Dietary non-nutrients and haemostasis in humans:

effects of salicylates, flavonoids and ginger

P.L.T.M. Karin Janssen

Promotoren: **Prof. Dr. M.B. Katan**
Bijzonder hoogleraar in de voedingsleer van de mens,
Katholieke Universiteit, Nijmegen.
Universitair Hoofddocent, Landbouwuniversiteit, Wageningen;

Prof. Dr. W.A. van Staveren
Bijzonder hoogleraar in de voeding van de oudere mens;

Co-promotor: **Dr. Ir. R.P. Mensink**
Universitair Docent, Universiteit Maastricht.

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Proefschrift

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Stellingen

1. De hoeveelheid acetylsalicylzuur in de dagelijkse voeding is verwaarloosbaar (*dit proefschrift*).

2. Er is geen wetenschappelijk bewijs voor de suggestie dat de inname van acetylsalicylaat uit de voeding bijgedragen kan hebben aan de afname van sterfte aan cardiovasculaire ziekten in de USA (*Ingster LM and Feinleib M. Abstract 36th Annual Conference on Cardiovascular Disease Epidemiology and Prevention, March 1996, San Francisco, California, USA; dit proefschrift*).

3. Er is geen wetenschappelijk bewijs voor de stelling dat consumptie van gember de thromboxaan-produktie door de bloedplaatjes vermindert (*Srivastava KC, Mustafa T. Prostaglandins Leukotrienes and Essential Fatty Acids 1989;38:255-66); dit proefschrift*).

4. Verplichte soberheid heeft een heilzame uitwerking op de gezondheid (uit: "Cubanen blaken van gezondheid dankzij crisis". Volkskrant 7/1/1997).

5. De eed van Hippocrates is hopeloos verouderd (Intermediair 1996;27:34-35, 37).

6. De vraag "Hoe gaat het?" is niet geschikt om te achterhalen hoe het werkelijk met iemand gaat.

7. Werksfeer is bij uitstek een onderschatte succes-factor voor produktiviteit, vooral in wetenschappelijk onderzoek.

8. De effectiviteit van een manager wordt meer bepaald door de kwaliteit van zijn/haar communicatie, dan door zijn/haar inhoudelijke kennis.

9. Als je jezelf geen concrete doelen stelt, behoed je jezelf voor mislukkingen, maar je ontneemt je ook de kans op succes en ontplooiing.

10. Significant is niet perse relevant.

Behorende bij het proefschrift van P.L.T.M.K. Janssen getiteld:
Dietary non-nutrients and haemostasis in humans: effects of salicylates, flavonoids and ginger.
Wageningen, 28 mei 1997.

Voor mam

"There is a bark of an English tree, which I have found by experience to be a powerful astringent, and very efficacious in curing anguish and intermitting disorders.

-

The Rev. Mr. Edmund Stone of Chipping-Norton in Oxfordshire, in a letter to the Right Honourable George, Earl of Macclesfield, president of the Royal Society, April 25, 1763."

(Weissmann G. Aspirin. Scientific American 1991; January: 58-64.)

These investigations were carried out at the:

Department of Human Nutrition,
Agricultural University,
Wageningen,
The Netherlands

State Institute for Quality Control of
Agricultural Products (RIKILT-DLO),
Wageningen,
The Netherlands

Department of Human Biology,
Maastricht University,
Maastricht,
The Netherlands

Department of Haematology,
Academic Hospital,
Utrecht,
The Netherlands.

Abstract

Dietary non-nutrients and haemostasis in humans:

effects of salicylates, flavonoids and ginger

PhD Thesis by P.L.T.M. Karin Janssen, Department of Human Nutrition, Wageningen Agricultural University, Wageningen, The Netherlands. 28th May, 1997.

In this thesis we studied the content of acetylsalicylate and total salicylates in foods, and we studied the effects of the dietary non-nutrients salicylates and flavonoids and of certain foods on haemostatic parameters in humans.

