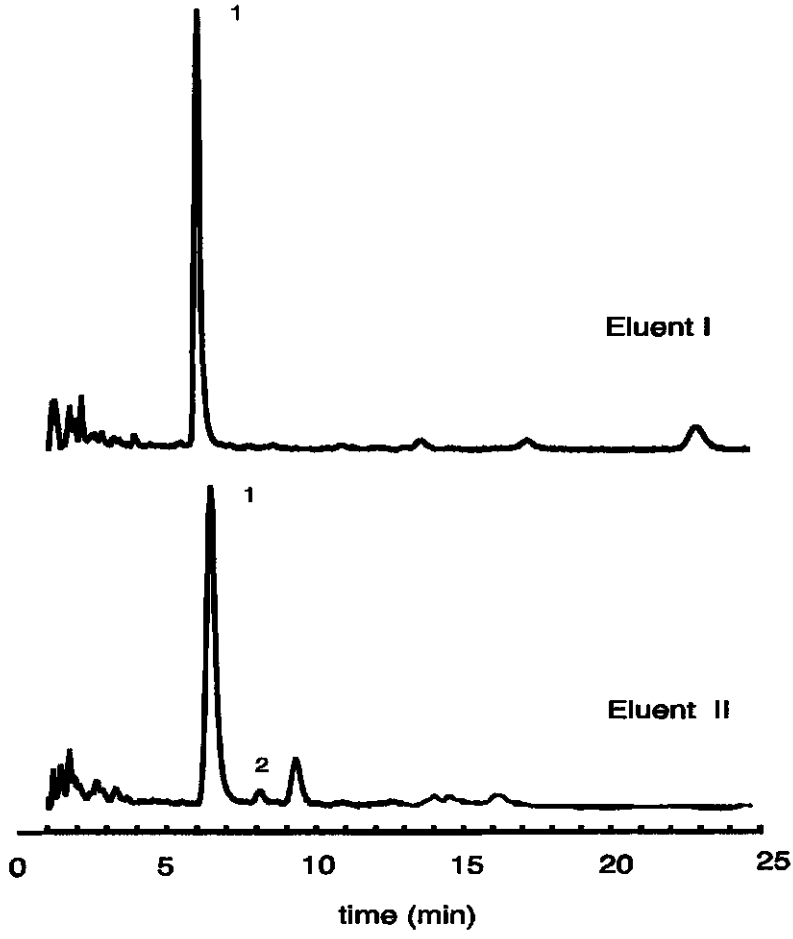

Figure 2b. Typical chromatograms of an onion extract in eluent I (25% acetonitrile in phosphate buffer (pH 2.4)) and eluent II (45% methanol in phosphate buffer (pH 2.4)). Hydrolysis conditions: 1.2 M HCl/2 hours (for details see text). Peaks: 1. quercetin; 2. unknown. Detection at 370 nm; 0.01 a.u.f.s. Flow-rate 0.9 mL/min.

We found quercetin (1485 mg/kg dry weight) and myricetin (662 mg/kg dry weight) in cranberries. As can be seen in Figure 3 the highest yields were found with 1.2 M HCl and a reaction period of 30 min. An increased acid concentration and an increased reaction period led to degradation of both quercetin and myricetin. After six hours, a loss of up to 30 % of myricetin and quercetin was observed.

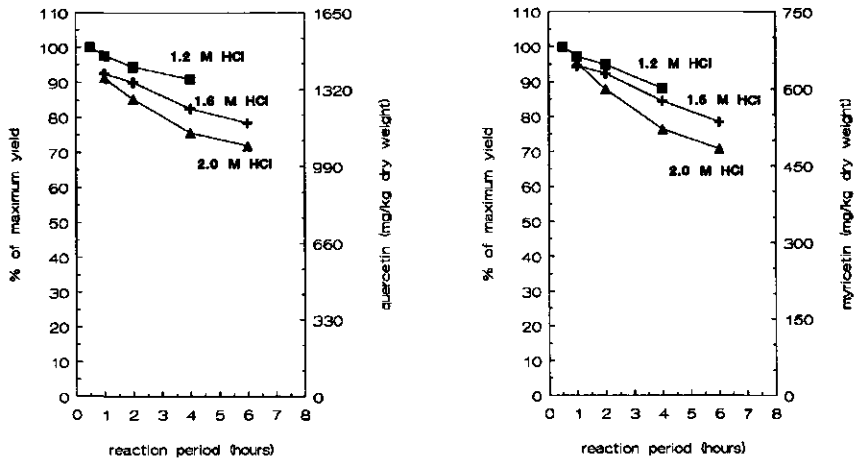

Figure 3. Influence of acid concentration and reaction period on quercetin and myricetin yield in ***cranberry***

Lettuce, endive: glucuronides of quercetin and kaempferol

Only quercetin (319 mg/kg dry weight) was detected in lettuce. Figure 4 shows that the highest yield was found using 2.0 M HCl and a reaction period of two hours. Similar yields were found using 1.6 M HCl and a reaction period of four hours. Increasing the reaction period up to 6 hours with 2.0 M HCl led to a degradation of quercetin of approximately 10 %. No luteolin was detected in this sample.

In endive, only kaempferol (271 mg/kg dry weight) was found. As shown in Figure 4 the highest yield was found using 2.0 M HCl and a reaction period of two hours. Similar yields were found with 1.6 M HCl and a reaction period of four hours. No significant additional yield could be gained by increasing the reaction period and/or hydrochloric acid concentration (2.5 M HCl, not shown).

Lettuce and endive samples were also treated with a (non-specific) ß-glucuronidase

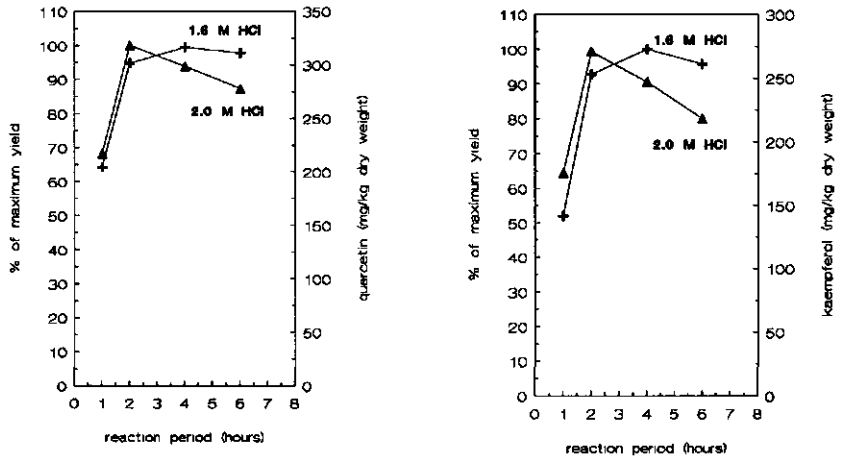

Figure 4. Influence of acid concentration and reaction period on quercetin yield in ***lettuce*** *and kaempferol yield in* ***endive***

solution (FLUKA #49310) using a method described by Kunzeman and Herrmann (1977). The amounts of quercetin and kaempferol liberated by ß-glucuronidase in lettuce and endive corresponded well with the amounts obtained after acid hydrolysis. About 69% of the glucosides in onions were liberated with ß-glucuronidase (Table V). The presence of flavonol glucuronides in both samples was confirmed by a method described by Sagara (1985). Flavonol glucuronides can be separated from other flavonoid compounds by adding an ion-pair reagent (5 mM tetra-n-pentylammoniumbromide, TPAB) to the eluent. At pH 4.0 only flavonol glucuronides will be partially ionised. By adding an ion-pair reagent, the retention of flavonol glucuronides will be enhanced, whereas the retention of the other, non-ionised glycosides will remain virtually unchanged. After addition of the ion-pair reagent the retention time of the major quercetin glycoside peak in lettuce changed from 8.8 to 18.2 min and the retention time of the major kaempferol glycoside peak in endive changed from 10.2 to 18.6 min. This confirmed that both glycosides are presumably glucuronides (Table V).

Table IV. Flavonoid content[a] (mg/kg dry weight) and corresponding optimum hydrolysis conditions in six food samples

Sample	conditions	mg/kg dry weight				
		quercetin	kaempferol	myricetin	luteolin	apigenin
cranberry	1.2 M HCl/0.5 hours	1485	<20[c]	662	<10[c]	<40[c]
onion	1.2 M HCl/2 hours	5076	<20[c]	<10[c]	<10[c]	<40[c]
leek	1.6 M HCl/4 hours	20[b]	295	<10[c]	<10[c]	<40[c]
lettuce	2.0 M HCl/2 hours	319	<20[c]	<10[c]	<10[c]	<40[c]
endive	1.6 M HCl/4 hours	<10[c]	271	<10[c]	<10[c]	<40[c]
celery	2.0 M HCl/4 hours	<10[c]	<20[c]	<10[c]	358	1787

[a] Mean for duplicate determination [b] conditions: 1.2 M HCl/2 hours [c] <limit of detection

Table V. Influence of ion-pair reagent (5 mM TPAB) on retention times of flavonol standards, glucosides and glucuronides.

Sample	component	$t_{(R)}$ (min)		mg/kg dry weight after hydrolysis	
		without ion-pair	with ion-pair	enzymatic	acid
standard	quercetin	15.6	17.2	--	--
standard	kaempferol	18.2	19.0	--	--
lettuce[a]	quercetin glucuronide	8.8	18.2	23	27
endive	kaempferol glucuronide	10.4	18.6	282	271
onion[a]	quercetin glucoside	11.8	14.2	1973	2880

Eluent A: 0.025 M phosphate buffer (pH 4.0)/methanol (90/10, v/v). Eluent B: 0.025 M phosphate buffer (pH 4.0)/methanol (20/80, v/v). Gradient: 20-70% B in 16 min. Detection at 370 nm, 0.02 a.u.f.s. Flow-rate 0.9 ml/min. Comparison between flavonol yield with enzymatic hydrolysis and acid hydrolysis (for conditions see text).

[a] samples not identical with samples reported in table IV

Celery: glucosides of apigenin and luteolin

Celery was prepared and analyzed as described before. An additional reaction period of three hours was used. We found luteolin (358 mg/kg dry weight) and apigenin (1787 mg/kg dry weight). Both gave the highest yields when 2.0 M HCl and a reaction period of four hours was used. Increasing the reaction period did not increase the flavone yield. Results are shown in Figure 5. Typical chromatograms of a celery extract using both

eluents are shown in Figure 6 together with the spectrum of the luteolin peak provided by diode-array.

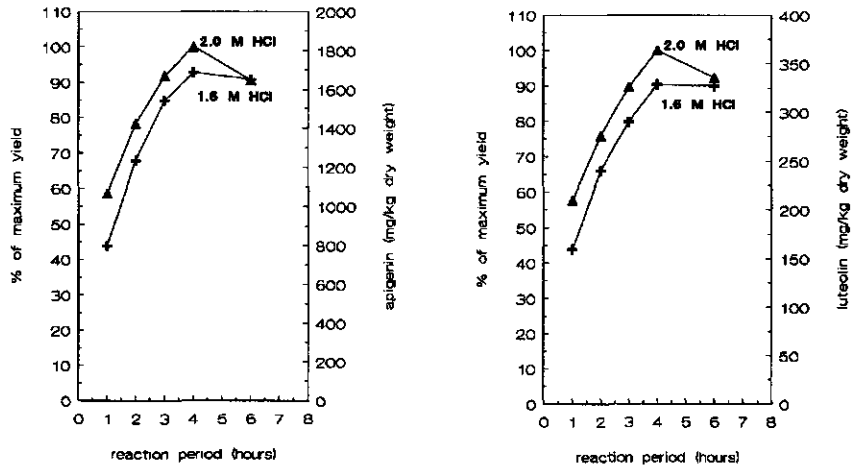

Figure 5. Influence of acid concentration and reaction period on luteolin and apigenin yield in celery

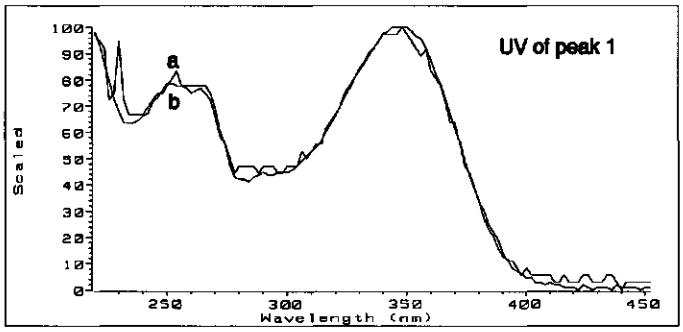

Figure 6a. Superimposed diode-array spectrum of luteolin standard (a) and luteolin in celery (b) both recorded in eluent I (see text).

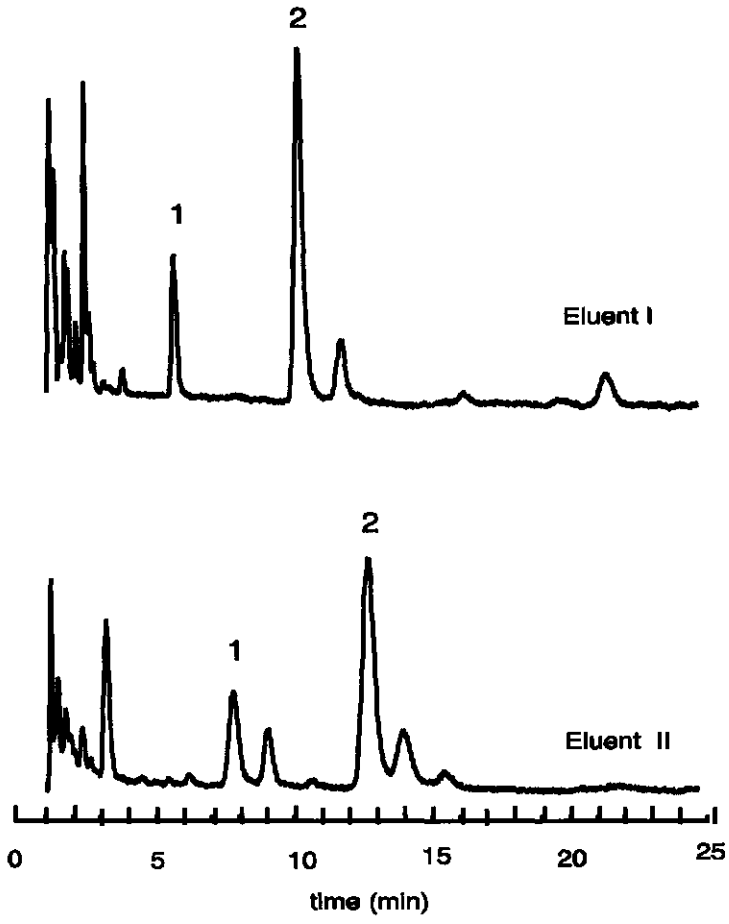

Figure 6b. Typical chromatograms of a celery extract in eluent I and eluent II (see text). Hydrolysis conditions: 2.0 M HCl/4 hours (for details see text). Peaks: 1. luteolin 2. apigenin. Detection at 370 nm; 0.01 a.u.f.s. Flow-rate 0.9 mL/min.

Precision

For quality control of future analysis of vegetables and fruits, control samples were composed of the foods investigated in this study containing the various flavonoid glycosides. Control sample A containing flavonol glucosides was made by mixing onions and leek (1:9, w/w). Unfortunately, addition of cranberries for myricetin glucosides to this control sample led to low flavonol levels and interfering peaks. Thus, a separate control sample for myricetin (cranberries, control sample B) was necessary. Lettuce and endive were mixed (1:1, w/w) to a control sample (C) containing flavonol glucuronides. Furthermore, celery was used as a control sample (D) for flavone glucosides. Repeatability of the method within our laboratory was determined by ten analyses of these four control samples. Hydrolysis and extraction conditions were based on the optimization curves established (Figure 1,3-5). However, as optimum conditions differ for all of the foods investigated we decided to define three "optimum" conditions depending on the following flavonoid and glycoside types: flavonol glucosides, flavonol glucuronides and flavone glucosides. For flavonol glucosides (control sample A and B) this choice (2 hours hydrolysis with 1.2 M HCl) was a compromise leading to an underestimation of the myricetin and kaempferol content of less than 5% and 15%, respectively. Recoveries were measured in the control samples by spiking pure standards to the extraction solutions at two levels (50% and 100% of the measured flavonoid content) prior to sample analysis. Within-laboratory reproducibility was established by five duplicate analyses on separate days within a period of one month. Results, together with the hydrolysis conditions are shown in Table VI.

Table VI. Mean content (mg/kg dry weight), coefficients of variation (CV) and recovery's of four control samples.

Sample	Conditions	Components	mean content n=10	CV		Recovery[a]	
				%[b]	%[c]	100% add	50% add
control sample A	1.2 M HCL/	quercetin	381.4	2.5	4.9	88%	98%
(onion/leek)	2 h hydrol	kaempferol	235.6	5.6	11.8	102%	110%
control sample B	1.2 M HCl/	quercetin	1411.4	3.1	4.1	91%	91%
(cranberries)	2 h hydrol	myricetin	601.6	4.6	6.1	94%	88%
control sample C	2.0 M HCl/	quercetin	121.4	3.1	7.6	77%	94%
(endive/lettuce)	2 h hydrol	kaempferol	142.4	4.6	8.3	96%	102%
control sample D	2.0 M HCl/	apigenin	1783.2	2.8	4.1	99%	104%
(celery)	4 h hydrol	luteolin	333.4	3.3	4.6	101%	106%

Composition of control samples is described under heading precision
[a] mean for duplicate determination
[b] within-laboratory repeatability (n=10)
[c] within-laboratory reproducibility (n=5)

DISCUSSION

From our results it clearly appears that completeness of hydrolysis depends on both reaction period and acid concentration. It is noteworthy that suboptimal hydrolysis conditions could therefore lead to an underestimation of up to 50% of the true flavonoid level in foods. In samples with flavonol glucosides, an optimum between flavonol yield and flavonol degradation was observed as prolonged extraction and hydrolysis showed a decrease in the flavonol contents measured (Figure 1,3). A reaction period of more than four hours with 1.6 M HCl in general led to loss of flavonols. This effect was enhanced when 2.0 M HCl was used in the extraction medium. However, flavonol degradation could not be observed in standard solutions treated under identical conditions. Therefore, the presence of some unknown compounds in the sample matrix accelerating the degradation of flavonoids should be considered.

Identical optimum hydrolysis conditions for flavonol glucosides in leek, onions as well as in cranberries could not be achieved. Time required for complete hydrolysis is dependent on the binding site of the sugar on the flavonoid nucleus: C7>C4'>C3 (Harborne, 1965). Glucose moieties of flavonol glucosides in cranberries are bound to C3 and in onions to C4' (Herrmann, 1976). This might explain different optimum conditions found for e.g. quercetin in onions and quercetin in cranberries. However, long hydrolysis conditions needed for complete hydrolysis of kaempferol-3-glucosides presumably present in leek could not be explained. Other kaempferol glycosides may be present in leek. Possibly, matrix composition is an important factor determining hydrolysis rate.

The following hydrolysis conditions for flavonoid glycosides are proposed. For analysis of foods containing predominantly flavonol glucuronides an extraction medium consisting of 2.0 M HCl in boiling 50% aqueous methanol (v/v) and a reaction period of 2 hours will result in complete hydrolysis of flavonols. Similar results are found with 1.6 M HCl and a reaction time of 4 hours. Foods containing flavonol glucosides should be hydrolysed with 1.2 M HCl in boiling 50% aqueous methanol (v/v) with a reaction period not exceeding 2 hours. Under these conditions possible degradation of flavonol-3-glucosides is less than 10%. Eventually, a second analysis under identical conditions with a reaction period of four hours should be applied for quantification of kaempferol glucosides. Flavone glucosides are more resistant to acid hydrolysis. Complete hydrolysis of flavones takes 4 hours, the extraction medium consists of 2.0 M HCl in 50% aqueous methanol (v/v).

The method employed to confirm the presence of flavonol glucuronides as the major glycosides in lettuce and endive, was shown to be sufficiently specific to distinguish between flavonol glucuronides and glucosides. As the available ß-glucuronidase preparations lack specificity (Markham, 1989) an enzymatic based distinction of these glycosides is not possible. Interestingly, amounts liberated by enzymatic hydrolysis corresponded well with the amounts found with the optimized acid hydrolysis.

In general recoveries of flavonoids in the samples spiked with 50% and 100% of the original level were good. However, in control sample B recoveries of myricetin spiked with 50% was somewhat inferior (88%). Probably, hydrolysis conditions were too severe. This also resulted in a lower mean level compared to the level found under optimal conditions in the optimization experiments (Table IV). In control sample C, recovery of quercetin was rather poor, possibly due to the low level of quercetin in that sample.

Coefficients of variation of the repeatability and within-laboratory reproducibility were good being less than 5% and 9%, respectively. Repeatability of kaempferol in control sample A is somewhat poor because hydrolysis conditions applied were not optimal. This is also reflected in the CV of within-laboratory reproducibility.

Table VII. Comparison of flavonoid levels in foods found in the present study and levels reported by Herrmann and co-workers and Bilyk and co-workers.

Products	Compounds	mg/kg fresh weight		
		Present study	Herrmann and co-workers	Bilyk and co-workers
lettuce	quercetin	9	6-273[a]	1-38[d]
leek	kaempferol	31	90-200[b]	20[d]
	quercetin	2	10-25[b]	ND
onions	quercetin	544[g]	104-1260[c]	15-62[c]
	kaempferol	<2.5[g]	21-235[c]	3-7[e]
cranberries	quercetin	172	N.A.	73-250[f]
	myricetin	77	N.A.	4-2[f]
endive	kaempferol	18	150[a]	N.A.
celery	apigenin	108	75[b]	N.A.
	luteolin	22	14[b]	N.A.

Findings are presented as mg/kg fresh weight in the whole foods unless otherwise stated.

[a] outer leaves, *Wöldecke and Herrmann, 1974*
[b] *Herrmann, 1976*
[c] open air, *Starke and Herrmann, 1976*
[d] green portion (leek), *Bilyk and Sapers, 1985*
[e] edible portions, *Bilyk, Cooper and Sapers, 1984*
[f] *Bilyk and Sapers, 1986*
[g] outer dry skin removed
N.D. Not detectable; N.A. no data available

In general, our findings compare well with findings reported earlier by Herrmann and co-workers and Bilyk and co-workers (Table VII). Differences found may be due to varietal or seasonal differences or by the fact that hydrolysis conditions were optimized in our study, but not in the studies reported by Herrmann *et al.* and Bilyk *et al.*

The HPLC column in this study, used for over one year on an almost daily basis, did not show any significant loss of plate numbers during this period. However, a long preconditioning period was necessary due to a high absorption of the new column. This period could be shortened by repeated injections of a concentrated quercetin solution. Furthermore, it was necessary to wash injection needle and tubings regularly with water in order to avoid blockings and damage to the autoinjector from the acid.

In conclusion, the method presented here allows a fast, quantitative and reproducible determination of the five flavonoid aglycones in freeze-dried foods after acid hydrolysis. As a large number of different glycosides are present in foods, the quantitative determination of individual flavonoid glycosides in vegetables and fruits commonly consumed, would be complicated. In addition, because of the low levels of individual flavonoid glycosides in foods and the limitations indicated in the Introduction section, only analysis of the aglycones after hydrolysis proved to be a practical method for the quantitative determination of flavonoids in foods. Currently, the above described method is applied in the study of a large group of fruits and vegetables at our laboratory.

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Chapter 3

Flavonol and Flavone Content of Vegetables and Fruits

This chapter is based mainly on:

Hertog MGL, Hollman, PCH, and Katan, MB.
Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. *Journal of Agricultural Food Chemistry*, 1992;40:2379-2383

ABSTRACT

The content of the potentially anticarcinogenic flavonoids quercetin, kaempferol, myricetin, apigenin and luteolin of 28 vegetables and 9 fruits was determined by RP-HPLC with UV-detection. Fresh foods were purchased in a supermarket, a grocery, and a streetmarket and combined to composites. Processed foods were purchased additionally. Sampling was carried out in spring, summer, winter and spring of the following year. Quercetin levels in the edible parts of most vegetables were generally below 10 mg/kg except for onions (284-486 mg/kg), kale (110 mg/kg), broccoli (30 mg/kg), french beans (32-45 mg/kg) and slicing beans (28-30 mg/kg). Kaempferol could only be detected in kale (211 mg/kg), endive (15-91 mg/kg), leek (11-56 mg/kg), and turnip-tops (31-64 mg/kg). In most fruits the quercetin content averaged 15 mg/kg, except for different apple varieties in which we found 21-72 mg/kg. The content of myricetin, luteolin and apigenin was below the limit of detection (<1 mg/kg) except for fresh broad beans (26 mg/kg myricetin) and sweet red pepper (13-31 mg/kg luteolin). Seasonal variability was low for most vegetables except for leafy vegetables with highest flavonoid levels in summer. These collective data provide a base for an epidemiological evaluation of possible anticarcinogenic effects of flavonoids.

INTRODUCTION*

Flavonoids are diphenylpropanes occurring ubiquitously in food plants and a common component in the human diet (see General Introduction). Average intake of all flavonoids is estimated to be 1 gram/day (Kühnau, 1976). Precise data on the occurrence of flavonoids in vegetables and fruits are, however, lacking. Food-derived flavonoids such as the flavonols quercetin, kaempferol and myricetin have antimutagenic and anticarcinogenic effects *in vitro* and *in vivo* (Kato et al., 1983; Huang et al., 1983; Fujiki et al. 1986; Mukhtar et al., 1988; Verma et al., 1988; Francis et al. 1989; Wei et al. 1990; Deschner et al., 1991). Epidemiological research on the relation between flavonoid intake and cancer risk in humans is needed to support the findings of these experimental studies.

So far, little attention has been paid to quantitative aspects of the determination of flavonoids in foods. Furthermore, the limited data published were mainly obtained with thin layer chromatography followed by a spectrophotometric measurement (Wöldecke and Herrmann, 1974; Herrmann, 1976; Starke and Herrmann, 1976). More recently, Bilyk and co-workers published results of flavonoid analyses in foods based on High Performance Liquid Chromatography (HPLC) and ultraviolet-detection (Bilyk et al., 1984; Bilyk and Sapers, 1985, 1986) in a limited number of foods.

Flavonoids consist mainly of anthocyanidins, flavonols, flavones, catechins, and flavanones (Herrmann, 1988). We selected three major flavonols, quercetin, kaempferol, and myricetin and two major flavones luteolin and apigenin because these flavonoids are most widely investigated in anticarcinogenesis studies. We developed a HPLC method for the identification and quantification of these flavonoids in freeze-dried foods (Hertog et al., 1992). We have now measured the content of these flavonoids of 28 types of vegetables and 9 types of fruit commonly consumed in the Netherlands and studied the effect of season and processing on flavonoid levels.

MATERIALS AND METHODS

Sources and Preparation of Samples

Vegetables and fruits were selected for sampling based on data provided by a nation-wide food consumption survey conducted among a representative population sample. Two-day dietary records were collected for 5898 subjects (2000 households) in 1987-1988 (Hulshof and Van Staveren, 1991). The survey provided data on food consumption, locations where particular foods were purchased and whether foods were bought fresh or processed i.e. canned, in glass jars, or frozen. 28 types of vegetables and 9 types of fruits commonly consumed in the Netherlands were selected (Table I and II). Citrus fruits were excluded because they contain almost exclusively flavanones (Herrmann, 1976).

