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PII: S0308-8146(96)00065-9

Food Chemistry, Vol. 57, No. 1, pp. 43–46, 1996

0308-8146/96 $15.00+0.00

Analysis and health effects of flavonoids

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Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. Over 4000 different flavonoids have been described. Flavonoids have a variety of biological effects in numerous mammalian cell systems, *in vitro* as well as *in vivo*. Recently much attention has been paid to their antioxidant properties and to their inhibitory role in various stages of tumour development in animal studies. Hitherto, analytical research on flavonoids was mainly aimed at identification and not at quantification. As a consequence, no data were available for epidemiological investigations of flavonoid intake and chronic diseases. Flavonoids in foods are mostly linked to sugars, the so-called glycosides. As one parent compound or aglycone, e.g. quercetin, may be linked to a number of different sugars, quantification in foods is complex. Hydrolysis of the glycosides and subsequent determination of the parent aglycones simplifies this task. Following this approach we developed and validated an HPLC method and determined the flavonol and flavone content of vegetables, fruits and beverages commonly consumed. Subsequent epidemiological evaluation showed that the intake of flavonols and flavones was inversely associated with coronary heart disease in both a prospective cohort study and in a cross-cultural study. However, no relation with cancer risk could be established. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

The overwhelming epidemiological evidence for a protective effect of vegetables and fruits against cancer (Steinmetz & Potter, 1991; Block *et al.*, 1992) has stimulated research on explanatory biological mechanisms. An exciting hypothesis is that vegetables and fruits contain compounds that have a beneficial effect independent of that of known nutrients and micronutrients. This is supported by *in vitro* and *in vivo* studies which show that naturally occurring plant compounds may inhibit various stages in the cancer process (Wattenberg, 1992). In these studies flavonoids have also been studied extensively.

Flavonoids, 2-phenyl-benzo-α-pyrones, are polyphenolic compounds that occur ubiquitously in foods of plant origin. A multitude of substitution patterns in the two benzene rings of the basic structure occur in nature. Variations in their heterocyclic ring gives rise to flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids. Over 4000 different naturally occurring flavonoids have been described (Middleton & Kandaswami, 1994) and this list is still growing.

Table 1 gives an overview of the occurrence of flavonoids in foods (Kühnau, 1976; Hertog *et al.*, 1992*a*, 1993*c*).

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We decided to study the potential role of flavonoids in cancer and coronary heart disease prevention using a prospective cohort study and a cross-cultural study.

BIOLOGICAL EFFECTS OF FLAVONOIDS

In 1936 Rusznyàk and Szent-Györgyi observed that a mixture of two flavanones decreased capillary permeability and fragility in humans and proposed the name vitamin P (Rusznyàk & Szent-Györgyi, 1936). However, the term vitamin P was abandoned because these flavonoids ultimately did not meet the definition of a vitamin. A multitude of *in vitro* studies has shown that flavonoids can inhibit, and sometimes induce, a large variety of mammalian enzyme systems. The effects of mainly flavones and flavonols on 24 different enzymes or enzyme systems were described in a review (Middleton & Kandaswami, 1994). Some of these enzymes are involved in important pathways that regulate cell division and proliferation, platelet aggregation, detoxification, and inflammatory and immune response. Thus, it is not surprising that effects of flavonoids have been found in cell systems and animals, on different stages in the cancer process, on the immune system and on hemostasis (Middleton & Kandaswami, 1994).

Table 1. Occurrence of flavonoids in common foods (Kühnau, 1976; Hertog *et al.*, 1992*a*,*c*)

Flavonoid subgroup	Major foods	Examples of foods
Flavones	Herbs	Parsley, thyme
Flavonols	Vegetables	Onions, kale, broccoli
	Fruits	Apples, cherries, berries
	Beverages	Tea, red wine
Flavanones	Fruits	Citrus
Catechins	Fruits	Apples
	Beverages	Tea
Anthocyanidins	Fruits	Cherries, grapes
Isoflavones	Vegetables	Soya beans, legumes

The anticarcinogenic and antiproliferative effects of quercetin and other flavonoids are becoming increasingly evident (Huang & Ferraro, 1992). Recently, much attention has been paid to their antioxidant properties which affect oxygen free radicals and lipid peroxidation. Oxygen free radicals and lipid peroxidation might be involved in several pathological conditions such as atherosclerosis, cancer and chronic inflammation (Halliwell, 1994). Most flavonoids are effective radical scavengers. This property by itself does not imply a beneficial effect because after scavenging a flavonoid radical is formed. A very reactive flavonoid radical would propagate rather than interrupt the deleterious events initiated by the radical attack. However, a flavonoid radical with high stability will not readily react and thus will act as an antioxidant. Several structural groups may enhance the stability of the flavonoid radical and thus its antioxidant capacity (Bors *et al.*, 1990).