Acetylsalicylic acid -aspirin- irreversibly inhibits platelet cyclo-oxygenase, leading to decreased platelet thromboxane A_2 production and decreased aggregation. Therefore it is effective as an anti-thrombotic drug in doses as low as 30 mg/d. Qualitative analyses by Swain *et al* suggested the presence of acetylsalicylate in foods. It was estimated that a normal mixed Western diet provides 10-200 mg/d of total salicylate and 3 mg/d of acetylsalicylate. We showed in 10 healthy subjects that 3 mg/d of acetylsalicylic acid decreased mean platelet thromboxane production by 39±8% (±sd). Thus, quantitative data on dietary acetylsalicylate deserved closer investigation. We determined acetylsalicylate and total salicylates in 30 foods using HPLC with fluorescence detection. Acetylsalicylate was lower than the detection limit (0.02 mg/kg for fresh and 0.2 mg/kg for dried products) in all foods. Total salicylates were 0-1 mg/kg in vegetables and fruits, and 3-28 mg/kg in herbs and spices. We showed that urinary excretion was a valid indicator for intake of pure (acetyl)salicylic acid (recovery 77-80%). We then studied urinary salicylate excretion in 17 subjects eating a variety of diets to estimate the content of bio-available salicylates of diets. Median excretion was 1.4 mg/24 h (range 0.8-1.6). Our data suggest that even purely vegetable diets provide less than 6 mg/d of salicylates, and no measurable acetylsalicylate. These amounts are probably too low to affect coronary heart disease risk, and worries about adverse effects of dietary salicylates on the behaviour of children may be unfounded.

Others found that dietary flavonoids were associated with a reduced risk of coronary heart disease and stroke. This might be due to effects on haemostasis, because flavonoids have been reported to inhibit platelet aggregation *in vitro*. We found that concentrations of 2.5 μM of the flavone apigenin inhibited collagen- and ADP-induced platelet aggregation *in vitro* by about 26%, whereas the flavonols quercetin and quercetin-3-glucoside had no effect. No effects were found on platelet aggregation, thromboxane production, or other haemostatic parameters in 18 healthy subjects after they had consumed large amounts of quercetin- (onions) and apigenin-rich (parsley) foods daily for 7 d each. We conclude that claims for anti-aggregatory effects of flavonoids are based on the *in vitro* use of concentrations that cannot be attained *in vivo*. Our findings suggest that it is unlikely that reported effects of dietary flavonoids on coronary vascular disease risk are mediated through platelet aggregation or cyclo-oxygenase activity. Possible effects on known risk indicators for coronary heart disease from the coagulation cascade or the fibrinolytic system should be examined in a larger study.

It has been claimed that ginger consumption exerts an anti-thrombotic effect by inhibiting platelet thromboxane production. We, however, found no effects on platelet thromboxane production in a placebo-controlled cross-over study in 18 healthy subjects after consumption of raw (-1±9%, mean±sd) or cooked ginger (1±8%).

We conclude that contents of (acetyl)salicylate in foods are too low to affect disease risk. We could not confirm the putative anti-thrombotic effect of ginger, onions and parsley on haemostatic parameters in humans.

Contents

Chapter 1

Introduction

Background

Coronary heart disease is a major health problem in the Western world (1). In 1994, about 92,000 people were hospitalized because of this disease, and it was the primary cause of more than 20,000 deaths in the Netherlands (2).

Coronary heart disease is caused by the narrowing of coronary arteries by atherosclerotic plaques followed by the formation of a thrombus in an atherosclerotic coronary artery, which may lead to an insufficient blood supply.

Blood platelets play an important role in the development of coronary heart disease: injury of endothelial cells in arteries exposes blood to sub-endothelial collagen and tissue factor. Collagen activates the enzyme cyclo-oxygenase in platelets to produce thromboxane A_2, which may cause platelet aggregation and early thrombosis. Aggregation is enhanced by the release of ADP from damaged platelets and vessel walls. Platelet-derived growth factor stimulates the migration and proliferation of smooth muscle cells and fibroblasts in the inner layer of the artery. Fatty streaks are formed, and these can turn into a complicated plaque. The plaque narrows the artery lumen, but is also prone to rupture. This, again, stimulates platelet aggregation, which can lead to occlusive thrombosis (3-4).

The activity of cyclo-oxygenase may be inhibited by dietary compounds (5), such as dietary non-nutrients, i.e. substances without apparent nutritive value for humans, but with pronounced biologic activity (6). This may lead to a decrease in platelet thromboxane A_2 production and aggregation, and to altered thrombotic tendencies (5). If such anti-thrombotic substances were present in foods, a diet rich in those non-nutrients might be important in coronary heart disease prevention. It has been suggested that dietary acetylsalicylate and flavonoids, as well as non-nutrients present in ginger reduce thrombotic tendencies by inhibiting platelet cyclo-oxygenase activity (7-12).

Acetylsalicylic acid, haemostasis and thrombosis

Acetylsalicylic acid, aspirin, is effective as an anti-thrombotic drug in doses as low as 30 mg/d (13-14). It irreversibly inhibits platelet cyclo-oxygenase activity by acetylation (15-16). This leads to a decreased conversion of arachidonic acid into platelet thromboxane A_2 -which is very unstable and is quickly converted into thromboxane B_2 (the inactive derivate of thromboxane A_2)- and to a decreased platelet aggregation (13-16). If acetylsalicylic acid were present in foods, a diet rich in acetylsalicylic acid might have an anti-thrombotic effect (7-8).