Foods were sampled in four periods (April 1991; August 1991; December 1991 and April 1992) unless, according to the survey, a particular food was bought less than 1% of the yearly total in that specific period. However, fresh garden beans and fresh peas were purchased only in July 1991 because they were not available in other periods. Although broccoli is not commonly consumed in the Netherlands, it is gaining popularity and was therefore sampled

* section shortened for publication in this thesis

only in April 1992. All foods were generally only purchased as fresh products; whenever the survey indicated that a particular food was consumed for more than 5 % as a processed product, this product was purchased additionally. No varietal differences were studied with the exception of apples (6 varieties) and pears (3 varieties) which were bought in the period when the specific variety was most easily available.

Fresh foods (1 kg, or a minimum of 3 units) were bought on the same day at three locations: an outlet of a nation-wide supermarket-chain (Albert Heijn), a local grocery and an open-air street market. Fresh foods were cleaned within 24 h, non-edible parts removed and samples were combined per product to a composite in proportions reflecting sales in the three locations. Typically, for most foods the sample bought at the supermarket accounted for 65 %, the grocery sample for 20 %, and the street market sample for 15 % of the composite sample. Food samples were chopped under liquid nitrogen and immediately stored at -20 °C for less than 2 weeks until they were lyophilized.

Processed foods were purchased in the supermarket within one week after the fresh products. Three major brands (1 unit each) of each product were bought in cans, glass jars or frozen as indicated in the survey. The three brands were mixed in equal portions (net weight) and immediately stored at -20 °C for less than 2 weeks until lyophilized. After lyophilisation samples were allowed to equilibrate in open air and ground to pass a 0.5 mm sieve. Sugar-containing foods were frozen in liquid nitrogen and ground to a fine powder. Moisture was measured by drying at 80 °C in a vacuum oven. The food samples were stored at -20 °C for less than 4 months until analyzed.

Methods of Analysis

Five major food flavonoids, viz. quercetin, kaempferol, myricetin, luteolin and apigenin were determined in freeze-dried foods after extraction and acid hydrolysis of the flavonoid glycosides. Development, optimization and validation of this method was described in detail before (Hertog et al., 1992). In brief, flavonoid glycosides were extracted and hydrolysed to their aglycones with HCl in 50 % aqueous methanol. Subsequently, the resulting aglycones were quantified by RP-HPLC on a NOVA-PAK C18 column using acetonitrile/phosphate buffer (25/75, v/v, pH 2.4) as mobile phase, and UV detection (370 nm). To confirm peak identity sample extracts were reinjected, using methanol/phosphate buffer (45/65, v/v, Ph 2.4) as mobile phase. In addition, peak identity and purity was confirmed using a photodiode-array detector to record UV-spectra of the flavonoids in samples on-line.

As completeness of hydrolysis depends on the type of glycoside, optimum hydrolysis conditions in samples with an unknown flavonoid glycosylation pattern had to be determined. All samples were first analyzed after hydrolysis in 1.2 M HCl for 2 h. Subsequently, samples containing flavonols were re-analyzed after 4 h of hydrolysis. A 10% higher flavonoid content in the second analysis than in the first indicated the presence of flavonol glucuronides. In that case the sample was analyzed a third time using 2.0 M HCl and a hydrolysis period of 2 h. Also, if the first analysis revealed the presence of the flavones apigenin and luteolin the samples were re-analyzed after 4 h of hydrolysis in 2.0 M HCl.

Limit of detection was defined as the amount of flavonoids resulting in a peak height of three times the standard deviation of the baseline noise.

Table I. Results of Analytical Quality Control Samples

	Control sample[a]					
	A *(flavonol glucosides)*		B *(flavone glucosides)*		C *(flavonol glucuronides)*	
	Quercetin	Kaempferol	Luteolin	Apigenin	Quercetin	Kaempferol
mean at start	381 (n=5)	344 (n=5)	333 (n=5)	1780 (n=5)	121 (n=5)	142 (n=5)
SD_R	19	28	15	73	9	12
CV_R	4.9 %	8.2 %	4.6 %	4.1 %	7.6 %	8.3 %
CV_r	2.5 %	5.7 %	3.3 %	2.8 %	3.1 %	4.6 %
mean series	375 (n=11)	354 (n=7)	236 (n=4)	1740 (n=4)	131 (n=5)	135 (n=5)
CV_R	5.1 %	9.4 %	14.0 %	6.2 %	7.2 %	5.2 %
CV_r	3.0 %	2.9 %	3.8 %	3.4 %	4.1 %	4.9 %

Mean (mg/kg dry weight), within-laboratory standard deviation of reproducibility (SD_R), within-laboratory coefficient of variation of reproducibility (CV_R) and repeatability (CV_r) at the start of the project (period of two weeks) and mean and coefficient of variation obtained in the whole project (1,5 years).

[a] each determination was carried out in duplicate

Analytical Quality Control

Control samples were included with each series of samples. Batches of three different control samples sufficient for the whole project, were made up of lyophilized vegetables containing the various flavonoid glycosides. Control sample A, containing flavonol glucosides, was made by mixing onion and leek (1:9, w/w). Celery, containing the flavone glycosides luteolin and apigenin was used as control sample B for the analysis of foods containing flavones. Control sample C, containing flavonol glucuronides, was composed of lettuce and endive (1:1, w/w). Control samples were stored at -20 °C. At the start of the project the flavonoid content of the control samples was determined by 5 duplicate analyses on different days within a period of two weeks to obtain within-laboratory standard deviation of reproducibility (SD_R).

For each series of analyses, stability of flavonoid calibration standards in methanol was checked spectrophotometrically at 375 nm (flavonols) and 340 nm (flavones) after dilution of the stock solution to 10 ug/mL in methanol. Standard solutions proved to be stable for over three months at 4° C. Myricetin had degraded up to 10 % after one month, and the stock solution was therefore made up freshly before every period of analysis.

Each series of analyses included one control sample corresponding to the glycosides expected and three calibration standards placed at begin and end of the series. All determinations were carried out in duplicate. Differences between duplicates of more than 15

% were not accepted. Series of analyses were repeated whenever flavonoid content of the control sample exceeded the confidence limits (mean ± 3 SD_R). Accepted results of the control samples are reported in Table I. Regression analysis of results of the control sample did not reveal any significant correlation between flavonoid content and time of analysis. An exception was luteolin in control sample B which, at the end of the project, had been degraded to 71% of the original level. This is also reflected in a high CV of the long-term variability. However, the apigenin content in the same control sample remained stable over the 1,5 year period. Control sample A was used for both analysis with hydrolysis periods of 2 and 4 h as described under the section Methods of Analysis. In general long-term variability of the method was low (CV's < 10%). It is concluded that stability of flavonoids in freeze-dried foods stored at -20 °C is adequate over a 1,5 year period. The low variation in flavonoid levels of the control samples demonstrates the absence of a significant long-term variability of flavonoid analysis in the laboratory.

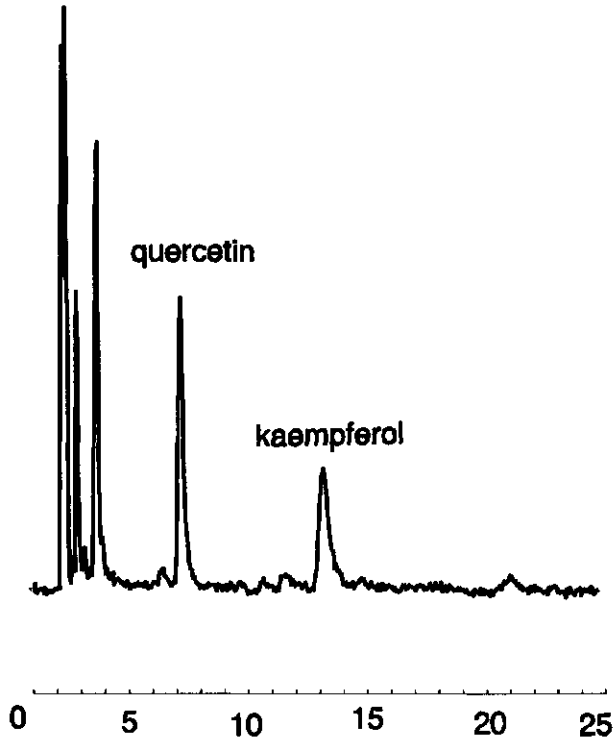

Figure 1. Typical chromatogram of a French bean extract monitored in 35% acetonitrile/phosphatebuffer (pH 2.4). Detection at 370 nm; 0.01 AUFS; flow-rate 0.9 mL/min

RESULTS AND DISCUSSION

For almost all products the initial step using 1.2 M HCl and a hydrolysis period of 2 h yielded the highest flavonoid levels. Only lettuce, endive, leek, broccoli, french beans and slicing beans had to be hydrolysed with 2.0 M HCl for 2 h. These results suggest that the major glycosides present in lettuce, endive, leek, broccoli, french beans and slicing beans are glucuronides. This is in accordance with Herrmann (1988) with the exception of broccoli and leek in which the major glycoside was reported to be quercetin-3-*O*-sophoroside-7-*O*-glucoside and kaempferol-3-*O*-ß-D-glucoside, respectively (Starke and Herrmann, 1976). A typical chromatogram of a vegetable extract (french beans) is shown in Figure 1.

Table I reports the flavonoid content of fresh and processed vegetables sampled in the four periods. As April 1991 and April 1992 represent the same season, results of these periods were first averaged before calculating the annual mean values and standard deviations given in Table II. Standard deviations are reported whenever a food was sampled in all four periods. The flavonoid content of fruits are reported in Table III. No standard deviations are given because fresh fruits were generally bought in August only. Different apple and pear varieties were bought in all periods. The mean flavonoid content of these fruits was calculated by averaging the values of all varieties. Standard deviation thus reflects standard deviation of the varieties.

The major flavonoid that we found in vegetables is quercetin followed by kaempferol. Myricetin could only be detected in fresh broad bean (26 mg/kg) and luteolin was only found in sweet red pepper (11 mg/kg). We could only detect apigenin in celery (108 mg/kg) that was used in developing our method of analysis and that was applied as control sample B in this study. The mean quercetin content of onions (347 mg/kg) and the mean kaempferol content of fresh kale (211 mg/kg) were five to ten-fold higher than in most other vegetables. High mean levels of quercetin were also found in kale (110 mg/kg), broccoli (30 mg/kg), fresh french beans (39 mg/kg), fresh slicing beans (29 mg/kg). The mean kaempferol content of endive (46 mg/kg), leek (30 mg/kg) and turnip-tops (48 mg/kg) were higher than in most other vegetables. None of the flavonoids investigated could be detected in chicory, spinach, pea, red beet, mushroom, cucumber, carrot and all brassicas, with the exception of broccoli, kale, red cabbage and turnip tops.

In fruits only quercetin was found with the exception of strawberries were also kaempferol is present (12 mg/kg) and white and black grapes were also a low content of myricetin (4.5 mg/kg) was found. None of the flavonoids investigated could be detected in peaches. Differences in quercetin content in three pear varieties and six apple varieties was low. The mean quercetin content for all varieties in apples and pears was 36 ± 19 mg/kg and 6.4 ± 3.4 mg/kg respectively. In general, apple varieties purchased in December had highest quercetin levels. Jonagold apples had a quercetin content twice that of other varieties. No significant differences could be noted between the quercetin content of white and black grapes. We failed to detect kaempferol in fruits, except for strawberries, as described by Wildanger and Herrmann (1973) for some fruits such as cherry, plum, peach and red currant. Quantities reported by Wildanger and Herrmann are however very low (<10 mg/kg fresh weight).

Table II. Flavonoid Content[a] (mg/kg fresh edible part) of 28 Vegetables Sampled in Four Periods

Product	Scientific name	Quercetin (mg/kg fresh edible part)					Kaempferol (mg/kg fresh edible part)				
		Apr 1991	*Aug 1991*	*Dec 1991*	*Apr 1992*	Mean[b]	*Apr 1991*	*Aug 1991*	*Dec 1991*	*Apr 1992*	Mean[b]
Mushroom	*Agaricus campester Fr.*	< 1	< 1	< 1	< 1	< 1	< 2	< 2	< 2	< 2	< 2
- processed		< 1	< 1	< 1	< 1	< 1	< 2	< 2	< 2	< 2	< 2
Onion	*Allium cepa L.*	332	347	284	486	347 ± 63	< 2	< 2	< 2	< 2	< 2
Leek	*Allium porrum L.*	< 1	< 1	< 1	< 1	< 1	31	56	11	16	30 ± 23
Red beet	*Beta vulgaris L. cv. rubra L.*	< 1	< 1	< 1	< 1	< 1	< 2	< 2	< 2	< 2	< 2
Turnip tops	*Brassica campretis L.*	10			4.6	7.3	64			31	48
Kale	*Brassica oleracea L. cv. acephala DC.*			110		110			211		211
- processed				47	43	45			156	212	184
Sauerkraut	*Brassica oleracea L. cv. alba DC.*	< 1	< 1	< 1	< 1	< 1	< 2	< 2	< 2	< 2	< 2
White cabbage	*Brassica oleracea L. cv. alba DC.*	< 1	< 1	< 1	< 1	< 1	< 2	< 2	< 2	< 2	< 2
Cauliflower	*Brassica oleracea L. cv. botrytis L.*	< 1	< 1	< 1	< 1	< 1	< 2	< 2	< 2	< 2	< 2
Brussels sprout	*Brassica oleracea L. cv. gemmifera DC.*			< 1		< 1			7.4		7.4
Broccoli	*Brassica oleracea L. cv. italica L.*				30	30				72	72
Swedish turnip	*Brassica napus L. cv. Napobrassica RCh*	< 1	< 1		< 1	< 1	< 2	< 2		< 2	< 2
Red cabbage	*Brassica oleracea L. cv. rubra DC.*	6.2	5.9	3.8	1.9	4.6 ± 1.1	< 2	< 2	< 2	< 2	< 2
- processed		2.4	2.6	2.7	3.9	2.8 ± 0.3	< 2	< 2	< 2	< 2	< 2
Green cabbage	*Brassica oleracea L. cv. sabellica Schulz*	< 1	< 1	< 1	< 1	< 1	< 2	< 2	< 2	< 2	< 2
Endive	*Chicorium endiva L.*	< 1	1.3	< 1	< 1	<1.3	15	95	21	30	46 ± 42
Chicory	*Chicorium intybus L.*	< 1	< 1	< 1	< 1	< 1	< 2	< 2	< 2	< 2	< 2
Cucumber	*Cucumus sativus L.*	< 1	< 1	< 1	< 1	< 1	< 2	< 2	< 2	< 2	< 2
Lettuce	*Lactuca Sativa L. cv. capitata L.*	1.9	30	6.7	7.3	14 ± 14	< 2	< 2	< 2	< 2	< 2
French bean	*Phaseolus vulgaris L.*	45	32	41	42	39 ± 6	14	< 2	13	8.8	<12

Product	Scientific name	Quercetin (mg/kg fresh edible part)					Kaempferol (mg/kg fresh edible part)				
		Apr 1991	*Aug 1991*	*Dec 1991*	*Apr 1992*	Mean[b]	*Apr 1991*	*Aug 1991*	*Dec 1991*	*Apr 1992*	Mean[b]
- processed		27	19	11	15	17 ± 5	< 2	3.8	< 2	< 2	<3.8
Slicing bean	*Phaseolus vulgaris L.*	28	30			29	< 2	< 2			< 2
Pea	*Pisum sativum L.*	< 1				< 1	< 2				
- processed		< 1	< 1	4.3	< 1	<4.3	< 2	< 2	< 2	< 2	< 2
Purslane	*Portulacea oleracea L.*	< 1	< 1			< 1	< 2	< 2			< 2
Radish	*Raphanus sativus L. cv. radicula Pers.*	< 1	< 1	< 1	< 1	< 1	3.9	7.7	6.2	5.5	6.2 ± 1.5
Tomato	*Solanum lycopersicum L.*	4.6	11	8.2	4.9	8.0 ± 3.1	< 2	< 2	< 2	< 2	< 2
Spinach	*Spinacia oleracea L.*	< 1	< 1	< 1	< 1	< 1	< 2	< 2	< 2	< 2	< 2
- processed		< 1	< 1	< 1	< 1	< 1	< 2	< 2	< 2	< 2	< 2
Broad bean	*Vicia Faba L.*	20				20	< 2				
- processed		6.6	6.3	3.9	6.2	5.5 ± 1.4	< 2	8.0	6.0	< 2	< 7
		Luteolin (mg/kg fresh edible part)					Myricetin (mg/kg fresh edible part)				
No vegetables contained luteolin, apigenin and myricetin except for:											
Sweet red pepper	*Capsicum annuum L.*	14	13	7.0	14	11 ± 4	< 1	< 1	< 1	< 1	< 1
Carrot	*Daucus carota L.*	1.4	< 1	< 1	< 1	<1.4	< 1	< 1	< 1	< 1	< 1
- processed		< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Broad bean	*Vicia Faba L.*	< 1				< 1	26				26

[a] mean of duplicate determinations
[b] annual mean ± SD, April 1991 and April 1992 were averaged prior to calculation of mean and SD
< 1, < 2 : limit of detection.
Blank spaces: not available/sampled in that period

Table III. Flavonoid Content[a] (mg/kg fresh edible part) of 9 Fruits (including peel) Sampled in Four Periods

Product	Scientific name	Quercetin (mg/kg fresh edible part)					Kaempferol (mg/kg fresh edible part)				
		Apr 1991	Aug 1991	Dec 1991	Apr 1992	Mean[b]	Apr 1991	Aug 1991	Dec 1991	Apr 1992	Mean[b]
Strawberry	*Fragaria ananassa Duch.*	10	8.4		7.7	8.6	7.0	16		12	12
Apple[c]	*Malus pumila Mill.*					36 ± 19					< 2
"granny smith"		24			24		< 2			< 2	
"james grieve"			21					< 2			
"golden delicious"			25					< 2			
"elstar"				32					< 2		
"jonagold"				72					< 2		
"cox's orange"				41					< 2		
Apple-sauce		22	18	21	20	20 ± 2	< 2	< 2	< 2	< 2	< 2
Red currant	*Ribes rubrum-hybriden*		13			13		< 2			< 2
Apricot	*Prunus armeniaca L.*		25			25		< 2			< 2
- processed		< 1	< 1	< 1	< 1	< 1	< 2	< 2	< 2	< 2	< 2
Pear[c]	*Pyrus communis L.*					6.4 ± 3.4					< 2
"conference"		3.3			3.2		< 2			< 2	
"beurré hardy"			10					< 2			
"doyenne du comice"				5.8					< 2		
Sweet cherry	*Prunus cerasus L.*		15			15		< 2			< 2
- processed		24	30	38	34	32 ± 5	< 2	< 2	< 2	< 2	< 2
Plum	*Prunus domestica L.*		9			9		< 2			< 2
Peach	*Prunus Persica Batsch*		< 1			< 1		< 2			< 2
- processed		< 1	< 1	< 1	< 1	< 1	< 2	< 2	< 2	< 2	< 2
Grape white	*Vitis vinifera L.*		12	12		12		< 2	< 2		< 2
- black			15			15		< 2			< 2
		Myricetin (mg/kg fresh edible part)									
No fruits contained luteolin, apigenin or myricetin except for:											
Grape white	*Vitis vinifera L.*		4.5	< 1		4.5					
- black			4.5			4.5					

[a] mean of duplicate determinations
[b] annual mean ± SD; April 1991 and April 1992 were averaged prior to calculation of mean and SD
[c] different varieties purchased in different periods
< 1, < 2 = limit of detection.
Blank spaces: not available/sampled in that period

In general, our values are somewhat lower than values reported earlier by Herrmann and co-workers (Wöldecke and Herrmann, 1974; Herrmann, 1976; Starke and Herrmann, 1977). However, Herrmann usually reported a large range of flavonoid levels which makes a comparison difficult. Besides, the thin layer chromatographic method with spectrophotometric measurement applied by Herrmann and co-workers lacks precision and accuracy. It should be noted that in our study only the edible parts were analysed, whereas Herrmann and co-workers generally analysed the whole foods. Our values for various foods are higher compared to values reported by Bilyk and co-workers (Bilyk, Cooper and Sapers, 1984; Bilyk and Sapers, 1985; Bilyk and Sapers, 1986). Discrepancies may be due to different european or american cultivars or to varietal differences. It should also be noted that hydrolysis conditions were not optimized in the studies reported by Bilyk and co-workers.

Variations in flavonoid levels due to seasonal influences were large in leafy vegetables such as lettuce (1.9-30 mg/kg quercetin), endive (15-95 mg/kg kaempferol) and leek (11-56 mg/kg kaempferol). Flavonoid quantities found in lettuce, endive and leek sampled in summer were three to five times higher than in other seasons. Seasonal variability was, however, low in red cabbage. As the formation of flavonoids is light-dependent, flavonoids occur predominantly in the leaves and growing plants in glass houses reduces the flavonoid content (Herrmann, 1976). Year to year variation measured by comparing results of April 1991 and April 1992 was generally within the range of the seasonal variability of the vegetables.

In general, flavonoid levels in processed foods were approximately 50% lower than in fresh products. However, processed sweet cherries had higher quercetin levels than fresh sweet cherries. Quercetin levels in apple-sauce corresponded well with those found in most varieties of apples. No quercetin could be found in processed apricots but 25 mg/kg quercetin was found in fresh apricots. Possibly, these discrepancies are due to varietal differences. No information was available on the variety used in fruit processing. Variation over the year was low in most processed foods.

In this study emphasis was placed on the identification and quantification of five major potentially anticarcinogenic flavonoids in the edible parts of various commonly consumed plant foods. Together with data on the content of flavonoids in several beverages, currently determined at our laboratory, a calculation of the daily intake of these potential anticarcinogens can be made. Our data thus provide a base for epidemiological studies investigating the relation between flavonoid intake and cancer risk.

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Chapter 4

Flavonol and Flavone Content of Beverages

This chapter is based mainly on:

Hertog MGL, Hollman PCH, van de Putte B.
Content of potentially anticarcinogenic flavonoids in tea infusions,
wines and fruit juices. *Journal of Agricultural and Food Chemistry* 1993;41:1242-1246

ABSTRACT

The content of the potentially anticarcinogenic flavonoids quercetin, kaempferol, myricetin, apigenin, and luteolin of commonly consumed beverages was determined by RP-HPLC with UV detection. Flavonoid levels in beer, coffee, chocolate milk, and white wine were below 1 mg/L. Twelve types of tea infusion, six types of wine, apple juice, tomato juice, grape juice, orange juice, grapefruit juice, and lemon juice were analyzed. No luteolin nor apigenin was detected in any of the beverages. In red wines and in grape juice we detected quercetin and myricetin levels varying from 4-16 mg/L and 7-9 mg/L, respectively. Quercetin levels in fruit juices were generally below 5 mg/L except for lemon juice (7 mg/L) and tomato juice (13 mg/L). In black tea infusions we detected quercetin (10-25 mg/L), kaempferol (7-17 mg/L), and myricetin (2-5 mg/L). Flavonoid levels in green tea were comparable to those in black tea. The flavonoid content of tea prepared with tea bags was generally higher than tea prepared with loose leaves. Together with data on the flavonoid content of vegetables and fruits published previously (Hertog et al., J Agric Food Chem 1992, 40, 2379-2383) these data provide a base for an epidemiological evaluation of the potentially anticarcinogenic effects of flavonoids.

INTRODUCTION*

Food-derived flavonoids such as quercetin, kaempferol, and myricetin inhibit carcinogen-induced tumors in rats and in mice (Mukhtar et al., 1988; Verma et al., 1988; Wei et al. 1990; Deschner et al., 1991). In addition, antioxidant flavonoids such as quercetin inhibited oxidation and cytotoxicity of low-density lipoproteins (LDL) in vitro (De Whalley et al., 1990; Negre-Salvagyre and Salvagyre, 1992) which may decrease their atherogenicity and subsequently risk on coronary heart disease (Steinberg et al., 1989) (see also General Introduction). An epidemiological evaluation of the effects of flavonoids on chronic diseases such as cancer and cardiovascular disease is needed to support the findings from experimental studies.

Important dietary sources of flavonoids are vegetables, fruits, and beverages, the latter accounting for at least 25-30 % of the total daily flavonoid intake (Kühnau, 1976). In a previous study we reported the flavonoid content of 28 vegetables and 9 fruits (Hertog et al., 1992b). Here, we report the flavonoid content of twelve types of tea, six types of wine, and seven types of fruit juice.

MATERIALS AND METHODS

Sources and preparation of beverages

All Dutch products were purchased in an outlet of a nationwide supermarket-chain (Albert Heijn) in the period February-August 1992. Products and brands were selected based on information obtained from the Dutch Commodity Board of Distillates and the Dutch Commodity Board of Tea and Coffee. We included typical types of English tea that are not commonly available in The Netherlands (Lay's English Breakfast, Jacksons Earl Grey, St Michael Extra Strong, and Lay's After Dinner) which were kindly provided by Dr. S. Bingham (Cambridge, UK). Although green tea is not commonly consumed in the Netherlands, we included it in our study because several reports on the cancer protective effects of green tea have been published (Conney et al., 1992; Katiyar et al., 1992; Stich, 1992).

Wine

All wines were averagely priced wines commonly consumed in the Netherlands. Three bottles (750 mL) of red French Bordeaux wines from different regions (Grand vin Bordeaux app. Lussac St Emilion contr. 1990, App. Bordeaux contr. Rineaux St. Loubes 1990, and App. Superieur Bordeaux contr. Rineaux St. Loubes 1990) were purchased and combined in equal portions to a composite. Three white German wines from different origins (Mosel-Saar Ruwer Graacher Himmelreich Riesling 1991, Mosel-Saar Ruwer Bernkasteler Kurfürst Lay 1990, and Rheinpfalz Scheigener Guttenberg Kabinett 1989) were mixed in equal portions to a composite. Furthermore, one red Italian Chianti wine (1990), one red Spanish Rioja wine (Otonal 1990), one red Californian wine (Dry Pinot Noir 1990), and one white French

* section shortened for publication in this thesis

Bordeaux wine (St Loubes Rineaux 1990) were purchased.

Tea

Twelve types of tea (*Camellia sinensis* L.), including black, green, and oolong tea, were prepared following the manufacturer's guidelines. Preparation was, unless otherwise indicated, as follows: 500 mL of boiling water was poured onto five grams of loose tea leaves. After 5 min the infusion was passed through a sieve, and allowed to cool prior to analysis. Tea in bags containing 4.0 g or 5.0 g, were placed during 5 min in 500 mL of boiling water. The tea bag was then removed, and the liquid was allowed to cool prior to analysis. The influence of brewing time on flavonoid yield was studied by varying the time before the infusion (Lay's after dinner tea) was passed through a sieve (5 min, 10 min, and 20 min). We also studied the effect the of amount of tea used for brewing on flavonoid yield (4.0 g, 5.0 g, and 6.0 g) using Lay's English Breakfast tea.

Flavonoid levels of infusions prepared with tea bags proved to be consistently higher than infusions prepared with loose tea leaves. We noted that the particle size of tea in tea bags (approximately 0.4-0.8 mm) was much smaller than that of loose tea leaves. In order to study the effect of particle size on flavonoid yield, loose tea leaves (Lays's English Breakfast tea) were ground and the fraction with a particle size between 0.4 mm and 0.8 mm were collected. We then compared flavonoid levels in tea infusions, prepared with untreated loose leaves, with those found in tea infusions prepared with the 0.4-0.8 mm fraction of the same tea leaves obtained after grinding.

Coffee, chocolate milk, and beer

Three bottles of Heineken beer (0.33 cL) were purchased and combined into a composite. One liter of commonly consumed chocolate milk (Albert Heijn's own brand) made of semi-skimmed milk was purchased. The coffee (Zilvermerk, Douwe Egberts, Utrecht, The Netherlands) was similar to the most popular types of coffee sold in The Netherlands. Coffee was made by pouring 125 mL of boiling water on 4.0 g of coffee, which then dripped through a paper filter into a cup.