QUANTITAVE DETERMINATION OF FLAVONOLS AND FLAVONES IN FOODS

Early interest in flavonoids focused on their unequivocal identification to study evolutionary and taxonomic relationships (Swain, 1980). However, in order to be able to evaluate the biological effects of flavonoids we needed reliable data on flavonoid contents of common Dutch vegetables and fruits. Such a database did not exist for The Netherlands, and data produced in other countries were fragmentary. In addition, the quality of these data was questionable because they were obtained with methods now considered obsolete. We decided to focus on the subgroups of flavonols and flavones because these flavonoids, including the flavonol quercetin, occur ubiquitously in plant foods and were the ones most frequently studied in model systems.

In edible parts of plant foods flavonols and flavones only occur as glycosides, i.e. bound to sugars. Sugar molecules can bind to various positions in the parent flavonoid, the so-called aglycon. More than 80 different sugars have been found bound to flavonoids in plants. The position in the ring can also vary. As a result, 179 different quercetin glycosides have been found in nature (Williams & Harborne, 1994). A multitude of glycosides also occurs in common foods such as tea (Finger *et al.*, 1991).

We aimed to determine three flavonols and two flavones in vegetables and fruits commonly consumed in The Netherlands. It would be an impossible task to determine all their glycosides separately. Therefore we decided to determine total flavonol and flavone contents after hydrolysis of the glycosides (Hertog *et al.*, 1992*b*). An additional advantage was that after hydrolysis low concentrations of individual glycosides with the same parent compound will add up and ease detection. We found that the rate of hydrolysis depended on the type of sugar and the type of aglycone. Our final method employed three different combinations of hydrochloric acid concentrations and hydrolysis times, and the optimal combination was determined experimentally for each food product.

After hydrolysis, the aglycones were separated and quantified by HPLC. To avoid false assignments caused by co-eluting substances we used two different eluents, and confirmed peak purity and identity by Diode Array Detection (Hertog *et al.*, 1992*b*).

Using this method we determined flavonols and flavones of vegetables, fruits and beverages commonly consumed in The Netherlands (Table 2) (Hertog *et al.*, 1992*a*, 1993*c*). Quercetin was by far the most important flavonol, followed by kaempferol. Flavones were only found in a few products, such as celery, sweet red pepper and broad beans.

With these data we were able to calculate the intake of flavonols and flavones in The Netherlands. To our surprise, tea turned out to be the major source in this population (48% of total intake), followed by onions (29%) and apples (7%) (Hertog *et al.*, 1993*b*). Thus, vegetables and fruits often associated with low rates of cancer in epidemiological studies are not major sources of dietary flavonols and flavones in this population. The average intake of flavonols and flavones was 23 mg/day, of which the flavonol quercetin contributed 16 mg/day. This is in contrast with data of Kühnau (1976) who estimated that the total intake of flavonoids in the United States was 1 g/day (expressed as glycosides). This would be equivalent to about 115 mg of flavonol and flavone aglycones per day, as opposed to 23 mg/day in our studies. Most probably the flavonoid content used by Kühnau (1976) was too high, not only because the levels were obtained with methods now considered obsolete, but also because the flavonoid content of non-edible parts were sometimes included.

Table 2. Flavonol and flavone* contents of common vegetables, fruits and beverages (Hertog *et al.*, 1992*a*, 1993c)

Flavonol and flavone contents	Foods
Low(< 10 mg/kg or < 10 mg/litre)	Cabbage, spinach, carrots, peas, mushrooms
	Peaches, strawberries
	Orange juice, white wine, brewed coffee
Medium(< 50 mg/kg or < 50 mg/litre)	Lettuce, broad beans, red pepper, tomato
	Apples, grapes, cherries
	Tomato juice, red wine, tea beverages
High(> 50 mg/kg or 50 mg/litre)	Broccoli, endive, kale, French beans, celery, onions
	Cranberries

*Sum of quercetin, kaempferol, myricetin, luteolin, and apigenin.

Finally it can be argued that the food disappearance values used by Kühnau (1976) tend to overestimate food intake.

FLAVONOLS AND FLAVONES AND DISEASE

We used the food composition data discussed in the preceding section to study associations between chronic diseases and flavonol and flavone intake. We had access to a population of elderly men in the Dutch town of Zutphen. In 1985 their food consumption was assessed using a dietary history method. A total of 805 men, aged 65–84 years, entered the study. The intake of flavonols and flavones was, on average, 26 mg/day. Major sources of flavonols and flavones were tea (61%), onions (13%) and apples (10%).

After 5 years, in 1990, their health records were collected, and the morbidity and mortality data were studied. Differences in baseline characteristics of these men between tertiles of flavonol and flavone intake were evaluated, and relative risks were calculated. No associations were found between flavonol and flavone intake and total cancer mortality. Also specific forms of cancer, such as lung cancer, were not found to be associated with flavonols and flavones (Hertog *et al.*, 1994). In contrast, coronary heart disease mortality was strongly and inversely associated with flavonol and flavone intake (Hertog *et al.*, 1993*a*); a reduction in mortality risk of more than 50% was found (Fig. 1). These results were not confounded by known risk factors for coronary heart disease and antioxidant vitamins.