Data on salicylate contents of foods are scarce (17-20). Based on food analyses and urinary excretion data, it was estimated that a normal mixed Western diet provides 10-200 mg/d of total salicylates (17,20). Those high values were, however, questioned by others (18). Qualitative analyses showed the presence of acetylsalicylate in 37 out of 56 foods studied (20), but quantitative analyses were absent. It was estimated that a normal mixed Western diet provides 3 mg/d of acetylsalicylic acid (17,20; Truswell personal communication). It was suggested recently that dietary salicylate intake might have contributed to the decline in cardiovascular disease mortality in the USA (8). To study this hypothesis data on acetylsalicylate contents of foods and data on effects of foods rich in acetylsalicylate on haemostasis in humans are needed.

Flavonoids, haemostasis and thrombosis

Flavonoids are polyphenolic compounds that occur ubiquitously in plant foods. Some subclasses of flavonoids are flavonols and flavones. Dietary intake of the flavonols quercetin, kaempferol and myricetin, and of the flavones luteolin and apigenin in the diet was associated with a reduced risk of coronary heart disease and stroke in some but not all studies. This association may be explained by the inhibition of LDL-oxidation and/or by effects on haemostasis (9-10). Incubations of human platelets or animal cells with isolated flavonoids suggest that flavonoids inhibit platelet aggregation, probably by inhibition of cyclo-oxygenase activity (10,21-22). It was also suggested that flavonoids may affect parameters for coagulation or fibrinolysis (23). However, conflicting observations on the effects of flavonols and flavones on *in vitro* platelet aggregation have been found (21-23). Flavonoid concentrations used in *in vitro* studies range between 10 and 1000 μM (21-22), which is 10-1000 times higher than plasma levels reached after oral intake (24). The effects of physiological flavonoid concentrations on *in vitro* platelet aggregation had not been studied, and human studies on the consumption of dietary flavonoids on platelet aggregation, cyclo-oxygenase activity, and parameters of coagulation and fibrinolysis were absent.

Ginger, haemostasis and thrombosis

It has been suggested that ginger exerts an anti-thrombotic activity by inhibiting platelet aggregation and thromboxane production *in vitro* (12). However, data on the effects of ginger consumption on blood platelet function, especially cyclo-oxygenase activity, are scarce and contradictive (25-26).

Objectives of the project and outline of the thesis

In this thesis we will focus on the content of total salicylates and acetylsalicylate in relevant foods. We will also study the effects of acetylsalicylic acid, flavonoids and ginger on haemostatic parameters as indicators for their anti-thrombotic tendency in healthy volunteers. Our main question is: ***Do dietary non-nutrients affect haemostatic parameters in humans?*** To answer this question we divided it into the following sub-questions.

Acetylsalicylic acid, haemostasis and thrombosis

- What is the effect of 3 mg/d of acetylsalicylic acid (the amount possibly present in a normal mixed Western diet) on platelet cyclo-oxygenase activity as assessed by thromboxane production in healthy volunteers (***Chapter 2***)?
- What are the contents of acetylsalicylate and total salicylates in relevant foods (***Chapter 3***)?
- Is the excretion of salicylates in urine a valid marker for intake of pure salicylates? What amount of salicylates is excreted in 24-h urine of subjects eating a variety of self-selected diets (***Chapter 4***)?
- What is the estimated amount of bio-available salicylates in diets, based on the food analyses and the urinary excretion data? Is this sufficient to prevent coronary heart disease (***Chapter 3-5***)?

Flavonoids, haemostasis and thrombosis

- Does a test tube addition of physiological concentrations of the flavonols quercetin and quercetin-3-glucoside and of the flavone apigenin affect *in vitro* platelet aggregation? Does daily consumption of large amounts of onions (quercetin-rich) or parsley (apigenin-rich) affect platelet aggregation, thromboxane production, and other haemostatic parameters in healthy subjects (***Chapter 6***)?

Ginger, haemostasis and thrombosis

- Does daily consumption of raw and cooked ginger affect platelet cyclo-oxygenase activity as assessed by thromboxane production in healthy subjects (***Chapter 7***)?

The answers to these questions, the conclusions of this thesis and suggestions for future research are described in ***Chapter 8***.