Fruit juice

Two major brands of tomato juice (1 L each) (Albert Heijn's own brand, Zaandam, The Netherlands and Zontomaatje, Riedel, Ede, The Netherlands) and two orange juice brands (1 L each) (Albert Heijn's own brand, and Appelsientje, Riedel), were each combined in equal portions to composites prior to analysis. One apple juice brand (Goudappeltje, Riedel) and one grape juice brand (Albert Heijn's own brand) (1 L each) were analyzed. All fruit juices consisted of 100 % fruit, as indicated by the producer. Fresh orange juice, fresh lemon juice, and fresh grapefruit juice were each made by squeezing one kg of fresh oranges (*Citrus sinensis L.*), one kg of fresh lemons (*Citrus medica L.*), and one kg of fresh grapefruits (*Citrus maxima Merr.*) with a common household fruit squeezer.

Methods of analysis

Five flavonoids, viz. quercetin, kaempferol, myricetin, luteolin and apigenin were determined in beverages after extraction and acid hydrolysis of the flavonoid glycosides. This method of analysis originally was developed for the determination of flavonoids in freeze-dried foods (Hertog et al., 1992a) and was therefore slightly adapted for analyses of beverages. In brief, 15 ml of sample was boiled with HCl in 50 % aqueous methanol leading to hydrolysis of the flavonoid glycosides to their aglycones and simultaneous extraction of the aglycones. The resulting aglycones were quantified by RP-HPLC on a NOVA-PAK C18 column using acetonitrile/phosphate buffer (25/75, v/v, pH 2.4) as mobile phase, and UV detection (370 nm). To confirm peak identity sample extracts were reinjected, using methanol/phosphate buffer (45/65, v/v, pH 2.4) as mobile phase. In addition, peak identity and purity was confirmed using a photodiode-array detector to record UV-spectra of the flavonoids in samples on-line. All determinations were carried out in duplicate. Limit of detection was defined as the amount of flavonoids resulting in a peak height of three times the standard deviation of the baseline noise.

As completeness of hydrolysis largely depends on the type of glycosides, we determined in a preliminary experiment optimum hydrolysis conditions in beverages as described elsewhere (Hertog et al. 1992b). Briefly, three HCl concentrations (1.2 M, 1.6 M, and 2.0 M) and a hydrolysis period of 2 h and 4 h at 90°C were tested. Optimum conditions were as follows: wines were hydrolyzed with 1.2 M HCl during 4 h at 90°C, other beverages were hydrolyzed with 1.2 M HCl during 2 h at 90°C (results not shown).

Analytical Quality Control

Precision of the method of analysis was reported before (Hertog et al, 1992a) for freeze-dried products. For beverages the following analytical variation was measured. Coefficient of variation of repeatability (CV_r) of quercetin in tea (n = 17), wines (n = 4), and fruit juices (n = 7) were 1.9 %, 4.5 %, and 4.5 %, respectively. CV_r of kaempferol in tea (n = 17) was 3.8 %, whereas the CV_r of myricetin in tea (n = 17) was 4.6 %, and in wine (n = 4) 2.4 %. Recoveries were determined in duplicate in black tea (melange, Douwe Egberts) by spiking pure standards to the extraction solution (100 % of the measured content) prior to sample analysis. Mean recovery was 86.4 % for quercetin, and 93.2 % for kaempferol.

One control sample, consisting of freeze-dried cranberries, was included in each series of samples. At the start of the project, which took about half a year, the quercetin and myricetin content was determined by four duplicate analyses on different days within a period of 4 weeks to determine within-laboratory standard deviation of reproducibility (SD_R). Each series of analysis (n = 7) consisted of the control sample and three calibration samples placed at the beginning and ending of the series. All determinations were carried out in duplicate. Differences between duplicates of more than 10 % were not accepted. Series of analysis were repeated whenever the control sample exceeded the confidence limits (mean ± 3 SD_R). Accepted results of the control sample are shown in Table I. Regression analysis of the results of the control sample did not reveal an association between flavonoid content and time of analysis. Long term variability of flavonoid analyses in the laboratory was low (CV_R's series < 5%).

Table I. Results of Analytical Quality Control Sample[a]

compound	control sample (cranberries)					
	mean[b] at start	SD_R	CV_R, %	mean[b] series	SD_R	CV_R, %
quercetin	1141 (n = 4)	60	5.3	1121 (n = 7)	36	3.2
myricetin	572 (n = 4)	30	5.2	590 (n = 7)	27	4.6

[a] each determination was carried out in duplicate
[b] mg/kg dry weight, within-laboratory standard deviation of reproducibility (SD_R), and within-laboratory coefficient of variation of reproducibility (CV_R) at start and in the whole project (0.5 years).

RESULTS

Table II reports the quercetin and myricetin content of wine and fruit juice. No apigenin, luteolin or kaempferol could be detected in any of these beverages (limit of detection 0.5 mg/L). In general myricetin was the most important flavonoid found in wines and grape juice, with the exception of the red Chianti and the red Californian wine in which quercetin predominated. In white wines we did not detect any quercetin and myricetin, except for the white German Mosel composite in which a very low level of myricetin (1 mg/L) was found. Quercetin levels varied between different types (4-16 mg/L) whereas the myricetin levels were similar in all red wines (7-9 mg/L). Highest flavonoid levels were found in the Italian Chianti wine. The quercetin and myricetin content of grape juice compared well with the levels found in red wines. In other fruit juices only quercetin was found with highest amounts found in tomato juice (13 mg/L). The quercetin level of fresh orange juice compared well with the quercetin level in industrially produced orange juice (fruit content: 100 %). No flavonoids were found in beer, and in brewed coffee. In chocolate milk we found a very low amount of quercetin (1 mg/L)

The quercetin, kaempferol and myricetin content of various types of tea are shown in Table III. A typical chromatogram of a black tea extract is shown in Figure 1. No apigenin nor luteolin could be detected in tea. Quercetin was in general the most important flavonoid found in tea, with the exception of Jacksons Earl Grey tea and Lay's After Dinner tea in which kaempferol levels were higher. Black tea infusions prepared with tea bags (4.0 or 5.0 g) contained 17-25 mg/L quercetin, 13-17 mg/L kaempferol and approximately 3 mg/L myricetin. The quercetin content of black tea infusions prepared with loose leaves are considerably lower (10-13 mg/L). Flavonoid yield in black tea (Lay's After Dinner) was slightly higher when extending the brewing time to 10 min, but it did not increase after 10 min. There was also an increase in flavonoid yield with increasing amount used for brewing but this increase was not linear. Grinding of the tea leaves had a pronounced effect on flavonoid yield. Quercetin levels increased by approximately 40 % from 11 mg/L in untreated tea to 16 mg/L in tea prepared with the 0.4-0.8 mm fraction of the ground leaves. However, this increase was less pronounced for kaempferol and myricetin.

Table II. Quercetin and Myricetin Content (mg/L) of Wine, Fruit Juices, and Other Beverages

Beverages	mg/L[a]	
	quercetin	myricetin
Wine		
Red Bordeaux (composite[b])	4.1	7.5
Red Rioja Otonal 1990	4.1	9.3
Red Chianti 1990	16	8.0
Red California Dry Pinot Noir 1990	8.8	6.9
White Bordeaux St Loubes Rineaux 1990	< 0.5	< 0.5
White Mosel (composite[c])	< 0.5	1.0
Fruit juice		
Apple juice (Albert Heijn, Zaandam)	2.5	< 0.5
Grape juice (Riedel, Ede)	4.4	6.2
Tomato juice (commercial composite[d])	13	< 0.5
Grapefruit juice (fresh)	4.9	< 0.5
Lemon juice (fresh)	7.4	< 0.5
Orange juice (fresh)	3.4	< 0.5
Orange juice (commercial composite[e])	5.7	< 0.5
Other beverages		
Beer (Heineken)	< 0.5	< 0.5
Chocolate milk (semi-skimmed milk)	1.3	< 0.5
Coffee	< 0.5	< 0.5

[a]mean of duplicate determination; < 0.5 below the limit of detection;
[b]1. Grand vin Bordeaux app. Lussac St Emilion contr. 1990; 2. App. Bordeaux contr. Rineaux St. Loubes 1990; 3. App. Superieur Bordeaux contr. Rineaux St. Loubes 1990;
[c]1. Mosel-Saar Ruwer, Graacher Himmelreich Riesling, 1991; 2. Mosel-Saar Ruwer, Bernkasteler Kurfürst Lay 1990; 3. Rheinpfalz Scheigener Guttenberg Kabinett 1989;
[d]Albert Heijn's own brand and Zontomaatje (1:1, v/v);
[e]Albert Heijn's own brand and Appelsientje (1:1, v/v)

Flavonoid levels in oolong tea were generally in the lower range (5-13 mg/L) of the flavonoid content of black tea. The amount of flavonoids found in both green tea infusions are comparable to the average levels found in black tea, except myricetin which was higher (5-12 mg/L) than in black tea (2-5 mg/L).

Table III. Content (mg/L) of Quercetin, Kaempferol, and Myricetin in Different Types of Tea Infusions[a].

Tea	amount	mg/L[b]		
	(b = teabag)	quercetin	kaempferol	myricetin
Pickwick DE melange[c] *black*	4.0 g (b)	19	15	3.2
Van Nelle melange[c] *black*	4.0 g (b)	17	14	2.8
Albert Heijn melange[c] *black*	4.0 g (b)	17	13	3.0
St. Michael extra strong *black*	5.0 g (b)	21	15	2.5
Lipton "Brisk" *black*	5.0 g (b)	25	16	5.2
Pickwick DE Earl Grey *black*	4.0 g (b)	21	17	3.5
Jacksons Earl Grey *black*	5.0 g	12	16	2.5
Lay's After Dinner *black*	5.0 g	10	12	2.5
brewing time: 10 min	5.0 g	12	14	3.0
brewing time: 20 min	5.0 g	13	14	2.5
Lay's English Breakfast *black*	4.0 g	10	6.3	1.7
	5.0 g	11	7.0	2.1
	6.0 g	16	9.5	3.1
ground fraction (0.4 mm - 0.8 mm)	5.0 g	16	9.2	2.9
Formosa, *oolong*	5.0 g	13	9.0	4.9
Japan, "Sencha" *green*	5.0 g	23	15	12
China, "Gunpowder" *green*	5.0 g	14	9.1	5.2

[a] brewing time: 5 min unless otherwise indicated
[b] mean of duplicate determinations
[c] mixture of English breakfast and afternoon tea

DISCUSSION

Most studies on the flavonoid content of beverages such as wine, fruit juices and tea have been carried out for taxonomic purposes. This implies that in general only the major flavonoid glycosides, or in fermented products such as tea and wine the major free flavonoid aglycones, were identified. Only little attention has been paid to the quantitative determination of flavonoids in beverages after hydrolysis of the glycosides to their corresponding aglycones. A quantitative comparison between our values and those reported in the literature is therefore difficult. The large variation in flavonoid content of different types of red wines is confirmed

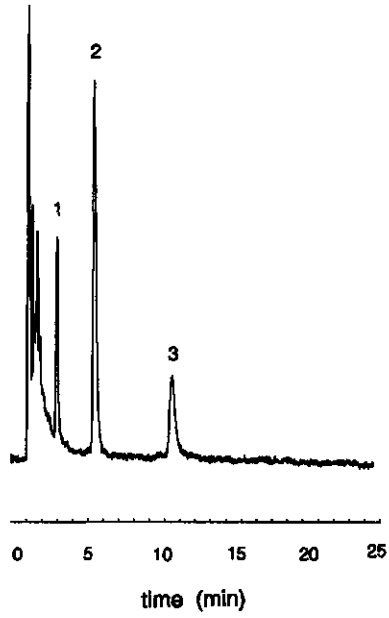

Figure 2. Typical chromatogram of a black tea (Lipton) extract monitored in 25% acetonitrile/phosphate buffer (pH 2.4). Peaks: Myricetin (1), Quercetin (2), and Kaempferol (3).

by Salagoïty-Auguste and Bertrand (1984), Revilla et al (1986), and Etievant et al. (1988). We could not detect flavonoids in white wines which is consistent with Revilla et al (1986) who reported quercetin levels in white Spanish wines below our limit of detection.

Unexpectedly, we detected quercetin in fresh orange, grapefruit, lemon juices, and in commercial orange juice. Herrmann (1976) and Balestieri et al. (1991) reported that citrus fruits contain almost exclusively flavanones. Flavanone analysis in fruit juices have been mainly carried out for tracking down of adulterations. The applied analytical techniques may thus have lacked sensitivity for detection of quercetin. To our knowledge no data on the flavonoid content of tomato juice, apple juice, and grape juice have been published previously. Quercetin was also found in fresh tomatoes, apples and grapes (Hertog et al., 1992b). We could not detect any of the investigated flavonoids in beer, and coffee, and only a very low amount in chocolate milk. Kühnau estimated in 1976 that these beverages were dietary sources of flavonoids, but this could not be confirmed in our study.

Flavonoid levels in different types of black tea varied only slightly. The size of tea leaves and consequently extraction surface seemed to be of far more importance. Particle size thus explains largely the differences in flavonoid yield between tea prepared with tea bags and tea prepared with loose leaves. A brewing time of tea of 20 min, as it is customary in some countries such as the UK did not result in an important increase in flavonoid yield. An ordinary brewed tea infusion which would be made by pouring 500 mL of boiling water on

5 g of tea leaves and a brewing time of 5 min) thus contains 30-40 mg/L of combined flavonoids and contributes therefore significantly to flavonoid intake in humans. Green tea infusions contained a similar amount of flavonoids as black tea. It should be noted that green tea used in this study had large size leaves compared to the loose black tea. The total content of combined flavonoids in Japanese green tea "Sencha" amounts up to 50 mg/L. In only one study the flavonoid content of tea infusion was reported. Fieschi et al. (1989) determined the total flavonoid glycoside and aglycone content of different types of black tea infusions using paper chromatography followed by spectrophotometric measurements. Total flavonoid content varied from 46-86 mg/L and the content of flavonoid aglycones from 0.8-1.1 mg/L. Total flavonoid content of green tea was 82 mg/L and aglycone content 2 mg/L. In most other studies loose tea leaves were extracted and analyzed directly for flavonoid glycosides. Biedrich et al (1989) reported 86-2140 mg/kg of rutin (quercetin-3-*O*-rhamnoglucoside) determined by HPLC in different types of black tea. Baily et al (1990) and Finger and Engelhardt (1991) reported that the content of quercetin and kaempferol rhamnodiglucosides determined by HPLC in different types of black tea was 410-950 mg/kg, and 500-1200 mg/kg respectively. These figures are generally lower than our values. We found approximately 2400-2875 mg/kg of quercetin and 1625-2125 mg/kg of kaempferol that is extracted in the black tea infusion.

In conclusion, the data of the present study and the results of the previous study (Hertog et. 1992b) provide a base for epidemiological studies investigating the relation between the intake of these antioxidant flavonoids and risk of chronic diseases such as cancer and coronary heart disease.

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Chapter 5

Intake of Flavonols and Flavones in The Netherlands

This chapter is based mainly on:

Hertog MGL, Hollman PCH, Katan MB, Kromhout D.
Estimation of daily intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutrition and Cancer* 1993;20:21-29

ABSTRACT

Flavonoids are strong antioxidants that occur naturally in foods and can inhibit carcinogenesis in rodents. Accurate data on population-wide intakes of flavonoids are not available. Here we, using data of the Dutch National Food Consumption Survey 1987-1988, report the intake of the potentially anticarcinogenic flavonoids quercetin, kaempferol, myricetin, apigenin and luteolin among 4,112 adults. The flavonoid content of vegetables, fruits, and beverages was determined by High-Performance Liquid Chromatography. In all subjects, average intake of all flavonoids combined was 23 mg/day. The most important flavonoid was the flavonol quercetin (mean intake 16 mg/day). The most important sources of flavonoids were tea (48% of total intake), onions (29%) and apples (7%). Flavonoid intake did not vary between seasons; it was not correlated with total energy intake ($r = 0.001$), and it was only weakly correlated with the intake of vitamin A (retinol equivalents, $r = 0.14$), dietary fiber ($r = 0.21$) and vitamin C ($r = 0.26$). Our use of new analytic technology, suggests that in the past flavonoid intake has been overestimated five-fold. However, on a milligram per day basis, the intake of the antioxidant flavonoids still exceeded that of the antioxidants β-carotene and vitamin E. Thus flavonoids represent an important source of antioxidants in the human diet.

INTRODUCTION*

Flavonoids are diphenylpropanoids occurring ubiquitously in plant foods and they form important constituents of the human diet. Flavonoids are strong antioxidants and scavengers of free radicals which are possibly involved in cell damage and tumor promotion. Quercetin and other related flavonoids inhibited carcinogen-induced tumors in rats and in mice and growth of cancer cells (see General Introduction). Kühnau (1976) estimated that the combined intake of flavonoids is approximately 1 g/day. However, this estimation was based mainly on food analyses using techniques of doubtful accuracy. Furthermore, no information is available on the intake of individual flavonoids such as quercetin.

We selected three major flavonols, quercetin, kaempferol, and myricetin, and two major flavones, luteolin and apigenin, because these flavonoids were most widely investigated in studies on anticarcinogenesis in experimental animals. We developed and validated a quantitative method for the determination of these flavonoids in foods (Hertog et al., 1992a), and we determined the flavonoid content of foods commonly consumed in The Netherlands (Hertog et al., 1992b, Hertog et al., 1993). We now report the intake of these flavonoids and their determinants in a large, representative population sample in The Netherlands.

MATERIALS AND METHODS

Food Consumption Survey

The Dutch National Food Consumption Survey was carried out among a representative sample of 5,898 individuals, aged 1-74 years, between April 1987 and March 1988. In the present study, we only used data of adults >19 years of age (n=4,112). Institutionalized subjects and subjects who could not speak Dutch were not eligible. Design and methods of this survey are described in detail elsewhere (Hulshof and van Staveren, 1991).
Information on food intake was collected by specially trained dietitians using a two-day record. Information on special dietary practices and smoking habits was also collected. Data collection was equally distributed over the seven days of the week and over the year (holidays excluded). Average nutrient intake per day was calculated using the 1986-1987 release of the Dutch Food Composition Data Bank. Table I reports intake of some nutrients and some selected food groups. These values are comparable to those obtained in other Western countries including the United States (USDA, 1984).

* section shortened for publication in this thesis

Table I. Daily Intake of Selected Nutrients and Consumption of Selected Food Groups (Dutch Food Consumption Survey, 1987-1988)[a]

Nutrients and Food Groups	Men (n = 1,896)	Women (n = 2,216)
Total energy (MJ/day)	11.5 ± 2.9	8.5 ± 2.4
(kcal/day)	2745 ± 692	2047 ± 570
Fat (% of energy)	40.5 ± 6.8	41.1 ± 7.4
Protein (% of energy)	13.1 ± 2.8	14.3 ± 3.6
Carbohydrate (% of energy)	41.2 ± 7.1	41.8 ± 7.6
Alcohol (% of energy)	5.2 ± 5.9	2.8 ± 4.7
Dietary Fiber (g/day)	27.0 ± 9.7	21.7 ± 7.2
Vitamin C (mg/day)	76.4 ± 56.6	76.8 ± 52.3
Retinol equivalents (mg/day)	1.1 ± 0.8	0.9 ± 0.7
Vegetables (g/day)	163 ± 105	154 ± 102
Fruits (g/day)	152 ± 155	186 ± 161
Legumes (g/day)	8 ± 33	7 ± 28
Non-alcoholic beverages (g/day)	1115 ± 429	1109 ± 447
Alcoholic beverages (g/day)	383 ± 520	94 ± 193

[a]Values are mean ± SD of 4,112 adults

Food analysis

Quercetin, kaempferol, myricetin, luteolin and apigenin were determined by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) in 28 types of vegetables, 12 types of fruits, and 9 types of beverages (Hertog et al., 1992a, 1992b, 1993). The sampling included foods that are often associated with low risk of cancer, such as green-yellow vegetables, cruciferous vegetables, and citrus fruits (Block et al., 1992), and are commonly consumed in The Netherlands. Sampling of each of the vegetables and fruits was carried out in three seasons: spring, summer, and winter. Each food was purchased in a supermarket, in a grocery, and in an open-air street market, combined per food into a composite, and analyzed. Canned and other processed foods (3 brands of each food) were bought in a supermarket and combined into composites before analysis. Six different types of tea (prepared according to Dutch customs), five types of wine and four types of fruit juice were analyzed. Coffee was analyzed additionally, but flavonoid levels in coffee were below the limit of detection (<0.5 mg/L) (Hertog et al., 1993).

The method of analysis (Hertog et al., 1992a, 1992b, 1993) consisted of extraction and acid hydrolysis of the flavonoid glycosides with HCl in 50% aqueous methanol, followed by RP-

HPLC and ultraviolet-detection of the flavonoid aglycones. Identification and quantification of the flavonoids was performed by co-chromatography with pure standards. In addition, identity and purity of the flavonoids were confirmed by diode-array detection on-line. Analytic quality control was performed by including control samples with a known amount of flavonoids in every series of analysis. Limit of detection for all flavonoids was approximately 1 mg/kg of fresh food. The mean flavonoid content, expressed as aglycons, of some selected foods is reported in Table II. Additional data have been published elsewhere (Hertog et al., 1992a, 1992b, 1993).

Table II. Flavonoid Content of Selected Fresh Vegetables, Fruits, and Beverages Commonly Consumed in The Netherlands.[a]

Food	Quercetin	Kaempferol	Myricetin	Apigenin	Luteolin
Lettuce	14 ± 14	<2	<1	<2	<1
Onion	347 ± 63	<2	<1	<2	<1
Endive	<1	46 ± 42	<1	<2	<1
Broad beans[b]	20	<2	25	<2	<1
Celery	<1	<2	<1	108	22
Apple[c]	36 ± 19	<2	<1	<2	<1
Wine (red)[c,d]	11 ± 5	<1	9 ± 3	<1	<0.5
Tea (black)[c,d]	20 ± 2	14 ± 3	2.5 ± 1.2	<1	<0.5
Apple Juice[b-d]	2.8	<1	<0.5	<1	<0.5

[a]values are mean ± SD in mg/kg <0.5, <1, and <2 are below limit of detection
[b]sampled only once
[c]mean ± SD of 5 varieties
[d]expressed in mg/L

Calculation of flavonoid intake

Individual flavonoid intake was calculated by multiplying the consumption of each food by its flavonoid content. Flavonoid content was known for approximately 95% of all flavonoid-containing foods that are commonly consumed in The Netherlands. Individual intake of a particular fresh food was calculated by adding the amounts of raw and prepared food consumed, with weight losses due to preparation taken into account. We did not correct for loss of flavonoids due to preparation, because in a preliminary study flavonoids proved to be heat stable, with losses <20 % (unpublished observations). For most foods, the year mean value of the flavonoid content was used because seasonal variability proved to be low. However, for fresh leafy vegetables such as lettuce, leek, and endive, season-specific values were used. Statistical analyses were carried out using the SAS statistical package (version 6, SAS Institute, Cary NC 1988). Spearman's rank correlations between flavonoid intake and selected nutrients were computed.

Table III. Flavonoid and Quercetin Intake by Sex and Age Group (Dutch National Food Consumption Survey, 1987-1988)[a]

		Flavonoids[b]				Quercetin			
		percentile				percentile			
	N	Mean	10th	50th	90th	Mean	10th	50th	90th
				Men					
All ages	1,896	22	3	17	46	16	2	11	35
20-39 yrs	901	21	3	15	47	16	1	10	36
40-59 yrs	641	22	3	17	45	15	2	11	35
>60 yrs	354	24	6	21	46	16	4	13	33
				Women					
All ages	2,216	23	5	20	45	16	3	13	33
20-39 yrs	1,083	23	4	18	48	16	2	12	34
40-59 yrs	706	24	5	20	45	16	3	13	32
>60 yrs	427	25	8	23	43	16	5	14	31

[a] Values are means in mg/day
[b] Sum of quercetin, kaempferol, myricetin, apigenin, and luteolin

RESULTS

Average intake of flavonoids was 23 mg/day (10th and 90th percentiles: 4 and 46 mg/day). Seventy-six participants (2%) had a flavonoid intake of 0 mg/day on the two particular days on which they were surveyed. Quercetin contributed about 70% of flavonoid intake, kaempferol 17%, myricetin 6%, and luteolin and apigenin 4% and 3%, respectively. Mean intake of quercetin was 16 mg/day (10th and 90th percentiles: 3 and 34 mg/day). Total flavonoid and quercetin intake varied only slightly by age and sex (Table III). Intake was highest in the oldest age strata and tended to be higher in women than in men. The median and percentiles indicate that flavonoid intake is strongly skewed toward the higher values. This is also reflected in Figure 1, which shows the distribution of flavonoid intake of the total population.

Flavonoid intake was approximately 21% lower in heavy smokers than in non-smokers or light smokers (Table IV). Male vegetarians had a 36% and female vegetarians a 43% higher intake of flavonoids than non-vegetarians. Mean flavonoid intake in summer and in fall was 22 mg/day, and in winter and in spring, it was 23 mg/day.

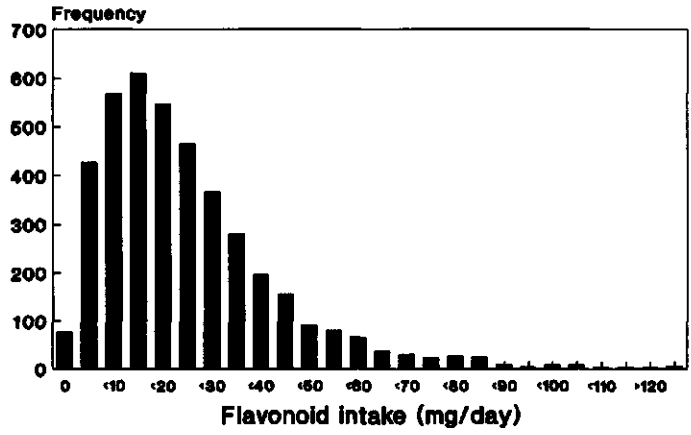

Figure 1: *Distribution of flavonoid intake of 4,112 adults in the Netherlands*

Table IV. Mean Flavonoid Intake Adjusted for Age According to Cigarette Use and Dietary Practice (Dutch National Food Consumption Survey, 1987-1988)

	Men (n = 1,896)		Women (n = 2,216)	
	Flavonoid Intake[a]		Flavonoid Intake[a]	
	N	mg/day	N	mg/day
Cigarettes/day				
0	1,000	23	1,413	25
1-9	318	23	242	22
>10	578	18	561	20
Vegetarians				
Yes	32	30	63	33
No	1,864	22	2,153	23

[a] Sum of quercetin, kaempferol, myricetin, apigenin, and luteolin

Table V lists the top ten foods contributing to total flavonoid intake. Tea was the major source of flavonoids at 48%, followed by onions at 29%, and apples at 7%. We also investigated the contribution of different foods in a subgroup of subjects consuming >50 mg of flavonoids per day. Mean flavonoid intake in this subgroup was 68 mg/day. Tea and onions remained the most important sources, followed by kale. All beverages combined accounted

for about 50% of flavonoid intake, whereas vegetables accounted for 40% and fruits for 10%. Flavonoid intake was not correlated with total energy intake ($r = 0.001$, $p > 0.05$), and only weakly with intake of vitamin A (retinol equivalents, $r = 0.14$, $p < 0.01$), dietary fiber ($r = 0.21$, $p < 0.01$) and vitamin C ($r = 0.26$, $p < 0.01$).