The Zutphen study cohort is one of the cohorts of the Seven Countries Study, a cross-cultural study of diet, lifestyle and disease. In 1987 the foods that represented the baseline diet as per 1960 of each cohort were bought locally. The foods were combined into food composites that represented the average daily food intake of each cohort. In these food composites flavonols and flavones were determined. The intake of flavonols and flavones ranged from 3 mg/day in a Finnish cohort to 70 mg/day in a Japanese cohort. The major dietary sources of flavonols and flavones varied substantially between cohorts. In the Japanese and Dutch cohorts the major source was tea, while red wine was the major source in Italy. Onions and apples were the predominant sources in the United States, Finland, Greece and the former

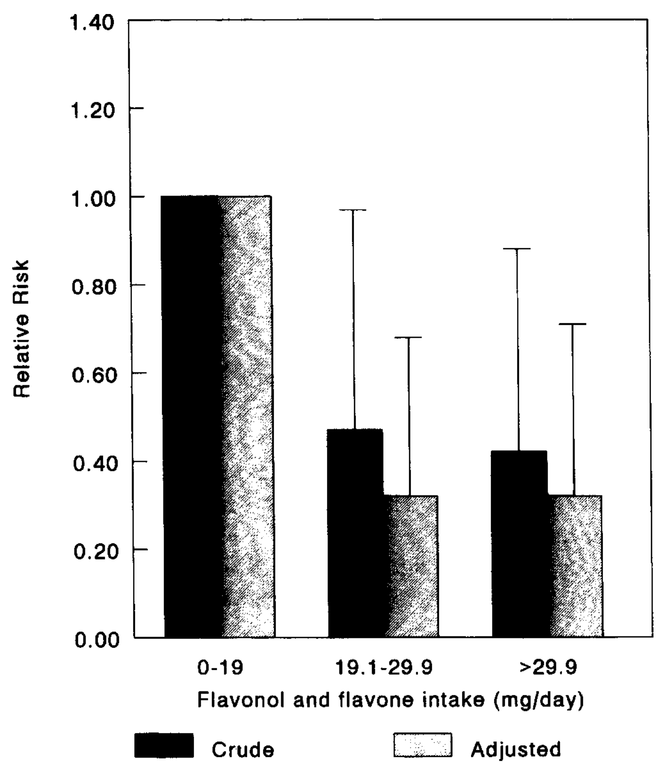

Fig. 1. Flavonol and flavone intake and relative risk (RR and 95% CI) of death from coronary heart disease in the Zutphen Elderly Study. Adjustment was made for age, diet and various risk factors (Hertog *et al.*, 1993*a*).

Yugoslavia. Figure 2 shows that there was a strong inverse association between flavonol and flavone intake and mortality from coronary heart disease (Hertog *et al.*, 1995). Again, no association with cancer mortality was found.

The discrepancy in cancer results with animal studies is puzzling. Possibly, anticarcinogenic effects only occur with the high doses used in animal studies. In addition, the animal model may have its limitations because possibly human tumours are not caused by the type and dose of carcinogens used in these models.

CONCLUSIONS

Flavonoids have many effects on mammalian biology. The assessment of flavonoids in foods is complex because of the multitude of glycosides; hydrolysis of

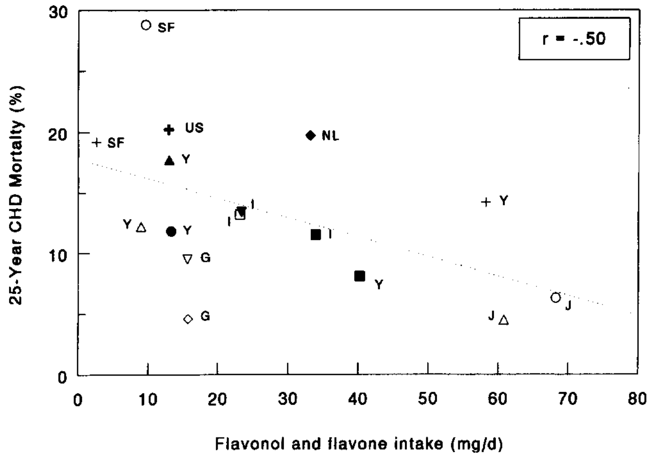

Fig. 2. Flavonol and flavone intake at baseline and mortality from coronary disease after 25 years of follow-up (the Seven Countries Study) (Hertog *et al.*, 1995). Flavonols and flavones were determined in food composites that represented the average daily food intake of each cohort. G, Greece; J, Japan; I, Italy; NL, The Netherlands; US, United States of America; SF, Finland; Y, the former Yugoslavia.

glycosides offers the most practical approach. Using data obtained for flavonols and flavones we found inverse associations with coronary heart disease in a prospective and in a cross-cultural study. Antioxidant and/or antithrombotic effects of flavonoids possibly explain these results. No associations with cancer mortality were found. These results need confirmation in other epidemiological studies.

At present flavonoids are considered to be non-essential non-nutrient dietary components. In view of their potentially health-promoting activities, this opinion might need to be modified in the future.

ACKNOWLEDGEMENTS

We thank Professor D. Kromhout, who took the initiative for these studies and was instrumental in bringing them to completion. We are grateful to Dr E. Feskens and Professor J. G. A. J. Hautvast for valuable discussions, and to D. P. Venema and B. van de Putte for technical assistance.

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