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Chapter 2

Pilot trial of the effects of low-dose acetylsalicylic acid on platelet thromboxane production

Janssen PLTMK, Akkerman JWN, Hollman PCH,
van Staveren WA, Zwaginga JJ, Katan MB.
Pilot trial of the effects of low-dose acetylsalicylic acid
on platelet thromboxane B_2 production.
Eur J Clin Nutr 1995;49:365-70.

Abstract

It has been suggested that certain foods of plant origin contain milligram-quantities of acetylsalicylate which could exert an anti-thrombotic effect. Acetylsalicylic acid prevents cardiovascular diseases through inhibition of platelet endoperoxyde thromboxane production and platelet aggregation. We investigated whether a daily intake of 3 mg acetylsalicylic acid causes a measurable decrease of platelet cyclo-oxygenase activity assessed by *in vitro* thromboxane B_2 production. We carried out a randomized, double-blind, placebo-controlled cross-over study. Ten healthy volunteers (5 men, 5 women) aged 22±3 years (mean±sd) participated in the study; there were no drop-outs. Participants took 3 mg/d of acetylsalicylic acid or a placebo for 2 weeks each. At the end of each treatment period venous blood was drawn, and platelet-rich plasma was stimulated with arachidonic acid. Treatment with acetylsalicylic acid caused a 39±8% decrease in maximal thromboxane B_2 production ($p=0.000$), which was independent of treatment order. Quantitative data on acetylsalicylate in foods and the possible anti-thrombotic action of a diet rich in acetylsalicylate deserves closer investigation.

Introduction

Acetylsalicylic acid is, even in low amounts, effective in the prevention of cardiovascular diseases (1-13). It inhibits platelet endoperoxyde thromboxane A_2 production and platelet aggregation by irreversible acetylation of platelet cyclo-oxygenase which converts arachidonic acid into prostaglandin G_2/H_2, leading to further formation of thromboxane A_2 (14-16). Low dose of acetylsalicylic acid, however, rarely influences the production of endothelial-cell-derived prostacyclin, a vasodilator and inhibitor of aggregation (17-22).

Swain and co-workers suggested that fruits, vegetables, herbs, spices and tea are good sources of salicylates. Qualitative results also hinted at the presence of acetylsalicylate in fruits, vegetables, beverages, herbs and spices (23-25). The content of acetylsalicylate in foods was thought to be 0.1-1.0 mg per 100 g (23,25; Truswell personal communication). However, quantitative data on acetylsalicylate in foods are as yet not available.

In addition to acetylsalicylic acid, flavonoids also inhibit *in vitro* platelet aggregation, but exact mechanisms are poorly understood (26). Intake of certain flavonoids, namely flavonols and flavones, is inversely associated with mortality from coronary heart diseases (27). The average daily intake of flavonols and flavones in the Netherlands is 23 mg, mainly provided by tea, apples, onions and red wine (28).

Data on the anti-thrombotic tendency of a diet rich in acetylsalicylate or flavonoids are as yet unknown. We studied whether a daily dose of 3 mg acetylsalicylic acid -the amount possibly provided by foods rich in acetylsalicylate- causes measurable changes in platelet function in man as assessed by a decrease in thromboxane B_2 production.

Subjects and methods

Subjects

Five men and five women, all students, participated in the study. Initial characteristics (mean ± sd) were as follows: body mass index 21±1 kg/m^2, age 22±3 years, platelet count 255±39 *10^9 platelets/L, and systolic and diastolic blood pressure 116±9 and 66±7 mm Hg, respectively. All participants were healthy, based on a medical questionnaire, with absence of glucose and protein in urine, and with normal blood chemistry

(haematocrit, haemoglobin, mean cell volume, erythrocyte sedimentation, alanine amino transferase, gamma-glutamyl transferase, creatinine, platelet count, thrombin time, prothrombin time, activated partial thromboplastin time). None of the subjects was hypertensive. The participants were all non-smokers and had not used any regular or homoeopathic medication for at least one month before the study. The subjects were asked not to use any medication during the study. Paracetamol was provided to the subjects for emergency pain relief, but none of the subjects made use of it. One person used Fucithalmic during the study; however there is no indication that this medication should have any influence on platelet activity.

The experimental protocol was approved by the Medical Ethics Committee of the Department of Human Nutrition. The protocol was fully explained to the subjects, but they were not told that the design of the study was cross-over, so as to minimize possible bias. All participants gave their written informed consent.

Methods

The study had a double-blind placebo-controlled cross-over design for 2x14 days; there was no wash-out period between the treatments. On day 1 the subjects were randomly assigned to daily treatment with 3 mg acetylsalicylic acid or a placebo. Four subjects started out on acetylsalicylic acid and six on placebo. Subjects swallowed one capsule a day before breakfast. Identical capsules of placebo and acetylsalicylic acid were prepared by the Department of Pharmacy of the Utrecht Academical Hospital.