Table V. Contribution of Various Foods to Flavonoid Intake[a] in Adults and in a Subgroup >50 mg/day

Food	All Subjects (N=4,112)		Subjects With Intake>50 mg/day (N=333)	
	Percent (rank)	Cumulative percentage	Percent (rank)	Cumulative percentage
Tea	48.1 (1)	48.1	38.8 (2)	38.8
Onions	28.9 (2)	77.0	43.4 (1)	82.2
Apples	7.1 (3)	84.1	3.2 (4)	85.5
Kale	3.6 (4)	87.7	9.0 (3)	94.5
French beans	2.9 (5)	90.6	1.1 (6)	95.6
Endive	2.2 (6)	92.8	1.5 (5)	97.1
Red wine	0.9 (7)	93.7	0.4 (7)	97.5
Apple-sauce	0.7 (8)	94.4	0.3 (8)	97.9
Oranges	0.6 (9)	95.0	0.3 (9)	98.2
Leek	0.5 (10)	95.5	0.3 (10)	98.5

[a] Flavonoid intake is sum of quercetin, kaempferol, myricetin, apigenin, and luteolin

DISCUSSION

To our knowledge, this is the first estimation of the intake of flavonoids in a large population sample using analytic data obtained by HPLC. Kühnau (1976) estimated that the average intake of all flavonoids combined was approximately 1 g/day, of which about 170 mg (expressed as glycosides) were flavonols, flavones, and flavanones. This is approximately 115 mg expressed as aglycons such as quercetin. This estimation was based on the average American diet as accounted for in the Food Consumption Statistics 1955-1971 of the Organization for Economic Cooperation and Development (OECD, Paris, 1973). These values have been widely quoted. Our results, expressed as aglycons, suggest that the intake of flavonols and flavones in a Western population is approximately fivefold lower than Kühnau's estimate, but a direct comparison between the two values is difficult. We did not include flavanones in our analysis, because no studies have reported anticarcinogenic activities of this class of flavonoids. Because the occurrence of flavanones is limited to citrus fruits at levels between 70 and 160 mg/kg (Balestieri et al., 1991), resulting in a mean intake of 5 mg/day on the basis of our data, they cannot account for the large difference between our results and

the estimation of Kühnau (1976). It is more likely that the high values reported by Kühnau are due to an overestimation of the flavonoid content of foods. The estimation of Kühnau was based on techniques now considered inappropriate, the most advanced being thin-layer chromatography followed by a spectrophotometric measurement. In addition, these data were based on analyses of the whole foods as opposed to analyses of the edible parts in our study. In general, our values obtained by HPLC (Hertog et al., 1992a, 1992b, 1993) are considerably lower than those cited by Kühnau (1976). Moreover, the OECD Food Consumption Statistics used by Kühnau are based on food disappearance values, which overestimate the intake of foods. Changes in dietary practices between 1971 and 1988 would, rather, suggest a higher intake of flavonoids in The Netherlands in 1988 because of the increased consumption of vegetables and fruits in this period. It is unlikely that we missed important potential sources of flavonoids, because we included all foods of vegetable origin consumed at an average level of >1 g/day.

Quercetin is quantitatively the most important of the five flavonoids investigated, followed by kaempferol. Myricetin, luteolin, and apigenin are far less important. Quercetin is the predominant flavonoid found in foods, and, as a consequence, it has been used in most studies investigating physiological and biologic effects of flavonoids. Tea was the most important contributor to total flavonoid intake, followed by onions and apples (cumulative percentage 84) in this population. Because consumption of tea, onions, and apples does not vary between the seasons, seasonal variability in flavonoid intake was low.

Average tea consumption of the total population was approximately 2 cups/day (294 ± 310 ml), average onion consumption was 16 ± 32 g/day, and average apple consumption was 45 ± 71 g/day. The range of flavonoid intake was very large; we found an approximately tenfold difference between the 10th and the 90th percentile. Participants with a zero flavonoid intake had a vitamin C intake of 29 mg/day, which is less than half the intake of vitamin C in the rest of the subjects (77 mg/day). The higher intake in women and in older age groups could be explained by a higher tea consumption. Onion and apple consumption was comparable in men and women. Consumption of onions was lower in older age groups, whereas consumption of apples was virtually constant over the four age groups. Heavy smokers had a lower flavonoid intake than nonsmokers or light smokers, which could be explained by a low consumption of tea and apples by heavy smokers. Onion consumption was approximately the same in the three smoking groups. Vegetarians consumed more tea, apples, and onions than nonvegetarians, which explains their high intake of flavonoids.

We conclude that flavonoids and especially quercetin are common components of the human diet. Unexpectedly, tea was the major contributor of flavonoids in this population and the contribution of all vegetables combined, including onions at 29%, was approximately 40 %. Thus the foods often associated with low rates of cancer in epidemiological studies, such as green-yellow vegetables and cruciferous vegetables (Block et al., 1992), are not major sources of flavonoids in this population. However, in populations with other dietary practices, the importance of contribution of various foods may be different. In experimental animal studies, polyphenolic compounds in tea, such as quercetin, had anticarcinogenic effects (Mukhtar et al., 1992; Stich, 1992). In epidemiological studies, little attention has been paid to the effects of tea on carcinogenesis, and the results are conflicting (La Vecchia et al., 1992).

In adults, mean intake of the five antioxidant flavonoids combined is 23 mg/day. Intake

of flavonoids thus exceeds that of other dietary antioxidants such as beta-carotene (2 - 3 mg/day) and vitamin E (7 - 10 mg/day), and equals approximately one-third of that of vitamin C (70 - 100 mg/day) (USDA, 1984). Furthermore, flavonoids are heat stable, and losses due to cooking and frying are low (unpublished observations). Thus flavonoids make a major contribution to the antioxidant potential of the diet in Western populations. Flavonoid intake was only weakly correlated with the intake of vitamin C, vitamin A (retinol equivalents), and dietary fiber, which have traditionally been regarded as cancer-protective factors in vegetables and fruits. This lack of intercorrelation offers the possibility to study the anticarcinogenic effects of flavonoids in epidemiological studies without the pitfalls of multicollinearity. Antioxidant flavonoids such as quercetin also inhibited oxidation and cytotoxicity of low-density lipoproteins *in vitro* (DeWhalley et al., 1990; Negre-Salvagyre et al., 1992) which may decrease their atherogenicity and, subsequently, risk of coronary heart disease (Steinberg et al., 1989). It is therefore of interest to study the relationship between flavonoid consumption and the risk of chronic diseases in an epidemiological context.

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Chapter 6

Flavonols, Flavones, and Cancer Risk

This chapter is based mainly on:

Hertog MGL, Feskens EJM, Hollman PCH, Katan, MB, and Kromhout, D.
Dietary flavonoids and cancer risk in The Zutphen Elderly Study
(*submitted for publication*)

ABSTRACT

Flavonoids are polyphenolic antioxidants naturally present in vegetable foods. Flavonoids are mutagenic in vitro, but recent studies show that some flavonoids such as quercetin strongly inhibit carcinogenesis in rodents. The effect of flavonoids on human carcinogenesis is unknown. We measured the flavonoids quercetin, kaempferol, myricetin, apigenin, and luteolin in foods, and assessed flavonoid intake in 1985 by dietary history in 738 men aged 65-84 years without a history of cancer, who were then followed for 5 years. Mean flavonoid intake was 25.9 mg/day. The major sources of flavonoid intake were tea at 61 % and vegetables and fruits (mainly onions, kale, endive, and apples) at 38 %. Between 1985 and 1990, 75 men developed cancer (all sites), and 34 men died from cancer. Twenty-six men had a first diagnosis of lung cancer in this period. Flavonoid intake in 1985 was not associated with either incidence of all cause cancer (P for trend = 0.54) or with mortality from all-cause cancer (P for trend = 0.51). Similar results were obtained when only cases with epithelial cancers of the alimentary and respiratory tract were used. Adjustment for age, body-mass index, smoking, physical activity, vitamin C, vitamin E, beta-carotene, and dietary fibre did not change the relative risks. Flavonoid intake was also not associated with lung cancer incidence and mortality. Flavonoid intake is not associated with risk of all-cause cancer in elderly men. The effect of flavonoids on risk of cancer at specific sites needs further investigation in prospective cohort studies

INTRODUCTION*

The role of flavonoids (Kühnau, 1976) in carcinogenesis is controversial (see General Introduction). Early studies showed that some major flavonoids, e.g. quercetin, kaempferol, and myricetin were mutagenic in bacterial test systems (Bjeldanes and Chang, 1977; Nagao et al.), but quercetin was reported not to be mutagenic *in vivo* (Aeschbacher et al., 1982). Quercetin at a level of 2 % in the diet was also found to induce bladder tumors in rats (Pamucku et al., 1980), but, these results were disproved in other studies using quercetin levels up to 10 % of the diet (Hirono et al., 1981; Morino et al., 1981; Saito et al., 1980). More recent studies in fact suggest that flavonoids protect against chemically-induced cancer in animals (Deschner et al., 1991; Mukhtar et al., 1988; Verma et al., 1988; Wei et al., 1990). Antioxidant flavonoids enhance the deactivation of potential carcinogens (Wattenberg, 1985), and scavenge free radicals which are possibly involved in cell damage and subsequently tumor development (Cerutti, 1985; Fujiki et al., 1986). Intake of flavonoids may therefore reduce cancer risk in humans. This is supported by epidemiologic findings that a high consumption of vegetables and fruits is associated with a reduced risk of cancer (Steinmetz and Potter, 1991a). Hitherto, no epidemiologic studies on the effects of flavonoids on cancer have been carried out due to a lack of accurate data on flavonoid content of foods.

We selected five major food flavonoids viz. quercetin, kaempferol, myricetin, apigenin, and luteolin, measured their content in the major types of vegetables, fruits, and beverages consumed in The Netherlands (Hertog et al., 1992a; Hertog et al. 1992b; Hertog et al., 1993a), and calculated baseline flavonoid intake of the participants of the Zutphen Elderly Study. We reported previously that flavonoid intake predicted a lower rate of coronary heart disease mortality in elderly men (Hertog et al., 1993c). Here we report the relation between flavonoid intake and 5-year cancer incidence and mortality.

MATERIAL AND METHODS

Flavonoid Analysis

We determined the content of the five major flavonoids quercetin, kaempferol, myricetin, luteolin and apigenin of 28 types of vegetables, 12 types of fruits, and 9 types of beverages (Hertog et al. 1992b; Hertog et al., 1993a). Together, these foods covered approximately 95 % of all vegetable foods commonly consumed in The Netherlands. Each food was purchased in each of the three seasons spring, summer, and winter in a supermarket, in a grocery, and in an open-air street market. Separate purchases were combined, and flavonoid content was determined by Reversed-Phase High Performance Liquid Chromatography with ultraviolet-detection. In plants a large number of flavonoid glycosides originating from the same parent aglycon (sugar-free flavonoid) occur. In order to simplify the analytical procedure and to enhance the limit of detection, glycosides were hydrolysed, and flavonoid levels are expressed as aglycons. The aglycon is, because of its polyphenolic character, the biologically active part of the flavonoid molecule. Control samples with a known amount of flavonoids were included in every series of analysis. Limit of detection for flavonoids was approximately 1 mg/kg or

* section shortened for publication in this thesis

0.5 mg/L. Other details have been published (Hertog et al., 1992a; Hertog et al. 1992b; Hertog et al., 1993a).

Population

The Zutphen Elderly Study is a longitudinal investigation of risk factors for chronic diseases (van Buchem et al., 1967) and forms the Dutch contribution to the Seven Countries Study (Keys et al., 1967). It was originally started in 1960 with a cohort of 878 men aged 40-59 years living in the town of Zutphen in the Netherlands. In 1985, 555 men of this cohort were still alive and were invited for new examinations. In addition, a random sample of all other men of the same age group (65-84 years) living in Zutphen and not part of the 1960 cohort were approached. This resulted in a target population of 1266 men. Sixty-two men (4.9 percent) could not participate because they had moved or could otherwise not be reached, 109 (9.0 percent) could not be examined because of serious illness, and 156 (12.0 percent) refused to participate. Hence, in 1985 a total of 939 men (74 %) aged 65-84 years entered the study. Complete information on diet and other risk factors was available for 801 participants.

Examinations

All examinations were carried out in the period between March and June in 1985 and a repeat examination was carried out in the same period in 1990. Information on the usual food intake of the participants in the month preceding the interview was collected by experienced dietitians using a cross-check dietary history method adapted to the Dutch situation (Bloemberg et al., 1989). Each participant was interviewed at home for about 1 h together with the person who prepared the food (usually the wife) about his usual food consumption pattern on weekdays and during weekends, and about food purchases. The habitual consumption of foods during a week was determined, and verified with the quantities of foods bought per week. This information was combined to calculate the participants food consumption on a typical weekday. The food intake data were encoded by the dietitians according to the Netherlands Uniform Food Encoding System and converted into energy and nutrient values using the 1985 release of the Netherlands nutrient data bank (Kommissie UCV, 1985) that was updated with 1993 values for beta-carotene and vitamin E, and with flavonoid data. Flavonoids refer to the sum of quercetin, kaempferol, myricetin, apigenin, and luteolin.

Medical examinations were carried out by trained physicians and included anthropometry and blood measurements. Information on amount and duration of smoking was assessed using a standardized questionnaire and converted into lifetime exposure of cigarettes ([number of cigarettes/day * 365] * years of smoking). Physical activity was assessed with a questionnaire specially designed for retired men and minutes of physical activity per week were calculated (Caspersen et al., 1991).

Incidence and Mortality Follow-up

Prevalence of a history of cancer was recorded during the medical examinations in 1985 and 1990 with a standardized questionnaire, and verified with written information from the

subject's general practitioner, with hospital discharge data, and with information of the cancer registry. For subjects that had died information on a possible history of cancer was collected from their general practitioner, from hospital discharge data, and from the cancer registry. All information was uniformly coded by a single physician, and the year of first diagnosis of each disease was recorded.

Information on the vital status of all participants up to July 1990 (5 year follow-up) was obtained from the municipal registries. No one was lost to follow-up. Information on primary cause of death was obtained from the Central Bureau for Statistics, verified by means of hospital discharge, cancer registry data, and from information from the general practitioner, and coded according to the *ninth revision of the International Classification of Diseases* (WHO, 1977). All-cause cancer refers to ICD 140-208, epithelial cancers of the alimentary and respiratory tract to ICD 140-165 and ICD 188-189, and lung cancer to ICD-162.

Table I. Baseline characteristics of 738 men[a] aged 65-84 years according to cancer incidence between 1985 and 1990 (The Zutphen Elderly Study)

	Cancer Incidence		
Characteristic	No (n=663)	Yes (n=75)	*P* value
	Mean ± SD		
Flavonoid intake (mg/day)	25.9 ± 14.6	26.2 ± 14.3	0.87
Age (yrs)	71.0 ± 5.2	72.5 ± 5.7	0.02
Lifetime cigarette smoking (x 1000)	489 ± 511	604 ± 509	0.03
Body-mass index (kg/m^2)	25.7 ± 3.1	25.1 ± 3.2	0.10
Physical activity (min/week)	788 ± 725	766 ± 799	0.66
Energy intake (MJ/day)	9.5 ± 2.2	9.6 ± 2.0	0.42
Alcohol (g/day)	13.1 ± 17.2	14.6 ± 16.4	0.23
Dietary fiber (g/day)	25.5 ± 7.7	25.7 ± 7.5	0.72
Vitamin C (mg/day)	95.5 ± 45.2	99.4 ± 61.4	0.96
Beta-carotene (mg/day)	1.37 ± 0.59	1.44 ± 0.82	0.56
Vitamin E (mg/day)	8.4 ± 2.6	8.3 ± 2.2	0.82

[a] participants free of a history of cancer

Statistical Analysis

Spearman rank correlation coefficients (r_s) were calculated between the intake of flavonoids and baseline variables, and differences in baseline characteristics according to cancer status were evaluated with the Mann-Whitney rank t-test. Relative risks (RR) and their 95%

confidence interval (95% CI) of incidence and mortality according to tertiles of flavonoid intake using the lowest tertile as reference, were calculated by Cox proportional-hazard (survival) analysis using the SAS procedure PHREG (SAS/STAT user's guide, 1990). Proportional hazard assumptions were checked (Kalbfleisch and Prentice, 1980) and found to be valid. *P* values for trend were calculated with the Mantel-Haenszel chi-square. Potential confounders were assessed in multivariate analysis. Sixty-three participants with a history of cancer at baseline were excluded from the analysis of cancer incidence and mortality. In order to increase statistical power we included men with a history of lung cancer at baseline in the lung cancer mortality analyses, and incorporated the prevalence of lung cancer at baseline (yes/no) as a covariate in the regression models. We first checked whether the association between flavonoid intake and lung cancer mortality was modified by a history of lung cancer using an interaction term in the regression models. No such interaction was found ($P = 0.58$). All *P*-values are two-sided and *P* values below 0.05 are considered statistical significant.

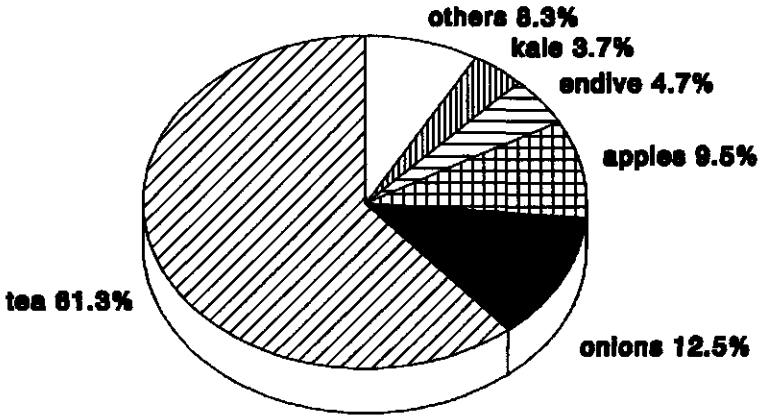

Figure 1. Contribution of various foods to flavonoid intake in 805 men aged 65-84 years participating in the Zutphen Elderly Study

RESULTS

Mean (± SD) flavonoid intake of the 738 participants was 25.9 ± 14.6 mg/day in 1985, and of the 509 men still alive and participating in 1990, 26.6 ± 13.2 mg/day. The correlation between flavonoid intake in 1985 and 1990 was 0.57 (P = 0.001) suggesting that individual intakes were measured with satisfactory precision. The most important flavonoid was quercetin at 16.4 ± 10.2 mg/day (63 %), followed by kaempferol at 8.2 ± 5 mg/day (32 %). Main source of flavonoids was tea at 61 %., whereas vegetables and fruits (mainly onions, endive, kale, and apples) accounted for approximately 38 % (Figure 1). Flavonoid intake was significantly (all P values < 0.01) inversely related to lifetime cigarette smoking (r_s = - 0.13), positively correlated with the consumption of tea (r_s = 0.83), and to a lesser extent with intake of vitamin C (r_s = 0.28), vitamin E (r_s = 0.16), beta-carotene (r_s = 0.26), and dietary fiber (r_s = 0.34). Other risk factors were not related to flavonoid intake.

Table II. Crude and adjusted relative risks of 5-year incidence and mortality from cancer according to tertiles of flavonoid intake in 738 men aged 65-84 years (The Zutphen Elderly Study)

	Flavonoid intake (mg/day)			
	0 to 19 (low)	19.1 to 29.9 (middle)	more than 29.9 (high)	P for trend
No. of Men	246	246	246	
Person-years	1047	1101	1118	
Cancer Incidence				
No. of Cases	21	27	27	
Relative Risk (95% CI)				
Crude	1.00	1.22 (0.69-2.16)	1.20 (0.68-2.13)	0.54
Adjusted[a]	1.00	1.17 (0.65-2.09)	1.21 (0.66-2.21)	0.54
Cancer Mortality				
No. of Deaths	9	12	13	
Relative Risk (95% CI)				
Crude	1.00	1.26 (0.53-3.00)	1.34 (0.57-3.14)	0.51
Adjusted[a]	1.00	1.20 (0.49-2.91)	1.43 (0.58-3.54)	0.44

[a] age, lifetime cigarette smoking, body-mass index, physical activity, intake of energy, alcohol, vitamin C, beta-carotene, vitamin E, and dietary fibre.

Table III. Crude and adjusted relative risks of 5-year incidence and mortality from lung cancer according to tertiles of flavonoid intake in men[a] aged 65-84 years (The Zutphen Elderly Study)

	Flavonoid intake (mg/day)			
	0 to 19 (low)	19.1 to 29.9 (middle)	more than 29.9 (high)	*P* for trend
Lung Cancer Incidence				
No. of Men	263	263	262	
Person-years	1116	1186	1194	
No. of Cases	7	11	8	
Relative Risk (95% CI)				
Crude	1.00	1.48 (0.58-3.82)	1.07 (0.39-2.96)	0.91
Adjusted[b]	1.00	1.66 (0.63-4.38)	1.35 (0.46-3.40)	0.58
Lung Cancer Mortality				
No. of Men	267	267	267	
Person-years	1182	1258	1266	
No. of Deaths	11	9	8	
Relative Risk (95% CI)				
Crude	1.00	0.77 (0.32-1.86)	0.68 (0.27-1.69)	0.40
Adjusted[bc]	1.00	0.90 (0.35-2.28)	0.66 (0.23-1.86)	0.43

[a] Analysis of incidence was based on participants without a history of lung cancer at baseline (n=788); analysis of mortality was based on all participants (n=801) [b] age, lifetime cigarette smoking, body-mass index, physical activity, intake of energy, alcohol, vitamin C, beta-carotene, vitamin E, and dietary fibre. [c] including prevalence of lung cancer in 1985 (yes/no) as covariate

After five years of follow-up (3,266 person-years), 75 men out of the 738 men had developed cancer, and 34 men had died from cancer. Cancer cases were older and smoked more than men who did not develop cancer (Table I). Flavonoid intake was not associated with all-cause cancer incidence (*P* for trend = 0.54), nor with cancer mortality (*P* for trend = 0.51) (Table II). Only age and cigarette smoking were significantly associated with cancer incidence. Adjustment for age, diet, and other risk factors did not affect the relative risks. After exclusion of 15 cancer cases diagnosed within the first two years of follow-up the adjusted relative risk of cancer incidence in the highest compared to the lowest tertile of flavonoid intake changed from 1.21 to 1.31 (95% CI 0.65-2.62, *P* for trend = 0.47).

Table IV. Crude and adjusted relative risks of 5-year incidence of all-cause cancer and lung cancer according to tertiles of flavonoids from tea and flavonoids from vegetables and fruit in men[a] aged 65-84 years (The Zutphen Elderly Study)

	All Cancer (n=75)				lung cancer (n=26)			
	No. of men	No. of cases	Relative Risk (95% CI) Crude	Adjusted[b]	No. of men	No. of cases	Relative Risk (95% CI) Crude	Adjusted[b]
Flavonoids from vegetables and fruits								
Low	246	29	1.00	1.00	263	12	1.00	1.00
Middle	246	25	0.81 (0.48-1.39)	0.74 (0.42-1.28)	263	8	0.64 (0.26-1.57)	0.68 (0.26-1.74)
High	246	21	0.65 (0.37-1.13)	0.57 (0.31-1.08)	262	6	0.47 (0.18-1.24)	0.51 (0.17-1.58)
P for trend			0.13	0.08			0.25	0.26
Flavonoids from tea								
Low	246	21	1.00	1.00	263	7	1.00	1.00
Middle	246	27	1.31 (0.74-2.32)	1.27 (0.71-2.26)	263	7	0.99 (0.35-2.83)	1.04 (0.36-2.99)
High	246	27	1.29 (0.73-2.28)	1.31 (0.73-2.35)	262	12	1.70 (0.67-4.32)	2.04 (0.78-5.33)
P for trend			0.41	0.44			0.25	0.22

[a] Analysis of all cancer based on 738 participants, and analysis of lung cancer on 788 participants (see text)
[b] age, lifetime cigarette smoking, body-mass index, physical activity, intake of energy, alcohol, vitamin C, beta-carotene, vitamin E, and dietary fibre.

Fifty-nine out of the 75 cancer cases had tumors of the alimentary or respiratory tract. Flavonoid intake was not related to incidence of these epithelial cancers (*P* for trend 0.78). After adjustment for age, diet, and risk factors, the relative risk of the highest tertile versus the lowest tertile of flavonoid intake was 1.10 (95% CI 0.55-2.17). Similar results were obtained when epithelial cancer mortality data were used.

Twenty-six out of 788 men free of a history of lung cancer at baseline developed lung cancer. No association was observed between flavonoid intake and lung cancer incidence (Table III). As indicated in the Method section we included prevalent cases of lung cancer in the lung cancer mortality analysis. The relative risk of lung cancer death decreased from the lowest to the highest tertile of flavonoid intake, but this decrease was not statistically significant (*P* for trend = 0.40). When prevalent cases of lung cancer were excluded 18 men died from lung cancer. The adjusted relative risk of lung cancer death in the highest compared to the lowest tertile of flavonoid intake was then 0.81 (95% CI 0.22-3.05, *P* for trend = 0.83).

In this population flavonoids were mainly provided by tea on the one hand and by vegetables and fruits on the other hand. We therefore decided to investigate separately the effects of flavonoids derived from tea and flavonoids derived from vegetables and fruits. Only

flavonoids derived from vegetables and fruits were inversely associated with cancer incidence, however, this association was borderline significant (adjusted *P* for trend = 0.08) (Table IV). A similar inverse association was observed with epithelial tumors of the alimentary or respiratory tract (*P* for trend = 0.12). A high intake of flavonoids from tea was associated with a doubling of the risk of lung cancer, whereas a high intake of flavonoids from vegetables and fruits with a 50 % reduced risk of lung cancer (Table IV). However, both associations were not statistically significant (*P* for trend = 0.22 and 0.26, respectively).

DISCUSSION

In these elderly men flavonoid intake is not associated with risk of all-cause cancer, nor with risk of lung cancer. The small decrease in lung cancer mortality rates from the lowest to the highest tertile of flavonoid intake suggests an inverse relation between flavonoid intake and lung cancer mortality. However, due to the limited number of lung cancer deaths this association could not be substantiated and it could therefore merely be a chance finding. To our knowledge no previous epidemiologic study investigated the relation between flavonoid intake and cancer. Indications for a protective effect of flavonoids on cancer has been provided by experimental studies in which flavonoids inhibited chemically-induced tumors in rodents. Dietary quercetin significantly reduced tumor multiplicity in azoxymethanolic-induced colonic neoplasia in mice (Deschner et al., 1991) and in *N*-nitrosomethylurea-induced mammary cancer in rats (Verma et al., 1988). Flavonoids also inhibited skin tumor promotion in mice (Fujiki et al., 1986). Antioxidant flavonoids such as quercetin also inhibited colonic cell proliferation *in vitro* (Hosokawa, et al, 1987; Raneletti et al., 1992).

Various explanations for the discrepancies between our findings and the experimental findings can be hypothesized. Cancer at different sites have different etiologies which may explain why no relation was found between flavonoid intake and all-cause cancer mortality. Epidemiologic studies suggest that the protective effect of vegetables and fruits is most marked on epithelial cancers of the respiratory and alimentary tract (Steinmetz and Potter, 1991). However, flavonoid intake was also not associated with cancer at these sites. Due to the limited number of cases, we were not able to examine the relation between flavonoid intake and mortality from cancer of specific sites, except lung cancer. In a cross-cultural comparison using data from 16 cohorts in seven countries we found that flavonoid intake was not related to all-cause cancer mortality, and weakly inversely related to lung cancer mortality. However, after adjustment for percentage of smokers and vitamin C intake, this relation was not statistically significant (Hertog et al., 1993, unpublished observations). On the other hand, in most animal studies high doses of a carcinogen are used for induction of tumors, and the inhibitory effect of high doses of flavonoids are then investigated. These results may therefore not easily be extrapolated to humans, and possibly flavonoids do not play a role in the etiology of human cancer. Clearly, more epidemiological studies on the effect of flavonoids on cancer at different sites are needed.

The presence of preclinical diseases in this elderly population may affect baseline dietary intakes. However, exclusion of cancer cases diagnosed in the first two years of follow-up did not result in a significant change of the risk ratios. The small inverse relation between flavonoid intake and lung cancer mortality risk was further weakened when prevalent cases were excluded. Although the prevalence of a history of lung cancer at baseline did not modify

the relation between flavonoid intake and lung cancer risk at the $P = 0.05$ significance level, the analysis excluding prevailing disease is probably the most reliable and free from such bias.

Selection bias due to selective survival of healthy men up to the age of 65-84 years may have occurred. However, other risk factors such as age and cigarette smoking were still associated with cancer risk. In contrast, intake of other dietary antioxidants with anticarcinogenic potential such as vitamin C, vitamin E, and beta-carotene (16), was also not related to cancer risk. Possibly, the association between dietary antioxidants and the occurrence of cancer present in middle-aged persons is less clear in the elderly. This may explain why we did not find an effect of flavonoids on cancer rates in the present study.

Flavonoids derived from tea, and flavonoids from vegetables and fruits had pronouncedly different effects on cancer rates. Flavonoids from vegetables and fruits were generally associated with a lower risk of cancer, whereas no such an effect was observed for flavonoids derived from tea. In fact, flavonoids from tea tended to be associated with an increased risk of (lung) cancer. These results suggest that other components present in specific dietary sources of flavonoids such as onions, endive, kale, and apples, but not in tea, may be responsible for the lower cancer incidence rates. On the other hand, tea in contrast to onions, endive, kale and apples, may contain unknown components that enhance cancer risk and therefore mask a potential protective effect of flavonoids. Epidemiologic studies on the effect of tea on cancer risk are scarce and the results are conflicting (IARC, 1991). Two prospective studies investigated the relation between tea consumption and cancer risk, and both reported a weak positive association between tea consumption and cancer (Kinlen et al, 1988; Heilbrun et al., 1986). In contrast, experimental studies reported inhibitory effects of tea polyphenols, especially those from green tea, on rodent carcinogenesis (Mukhtar et al, 1992; Stich et al, 1992). The role of tea in human carcinogenesis therefore deserves more attention.

Mean intake of flavonoids in this population was approximately 26 mg/day, which compares well with the 24 mg per day that we found for Dutch men aged over 60 years (Hertog et al., 1993b). Misclassification of exposure is not likely to have occurred. The cross-check dietary history provides extensive information on all consumed foods and its validity and reproducibility is well-established (Bloemberg et al., 1989). Also individual flavonoid intake was reproducible over a 5-year interval. Moreover, our method of flavonoid analysis, and our extensive food sampling provided accurate figures on the flavonoid content of foods commonly consumed in The Netherlands. Previous estimations reported that the average intake of all flavonoids combined in the US was approximately 1 gram/day, of which about 100 mg consisted of the group of flavonoids investigated in the present study (Kühnau, 1976). This estimation was based on food analysis techniques now considered inappropriate, which presumably resulted in an overestimation of the flavonoid content of foods. Quercetin was the most important flavonoid, and intake of quercetin was highly correlated with total flavonoid intake ($r_s = 0.98$). Our results can therefore also be interpreted as the relation between quercetin intake and cancer.

We conclude that flavonoid intake is not associated with risk of all-cause cancer in elderly men. However, the effect of flavonoids on cancer at different sites merits further investigation in other prospective epidemiologic studies.

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Chapter 7

Flavonols, Flavones, and Risk of Coronary Heart Disease

This chapter is based mainly on:

Hertog MGL, Feskens EJM, Hollman PCH, Katan, MB, Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease. The Zutphen Elderly Study. *The Lancet* 1993;342:1007-11

ABSTRACT

Flavonoids are polyphenolic antioxidants naturally present in vegetables, in fruits, and in beverages such as tea and wine. Flavonoids inhibited LDL oxidation in vitro and reduced thrombotic tendencies, but their effects on atherosclerotic complications in humans are unknown. We measured the flavonoids quercetin, kaempferol, myricetin, apigenin, and luteolin in foods, and assessed flavonoid intake in 1985 by cross-check dietary history in 805 men aged 65-84 years, who were then followed for 5 years. Mean flavonoid intake was 25.9 mg/day. The major sources of intake were tea (61 %), onions (13 %), and apples (10 %). Between 1985 and 1990, 43 men died of coronary heart disease. A fatal or non-fatal myocardial infarction occurred in 38 out of 693 men free of myocardial infarction at baseline. Flavonoid intake was inversely associated with both mortality from coronary heart disease (p for trend = 0.015) and with incidence of myocardial infarction (p for trend = 0.08). The relative risk of coronary heart disease mortality in the highest versus the lowest tertile of flavonoid intake was 0.42 (95% CI 0.20-0.88). Adjustment for age, body-mass index, smoking, total- and HDL serum cholesterol, blood pressure, physical activity, coffee consumption, and intake of energy, vitamin C, vitamin E, beta-carotene, and dietary fibre changed this risk to 0.32 (95% CI 0.15-0.71). Tea, onions, and apples were also inversely related to coronary heart disease mortality, but these associations were weaker than those observed with flavonoid intake. Flavonoids present in ordinary consumed foods might reduce the risk of death from coronary heart disease in elderly men.

INTRODUCTION*

Flavonoids are a large group of polyphenolic antioxidants that occur naturally in vegetables, in fruits, and in beverages such as tea and wine (Kühnau, 1976; Hertog et al., 1993b). Flavonoids are antioxidants and quercetin, a major flavonol, inhibited oxidation and cytotoxicity of low density lipoproteins in vitro (De Whalley et al., 1992; Negre-Salvagyre A and Salvagyre, 1992) (See also General Introduction). Oxidized low-density lipoproteins are atherogenic, and are considered to be a crucial intermediate in the formation of atherosclerotic plaques (Steinberg et al., 1989). Flavonols and flavones also inhibited cyclo-oxygenase leading to reduced platelet aggregation and reduced thrombotic tendencies. However, their effects on human atherosclerotic complications are unknown.

We selected five major antioxidant food flavonoids, viz. the flavonols quercetin, kaempferol, myricetin, and the flavones apigenin, and luteolin, and measured their content in the major types of vegetables, fruits, and beverages consumed in The Netherlands[11,12]. Here, we report the relation between baseline intake of flavonoids and subsequent coronary heart disease mortality, and incidence of myocardial infarction in the Zutphen Elderly Study.

METHODS

Study Population

The Zutphen Elderly Study is a longitudinal investigation of risk factors for chronic diseases in elderly men (van Buchem et al., 1967). It is an extension of the Zutphen Study, the Dutch contribution to the Seven Countries Study (Keys et al., 1967). The Zutphen Study was started in 1960 with a cohort of 878 men then aged 40-59 years and living at least five years in Zutphen, a town in the eastern part of the Netherlands. In 1985, 555 men of the original cohort were still alive and were invited for new examinations. In addition, a random sample of all other men of the same age group (65-84 years) living in Zutphen and not part of the 1960 cohort were approached. This resulted in a target population of 1266 men. Sixty-two men (4.9 %) could not participate because they had moved or could not be reached, 109 (9.0 %) could not be examined because of serious illness, and 156 (12.0 %) refused to participate. Hence, in 1985 939 men (74 %) aged 65-84 years entered the study, and complete information on diet and risk factors was available for 805 men.

Flavonoid Analysis

We determined the content of the three major flavonols, quercetin, kaempferol, myricetin, and two major flavones luteolin, and apigenin of 28 types of vegetables, 12 types of fruits, and 9 types of beverages commonly consumed in The Netherlands (Hertog et al., 1992b, 1993a). Each food was purchased in each of three seasons in a supermarket, in a grocery, and in an open-air street market. Separate purchases from each site were combined, and flavonoid content was determined by Reversed-Phase High Performance Liquid Chromatography with ultraviolet-detection (Hertog et al., 1992a). Identity and purity of the flavonoids were confirmed by diode-array detection on-line. In plants a large number of flavonoid glycosides

* section shortened for publication in this thesis

originating from the same parent aglycon (sugar-free flavonoid) occur. In order to simplify the analytical procedure and to enhance the limit of detection, glycosides were hydrolysed, and flavonoid levels are expressed as aglycons. The aglycon is, because of its polyphenolic character, the biologically active part of the flavonoid molecule. Control samples with a known amount of flavonoids were included in each series. The limit of detection was approximately 1 mg of flavonoids per kg or 0.5 mg/L. Other details have been published (Hertog et al., 1992a, 1992b, 1993a)

Examinations

Dietary and medical examinations were carried out between March and June 1985, and again in the same period in 1990. Usual food intake of the participants in the month preceding the interview was determined by trained dietitians using a cross-check dietary history method adapted to the Dutch situation (Bloemberg et al., 1989). Each participant was interviewed at home for about 1 h together with the person who prepared the food (usually the wife) about his usual food consumption pattern on weekdays and during weekends, and about food purchases. The habitual consumption of foods during a week was determined, and verified with the quantities of foods bought per week. This information was combined to calculate the participants food consumption on a typical weekday. Quantities of foods were estimated by the dietitians, using a portable scale if necessary. The food intake data were encoded by the dietitians according to the Netherlands Uniform Food Encoding System, and converted into energy and nutrient values using the 1985 release of the Netherlands nutrient data bank[17], updated with 1993 data for beta-carotene and vitamin E, and with flavonoid data. The flavonoid content was defined as the sum of quercetin, kaempferol, myricetin, apigenin, and luteolin. Seasonal variability proved to be low (Hertog et al., 1992b), and the average flavonoid content measured in three seasons was used in the analyses. Flavonoid content was known for approximately 95 % of all foods of vegetable origin that are commonly consumed at more than 1 g/day in The Netherlands (Hertog et al., 1993b)

Trained physicians took non-fasting venous blood samples during the medical examinations, and measured weight and height while the participants were in their underwear. Blood pressures were measured in duplicate at the end of the medical examination. Total cholesterol and high-density lipoprotein cholesterol (HDL cholesterol) were determined enzymatically (Sidel et al., 1981; Warnick et al., 1982). Information on amount and duration of smoking was assessed using a standardized questionnaire, and converted into lifetime exposure of cigarettes using the formula: exposure = (365 * number of cigarettes/day) * years of smoking. Minutes of physical activity (mainly walking, cycling, gardening, sports, hobbies, and work) per week were calculated using a questionnaire specially designed for retired men (Caspersen et al., 1991)

Follow-up

Information on the occurrence of cerebrovascular accident, diabetes mellitus, chronic non-specific lung disease, and cancer was obtained by a standardized medical questionnaire during the medical examinations in 1985 and 1990. Information on a history of myocardial infarction, angina pectoris, and intermittent claudication was obtained from a separate

questionnaire developed at the London School of Hygiene and Tropical Medicine (Rose and Blackburn, 1968). Diagnosis of each disease was verified with hospital discharge data, and with written information from the subjects' general practitioners. All information was uniformly coded by a single physician, and the year of first diagnosis of each disease was recorded. Myocardial infarction was assumed to be present when two out of the following three criteria were fulfilled: a specific medical history e.g. severe chest pain lasting for more than 20 minutes and not disappearing in rest, characteristic ECG changes (e.g. major Q wave, and major T wave findings), and specific enzyme elevations.

Table I. Baseline Characteristics of 805 Men Initially Aged 65-84 Years According to Tertiles of Flavonoid Intake (The Zutphen Elderly Study)

Characteristic	Flavonoid intake (mg/day)			p value
	0 to 19 (low)	19.1 to 29.9 (middle)	more than 29.9 (high)	
No. of men	268	268	269	
Men with history of myocardial infarction	36	40	36	0.84
		Mean (SD)		
Flavonoid intake (mg/day)	12.0 (4.8)	23.9 (3.2)	41.6 (12.4)	
Age in 1985 (years)	71.0 (5.2)	71.8 (5.4)	71.0 (5.1)	0.14
Lifetime cigarette smoking (x 1000)	216 (191)	167 (164)	174 (195)	0.007
Body-mass index (kg/m^2)	25.4 (3.5)	25.3 (2.9)	25.8 (3.0)	0.13
Systolic blood pressure (mmHg)	149 (20)	152 (21)	151 (22)	0.32
Serum total cholesterol (mmol/L)	6.16 (1.16)	6.09 (1.13)	6.11 (1.03)	0.72
Serum HDL cholesterol (mmol/L)	1.15 (0.35)	1.10 (0.26)	1.11 (0.27)	0.14
Physical activity (min/week)	720 (693)	816 (785)	805 (713)	0.26
Energy intake (MJ/day)	9.1 (2.2)	9.4 (2.1)	9.9 (2.2)	0.0005
Saturated fatty acids (g/day)	43.4 (15.0)	43.3 (13.5)	44.5 (15.1)	0.54
Dietary cholesterol (mg/day)	334 (111)	336 (119)	347 (130)	0.40
Vitamin C (mg/day)	83.1 (40.0)	92.9 (42.7)	112.2 (51.3)	0.0001
Vitamin E (mg/day)	8.1 (2.6)	8.2 (2.4)	8.9 (2.6)	0.0002
Beta-carotene (mg/day)	1.2 (0.5)	1.4 (0.6)	1.6 (0.7)	0.0001
Coffee consumption (mL/day)	508 (300)	407 (220)	392 (286)	0.0001

Information on the vital status of all participants up to July 1990 was obtained from the municipality registries. No man was lost to follow-up. Information on the primary cause of

death was obtained from the Central Bureau for Statistics, and verified by means of hospital discharge data and information from the general practitioner. The primary cause of death was coded according to the *International Classification of Diseases, 9th Revision* (WHO, 1977). Coronary heart disease refers to ICD-9 codes 410-414.

Statistical Methods

Differences in baseline characteristics of the participants between tertiles of flavonoid intake were evaluated using the one-way analysis of variance SAS procedure ANOVA (SAS Institute, 1990). Spearman rank correlation coefficients (r_s) were calculated between the intake of flavonoids and other dietary variables. Crude and adjusted relative risks of mortality and incidence according to tertiles of flavonoid intake were calculated by Cox proportional-hazard (survival) analysis using the SAS procedure PHREG. Proportional hazard assumptions (Kalbfleisch and Prentice, 1980) were satisfied. P values for trend were calculated with Mantel-Haenszel chi-square statistics. We checked whether the association between flavonoid intake and coronary heart disease mortality was modified by a history of myocardial infarction using an interaction term in the regression models. No such interaction was found (p= 0.58). We therefore included men with myocardial infarction at baseline in the mortality analyses, and incorporated the prevalence of myocardial infarction at baseline (yes/no) as a covariate in the regression models. All P-values are two-sided.

RESULTS

Mean (± SD) flavonoid intake in the 805 participants was 25.9 ± 14.5 mg/day in 1985, and in the 509 men still alive and participating in 1990, 26.6 ± 13.2 mg/day. The correlation between flavonoid intake in 1985 and 1990 was 0.57 suggesting that individual intakes were measured with satisfactory precision. The most important flavonoid was quercetin at 16.3 ± 10.1 mg/day or 63 %, followed by kaempferol at 8.2 ± 5 mg/day or 32 %. Main sources of flavonoids in this population were regular black tea at 61 %, followed by onions at 13 %, and apples at 10 % of flavonoid intake. Mean consumption of tea was 427 ± 319 mL/day or 3.4 ± 2.5 cups/day, mean consumption of onions was 9.4 ± 20.9 g/day, and of apples 68.8 ± 70.3 g/day. Flavonoid intake was highly correlated with consumption of tea (r_s= 0.83, p= 0.001), but weaker with consumption of onions (r_s= 0.32, p= 0.001), and apples (r_s= 0.27, p= 0.001). Similar associations were observed with the consumption of total vegetables (r_s= 0.31, p= 0.001) and total fruits (r_s= 0.23, p= 0.001).

Lifetime cigarette smoking and coffee consumption was highest among men in the lowest tertile of flavonoid intake (table I). Intake of energy, vitamin C, vitamin E, and beta carotene increased significantly from the lowest to the highest tertile of flavonoid intake. Flavonoid intake was inversely associated with fat intake (r_s= -0.11, p= 0.009) and positively related to intake of carbohydrates (r_s= 0.16, p= 0.001), and dietary fibre (r_s= 0.34, p= 0.001).

*Table II. Crude and Adjusted Relative Risks of Mortality from Coronary Heart Disease, and Incidence of Fatal and Non-Fatal Myocardial Infarction, According to Tertiles of Flavonoid Intake in 805 Men Aged 65-84 Years (The Zutphen Elderly Study)**

	Flavonoid intake (mg/day)			
	0 to 19 (low)	19.1 to 29.9 (middle)	more than 29.9 (high)	p for trend
Mortality from coronary heart disease				
No. of men	268	268	269	
No. of Deaths	22	11	10	
Mortality Rate (per 1000 person-years)	18.5	8.7	7.8	
Relative Risk (95% CI)				
Crude	1.00	0.47 (0.23-0.97)	0.42 (0.20-0.88)	0.015
Adjusted for age and diet†§	1.00	0.34 (0.16-0.73)	0.34 (0.15-0.79)	0.008
Adjusted for age, diet, and risk factors‡§	1.00	0.32 (0.15-0.68)	0.32 (0.15-0.71)	0.003
Incidence of fatal and non-fatal first myocardial infarction				
No. of men	231	231	231	
No. of Cases	16	14	8	
Incidence Rate (per 1000 person-years)	16.2	13.8	7.6	
Relative Risk (95% CI)				
Crude	1.00	0.85 (0.42-1.75)	0.47 (0.20-1.09)	0.08
Adjusted for age and diet†	1.00	0.87 (0.41-1.84)	0.49 (0.19-1.25)	0.15
Adjusted for age, diet, and risk factors‡	1.00	0.89 (0.43-1.87)	0.52 (0.22-1.23)	0.15

* Analysis of mortality was based on all participants (n=805); analysis of incidence was based on participants free of myocardial infarction at baseline (n=693)
† intake of total energy, saturated fatty acids, cholesterol, alcohol, coffee, vitamin C, vitamin E, beta-carotene, and dietary fibre.
‡ intake of total energy, saturated fatty acids, physical activity, body-mass index, smoking, serum total and HDL cholesterol, and systolic blood pressure.
§ history of myocardial infarction in 1985 (yes/no) was included as additional covariate.

After five years of follow-up (3727 person-years) a total of 185 men had died, 43 of them from coronary heart disease. Twenty of these men did not have a history of myocardial infarction at baseline. Of the 693 men free of myocardial infarction at baseline, 38 men

suffered a myocardial infarction which was fatal for 13 out of them. Flavonoid intake was inversely associated with both mortality from coronary heart disease (p for trend = 0.015) and incidence of a first fatal and nonfatal myocardial infarction (p for trend = 0.08) (table II). Incidence data were calculated only for subjects free of myocardial infarction at baseline. Relative risks of both mortality from coronary heart disease, and incidence of a first myocardial infarction were approximately 50 % lower in the highest tertile than in the lowest tertile. In the initial multivariate model, the estimated relative risks were adjusted for age and dietary variables. Only age, prevalence of myocardial infarction, flavonoid intake, and intake of saturated fatty acids were significantly related to incidence of myocardial infarction and death from coronary heart disease and the effect of flavonoid intake on mortality from coronary heart disease became more pronounced after adjustment (p for trend = 0.008). After further adjustment in the final model for non-dietary risk factors the relation between flavonoid intake and coronary mortality remained unchanged (p for trend = 0.003). The effect of flavonoids on relative risks of incidence of myocardial infarction were unaltered after these adjustments (table II). Adjustment for total consumption of fruits (200 ± 141 g/day) and vegetables (177 ± 73 g/day) did not affect the strength of the relation of flavonoid intake with myocardial infarction incidence or coronary heart disease mortality.

We also calculated relative risks of mortality from coronary heart disease in 693 men free of myocardial infarction at baseline. Relative risks were 0.31 (95% CI 0.10-0.99) in the highest compared to the lowest tertile of flavonoid intake, and 0.29 (95% CI 0.09-0.93) after adjustment for age, diet, and risk factors.

Mortality rates from coronary heart disease decreased from the lowest to the highest tertile of tea consumption (p for trend = 0.08) (table III). Tea consumption was inversely related to coffee consumption (r_s = -0.27). However, coffee drinking was not associated with coronary heart disease mortality (p for trend = 0.79), and the inverse association between tea consumption and coronary heart disease mortality was more pronounced after adjustment for potential confounders including coffee (table III). Apple consumption was also inversely related with mortality from coronary heart disease but this relation did not reach statistical significance (table III). Onion users had an adjusted relative risk of 0.85 (95% CI 0.46-1.61) of mortality from coronary heart disease compared to non-users. Incidence of a first myocardial infarction was not related to the consumption of tea (p for trend = 0.58), apples (p for trend = 0.39), or onions (p = 0.28).

All-cause mortality rates decreased with increasing flavonoid intake. This decrease was borderline significant, and persisted after adjustment for potential confounders (p for trend = 0.084) (table IV).

Table III. Crude and Adjusted Relative Risks of Mortality from Coronary Heart Disease According to Tertiles of Tea Consumption and Tertiles of Apple Consumption in 805 Men Aged 65-84 Years (The Zutphen Elderly Study)

	Tertiles of intake*			
	low	middle	high	p for trend
No. of Men	268	268	269	
Tea consumption				
No. of Deaths	21	10	12	
Mortality Rate (per 1000 person-years)	17.1	8.1	9.5	
Relative Risk (95% CI)				
Crude	1.00	0.48 (0.22-1.01)	0.55 (0.27-1.13)	0.081
Adjusted for age and diet†§	1.00	0.39 (0.18-0.85)	0.44 (0.20-0.96)	0.033
Adjusted for age, diet, and risk factors‡§	1.00	0.38 (0.18-0.82)	0.45 (0.22-0.93)	0.024
Apple consumption				
No. of Deaths	17	16	10	
Mortality Rate (per 1000 person-years)	13.7	13.2	7.9	
Relative Risk (95% CI)				
Crude	1.00	0.97 (0.49-1.92)	0.58 (0.26-1.26)	0.18
Adjusted for age and diet†	1.00	0.86 (0.43-1.75)	0.50 (0.21-1.19)	0.13
Adjusted for age, diet, and risk factors‡	1.00	0.90 (0.45-1.82)	0.51 (0.23-1.16)	0.12

* Cut-off points tea: 0 - 250 mL/day (low); 251 - 500 mL/day (middle); > 500 mL/day (high)
apples: 0 - 18 g/day (low); 19-110 g/day (middle); >110 g/day (high)

† history of myocardial infarction in 1985, intake of total energy, saturated fatty acids, cholesterol, coffee, alcohol, vitamin C, vitamin E, beta-carotene, and dietary fibre.

‡ history of myocardial infarction in 1985, intake of total energy, and saturated fatty acids, physical activity, body-mass index, smoking, serum total and HDL cholesterol, and systolic blood pressure.

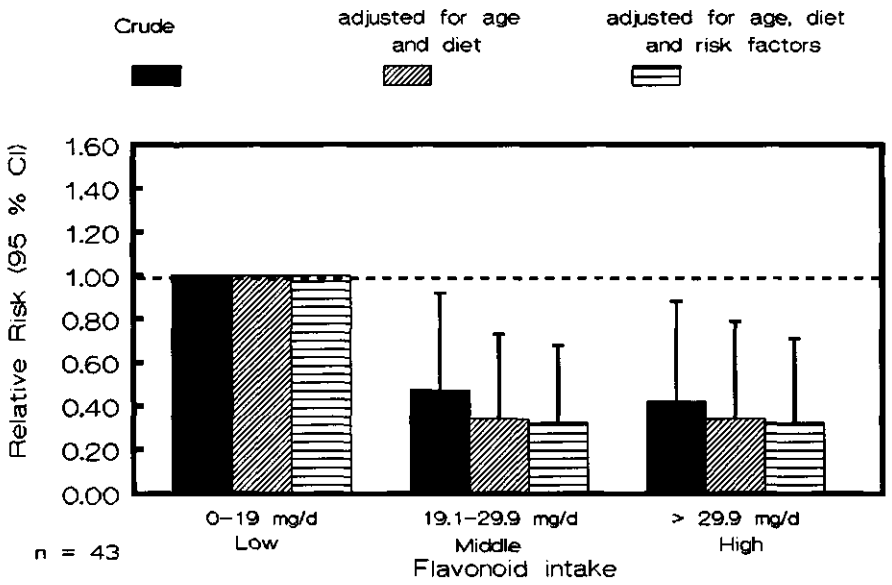

Figure 1. Flavonoid intake and relative risk (RR and 95 % CI) of coronary heart disease mortality in the Zutphen Elderly Study

DISCUSSION

In these elderly men, high intakes of flavonoids predicted a lower rate of mortality from coronary heart disease and of incidence of myocardial infarction. The association was stronger for coronary heart disease mortality than for myocardial infarction incidence. Although the power of this study is rather limited due to relatively small number of deaths and incident cases, the associations with coronary heart disease mortality were highly statistically significant. Coronary heart disease mortality rates was approximately 60 % reduced in the highest compared with the lowest tertile of flavonoid intake. This inverse relation persisted after excluding prevalent cases of myocardial infarction. The predominant flavonoid found in foods is quercetin. Quercetin intake was highly correlated with total flavonoid intake (r_s= 0.98), and tertiles of quercetin intake yielded essentially the same relative risks as tertiles of total flavonoid intake. Our results can also be taken as evidence for an inverse relation between quercetin intake and coronary heart disease.

The decrease in coronary heart disease mortality was not accompanied by increases in the rates of death from other causes: the highest tertile of flavonoid intake had 12 fewer men dying from coronary heart disease than the lowest tertile, and 15 fewer dying altogether. Unlike the difference in coronary mortality, the difference in total mortality failed to reach the p= 0.05 level of significance in the final multivariate model, because the contrast due to coronary heart disease mortality was diluted by a constant, but larger number of deaths from

other causes.

Flavonoid intake was not clearly associated with a baseline history of myocardial infarction, possibly because of a higher mortality in the lowest tertile of flavonoid intake. Adjustment for baseline history of myocardial infarction did not attenuate the inverse relation between flavonoid intake and coronary heart disease mortality, in fact, the relation became more pronounced, and the relative risk estimates were similar to the results when participants with a baseline history of myocardial infarction were excluded from the analysis.

Table IV. Crude and Adjusted Relative Risks of All-Cause Mortality According to Tertiles of Flavonoid Intake in 805 Men Aged 65-84 years (The Zutphen Elderly Study)

	Flavonoid intake (mg/day)			
	0 to 19 (low)	19.1 to 29.9 (middle)	more than 29.9 (high)	p for trend
All-cause mortality				
No. of men	268	268	269	
No. of Deaths	70	60	55	
Mortality Rate (per 1000 person-years)	59.0	47.7	42.9	
Relative Risk (95% CI)				
Crude	1.00	0.81 (0.57-1.14)	0.72 (0.51-1.03)	0.071
Adjusted for age and diet*	1.00	0.68 (0.47-0.96)	0.67 (0.45-0.98)	0.039
Adjusted for age, diet and risk factors†	1.00	0.75 (0.52-1.07)	0.72 (0.50-1.05)	0.084

* prevalence of chronic diseases (yes/no), intake of total energy, saturated fatty acids, cholesterol, coffee, alcohol, vitamin C, vitamin E, beta-carotene, and dietary fiber.