Subjects were requested not to eat fatty fish (salmon, trout, herring, mackerel), and to maintain their usual physical activity patterns, alcohol consumption and eating habits. Deviations from activity patterns or eating and drinking habits, and consumption of tea, fish, licorice, herbs and spices, alcoholic drinks, honey, onions and garlic were recorded in a diary, as were any signs of illness.

Subjects visited the Department on days 0, 7, 15, 21 and 29 to enhance compliance and check maintenance of activity patterns and eating and drinking habits. On these visits we checked the diary and the body weight, and asked about adverse effects, illness, medication and visits to a dentist or doctor. Returned capsules were counted and a new supply was distributed.

Random codes were assigned to all blood samples. Venous blood was drawn into 3.8% sodium-citrate tubes 1:10 v/v (Sarstedt, Etten-Leur, The Netherlands) on days 11, 14, 25 and 28 at 8.30 a.m. after an overnight fast. Platelet-rich plasma was prepared by centrifugation at room temperature for 10 min at 200 g (Hettich Rotanta, Depex, De Bilt, The Netherlands), removed and stored at room temperature in a capped tube. The residual blood was centrifuged at room temperature for 10 min at 1500 g to prepare platelet-poor plasma. Platelets were counted (Sequoia Turner, Abbot, Santa Clara, USA) and the platelet-rich plasma was diluted by adding autologous platelet-poor plasma to $250*10^9$ platelets/L. The diluted platelet-rich plasma (450 µL) was stimulated in duplicate with arachidonic acid (1.5 mM final concentration) (Bio Data Corporation, Horsham, USA) in an aggregometer at 37 ^{0}C (Bio Data Corporation, Horsham, USA). After 10 minutes 50 µL of the aggregate was added to 950 µL buffer (0.9% NaCl, 0.01 M EDTA, 0.3% bovine gamma-globulin, 0.005% Triton-X-100, and 0.05% sodium-azide in 50 mM phosphate buffer, pH 6.8; NEN Research Products, Du Pont, Boston, USA), the samples were immediately submerged in liquid nitrogen, and stored at -80 ^{0}C until analysis. Thromboxane B_2 was measured in

duplicate (Thromboxane B_2 [^{125}I] RIA kit, NEN Research Products, Du Pont, Boston, USA). Venous blood, taken from 2 healthy volunteers who had not used any medication for a month preceding blood donation, served as control pools. Preparation of the citrate-plasma, aggregations and storage were carried out as described. Within-run variation was 8%.

Data were analyzed using the Statistical Package for Social Sciences (29). Mean values of thromboxane production at d 11 and 14, respectively and d 25 and 28, respectively were calculated. Those mean values were used to evaluate changes in thromboxane B_2 production after treatment with acetylsalicylic acid and placebo using a paired Student's t-test with a probability level of 5% and a power of 90% for normally distributed data; Wilcoxon's Signed Rank Test was used for not normally distributed data. Within-person within-period variation of thromboxane production was calculated. Treatment order effects were checked using analysis of variance (30).

Results

Compliance, expressed by the proportion of capsules not returned, was 100% during placebo treatment, and 99% during acetylsalicylic acid treatment. The diaries did not reveal relevant changes between the treatment and placebo period in physical activity patterns, eating or drinking habits. Body mass index was stable (95%-confidence interval -0.1 to 0.5 kg/m^2) and no adverse reactions were reported.

There was no effect of treatment order (p=0.207). Within-person variation of thromboxane B_2 production between blood samples taken 3 days apart was 8% after placebo and 10% after aspirin treatment. Consumption of 3 mg/d of acetylsalicylic acid for 14 days significantly decreased (p=0.000) platelet thromboxane B_2 production by 783±77 nmol/10^{11} platelets or 39±8% (mean±sd, n=10). Mean thromboxane B_2 production was 2016±299 and 2062±356 nmol/10^{11} platelets after daily treatment with placebo for 11 and 14 days, respectively, and 1216±294 and 1294±326 after daily treatment with 3 mg aspirin for 11 and 14 days, respectively (Fig 1).

Discussion

There is a growing interest in food components that affect cardiovascular risk through mechanisms other than the classical intermediates, blood pressure and lipoproteins. Dietary effects on platelet function are of special interest because acetylsalicylic acid has been convincingly shown to reduce the incidence of cardiovascular diseases (7,12,31). Contents of acetylsalicylate in foods are unknown yet, and need further investigation.