† prevalence of chronic diseases (yes/no), physical activity, BMI, smoking, serum total and HDL cholesterol, systolic blood pressure, intake of total energy, and saturated fatty acids.

It could be hypothesized that a high flavonoid intake is merely an indicator of a healthy lifestyle or of a diet high in vegetables and fruits, and low in fat. However, adjustment for dietary variables such as vitamin C, vitamin E, beta-carotene, dietary fibre, or fruits and vegetables, or for non-dietary risk factors such as physical activity, blood lipids, or obesity did not attenuate the relation between flavonoid intake and coronary heart disease. Also, the major source of flavonoids in this population is not fruits or vegetables but tea, which provided about 61 %.

Flavonoid intake and tea consumption were highly correlated (r_s = 0.83) and both were inversely associated with mortality from coronary heart disease. The inverse association between flavonoid intake and death from coronary heart disease might therefore be due to substances in tea other than flavonoids, such as the antioxidant tea polyphenols (-)epicatechin gallate, and (-)epigallocatechin gallate (Conney et al., 1992; Katiyar et al., 1992). However,

the observed effects of total flavonoids on coronary heart disease mortality in our study were stronger than those of tea itself. Onion and apple consumption were less strong related to coronary heart disease mortality than flavonoid intake. In addition, neither tea, apples, or onions were related to incidence of myocardial infarction. This suggests that quercetin and other flavonoids themselves rather than unknown substances in these foods are responsible for the lower coronary heart disease mortality rates. Coffee consumption has been associated with coronary heart disease in some studies (Wilson et al., 1989) and there was an inverse relation between coffee consumption and both, flavonoid and tea intake in our population. However, coffee consumption was not related to coronary heart disease in the present study, and adjustment for coffee did not change the association of flavonoid intake and tea consumption with coronary heart disease mortality.

The average flavonoid intake of the Zutphen elderly men was about 26 mg per day, which is comparable with the 24 mg per day that we found for Dutch men over 60 years of age (Hertog et al., 1993b). These values are much lower than previous estimation made by Kuhnau who reported that in the US the daily intake of all flavonoids combined was 1 gram (expressed as glycoside), of which about 100 mg (expressed as aglycons) consisted of flavonols and flavones investigated in the present study (Kühnau, 1976). Kühnau's estimation was based on food analysis techniques now considered inappropriate, which presumably resulted in an overestimation of their flavonoid content. Also these results are based on analyses of whole foods in contrast to our analyses of the edible parts. In addition, the estimate made by Kühnau is based on food disappearance data, which overestimates the real intake of foods. In the Dutch diet tea, onions, and apples contributed about 84 % to total flavonoid intake (Hertog et al., 1993b). In other cultures, e.g. mediterranean countries, red wine, which contains 10 to 25 mg/L of combined flavonoids (Hertog et al., 1993a) could also be an important source of flavonoids. Recently it was suggested that polyphenolic flavonoids present in wines may be partly responsible for the reduced risk of coronary heart disease of wine drinkers ("French Paradox") (Frankel et al., 1993).

There is evidence that free-radical oxidation of LDL plays an important role in atherogenesis (Steinberg et al., 1989). Flavonoids are scavengers of free radicals such as superoxide anions, and lipid peroxy radicals, and will thus interrupt radical chain reactions. Some flavonoids, including quercetin, inhibited the oxidative modification of LDL by macrophages *in vitro*, probably by inhibiting the generation of hydroperoxides and by protecting α-tocopherol present in lipoproteins from oxidation (De Whalley et al., 1992). Quercetin also inhibits the cytotoxicity of oxidized LDL *in vitro* (Negre-Salvagyre A and Salvagyre, 1992). Our observations can thus be rationalized by assuming that quercetin and other flavonoids reduce the rate of formation of oxidized LDL and thus inhibit the growth of atherosclerotic plaques. In addition, flavonoids inhibited cyclo-oxygenases which may reduce thrombosis. Flavonoids possibly act through both mechanisms, which could explain why the association between flavonoid intake and coronary heart disease mortality was stronger than the association with incidence of myocardial infarction. Data on the absorption and metabolism of flavonoids are scarce and inconclusive (Gugler et al., 1975; Ueno et al., 1975; Kleijnen and Knipschild, 1992), and more studies are needed to elucidate the mechanisms involved.

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Chapter 8

Flavonols, Flavones, and Cross-Cultural Risk of Coronary Heart Disease and Cancer Mortality

This chapter is based mainly on:

Hertog MGL, Kromhout D, Keys A, et al...
Flavonoid Intake and Risk of Coronary Heart Disease and Cancer Mortality in The Seven Countries Study
(*submitted for publication*)

ABSTRACT

Flavonoids are antioxidants present in e.g. onions, apples, tea, and red wine. A high intake of flavonoids predicted a lower mortality from CHD, but not from cancer in a prospective cohort study. Flavonoid intake may therefore also explain differences in chronic disease mortality rates between populations. We calculated average flavonoid intake of the 16 cohorts of The Seven Countries Study at baseline around 1960 by flavonoid analysis of food equivalent composites that represented the average diet in the cohorts. We then related in a cross-cultural regression analysis average flavonoid intake in 16 cohorts to age-adjusted mortality of coronary heart disease (CHD) and cancer after 25 years of follow-up. Average intake of antioxidant flavonoids was inversely associated with mortality from CHD and explained about 25 % of the variance in CHD rates. Intake of saturated fat (73 %, P = 0.0001), flavonoid intake (9 %, P = 0.01), and percentage of smokers per cohort (8 %) explained together, independently from intake of alcohol and antioxidant vitamins, 90 % of the variance in CHD rates. Flavonoid intake was not independently associated with mortality from other causes. Average flavonoid intake may partly contribute to differences in CHD mortality rates across populations, but it does not seem to be an important determinant of cancer mortality.

INTRODUCTION*

Flavonoids are polyphenolic antioxidants that occur in a variety of foods from vegetable origin such as apples, onions, tea, and red wine (Kühnau, 1976; Hertog et al., 1993b). Intake of antioxidant flavonoids might conceivably reduce coronary heart disease and cancer risk in human beings (see General Introduction). We have indeed shown that intake of quercetin and other related flavonoids was inversely associated with coronary heart disease (CHD) risk in Dutch elderly men (Hertog et al., 1993c). However, no association with total cancer risk was observed (Hertog et al., *Chapter 6*).

Countries differ largely in the consumption of tea, red wine, apple, and onions, the major contributors to flavonoid intake, and a wide range in intake of flavonoids can be expected in cross-cultural studies. We therefore decided to study the association between flavonoid intake and longterm mortality at the average cohort level in the Seven Countries Study. We determined the mean intake of flavonoids at baseline in the 16 participating cohorts by chemical analysis of food equivalent composites and related it to 25 year mortality from CHD, cancer at various sites, and all causes.

MATERIALS AND METHODS

Seven Countries Study Cohorts

Between 1958 and 1964 12,763 men aged 40-59 years from 16 different cohorts were enrolled in the Seven Countries Study. Eleven cohorts consisted of men living in rural parts of Finland, Italy, Greece, former Yugoslavia, and Japan, one of workers in a large cooperative in Zrenjanin, Serbia, one of university professors in Belgrade, one of inhabitants of Zutphen, a small industrial town in The Netherlands, and two cohorts of railroad employees in the USA and Italy, respectively. Other characteristics of the cohorts have been described (Keys et al., 1967). Information on smoking and alcohol consumption was collected by questionnaire and percentage of cigarette smokers per cohort was determined. Dietary information was collected at baseline in small random samples (8-49 men) of each cohort using the dietary weighed record method. In 1985 and 1986 the original dietary intake data were recoded by one dietitian (A. Jansen), and the average food intake of the men in the 16 cohorts was calculated (Kromhout et al., 1989). In 1987, the foods representing the baseline diet were bought locally and sent by air in cooling boxes to The Netherlands. Within one day after arrival, the foods were washed, cleaned, and combined into food equivalent composites representing the average food intake of each cohort. Vegetables, fruits, and fruit juices were combined into two separate food equivalent composites. Food equivalent composites were subsequently homogenized, freeze-dried, and stored at -20°C until analyzed. Macro- and micro nutrient contents of the food equivalent composites were determined and used to calculate mean intake of nutrients per cohort. The vital status of all men was checked after 25 years of follow-up, and the primary cause of death of the men who died was established centrally by H.B. and A.M as described (Keys et al., 1986). The end points in the present study are mortality from coronary heart disease (ICD 410-414), cancer (ICD 140-209), lung cancer (ICD 162), colorectal cancer (ICD 152-154), and stomach cancer (ICD 151), and all-cause mortality (8th

* section shortened for publication in this thesis

Revision of the International Classification of Diseases). Age-adjusted mortality rates were calculated using the age distribution of the whole study population as standard.

Flavonoid analysis

The food equivalent composites had been stored at - 20°C from the middle of 1987 to 1992 and we first determined the stability of flavonoids by comparing the quercetin content of the food equivalent composites measured in early 1988 with values obtained in 1992. Paired comparison showed that loss of quercetin was less than 10 % and that quercetin levels in both measurements were highly correlated (r = 0.91) (MGL Hertog, unpublished observations). We concluded that the food equivalent composites were suitable for flavonoid analysis with a method that was newly developed, optimized, and validated in 1992 (Hertog et al., 1992a). We determined the content of five major food flavonoids, e.g. quercetin, kaempferol, myricetin, luteolin, and apigenin in the food equivalent composites by extraction and hydrolysis of the flavonoids with 50 % boiling aqueous methanol in 1.2 M HCl. Flavonoid aglycones were subsequently identified and quantified using High Performance Liquid Chromatography (HPLC) by co-chromatography with pure standards. Additional identification was carried out by diode-array detection on-line. The total flavonoid content of the food equivalent composites was determined as the sum of its quercetin, kaempferol, myricetin, luteolin, and apigenin content.

Flavonoid intake

The main sources of flavonoids in the diet are vegetables, fruits, fruit juices, tea, and wine (Kühnau, 1976; Hertog et al., 1993b). As the food equivalent composites did not contain tea or wine, their contribution to total flavonoid intake had to be estimated separately. Data on the average quantity and the type of wine consumed around 1960 was derived in all cohorts from the dietary weighed records. The dietary records did not include information on tea consumption except for the Dutch Zutphen cohort: mean of 4 cups (150 mL each) of black tea per day. Average tea consumption was estimated to be about 0.5 cups of black tea in the US Railroad cohort (D. Jacobs, personal communication), and 7 cups of green tea per day in both Japanese cohorts (H.Toshima). FAO food balance sheets showed that tea consumption was negligible around 1960 in the Italian, former Yugoslavian, Greek, and Finnish cohorts. Wine (red and white) and tea (black and green) specific flavonoid data (Hertog et al., 1993a) were used to calculate their contribution to total flavonoid intake, and together with flavonoid values obtained by chemical analysis of the food equivalent composites the average flavonoid intake per cohort was calculated.

Statistical methods

Pearson correlation coefficients were calculated between mean flavonoid intake in the 16 cohorts and dietary and other lifestyle-related risk factors for chronic diseases. This was followed by regression analysis (PROC REG, SAS statistics), with age-adjusted 25 year mortality from several causes as dependent variable and flavonoid intake plus various potential confounders as independent variables. The potential confounders consisted of known

cross-cultural risk factors for chronic diseases and antioxidant vitamins. Due to the limited number of degrees of freedom, the multiple regression models never included more than four independent variables. Two-sided P-values below 0.05 were considered statistically significant.

RESULTS

Mean cohort flavonoid intake varied from 2.6 mg/day in West-Finland and 68.2 mg/day in Ushibuka (Japan). Quercetin represented 100 % (e.g. West-Finland) to 39 % in Zutphen-the Netherlands of total flavonoid intake. Flavonoid and quercetin intake were highly correlated (r = 0.92) (Table I). At baseline around 1960 the percentage of cigarette smokers ranged from 44% in Belgrade and 78% in Ushibuka (Japan). Mean flavonoid intake was positively associated with percentage of smokers (r = 0.52, p =0.04), with the logarithm of alcohol intake (r = 0.43, p=0.09), and with the intake of beta-carotene (0.37, p=0.16), and inversely with the intake of saturated fat (r = - 0.44, p = 0.09), dietary fiber (r = -0.50, p = 0.03), vitamin C (r = - 0.43, p = 0.10), and vitamin E (r = -0.29, p = 0.26).

Coronary Heart Disease Mortality

The average flavonoid intake was inversely related with CHD mortality. Variance in flavonoid intake explained about 25 % of the total variance in CHD mortality across the 16 cohorts (Table II, Figure 1). Previous studies of the Seven Countries Study showed that intake of saturated fat was a strong predictor of CHD mortality rates (Keys et al., 1986; Kromhout et al., submitted). In multiple regression analysis flavonoid intake, average saturated fat acid intake, and the percentage of smokers were used as independent variables. This resulted in a total explained variance of 90 % (Table II, Figure 2). Flavonoid intake contributed significantly to this model (P=0.01) and explained about 9 % of the total variance, whereas saturated fat intake explained 73 % and percentage of smokers 7 %. The regression coefficient of flavonoid intake (β_{flav}) changed from - 0.16 in the univariate model to - 0.12 in the multiple regression model including average saturated fat acid intake and the percentage of smokers. Additional adjustment for intake of either vitamin E, vitamin C, beta-carotene, or alcohol did not increase the total explained variance and did not change the regression coefficient of flavonoid intake: $-0.12 < \beta_{flav} < -0.13$, all P values <0.01.

Cancer mortality

The average flavonoid intake was weakly and inversely associated with lung cancer mortality (Table II). A recent cross-cultural study suggest that saturated fat intake also is a determinant of lung cancer mortality (Jinxiang et al., 1991). After introducing flavonoid intake, saturated fat intake, and percentage of smokers as independent variable the total explained variance increased to 74 %. The contribution of flavonoids to this model was borderline significant ($\beta_{flav} = 0.37 \times 10^{-3}$, P= 0.06). Additional separate adjustment for vitamin E or beta-carotene intake did not affect the regression coefficient of flavonoid intake, but adjustment for vitamin C intake significantly weakened the association between flavonoid intake and lung cancer mortality (Table II).

Table I. Flavonoid intake, quercetin intake, and mortality from several causes after 25 year of follow-up in 16 cohorts of the Seven Countries Study

Cohorts	Flavonoid intake	Quercetin intake	25-year mortality in %					
			CHD	Cancer	Lung-	Colorectal-	Stomach-	Total
West-Finland	2.6	2.6	19.2	12.3	4.4	0.9	2.3	50.3
Velika Krsna-Serbia	9.0	9.0	12.2	10.3	1.6	1.7	2.6	50.0
East-Finland	9.6	9.6	28.8	12.7	7.3	0.1	2.9	59.7
US Railroad	12.9	11.0	20.2	11.4	3.3	2.0	0.5	45.1
Zrenjanin-Serbia	13.0	13.1	17.7	13.1	2.1	1.4	1.8	57.9
Belgrade-Serbia	13.3	7.7	11.8	8.4	2.1	0.6	0.2	29.5
Corfu-Greece	15.6	14.1	9.5	10.9	2.8	0.4	1.3	40.4
Crete-Greece	15.7	15.0	4.6	8.8	2.0	0.9	0.5	31.4
Rome Railroad-Italy	23.1	17.2	13.2	12.2	3.3	1.4	1.6	39.7
Crevalcore-Italy	23.3	18.3	13.4	17.0	3.0	1.6	3.2	49.8
Zutphen-The Netherlands	33.1	13.1	19.7	17.8	7.2	2.0	1.7	48.0
Montegiorgio-Italy	33.9	26.8	11.5	12.2	1.0	1.1	4.0	46.2
Dalmatia-Croatia	40.2	21.0	8.1	10.0	3.5	0.5	1.0	43.3
Slavonia-Croatia	58.2	38.2	14.2	10.8	1.8	1.1	3.8	61.0
Tanushimaru-Japan	60.8	27.2	4.5	13.1	1.0	1.6	5.1	39.4
Ushibuka-Japan	68.2	34.6	6.3	18.1	2.6	1.0	5.1	51.5

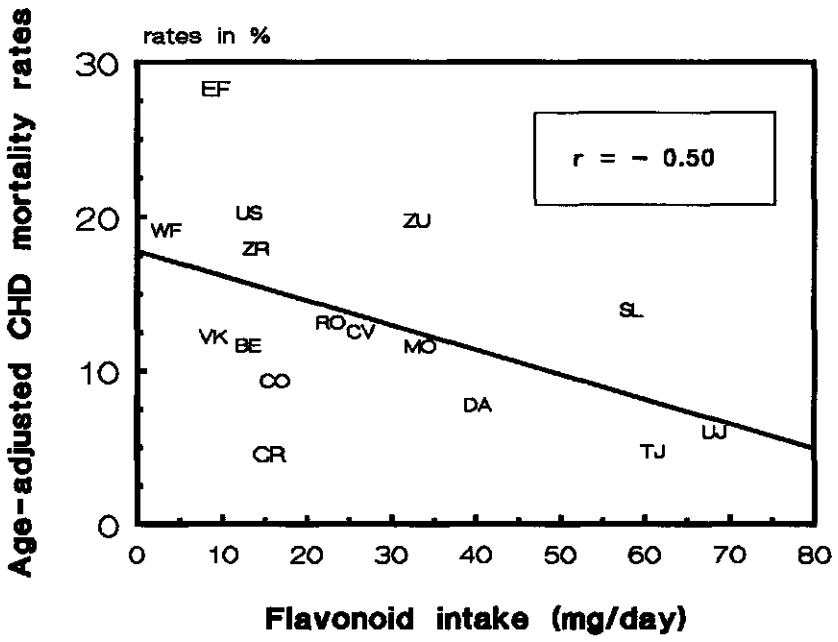

Figure 1. Flavonoid intake and mortality from coronary heart disease after 25 year of follow-up (The Seven Countries Study) BE: Belgrade-Serbia; CO: Corfu-Greece; CR: Crete-Greece; CV: Crevalcore-Italy; DA: Dalmatia-Croatia; EF: East Finland; MO: Montegiorgio-Italy; RO: Rome-Italy; SL: Slavonia-Croatia; TJ:: Tanushimaru-Japan; US; USA Railroad; UJ; Ushibuka-Japan; VK: Velika-Krsna-Serbia; WF: West-Finland; ZR: Zrenjanin-Serbia; ZU: Zutphen-The Netherlands

No association was observed between flavonoid intake and colorectal cancer mortality. Adjustment for the potential confounders intake of dietary fibre, total fat, and antioxidant vitamins (Willet, 1989), did not change the regression coefficient of flavonoid intake (Table II).

Flavonoid intake was strongly and positively correlated to stomach cancer mortality. Sodium intake was not related to stomach cancer mortality and adjustment for sodium intake did not affect the regression coefficient of flavonoid intake. Smoking has been associated with stomach cancer in prospective studies (Nomura et al., 1990) and percentage of smokers was a strong predictor of stomach cancer mortality in the present study. After including the percentage of smokers and flavonoid intake in the multiple model the total explained variance increased from 46 % to 56 %, but the regression coefficient of flavonoid intake did not change ($\beta_{flav} = 0.5 \times 10^{-3}$, $P = 0.02$), nor did it change after additional separate adjustment for intake of vitamin E or beta-carotene. After additional adjustment for vitamin C intake the association between flavonoid intake and stomach cancer mortality was not longer statistically

significant (Table II).

Average flavonoid intake was weakly positively related to all-cause cancer mortality but after adjustment for fat intake and percentage of smokers this association was further weakened (Table II). Additional adjustment for intake of antioxidant vitamins or alcohol intake did not further change the regression coefficients ($0.02 < \beta_{flav} < 0.03$, all P values>0.50).

Table II. Relation between average intake of flavonoids in 1960 and mortality from various causes during 25 years of follow-up across 16 cohorts participating in the Seven Countries Study

	Independent variables							
	Flavonoid intake				Flavonoid intake and potential confounders[a-e]			
Dependent variable	Intercept	ß (SE)	*P*	r	Intercept	ß (SE)	*P*	r^2
Coronary heart disease	17.78	- 0.16 (0.07)	0.048	-0.50	- 15.96	- 0.12 (0.04)[a]	0.01	0.90
Total cancer	10.94	0.06 (0.04)	0.14	0.39	- 5.46	0.006 (0.032)[b]	0.86	0.63
Lung cancer	0.037	-0.25×10^{-3} (0.2×10^{-3})	0.31	-0.27	- 0.084	-0.33×10^{-3} (0.23×10^{-3})[c]	0.17	0.74
Colorectal cancer	0.011	0.03×10^{-3} (0.07×10^{-3})	0.68	0.11	0.03	-0.07×10^{-3} (0.08×10^{-3})[d]	0.35	0.40
Stomach cancer	0.009	0.5×10^{-3} (2×10^{-3})	0.004	0.68	-0.025	0.15×10^{-3} (0.15×10^{-3})[e]	0.34	0.79
All-causes	44.93	0.06 (0.12)	0.64	0.15	2.11	0.13 (0.11)[a]	0.27	0.59

[a] adjusted for saturated fat intake and percentage of smokers
[b] adjusted for fat intake and percentage of smokers
[c] adjusted for saturated fat intake, percentage of smokers, and vitamin C intake
[d] adjusted for fat intake and intake of dietary fibre
[e] adjusted for sodium intake, percentage of smokers, and vitamin C intake

All-cause mortality

Average flavonoid intake was not related to all-cause mortality in the 16 cohorts. Adjustment for saturated fat intake, percentage of smokers, and either intake of antioxidant vitamins or alcohol intake did not affect this relation (Table 2).

DISCUSSION

The cross-cultural comparison suggests that the intake of antioxidant flavonoids is associated with lower long term mortality from CHD in different countries. Although the intake of saturated fat remains the major determinant of CHD mortality in the Seven Countries Study cohorts, flavonoid intake contributed another 9 % the explanation of the total variance in CHD mortality between the 16 cohorts. Flavonoid intake was not independently associated with mortality from other causes. Quercetin intake and flavonoid intake were highly correlated and the relations between quercetin intake and disease mortality were similar to those observed for total flavonoid intake.

The results of this cross-cultural comparison are consistent with those of a prospective cohort study involving 800 elderly men, The Zutphen Elderly Study (Hertog et al., 1993c; Hertog et al., *Chapter 5*). In that study flavonoid intake was inversely associated with CHD mortality, but not with mortality from cancer at all sites. A weak inverse relation with lung cancer mortality was observed, but it was based on a very low number of cases and not statistically significant. We are not aware of any other epidemiological studies on the relation between flavonoid intake and mortality from chronic diseases.

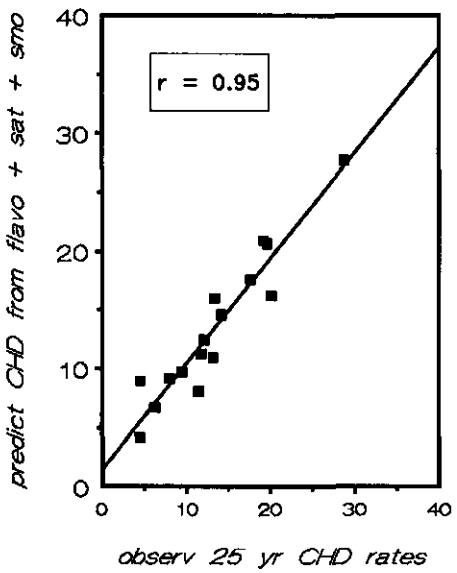

Figure 2. Observed and predicted 25-year coronary heart disease mortality from intake of saturated fat, flavonoids, and percentage of smokers in 16 cohorts

Indications for possible protective effects of flavonoids on CHD are provided by *in vitro* studies in which antioxidant flavonoids scavenge free radicals, and protect LDL against

oxidative modifications. The susceptibility of LDL for oxidation and the level of oxidized LDL in serum is associated with severity of atherosclerosis in man (Salonen et al., 1992). Our results could therefore be explained by assuming that diets with a high amount of flavonoids protect LDL against oxidation by free radicals.

Flavonoids have been reported to reduce colonic cell proliferation in vitro and to inhibit chemically induced tumors in rodents. These experimental results could not be confirmed in the present study in that average flavonoid intake was not related to colorectal cancer rates and all-cause cancer mortality rates across the cohorts. Lung cancer mortality rates were somewhat lower in populations with a high intake of flavonoids, but those results were not independent from vitamin C intake. Flavonoid intake was positively related to mortality from stomach cancer, but again this effect was not independent from vitamin C intake and percentage of smokers. It was recently found that infections with *Helicobacter Pylori* are an important cross-cultural determinant of stomach cancer mortality (The Eurogast Studygroup, 1993). Infections with *H Pylori* are more common in countries with a high flavonoid intake such as Japan, and less common in countries with a low flavonoid intake such as the USA and Scandinavian countries. Possibly, the positive association between flavonoid intake and stomach cancer mortality is confounded by the prevalence of *H Pylori* infections. However, no information on this infection is available for men who participated in the present study.

The main sources of flavonoid intake differed largely in the 16 cohorts. Tea was the major source of flavonoid intake in Japan at more than 80 % and in the Zutphen cohort at 60 %. Red wine was the major source in the Italian cohorts in which it contributed to approximately 40 %, whereas vegetables and fruits (mainly onions and apples) were the most important sources in the US, Finland, former Yugoslavia, and Greece. It may be questionable whether the average intake of flavonoids at baseline around 1960 reflects long-term average intake in the 16 cohorts. We showed previously that, although the characteristic food consumption patterns in the 16 cohorts have changed during the 25 years of follow-up, the relative position of the cohorts in the distribution of foods was maintained (Kromhout et al., 1989). Possibly, flavonoid levels in foods may have changed during the 25 years due to changes in cultivation and breeding conditions of food plants. We could not investigate this because no reliable quantitative data on the flavonoid content of foods in 1960 is available. The variability in flavonoid sources between cohorts explain why cohorts with a high average flavonoid intake did not necessarily have a high intake of dietary fibre and vitamin E and C. The fact that in different cohorts flavonoid intake was derived from various sources also suggests that specific intake of flavonoids rather than that of flavonoid containing foods are responsible for the associations with CHD. Our findings also support the recent suggestion that flavonoids present in red wine could be partly responsible for low CHD mortality in red wine drinkers ("French paradox") (Frankel et al., 1993). Tea, especially green tea as it is typically consumed in Japan, also contains other antioxidative polyphenols such as epicatechin gallate, and epigallocatechin gallate (Conney et al., 1992), which contribute greatly to the antioxidative potential of this beverage. Possibly, the high consumption of antioxidant polyphenols from tea in Japan in combination with a low saturated fat diet contributes to the low CHD rates in Japan despite a very high percentage of smokers.

We conclude that average flavonoid intake may contribute to differences in mortality rates from coronary heart disease across populations, but not to differences in cancer mortality rates. Although these cross-cultural comparisons cannot prove causal relations between

flavonoid intake and disease mortality, the consistency of these results with the results obtained on the individual level are promising. However, more experimental, epidemiological, and clinical studies on the role of flavonoids in chronic disease risk are needed before firm conclusions can be drawn.

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Chapter 9

General Discussion

Da steh ich nun, ich armer Tor!
Und bin so klug als wie zuvor;
Heiße Magister, heiße Doctor gar,
Und Ziehe schon an die Zehen Jahr
Herauf, herab und quer und krumm
Meine Schüler an der Nase herum-
Und sehe, daß wir nichts wissen können !

Johann Wolfgang Goethe. Faust: Der Tragödie erster Teil, p.13;358-364
Philipp Reclam Jun. 1971 Stuttgart, West-Germany

General Discussion

In this thesis the rationale, design, and results of an epidemiological evaluation of the anticarcinogenic and antioxidant flavonoids quercetin, kaempferol, myricetin, luteolin and apigenin is described. After developing and validating an analytical method for the determination of these flavonoids in foods, we determined the content of these flavonoids in 28 types of vegetables, 9 types of fruits, and 12 types of beverages commonly consumed in The Netherlands. We found that intake of these flavonoids was inversely related to subsequent coronary heart disease risk in men participating in the Zutphen Elderly Study, but not with cancer risk. Average flavonoid intake around 1960 in 16 cohorts from seven countries was also inversely associated with coronary heart disease mortality after 25 years of follow up, but not with cancer mortality at various sites. These results suggest that intake of these flavonoids may be an important determinant of coronary heart disease risk, but that its role in cancer etiology is less clear.

Chemical analysis

Flavonoids occur in foods as glycosides, and over 2000 different flavonols and flavones have been identified, which occur mainly in very low levels in foods. Consequently, the quantitative determination of individual flavonol and flavone glycosides in foods is complicated. We therefore decided not to quantify the individual flavonol and flavone glycosides but, instead, to hydrolyse the glycosides to their parent aglycons (sugar-free flavonoid) and quantify the aglycons as discussed in Chapter 2. This is justified because flavonol and flavone aglycons presumably are the biologic active part of the molecule. In addition, in the human gastro-intestinal tract bacterial enzymes have been found that hydrolyse flavonoid glycosides to their aglycons (Kühnau, 1976; Tamura et al., 1980). Much effort was given to the unequivocal identification of the flavonoid aglycons. Two mobile phases, each giving a different selectivity of the HPLC column were used. All foods were sequentially chromatographied using these two mobile phases. Misinterpretation of the chromatograms was minimized as the retention times of flavonoids peaks had to match the retention times of the reference flavonoids in both mobile phases. In addition peak identity and peak purity of the flavonoids eluting from the HPLC column was checked for each food with diode-array detection. Possibly our hydrolysis conditions were not optimum for all glycosides because optimization of the analytical method was targeted at hydrolysis of *O*-glucosides, glucuronides, and sulphates only. For instance, *C*-glycosyl flavones which have a sugar residue attached to the aromatic carbon atom, are not hydrolyzed with our method of analysis. Similarly methoxy-flavonoids are more resistant to acid hydrolysis than flavonoid glycosides and consequently escaped our method of analysis. However, C-glycosyl flavones occur mainly in the leaves of cereal crops whereas methoxyflavones occur in citrusfruits peel (Herrmann, 1976). These parts of the plant are usually not consumed and their relative importance in the diet is questionable. We therefore concluded that our method of analysis allows a quantitative determination of the five flavonoid aglycones in freeze-dried foods after acid hydrolysis of the parent glycosides. These data can thus be used to determine flavonol and flavone intake in humans.

Flavonoids in foods

Flavonols and flavones were found in most vegetables, with the exception of some brassicas, mushrooms, chicory, cucumber, and carrots. As expected, flavonoid levels were highest in the aerial parts (leaves) of most vegetables, and lowest in vegetables that grow below the soil surface. An exception were onions in which very high levels of the flavonol quercetin were found. Potatoes, coffee, and beer have been reported to be important sources of flavonoids, notably in Kühnau's review from 1976, however this was not confirmed in our study. For instance flavonols, flavones, and flavanones in potatoes would according to Kühnau contribute about 8 % to total flavonoid intake (Kühnau, 1976). However, even after increasing the detection limit to 0.1 mg/kg no flavonoids were found in potatoes (unpublished observations). As it is established that these flavonoids occur in the leaves of the potato plant, possibly Kühnau's estimations were based on data of the whole food plants, including the leaves. It should therefore be noted that our flavonoid content data are based only on the edible part of foods.

Flavonols and flavones in herbs and spices may contribute to flavonol and flavone intake in humans. Black pepper contains about 2 g/kg of kaempferol and 4 g/kg of quercetin and kaempferol combined was found in clove (Gerhardt, 1984; Vösgin and Herrmann, 1980), but no quantitative data on herbs have been published. The estimated intake of herbs and spices in The Netherlands is probably less than 2 g/day, and supposing that spices and herbs contain an average of 1 g/kg, herbs and spices would contribute approximately 2 mg to total flavonoid intake. This figure, which is obviously only a very rough estimate, probably overestimates the contribution of herbs and spices to flavonol and flavone intake in The Netherlands. We therefore concluded that the contribution of herbs and spices is negligible in The Netherlands.

Some vegetables are usually not consumed as raw products but only after cooking or baking. In order to investigate the effect of household preparation on flavonoid levels in foods we selected six vegetables that are commonly consumed after preparation, e.g. onions, leek, endives, red cabbage, French beans, and Brussels sprouts. These vegetables were cooked or baked according to Dutch customs and flavonol and flavone content of the raw products were compared to the flavonol and flavone content after preparation. We found that mean loss of these flavonoids after cooking for all foods was about 0 %, whereas mean loss after baking was approximately 11 %. We concluded that loss of these flavonoids due to preparation is negligible (<10 %) and that a correction of flavonoid levels due to losses from preparation is not necessary (unpublished observations).

Dietary surveys

A major concern in nutritional epidemiology is the quality of food intake data which is determined by its validity and reproducibility. Although we did not perform a validity study on the flavonoid intake data, several indications for the validity of our flavonoid intake data is provided. First, the data on the flavonol and flavone content of foods commonly consumed in The Netherlands are derived from an extensive food sampling and analysis programme specifically designed for an epidemiological evaluation of flavonols and flavones. Much attention was paid to the validity of the method of analysis which used the newest analytical technology (see above). The relative validity of dietary history method, which was used in

the Zutphen Elderly Study, has been investigated by several authors including Block (1982) and by Bloemberg et al. (1989a) and is generally considered to adequately reflect usual food intake of individuals. Mean intake of flavonols and flavones among the elderly was approximately 25 mg daily in the Dutch National Food Consumption Survey 1987-88 and in the Zutphen Elderly Study 1985 and in 1990, whereas it was 33 mg daily in the Zutphen cohort of the Seven Countries Study in 1960 among middle-aged men. Although these data provide no evidence on the validity of this estimation, the similarity between these results is very much encouraging. In only one previous study an estimation of mean flavonoid intake in humans is described. Kühnau estimated in 1976 that daily intake of flavonols, flavones, and flavanones in the US was approximately 100 mg. As pointed out before there are some serious limitations as to the validity of Kuhnau's estimation which have been extensively discussed in Chapter 4. One of the suggested explanation was that flavonoid intake in the US may be higher than in the Netherlands. However, in the US Railroad cohort from the Seven Countries Study average intake of flavonoids around 1960, determined by chemical analysis of food composites, was only 13 mg/day. Together with flavanones, which occur mainly in citrus fruits, total intake of these flavonoids in this cohort would approximately be 18 mg/day. This still is considerably lower than the values reported by Kühnau.

The reproducibility of the 1985 dietary intake data of the Zutphen Elderly Study was investigated by Bloemberg and co-workers (Bloemberg et al., 1989b). The correlation between the first measurements and the measurements after 1 year varied for vegetables, fruits, and beverages between $r = 0.51$ and $r = 0.82$. These values are generally considered acceptable. Flavonoid intake of 501 men participating in 1985 and in 1990 in the Zutphen Elderly Study correlated well ($r = 0.57$) and about 55 % percent of the men were divided into the same tertiles of intake of flavonoid intake whereas about 7 % were divided into opposite tertiles in 1985 and 1990. These results can be considered satisfactory taking into account the period of five years between both measurements.

We concluded that our data on flavonoid intake in the Zutphen Elderly Study and Seven Countries Study provided a suitable base for an epidemiological evaluation of flavonols and flavones. In addition seasonal variability of flavonol and flavone intake proved to be low (Chapter 5) and losses due to household preparation are negligible.

Flavonoids and cancer

We found that flavonol and flavone intake was not related to cancer mortality in a prospective study and a cross-cultural study. We could therefore not confirm our first hypothesis on the cancer protective effects of these flavonoids in humans. Possible explanations for the discrepancies between the cancer protective effects of these flavonoids as suggested by several experimental (animal) studies and our epidemiological results have already been discussed in Chapter 6 and Chapter 8.

Fruits and vegetables were thought to be the main sources of flavonols and flavones in the human diet. Experimental studies on the anticarcinogenic properties of these flavonoids have also been partly justified by the epidemiological evidence on a protective effect of vegetables and fruits. However, tea was the major source of these flavonoids in the Netherlands (about 60 %) and in Japan (about 90 %), whereas wine was the major single source in Italy (about 40 %), and onions and apples in other countries such as e.g. the US and

Finland. Thus the foods often associated with low cancer rates in a large variety of epidemiological studies, such as green-yellow vegetables and cruciferous vegetables, are not important sources of flavonols and flavones. Our results would therefore suggest that these flavonoids probably only play a minor role in the explanation of the protective effect of vegetables and fruits. In the Zutphen Elderly Study intake of flavonols and flavones from onions, endives, kale, and apples combined was inversely related to cancer risk whereas intake of flavonoids from tea was not. This strongly supports that other components present in these vegetables and fruits, but not in tea, may be effective in reducing cancer risk.

Reactive oxygen species are also thought to be involved in the several stages of tumor development, and a cancer protective effect of oxidant scavenging flavonoids could therefore be expected. However, intake of antioxidant flavonols and flavones was not related to cancer risk in the Zutphen Elderly Study, nor was the intake of other antioxidant vitamins. When considering the three step mechanism of carcinogenesis in humans, initiation phase presumably takes place early in life. Since The Zutphen Elderly Study population comprises only elderly men it could be speculated that intake of these antioxidant flavonoids has only a minor impact on the later stages of carcinogenesis, e.g. promotion or progression in humans. However, in experimental studies flavonols and flavones showed to be particularly effective in inhibiting promotion and progression. There was a suggestion for a weak inverse relation between intake of these antioxidant flavonoids and lung cancer mortality in the Zutphen Elderly Study and in the Seven Countries Study. Vitamin C intake was also inversely related to lung cancer mortality after 25 years of follow-up in the original Zutphen cohort (Kromhout et al., 1987), and a large number of other epidemiologic studies suggested inverse associations between orange and green-yellow vegetables rich in the antioxidant beta-carotene and lung cancer risk (Block et al., 1992). Oxidative stress may be especially important in lung cancer etiology, because reactive oxygen radicals produced by cigarette smoke with short half-times will react locally. Oxidant scavengers such as flavonols and flavones and vitamin C could therefore reduce lung cancer risk, but be less effective in inhibiting other cancers. Possibly, this explains why flavonoid intake was not related to all-cause cancer in our study.

Flavonoids and coronary heart disease

We found that intake of flavonols and flavones was inversely related to coronary heart disease in a prospective study and a cross-cultural study. These results confirm our second hypothesis that, presumably due to its antioxidant and antithrombotic activity, intake of these flavonoids may reduce coronary heart disease risk.

Our study is the third prospective study in which an inverse relation between intake of an antioxidant and coronary heart disease risk was found. Rimm, Stampfer, and coworkers found earlier in a study involving about 40,000 middle-aged men and 88,000 middle-aged women, a reduced risk for a coronary event of 30-40 % in the highest quintile of vitamin E intake (Rimm et al., 1993; Stampfer et al., 1993). The highest quintile of vitamin E consumption consisted in both studies only out of vitamin E supplement users, which possibly indicates also a generally healthy lifestyle of these participants. It should be noted that in our studies a considerable risk reduction was achieved with the consumption of ordinary foods such as tea, apples, and onions. We can not make a firm statement on the magnitude of the risk reduction as our relative risk estimates are based on only 43 cases of death from coronary

heart disease and 38 cases of a first myocardial infarction, and the 95 % confidence interval ranged from a reduction in mortality risk between 85 % and 29 %, with a best estimate of 68 %. In the Zutphen Elderly Study adjustment for vitamin E, vitamin C, and betacarotene intake did not alter the relation between flavonol and flavone intake and coronary heart disease. It should be noted that the range of vitamin intake in The Netherlands is low compared with e.g. the US, because the use of vitamin supplements is not very common. In the Zutphen Elderly Study only 1 % used vitamin E supplements and 6 % vitamin C supplements, which prohibits a separate investigation of vitamin supplement users.

The variance in coronary heart disease mortality rates among countries can, in general, partly be explained by well-known risk factors such as for instance average intake of saturated fat and percentage of smokers. This was also shown in the Seven Countries Study. However, some inconsistencies in these relations have been observed, notably the low coronary heart disease mortality among French wine drinkers ("French Paradox"), in spite of a similar level of saturated fat intake as in northern European countries. In Japan coronary heart disease mortality rates are very low, possibly due to a low saturated fat intake, but which contrasts with the very high percentage of smokers (more than 70 % of adult males) in Japan. Our study suggests that the high consumption of tea and red wine, both rich in antioxidant flavonols and flavones partly explains the low coronary heart disease rates in Japan and in Italy, respectively. However, our study also suggests that intake of these flavonoid has only a moderate impact on cross-cultural coronary heart disease mortality rates and that saturated fat intake remains the main determinant. The high intake of saturated fat probably also explains why the United Kingdom has one of the highest coronary heart disease mortality rates in the world in spite of a very high average tea consumption. It may also be hypothesized that due to the custom in the UK to add milk to tea, a binding of tea flavonoids to milk proteins may occur. Possibly flavonoids bound to proteins are less easily absorbed in the gastro-intestinal tract, which could in turn result in a low bioavailability. However, information on bioavailability of flavonoids and its determinants is lacking.

Metabolism of flavonoids

A drawback of our study is the lack of data on the absorption and metabolism of flavonoids. The information on the pharmacokinetics of flavonoids is scarce and conflicting. Gugler et al. (1975) investigated the disposition of oral administered quercetin (4 g) in humans, and failed to detect any quercetin in plasma or in urine. Recovery in faeces was approximately 50 %, thus suggesting extensive degradation. Ueno et al (1983) fed radiolabeled quercetin to rats (620 mg/kg body weight), and found that about 20 % was absorbed from the digestive tract. More recently, Nieder and coworkers measured flavonol levels in plasma after oral suppletion of two volunteers of 50-300 mg flavonol-containing *Ginkgo biloba*. A dose-dependent rise in plasma levels of flavonoids was found with a peak at 2-3 hours and half time of 2-4 hours (Kleijnen and Knipschild, 1992). Results of a pilot study involving 9 volunteers showed that after dietary quercetin supplementation quercetin levels in plasma rose to approximately 1 µmol/L (Hollman et al., 1993). The evidence until today therefore strongly suggest that some of dietary quercetin is absorbed from the intestines in humans but that the degree of absorption is unclear. Indirect evidence for absorption of flavonoids is provided by experimental animal studies in which orally administered quercetin

resulted in a reduced number of mammary tumors in rats (Verma et al., 1988).

Conclusion

We found that intake of antioxidant flavonols and flavones is inversely related to coronary heart disease risk in men. These results do not prove a causal relation between intake of flavonols and flavones and coronary heart disease risk. However, the consistency of our findings in both a prospective cohort study and a cross-cultural study, in addition to the indications from experimental studies supports the hypothesis that intake of these flavonoids might indeed reduce coronary heart disease risk in humans. Flavonol and flavone intake was not related to all-cause cancer risk in individuals and in populations. However, the relation between intake of these flavonoids and cancer risk at different sites could not be studied adequately due to small number of cases and merits further investigation in large prospective cohort studies.

Additional epidemiological, experimental, and clinical evidence is needed before firm conclusions as to the protective effects of flavonols and flavones against chronic diseases can be drawn. Nevertheless, a large number of other epidemiological studies in addition to ours strongly suggest that foods of vegetable origin, especially fresh vegetables and fruits, contain a large number of protective compounds. Our results suggest that these compounds embrace not only the well-known vitamins but also so-called non-nutritive factors such as flavonols and flavones. General recommendations for an increased consumption of vegetables and fruits seem therefore justified.

Suggestions for further research

Results from additional prospective studies on the relation between flavonol and flavones intake and different types of cancer and coronary heart disease risk are needed and are currently in progress. In addition results from experimental and clinical studies are warranted for the investigation of the relation between dietary flavonol and flavone intake and various biologic markers such as for instance DNA adducts, resistance to LDL oxidation, platelet functions...etc. Another important issue is the study of the metabolism of flavonoids in humans, their bioavailability, and their pharmacokinetics. An important group of flavonoids, the catechins, have recently attracted much attention because of their strong anticarcinogenic and antioxidant effects (Conney et al., 1992). The quantitation of catechins in foods and subsequent epidemiological evaluation of their effects on cancer and coronary heart disease risk seems therefore warranted.

Epilogue

It seems important to note that we are not the first to suggest that non-nutritive flavonoids may have protective effects in humans. Already in the late thirties Szent-György an co-workers suggested that flavonoids may influence the permeability of capillary membranes and may have important vitamin C sparing activities. They thus proposed that flavonoids were essential vitamins. In 1976, Kühnau stated that flavonoids should, due to their biologic activities in humans, be called semi-essential food components, because the traditional

distinction between essential (nutritive) and non-essential (non-nutritive) food components seemed obsolete. Currently, the results of several investigators and our own results strongly suggest that some flavonoids may indeed have protective effects in humans and that their presence in foods contributes to a good health. A re-evaluation of the distinction between nutrients and non-nutritive substances as suggested by Kühnau in 1976 seems therefore warranted. First steps in this direction have already been made at a ILSI conference in Washington, DC (USA) in 1993 entitled "Substantiation of the impact of nutrient and nonnutrient antioxidants on health".

Clearly the complexity of the relation between nutrients, non-nutrients, cancer, and coronary heart disease is, though very fascinating, also extremely daunting. Multidisciplinary research is needed to adequately investigate these relations. The study described in this thesis was such a multidisciplinary project and the cooperation between three different institutes, each with their own area of expertise, e.g. nutrition, analytical chemistry, and epidemiology, proved to be very successful in addressing some of these complicated research issues.

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Summary

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Summary

Flavonoids are polyphenols ubiquitously present in foods of vegetable origin. Some flavonoids such as the flavonols quercetin and kaempferol inhibit chemically-induced carcinogenesis in rodents. Flavonoids are strong antioxidants and quercetin inhibits oxidation and reduces cytotoxicity of low-density lipoproteins (LDL) *in vitro*. Oxidized LDL are atherogenic and may be important in the formation of atherosclerotic plaques. Some flavonoids also inhibit platelet aggregation leading to reduced thrombotic tendencies. The intake of flavonoids from vegetables and fruits could therefore reduce risk of cancer and coronary heart disease in human beings. However, the biologic effects of flavonoids in humans are unknown. We therefore decided to determine the flavonoid content of several foods and investigate the relation between flavonoid intake and risk of cancer and coronary heart disease in humans. The present thesis contains the results of these studies *(Chapter 1)*.

We selected the three flavonols quercetin, kaempferol, and myricetin, and two flavones luteolin and apigenin, because of their anticarcinogenic and antioxidant effects and because of their ubiquitous occurrence in foods. We developed, optimized, and validated an analytical method for the quantitative determination of these flavonoids in foods. Flavonoids were extracted and hydrolyzed by boiling with hydrochloric acid containing 50 % (v/v) methanol, and analyzed by High Performance Liquid Chromatography followed by ultraviolet detection *(Chapter 2)*. We then determined the flavonoid content of 28 types of vegetables, 12 types of fruits, and 9 types of beverages commonly consumed in The Netherlands. The quercetin content of most vegetables was below 10 mg/kg except for onions (284-486 mg/kg), kale (110 mg/kg), broccoli (30 mg/kg), french beans (32-45 mg/kg) and slicing beans (28-30 mg/kg). Kaempferol could only be detected in kale (211 mg/kg), endive (15-91 mg/kg), leek (11-56 mg/kg), and turnip-tops (31-64 mg/kg). In most fruits we detected only quercetin in levels averaging 15 mg/kg, except for different apple varieties in which we found 21-72 mg/kg. The content of myricetin, luteolin and apigenin was below the limit of detection (<1 mg/kg) except for fresh broad beans (26 mg/kg myricetin) and sweet red pepper (13-31 mg/kg luteolin) *(Chapter 3)*. In red wines and in grape juice quercetin and myricetin levels varied between 4-16 mg/L and 7-9 mg/L, respectively. Quercetin levels in various fruit juices were about 5 mg/L except tomato juice (13 mg/L). Black tea infusions contained quercetin at 10-25 mg/L, kaempferol at 7-17 mg/L, and myricetin at 2-5 mg/L *(Chapter 4)*.

Using data of 4,112 adults interviewed in the Dutch National Food Consumption Survey 1987-88 we calculated that average intake of all flavonoids combined was 23 mg/day. The most important flavonoid was the flavonol quercetin (mean intake 16 mg/day) whereas the main sources of flavonoids were tea (48 % of total intake), onions (29 %) and apples (7 %). Flavonoid intake did not vary between seasons and it was not correlated with total energy intake ($r = 0.001$), only weakly with the intake of vitamin A (retinol equivalents, $r = 0.14$), dietary fiber ($r = 0.21$), and vitamin C ($r = 0.26$) *(Chapter 5)*.

We determined flavonoid intake at baseline in 1985 of 805 men aged 65-84 years participating in the Zutphen Elderly Study. Mean flavonoid intake was 26 mg/day. The major sources of flavonoid intake were tea at 61 % and vegetables and fruits (mainly onions, kale, endive, and apples) at 38 %. We then related flavonoid intake to cancer and coronary heart disease risk after five years of follow-up. Between 1985 and 1990 75 men developed cancer of which 26 lung cancer. During this period 34 men died from cancer. Flavonoid intake in

1985 was not associated with either incidence of all cause cancer (*P* for trend = 0.54) or with mortality from all-cause cancer (*P* for trend = 0.51). Flavonoid intake was also not associated with lung cancer incidence (*P* for trend = 0.43). Similar results were obtained when only epithelial tumors of the gastro-intestinal and respiratory tract were investigated *(Chapter 6)*.

During the five year follow-up 43 men died from a coronary heart attack whereas 38 men had a first myocardial infarct. Flavonoid intake was inversely related with coronary heart disease mortality (*P* for trend = 0.015) and to a lesser extent with incidence of first myocardial infarction (*P* for trend = 0.08). Coronary heart disease mortality was approximately 60 % reduced in the highest compared with the lowest tertile of flavonoid intake. These results were independent of known risk factors such as age, body-mass index, smoking, serum cholesterol, blood pressure, physical activity, coffee consumption, and of intake of energy, vitamin C, vitamin E, beta-carotene, and dietary fibre. Flavonoid intake was also independently inversely associated with all-cause mortality although this association was only of borderline significance (*P* for trends 0.08) *(Chapter 7)*.

We also calculated average flavonoid intake around 1960 of 16 cohorts participating in the Seven Countries Study. Main sources of flavonoids were tea in Japan and the Netherlands, red wine in Italy, and onions in the US, former Yugoslavia, Greece, and Finland. Average flavonoid intake was inversely related to coronary heart disease mortality rates across the cohorts after 25 years of follow-up, and explained about 25 % of the variance. However, it was not independently related to mortality from cancer at various sites. Although intake of saturated fat remained at 73 % the main cross-cohort determinant in coronary heart disease mortality rates in the Seven Countries Study, flavonoid intake explained, independently of vitamin E, vitamin C and alcohol intake, another 9 % of the total variance in coronary heart disease mortality rates between the cohorts *(Chapter 8)*.

We conclude that flavonoids contribute to the antioxidant potential of the human diet. Unexpectedly not vegetables and fruits in general, but specifically tea, red wine, and onions were the main dietary sources of flavonoids. The results of the prospective cohort study and the cross-cultural study both suggest that flavonoid consumption may reduce risk of coronary heart disease, but that it does not seem to be an important determinant of cancer risk. Information on the absorption and metabolism of flavonoids in humans is scarce and additional experimental, clinical, and epidemiological evidence is needed before firm conclusions on the health benefits of flavonoids can be drawn *(Chapter 9)*.

Samenvatting

Flavonoiden zijn polyfenolen die van nature in plantaardige voedingsmiddelen voorkomen. Een aantal flavonoiden, waaronder de flavonolen quercetine en kaempferol remmen de chemische inductie van tumoren in proefdieren. Flavonoiden zijn sterke antioxidanten en quercetine remt in in vitro-onderzoekingen de oxydatie en verlaagt de cytotoxiciteit van LDL-cholesterol (low-density lipoproteins). Geoxideerd LDL is atherogeen en lijkt betrokken te zijn bij de vorming van atherosclerotische plaques. Flavonoiden remmen ook de bloedplaatjesaggregatie en verlagen trombosevorming. De consumptie van flavonoiden uit groenten en fruit zou dus zowel het kankerrisiko als het risiko van coronaire hartziekten kunnen verminderen. De effecten van flavonoiden in de mens zijn echter niet bekend. Daarom is besloten om eerst het flavonoidgehalte van diverse voedingsmiddelen te bepalen en vervolgens de relatie tussen flavonoidinname en het risiko op kanker en coronaire hartziekten bij de mens te onderzoeken. Dit proefschrift bevat de resultaten van deze onderzoeken *(Hoofdstuk 1)*.

Wij kozen drie flavonolen quercetine, kaempferol en myricetine, en twee flavonen apigenine en luteoline omdat deze flavonoiden anticarcinogenen en antioxidanten zijn en omdat ze in vrijwel alle voedingsmiddelen voorkomen Voor deze flavonoiden werd een analysemethode ontwikkeld, geoptimaliseerd en gevalideerd. Flavonoiden werden geëxtraheerd en gehydrolyseerd met zoutzuur in 50 % (v/v) methanol, en vervolgens gedetecteerd met hoge druk vloeistofchromatografie en ultraviolet detectie *(Hoofdstuk 2)*. Vervolgens is het flavonoidgehalte van 28 soorten groenten, 12 soorten fruit, en 9 soorten dranken bepaald. Het quercetinegehalte van de meeste groentesoorten was lager dan 10 mg per kg. Uitzonderingen hierop vormden uien (284-486 mg/kg), boerenkool (110 mg/kg), broccoli (30 mg/kg), sperziebonen (32-45 mg/kg) en tuinbonen (28-30 mg/kg). Kaempferol kwam alleen voor in boerenkool (211 mg/kg), andijvie (15-91 mg/kg), prei (11-56 mg/kg), en raapstelen (31-64 mg/kg). Verschillende soorten fruit bevatten vooral quercetine, ongeveer 15 mg/kg, alleen in diverse appelrassen bleek het quercetinegehalte tussen de 21 en 72 mg/kg te liggen. Myricetine, luteoline en apigenine konden niet gedetecteerd worden (<1 mg/kg), met uitzondering van 26 mg/kg myricetine in tuinbonen en 12-31 mg/kg luteoline in rode paprika. De seizoensverschillen waren over het algemeen klein, al was het flavonoidgehalte in bladgroenten in de zomer het hoogst *(Hoofdstuk 3)*. Het quercetine- en myricetinegehalte van rode wijn en druivesap varieerde respectievelijk tussen 4-16 mg/L en 7-9 mg/L. In diverse vruchtesappen was het quercetinegehalte ongeveer 5 mg/L, met uitzondering van tomatensap (13 mg/L). Diverse soorten zwarte thee bevatte 10-25 mg/L quercetine, 7-17 mg/L kaempferol en 2-5 mg/L myricetine *(Hoofdstuk 4)*.

Met behulp van de Nederlandse Voedsel Consumptie Peiling 1987-88 is vervolgens de flavonoidinname van 4112 volwassenen berekend. De gemiddelde flavonoidinname bedroeg 23 mg/dag waarvan quercetine het grootste deel leverde (16 mg/dag). De belangrijkste voedingsbronnen van flavonoiden waren thee (48 % van totale inname), gevolgd door ui (29 %) en appels (7 %). De flavonoidinname varieerde niet per seizoen en was niet gecorreleerd aan energieinname (r=0.001) en maar matig met de inname van retinol equivalenten (r=0.14), voedingsvezel (r=0.21) en vitamine C (r=0.26) *(Hoofdstuk 5)*.