As far as we are aware the only previous studies which used a daily dose of acetylsalicylic acid in amounts of 3 mg or less were uncontrolled trials (21,32-34). We now found that 3 mg acetylsalicylic acid daily reduced *in vitro* platelet thromboxane B_2 production by almost 40%. By applying careful standardization we were able to reduce the combined analytical and biological within-subject variation to 8% after placebo treatment and 10% after aspirin treatment. Using our methodology we may therefore be able to detect even a 10% reduction in platelet thromboxane B_2 production at p=0.05 with a power of 90%, corresponding to the effect of less than 1 mg acetylsalicylic acid daily (21-22).

According to Swain, fruits, vegetables, herbs, spices, honey, and tea are good sources of salicylate and a normal mixed daily diet provides 10-200 mg of total salicylates.

Qualitative results also hinted at the presence of acetylsalicylate in fruits, vegetables, beverages, herbs, and spices (23,25), but quantitative data are absent. Our data suggest that if the content of acetylsalicylic acid in foods were as high as can be inferred from the results of Swain *et al.* (23,25), a normal mixed daily diet might have measurable effects on platelet function in man. Studies on the possible anti-thrombotic action of a diet rich in acetylsalicylate or flavonoids deserve closer investigation.

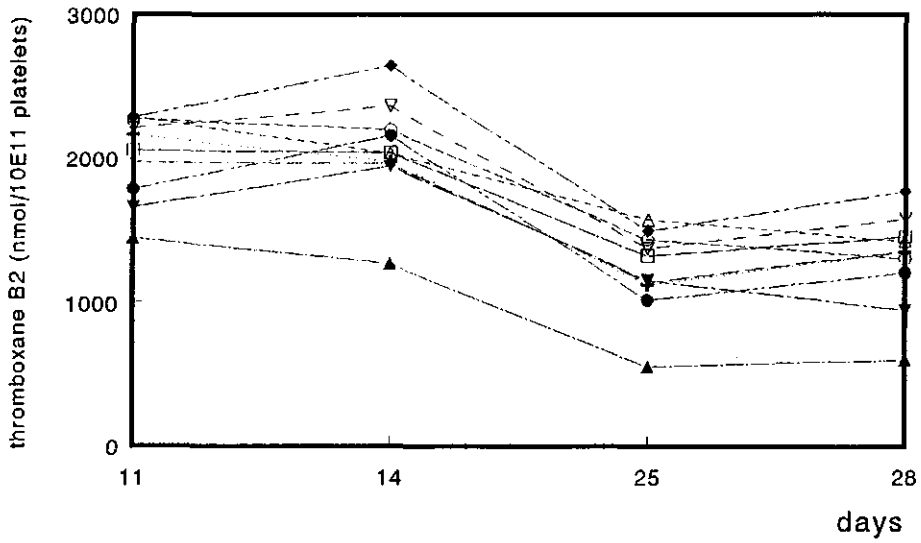

Fig 1: Maximally stimulated thromboxane B_2 production (nmol/10^{11} platelets) in 10 healthy volunteers treated with placebo (d 11 and 14) or 3 mg/d of acetylsalicylic acid (d 25 and 28) daily, for 2 wks each. Treatments were given in random order in a double-blind cross-over trial. Thus 4 out of the 10 subjects actually received aspirin on d 1 to 15, and 6 out of the 10 subjects received placebo on d 1 to 15.

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Chapter 3

Salicylates in foods

Venema DP, Hollman PCH, Janssen PLTMK, Katan MB.
Determination of acetylsalicylic acid and salicylic acid in foods, using HPLC with fluorescence detection.
J Agric Food Chem 1996;44:1762-7.

and

Janssen PLTMK, Katan MB, Hollman PCH, Venema DP.
No aspirin in red wine.
Lancet 1994;344:762.

3.1 Determination of acetylsalicylic acid and salicylic acid in foods, using HPLC with fluorescence detection

Abstract

We developed a specific and sensitive HPLC method with fluorescence detection for the determination of free acetylsalicylic acid, free salicylic acid, and free salicylic acid plus salicylic acid after alkaline hydrolysis (free-plus-bound) in foods. Acetylsalicylic acid was detected after post-column hydrolysis to salicylic acid. With the method for free acetylsalicylic acid and salicylic acid recovery was 95-98% for acetylsalicylic acid added to foods, and 92-102% for salicylic acid. Recovery of added salicylic acid was 79-94% for the free-plus-bound salicylic acid method. The limit of detection was 0.02 mg/kg for fresh and 0.2 mg/kg for dried foods for all substances. We did not find acetylsalicylic acid in any of 30 foods previously thought to be high in salicylates. The contents of free-plus-bound salicylic acid and of free salicylic acid ranged from 0 to 1 mg/kg in vegetables and fruits and from 3 to 28 mg/kg in herbs and spices. Thus the tested foods did not contain acetylsalicylic acid and only small amounts of salicylic acid. Our data suggest that the average daily intake of acetylsalicylic acid from foods is nil and that of salicylic acid is 0-5 mg/day.

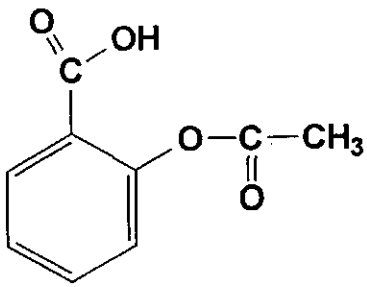
Introduction

Acetylsalicylic acid or 2-acetoxybenzoic acid (Fig 1) are the formal names of a drug commonly known as aspirin. Aspirin is effective as a prophylactic against coronary heart disease in doses as low as 30 mg/day (1-4). Acetylsalicylic acid may also prevent colon cancer (5) and pregnancy induced pre-eclampsia (6). If acetylsalicylic acid were present in foods, a diet rich in acetylsalicylic acid might have an anti-thrombotic effect. Not much is known about the presence of acetylsalicylic acid in foods. Swain (7) suggested that acetylsalicylic acid was present in 37 out of 56 foods studied. However, only qualitative data were given, obtained with thin layer chromatography.

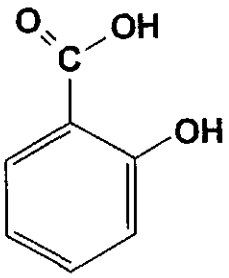
Feingold (8) suggested that the Kaiser-Permanente diet, which eliminates all artificial food colours and flavours, as well as foods containing natural salicylates, improves behavioral disturbance of children. Although this could not be substantiated in properly controlled trials, belief in the efficacy of dietary treatment with salicylate-free diets is firmly held (9).

Hypersensitivity is one of the reported adverse reactions of medication containing salicylates, but improvement by a salicylate-free diet is highly unlikely (10). In spite of this, there is still much interest in salicylate contents of foods (11).

The number of publications regarding the content of salicylates in foodstuffs is limited and contradictive (12-14). Previous food analyses were performed using HPLC with UV detection at 235-245 nm, which is prone to interferences. We therefore adapted an HPLC method with fluorescence detection (15) for food analysis and measured free acetylsalicylic acid, free salicylic acid and free-plus-bound salicylic acid, i.e. free salicylic acid plus salicylic acid after alkaline hydrolysis, in 30 common foods previously reported to contain salicylates.



ACETYLSALICYLIC ACID



SALICYLIC ACID

Figure 1: Structure of acetylsalicylic acid and salicylic acid.

Materials and methods

Foods and sample preparation

We selected foods that were earlier reported to have a high content of salicylic acid and/or are known to be consumed in large amounts.

We purchased 3 samples of 500 g of each type of fresh fruit or vegetable at different local supermarkets in the summer of 1994. Fresh foods were processed within 24 h, non-edible parts were removed and the 3 samples were combined per product to a composite sample by mixing equal amounts. One packing of each of 3 different brands of processed foods was purchased, again in local supermarkets in the summer of 1994, and combined to a composite sample.

Fresh and canned foods were chopped under liquid nitrogen immediately after cleaning. The frozen samples were ground using a food processor, and extracted immediately. None of the samples were freeze-dried because of the risk of sublimation of the salicylic acid. Dried foods were ground without pretreatment.

We prepared tea from 1500 mL of boiling water and 3 tea bags of different brands each containing 4 g of tea. Tea bags were removed after 5 min, and the liquid was allowed to cool. We prepared coffee by pouring 1 L of boiling water on 50 g of a mixture of 3 brands of ground coffee contained in a paper filter. The liquid was allowed to cool.

Extraction and hydrolysis

Samples were protected from direct daylight during the entire extraction procedure (Fig 2). We weighed out 10.00 g of the freshly ground fresh foods or 1.00 g of the dried foods and added 9 mL of water to the dried foods.

Determination of free acetylsalicylic acid and salicylic acid

Samples were homogenized with 20 mL of acetonitrile/water/acetic acid (25/75/5) for 30 s in a Waring Blender at high speed and sonicated for 5 min.