Vervolgens is de flavonoidinname berekend van 805 mannen tussen de 65 en 84 jaar die participeerden in de Zutphen Ouderen Studie. De gemiddelde flavonoidinname was 26 mg/dag

en de belangrijkste bronnen waren thee (61 %), en groenten en fruit (38%) (waaronder vooral uien, appels, boerenkool en andijvie). De flavonoidinname in 1985 is gerelateerd aan zowel het kankerrisiko als het risico op coronaire hartziekten gedurende vijf jaar follow-up. Tussen 1985 en 1990 kregen 75 mannen kanker waarvan 34 longkanker. In deze periode overleden 34 mannen aan kanker. De flavonoidinname in 1985 hing niet samen met het risico om kanker te krijgen (P trend = 0.58), of om hieraan te overlijden (P trend = 0.51). De flavonoidinname hing ook niet samen met het risico om longkanker te krijgen (P trend = 0.43). Vergelijkbare resultaten werden verkregen als alleen epitheliale tumoren van het maag-darm kanaal en van de luchtwegen onderzocht werden *(Hoofdstuk 6)*.

Gedurende de vijf jaar follow-up kregen 38 mannen een eerste myocardinfarct en overleden 43 mannen aan coronaire hartaandoeningen. Flavonoidinname in 1985 hing omgekeerd samen met zowel het optreden van een eerste myocardinfarct (P trend = 0.08) als aan de sterfte aan coronaire hartziekten (P trend = 0.015). Het risico om aan een coronaire aandoening te overlijden was in het hoogste tertiel van flavonoidinname ongeveer 60 % minder dan in het laagste tertiel. Dit resultaat was onafhankelijk van bekende risicofactoren zoals leeftijd, Quetelet Index (gewicht in kg/lengte in m^2), roken, serum cholesterol, bloeddruk, lichamelijke activiteit, koffie consumptie, en inname van energie, vitamine C, vitamine E, ß-caroteen en voedingsvezel. De flavonoidinnname hing ook omgekeerd samen met de totale sterfte in deze populatie, al was deze associatie zwakker *(Hoofdstuk 7)*.

De gemiddelde flavonoidinname van de 16 cohorten rond 1960 die participeerden aan de Zeven Landen Studie is vervolgens gerelateerd aan de sterfte aan verschillende aandoeningen gedurende 25 jaar follow-up. De belangrijkste bronnen voor flavonoiden waren thee in Japan en Nederland, rode wijn in Italië, en uien in de VS, voormalig Joegoslavië, Griekenland en Finland. De gemiddelde flavonoidinname in de 16 cohorten was invers gerelateerd aan de sterfte aan coronaire hartziekten en verklaarde ongeveer 25 % van de variantie tussen de cohorten. De flavonoidinname was niet onafhankelijk gerelateerd aan sterfte door andere aandoeningen. Uit multiple regressieanalyse bleek dat de belangrijkste voorspeller voor coronaire sterfte in de 16 cohorten de consumptie van verzadigd vet was (73 % van de variantie). De gemiddelde flavonoidinname voegde hier nog, onafhankelijk van de gemiddelde consumptie van vitamine C, vitamine E, en betacaroteen, ongeveer 9 % verklaarde variantie aan toe *(Hoofdstuk 8)*.

Wij concluderen dat flavonoiden een belangrijke bijdrage leveren aan het antioxidantgehalte van de voeding. Onverwacht waren niet groenten en fruit in het algemeen, maar vooral thee, rode wijn en uien de belangrijkste bronnen van flavonoiden in de voeding. De resultaten van het prospectieve cohort onderzoek en van het cross-culturele onderzoek wijzen beide erop dat de consumptie van flavonoiden het risico op coronaire hartaandoeningen verlaagt, maar niet het kankerrisiko. Er is weinig informatie beschikbaar over de absorptie en omzettingen van flavonoiden in het lichaam en meer aanvullend klinisch en epidemiologisch onderzoek is nodig voordat harde conclusies over de gezondheidseffecten van flavonoiden getrokken kunnen worden *(Hoofdstuk 9)*.

Résumé

Les flavonoïdes sont des polyphénols qui se trouvent naturellement dans la plupart des aliments d'origine végétale. Certains de ces flavonoïdes, notamment les flavonols quercétine et kaempferol, inhibent le développement de tumeurs induites chimiquement lors d'expérimentations animales. Les flavonoïdes sont des antioxydants et la quercétine inhibe aussi, *in vitro*, l'oxydation et réduit la cytotoxicité des lipoprotéines LDL (low-density lipoproteins). Sous forme oxydée, cette lipoprotéine semble jouer un rôle important dans l'initiation et le développement de l'athérosclérose. Les flavonoïdes inhibent également l'agrégation des plaquettes et réduisent la thrombose. La consommation de légumes et fruits renfermant les flavonoïdes pourrait ainsi réduire le risque de cancers et de maladies cardiaques. Néanmoins les effets des flavonoïdes sur la santé de l'homme sont encore mal connus. Nous avons alors décidé de déterminer la composition des aliments en flavonoïdes et d'examiner ensuite la relation entre la consommation de flavonoïdes et le risque de cancer et de maladies cardiaques chez l'homme. La présente thèse contient les résultats de ces études *(Chapitre 1)*.

Nous avons sélectionné les trois flavonoles quercétine, kaempferol et myricétine et les deux flavones apigenine et lutéoline pour leurs effets anticancérogènes et antioxydants et pour leur omniprésence dans la plupart des aliments d'origine végétale. Nous avons ensuite développé, optimalisé et validé une méthode d'analyse quantitative du contenu en flavonoïdes des aliments. Les flavonoïdes ont été extraits et hydrolysés avec de l'acide chlorhydrique dans 50 % de méthanol (v/v), et ensuite détectés par chromatographie liquide haute pression suivi de détection ultraviolette *(Chapitre 2)*. Nous avons ainsi déterminé le contenu de flavonoïdes dans 28 légumes, 12 fruits et 9 boissons diverses. La plupart des légumes renfermaient moins de 10 mg/kg de quercétine, á l'exception des oignons contenant 284-486 mg/kg, du choux frisé contenant 110 mg/kg, la brocoli contenant 30 mg/kg, et les haricots verts contenant 32-45 mg/kg. Nous avons détecté le kaempferol seulement dans le choux frisé à 211 mg/kg, dans la chicorée frisée à 15-91 mg/kg, dans les poireaux à 11-56 mg/kg et dans les navets à 31-64 mg/kg. La plupart des fruits renfermaient essentiellement la quercétine à environ 15 mg/kg, à l'exception des différentes variétés de pommes renfermant de 21 à 72 mg de quercétine par kg. Nous n'avons détecté ni myricétine, ni lutéoline, ni apigenine dans les légumes et fruits divers sauf de la myricétine dans les poix (26 mg/kg) et la lutéoline dans les poivrons rouges (12-31 mg/kg) *(Chapitre 3)*. Le vin rouge et le jus de raisins contenaient de 4 à 16 mg/L de quercétine et de 7 à 9 mg/L de myricétine. Le niveau de quercétine dans les autres jus de fruit était environ de 5 mg/L, sauf dans du jus de tomate (13 mg/L). Le thé noir ordinaire contenait environ 10-25 mg/L de quercétine, 7-17 mg/L de kaempferol et 2-5 mg/L de myricétine *(Chapitre 4)*

Nous avons calculé la consommation moyenne de flavonoïdes aux Pays-Bas en utilisant les données de l'Etude Nationale sur la Consommation Alimentaire 1987-88 portant sur 4112 personnes âgées de 19-74 ans. La consommation de flavonoïdes était environ de 23 mg par jour et la quercétine était, á 16 mg par jour, le flavonoïde principal. Le thé constituait la principale source de flavonoïdes à 48 %, suivie des oignons à 29 % et des pommes à 7 %. La consommation de flavonoïdes était indépendante de la variation saisonnière et elle n'était pas correlée avec l'apport énergétique ($r = 0.001$), et seulement faiblement avec l'apport en

vitamine A (r = 0.14), en fibres alimentaires (r=0.21), et en vitamine C (r=0.26) *(Chapitre 5)*.

Par la suite nous avons calculé la consommation moyenne de flavonoïdes de 805 hommes âgés de 65 à 84 ans participant à l'étude de Zutphen (Pays-Bas). L'apport moyen en flavonoïdes était de 26 mg par jour et les sources principales de flavonoïdes étaient le thé à 61% et les légumes et fruits (particulièrement oignons, pommes, choux frisé et chicorée frisée) à 38 %. La consommation moyenne en 1985 a ensuite été relaté au risque de cancer et maladies cardiaques après cinq ans de suivi. Entre 1985 et 1990 75 hommes ont présenté un cancer dont 26 un cancer du poumon. Durant cette période 34 hommes sont décédés d'un cancer. L'apport en flavonoïdes en 1985 n relaté ni à la morbidité (*P* trend =0.54) ni à la mortalité due au cancer (P trend = 0.51). La consommation de flavonoïdes en 1985 n également pas relaté au risque de cancer du poumon (*P* trend = 0.43). Des résultats similaires ont été observés en considérant uniquement les cas de cancers épithéliaux du tractus digestif et respiratoire *(Chapitre 6)*. Durant les cinq années de suivi, 43 hommes sont décédés d'une maladie cardiaque et 38 hommes on eu un infarctus du myocarde. La consommation de flavonoïdes en 1985 était inversement associée au risque de décès par maladie cardiaque (*P* trend 0.015), et dans une moindre mesure au risque d'un infarctus du myocarde (*P* trend = 0.08). Le taux de mortalité associé à des maladies cardiaques était diminué d'environ 60 % dans le tertile des individus ayant les consommations en flavonoïdes les plus élevées par rapport au tertile des consommations les plus basses. Ces effets se revélaient indépendants de divers facteurs de confusions tel que l'age, l'indice de masse corporelle, le tabagisme, le niveau de cholestérol, la tension artérielle, les activités sportives, la consommation de café, l'apport énergétique, l'apport en vitamine C, vitamine E, beta-carotène et fibres alimentaires. La consommation de flavonoïdes était également inversement relatée á la mortalité totale dans cette étude, bien que ces résultats ne soient pas tout à fait significatifs *(Chapitre 7)*.

Nous avons ensuite déterminé la consommation moyenne de flavonoïdes de 16 cohortes en 1960 dans une étude internationale portant sur sept pays (Seven Countries Study). La source principale de flavonoïdes était le thé au Japon et aux Pays-Bas, le vin rouge en Italie et les oignons dans les Etats-Unies, l'ex-Yougoslavie, la Grèce et la Finlande. La consommation moyenne de flavonoïdes des 16 cohortes était inversement relatée avec le taux de mortalité de maladies cardiaques après 25 ans de suivi, et expliquait près de 25% de la variation totale. La consommation moyenne de flavonoïdes n'était cependant pas associée indépendamment au taux de mortalité de cancer. Bien que la consommation moyenne de graisses saturées reste à 73 % le principal facteur déterminant la variation du taux de mortalité cardiaque, la consommation moyenne de flavonoïdes explique un autre 9% de la variation dans cette étude internationale *(Chapitre 8)*.

Nous concluons que les aliments ordinaires contiennent des quantités significatives de flavonoïdes qui contribuent ainsi à la capacité antioxydante de l'alimentation. Contrairement à nos suppositions les plus importantes sources alimentaires de flavonoïdes ne sont pas seulement les fruits et légumes mais plus particulièrement le thé ordinaire, le vin rouge, et les oignons. Les résultats de l'étude prospective et de l'étude internationale suggèrent que la consommation de flavonoïdes pourrait réduire le risque de maladies cardiaques, mais qu'elle ne semble pas jouer un rôle important dans la prévention du cancer. Une meilleure connaissance de l'absorption, le métabolisme et la bio-disponibilité des flavonoïdes et les résultats d'autres études cliniques et épidémiologiques sont encore nécessaires pour pouvoir conclure sur les effets protecteurs des flavonoïdes.

Zusammenfassung

Flavonoide sind Polyphenole, die in pfanzlichen Nahrungsmitteln vorkommen. Einige Flavonoide, unter ihnen die Flavonole Quercetin und Kämpferol, hemmen die Entstehung und Entwickelung von chemisch induzierten Tumoren in Nagetieren. Flavonoide sind starke Antioxidantien, so hemmt Quercetin z.B. die Oxidation bzw. vermindert die Zytotoxizität von LDL-Cholesterin (low-density lipoproteins) *in vitro*. Oxidiertes LDL-Cholesterin ist atherogen und steht im Verdacht, eine wichtige Rolle bei der Bildung von atherosklerotischen Plaques zu spielen. Flavonoide hemmen ebenfalls die Blutplättchenaggregation und verringern die Thromboseneigung. Der Konsum von Flavonoiden durch Gemüse und Obst könnte daher sowohl das Krebsrisiko als auch das Herzinfarktrisiko beim Menschen senken. Die biologischen Wirkungen von Flavonoiden im Menschlichen Körper sind allerdings noch unbekannt. Es wurde darum beschlossen zuerst die Flavonoidkonzentrationen von verschiedenen Nahrungsmitteln zu bestimmen und dann den Zusammenhang zwischen Flavonoidkonsum und dem Krebsrisiko bzw. dem Risiko koronarer Herzkrankheiten beim Menschen zu untersuchen. In dieser Dissertation werden die Ergebnisse dieser Untersuchungen beschrieben *(Kapitel 1)*.

Die drei Flavonole Quercetin, Kämpferol und Myricetin und die zwei Flavone Luteolin und Apigenin wurden ausgewählt, da diesen Flavonoiden antikarzinogene und antioxidative Eigenschaften zugeschrieben werden und da diese ubiquitär in Nahrungsmitteln zu finden sind. Für diese Flavonoide wurde eine Analysemethode entwickelt, optimiert und validiert. Die Flavonoide wurden mit Salzsäure in 50%igem (v/v) Methanol extrahiert und hydrolysiert und mittels Hochleistungsflussigchromatographie und UV detektiert *(Kapitel 2)*. Unter Verwendung dieser Methode wurde der Flavonoidgehalt von 28 Gemüsesorten, 12 Fruchtsorten und 9 verschiedenen Getränken bestimmt. Die meisten Gemüsesorten enthielten weniger als 10 mg/kg Quercetin mit Ausnahme von Zwiebeln (284-486 mg/kg), Krauskohl (110 mg/kg), Broccoli (30 mg/kg), Prinzeßbohnen (32-45 mg/kg) sowie Saubohnen (28-30 mg/kg). Kämpferol dagegen wurde nur in Krauskohl (211 mg/kg), Endivien (15-91 mg/kg), Porree (11-56 mg/kg) und Rübenstrunk (31-64 mg/kg) gefunden. Die Obstsorten enthielten um 15 mg/kg Quercetin mit Ausnahme verschiedener Apfelsorten die 21-72 mg/kg Quercetin enthielten. Myricetin, Luteolin und Apigenin konnten in keine der Gemüse- oder Obstsorten nachgewiesen werden mit Ausnahme der Saubohnen mit 26 mg/kg Myricetin und rotem Paprika mit 12-31 mg/kg Luteolin. Saisonale Gehaltsunterschiede zeigten sich beim Blattgemüse wobei der Flavonoidgehalt im Sommer am höchsten war *(Kapitel 3)*. Rotwein und Traubensaft enthielten jeweils 4-16 mg/L Quercetin und 7-9 mg/L Myricetin. In verschiedenen anderen Fruchtsäften lag der Quercetingehalt bei etwa 5 mg/L, mit Ausnahme von Tomatensaft, welcher 13 mg/L enthielt. Diverse Teesorten enthielten jeweils 10-25 mg/L Quercetin, 7-17 mg/L Kämpferol und 2-5 mg/L Myricetin *(Kapitel 4)*.

Unter Verwendung der Daten einer repräsentativen Ernährungsstudie bei 4112 niederländischen Erwachsenen im Alter von 19-74 Jahren wurde der Flavonoidkonsum berechnet. Der durchschnittliche tägliche Flavonoidkonsum betrug 23 mg, wovon Quercetin den Hauptanteil stellte (16 mg täglich). Die wichtigsten Flavonoidquellen waren Tee (48 %), Zwiebeln (27 %) und Äpfel (7 %). Der Flavonoidkonsum war nicht korreliert mit der Energieaufnahme (r = 0.001), und nur schwach positiv korreliert mit dem Konsum vom

Vitamin A (r = 0.14), Vitamin C (r= 0.21), und Balaststoffen (r = 0.21) *(Kapitel 5)*.

Der Flavonoidkonsum von 805 Männern im Alter zwischen 65 und 84 Jahren die im Jahr 1985 teilnahmen an der Zutphen Studie wurde berechnet und anschließend der Zusammenhang zwischen Flavonoidkonsum im Jahr 1985 und dem Krebsrisiko bzw. dem Risiko koronarer Herzkrankheiten untersucht. Der durchschnittliche Flavonoidkonsum in 1985 betrug 26 mg pro Tag und die wichtigsten Flavonoidquellen waren Tee (61 %), und Gemüse und Obst (38%) (vor allem Zwiebeln, Äpfel, Krauskohl und Endivien). Zwischen 1985 und 1990 erkrankten 75 Männer an Krebs wobei als häufigste Diagnose Lungenkrebs (26 Fälle) festgestellt wurde. In diesem Beobachtungszeitraum starben vierunddreißig Männer an Krebs. Der Flavonoidkonsum war weder mit dem Risiko für Krebs insgesamt(*P* trend 0.58) noch mit dem Lungenkrebsrisiko (*P* trend 0.43) verbunden. Der Flavonoidkonsum wies ebenfalls keinen Zusammenhang mit dem Risiko an Krebs zu sterben auf (*P* trend 0.58). Vergleichbare Ergebnisse ergaben Berechnungen zu epithelialen Tumoren des Magen-Darm Traktes und der Atemwege *(Kapitel 6)*. Während der fünf Beobachtungsjahre erlitten 38 Männer erstmalig einen Herzinfarkt und 43 Männer starben an koronaren Herzkrankheiten. Der Flavonoidkonsum im Jahr 1985 war sowohl umgekehrt verbunden mit dem Herzinfarktrisiko (*P* trend 0.08) als auch mit dem Risiko an koronaren Herzkrankheiten zu sterben (*P* trend 0.015). Das Risiko an koronaren Herzkrankheiten zu sterben war für die Männer in dem höchsten Flavonoidterziel etwa 60 % niedriger als für Männer in dem niedrigsten Terziel. Diese Befunde waren unabhängig von anderen bekannten Risikofaktoren wie Alter, Rauchgewohnheiten, Body Mass Index, Serum-Cholesterin, Blutdruck, sportliche Aktivität, Kaffeekonsum und von der Aufnahme von Energie, Vitamin C, Vitamin E, Beta-Karotin und Ballaststoffen *(Kapitel 7)*.

Weiter wurde der Flavonoidkonsum aus der Zeit um 1960 in 16 Kohorten, die an einer multizentrischen geographischen Vergleichsstudie in sieben Ländern durchgeführt wurde, berechnet und der Zusammenhang zwischen Flavonoidkonsum und der Krebs- und Herzkrankheitenmortalität nach 25 Jahren untersucht. Die wichtigsten Quellen für Flavonoide in den verschiedenen Kohorten waren in Japan und in den Niederlanden Tee, in Italien Rotwein und in den Vereinigten Staten, im Gebiet des ehemaligen Jugoslawien, Griechenland und Finnland Zwiebeln. Der Flavonoidkonsum um 1960 war negativ korreliert mit der Herzmortalität in den 16 Kohorten und erklärte ungefähr 25 % der Varianz der Sterberaten. Der Flavonoidkonsum in den 16 Kohorten war dagegen nicht unabhängig korreliert mit den organspezifischen Krebssterberaten. In multivariaten Modellanalysen zeigte sich daß der Verzehr von gesättigten Fettsäuren etwa 73 % der Varianz der Herzmortalität in den Kohorten erklärte. Der Flavonoidkonsum konnte unabhängig vom Verzehr an Vitamin E, Vitamin C, Beta-Karotin und Alkohol, weitere 9 % der Varianz erklären *(Kapitel 8)*.

Unsere Befunde zeigen daß Flavonoide einen wichtigen Beitrag an dem Antioxidantiengehalt der Ernährung liefern. Tee, Rotwein und Zwiebeln erwiesen sich als die wichtigsten Quellen für Flavonoide und nicht, wie erwartet, hauptsächlich Gemüse und Obst. Die Befunde der prospektiven Studie und der geographischen Studie weisen beide daraufhin, daß der Konsum von Flavonoiden das Risiko für Herzkrankheiten verringert, aber keinen großen Einfluß auf das Krebsrisiko hat. Der Metabolismus und die Resorption von Flavonoiden im menschlichen Körper sind jedoch noch weitgehend unbekannt. Weitere Laborversuche, epidemiologische und klinische Studien sind erforderlich, bevor eine klare Aussage über die Wirkung von Flavonoiden auf die Gesundheit gemacht werden kann.

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Appendix

Summary of Workshop Non-nutritive anticarcinogens in the diet: state of the art and future developments

Non-nutritive anticarcinogens in foods.
State of the art and future developments.
26-27 March 1990 Wageningen, The Netherlands
State Institute for Quality Control of Agricultural Products (RIKILT-DLO)
Report 90.32. Michaël G.L. Hertog and Peter C.H. Hollman

Summary of general discussion

- Polyphenols
- Indoles
- Aromatic isothiocyanates
- Terpenes
- Sulphur compounds of Allium species
- Epidemiology

Concluding remarks

Summary of general discussion

The aim of this workshop was to establish priorities for epidemiological research on anticarcinogens. In the following discussion and conclusions an emphasis has been laid on this type of research. It has to be mentioned that for other types of research, for instance elucidating mechanisms or application of anticarcinogens as therapeutic agents, other priorities may be relevant.

Polyphenols

Polyphenols were identified as a most promising group of anticarcinogens. Polyphenols are a vast class of different compounds present in a large variety of vegetables and fruits. Polyphenols prove to be effective in a number of experimental systems, and at least some of them effect the arachidonic acid cascade, which is clearly important in the promotional phase of carcinogenesis. A great deal of animal data show that these effects on the arachidonic acid cascade are associated with inhibition of carcinogenesis. There is sufficient analytical support for small molecular polyphenols, however, only limited chemical information is available on so called "tannins". The group of polyphenols is very large, so guidance in the choice of the most promising polyphenolic anticarcinogens would be very helpful. Only structure-activity relations of inhibitory effects on ultimate carcinogens and the arachidonic acid cascade have been reported. However, extrapolation from these model systems to the human situation is very difficult. The following promising compounds for epidemiological studies were suggested: flavonols, phenolic acids, catechin derivatives (green tea polyphenols).

Indoles

In experimental studies, indoles can either have an inhibitory effect or a stimulating effect on carcinogenesis depending on the type of carcinogen applied. Indoles originate from indolyl glucosinolates in cruciferous vegetables and, depending on the pretreatment of the vegetable, there is a direct exposure or an indirect exposure after metabolism in the digestive tract. Epidemiological studies on indoles are therefore difficult.

Aromatic isothiocyanates

Aromatic isothiocyanates could be interesting compounds in epidemiological studies on smokers, as in experimental systems ,these compounds were able to inhibit lung cancer induced by a tobacco specific nitrosamine. Only a minor fraction of the glucosinolates of vegetables, the parent compounds of isothiocyanates, release aromatic isothiocyanates after hydrolysis. So chemical analysis of total diets in order to determine the intake of aromatic isothiocyanates is not feasible. Intake could be determined by analyzing specific vegetables relatively rich in the parent glucosinolate (gluconasturtiin). It was suggested to use a biological marker to be able to measure the human exposure. However, the thiocyanate ion as a metabolite of the isothiocyanate is no specific marker for the exposure to romatic isothiocyanates.

Terpenes

The major source for terpenes like d-limonene in the diet are soft drinks. Part of the consumption of terpenes is caused

by flavoring agents which will be of increasing importance in the future. Terpenes are readily absorbed and their biological half life is rather long. Sensitive GC methods are available and determination of terpenes in serum is feasible. No data are available about amounts of terpenes that are ingested, and that might be biologically active. In conclusion, no top priority for epidemiological studies should be given to terpenes.

Sulphur compounds of allium species

Sulphur compounds of onions and garlic might be very potent, but they appear analytically to be a very difficult class of substances. As these compounds are restricted to onions and garlic, the need for chemical analyses is less urgent. For epidemiological studies it will be easy to determine the users and non-users of for instance garlic. However, this approach will not allow to quantify the intake of these compounds, so dose-response relations are not possible. A more promising approach compared to analyses of foods, would be to determine these sulphur compounds in the breath or sweat of the subjects, and use these data as indicators for the exposure.

Epidemiology

Concerning epidemiological studies on the effects of anticarcinogens, between populations studies only can give indications of possible effects of certain anticarcinogens or types of foods. In this way between populations studies compared to within population studies can be very valuable as they show more variations in dietary patterns and cancer rates. However, these studies never can prove causality. Individual based studies will always be necessary. If large cohorts are involved in individual based studies, food analyses are much to complicated because there may be hundreds of components involved. One solution would be only to analyze the diets of individuals that developed cancer, and compare them with controls. It was recognized that the development of biological markers for the intake and or the effect of anticarcinogens in the human body should deserve a high priority. Especially markers for effects of anticarcinogens (polyphenols) on the arachidonic acid cascade in the human body would be very valuable. Possibilities of measuring these effects in for instance blood cells were discussed.

Concluding remarks

At the end of the discussion Wattenberg made the following concluding remarks. Each of the groups of compounds presented in this workshop have great potential. The importance of these compounds to the inhibition of human cancer remains to be determined. Epidemiology could be very important in this, but is only one part of the whole picture. Nevertheless, positive epidemiological data would be very encouraging. The group of researchers that were present in this workshop represent almost the total range of the known natural occurring non-nutritive inhibitors. This shows how small the effort has been until now in identifying compounds that may have inhibitory effects. There may be many anticarcinogens that are more important than the ones that were discussed during this workshop.

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About the author

Michaël Gerard Leo Hertog was born in Rome, Italy, on March 20th, 1965. After French primary school, first in Belgrade, former Yugoslavia (Petite Ecole Francaise), and then in Rome, Italy (Lycee Chateaubriand) he visited from 1977 to 1984 German secondary school, Kardinal Frings Gymnasium, in Bonn, Federal Republic of Germany. He passed his final exam (Abitur) in June 1984, and started studying Health Sciences at the State University of Limburg in Maastricht, The Netherlands in September of that year. In 1988 he obtained his M.Sc. degree in Biological Health Sciences (majors: nutrition, toxicology, epidemiology) and he received the annual M.Sc. students thesis award from the Dutch Association for Nutrition and Food Technology (NVVL). In 1989 he obtained for the period of seven months a Special Training Award to do research on nutrition and cancer at the International Agency for Research on Cancer (IARC) in Lyon, France. In October 1989 he became a PhD fellow at the Department of Human Nutrition, Wageningen Agricultural University, in the Netherlands. The work described in the present thesis was carried out at the State Institute for Quality Control of Agricultural Products in Wageningen (DLO-RIKILT) and the Department of Chronic Diseases and Environmental Epidemiology of the National Institute of Public Health and Environmental Protection (RIVM) in Bilthoven. In 1993 he was registered as Master of Science in Epidemiology by the Council of the Netherlands Epidemiological Society. In February 1994 he started a three-year post-doc project at the Netherlands Institute of Health Sciences (NIHES) and at the National Institute of Public Health and Environmental Protection in Bilthoven.