Free-plus-bound salicylic acid

We essentially followed the method of Swain (7): food samples were mixed with 10 mL of NaOH (250 g/L) for 1 h on a rotary-shaker (New Brunswick Sc.), at 250 rpm. Extracts were left to stand overnight at room temperature, shaken for 1 h, acidified to pH 1-2 with 10 M HCl, and transferred quantitatively with water into a liquid/liquid extractor (Fig 3). The extractor was placed onto a heated flask, and diethyl ether was added in the extractor until about 50 mL flowed over into the heated flask. We extracted the samples on 2 successive days for 6 h each day. Diethyl ether was evaporated carefully until almost dry, and the last bit of ether was evaporated at room temperature, to avoid sublimation of salicylic acid. The residue was taken up in 25 mL of acetonitrile/water/acetic acid (25/75/5) and sonicated for 5 min.

We filtered approximately 2 mL of each sample extract through a 0.45 µm filter for organic solvents (Acrodisc CR) prior to injection into the HPLC system.

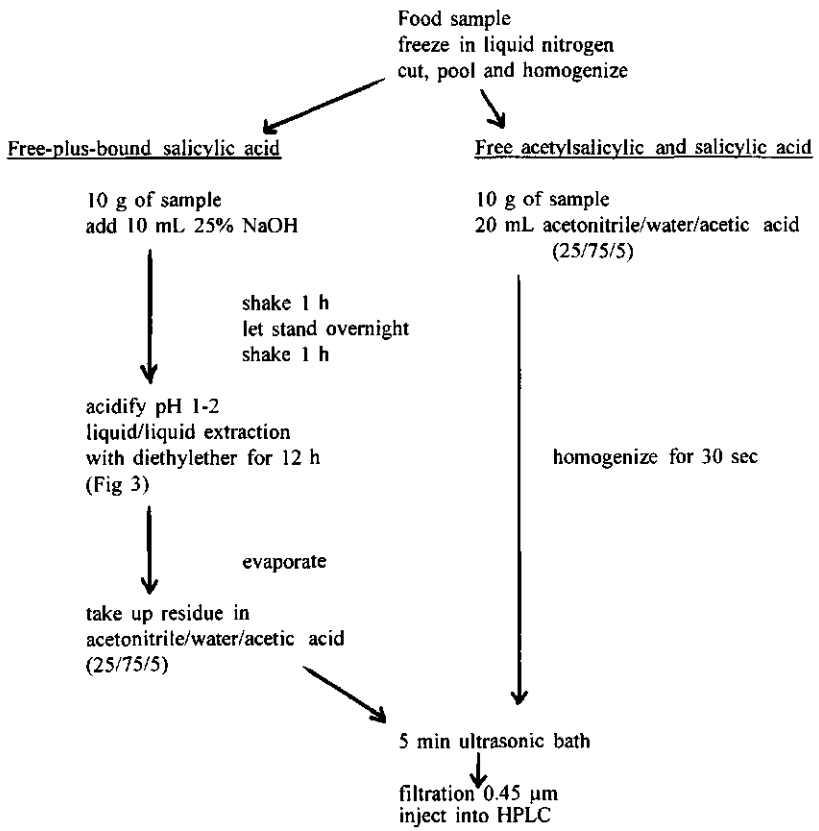


Figure 2: Methods for extraction of free-plus-bound salicylic acid and of free acetylsalicylic and salicylic acid.

Standards

Acetylsalicylic acid (Sigma A-5376) and salicylic acid (Sigma S-0875), were dissolved in acetonitrile/acetic acid (99/1) to a concentration of 1 g/L. Acetylsalicylic acid standards were made daily and salicylic acid standards once a week. Further dilutions were made daily in acetonitrile/water/acetic acid (25/75/5) which is similar to the eluent, thus preventing injection problems. Calibration curves were constructed in the range of 2-20 μg/L for acetylsalicylic acid and 10-300 μg/L for salicylic acid. We identified the peaks by comparing retention times of samples with those of standards. Occasionally, peaks were confirmed by addition of standards to the sample extracts.

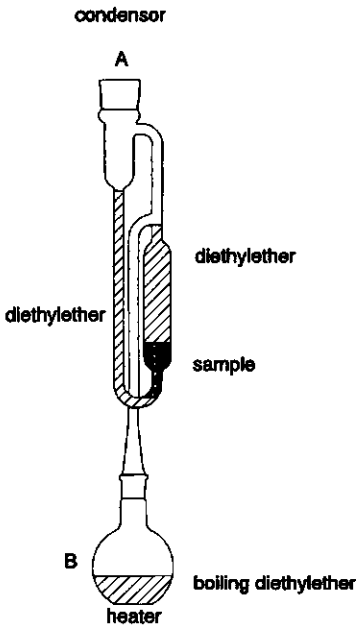


Figure 3: Liquid/liquid extraction apparatus for the continuous water/diethyl ether extraction of salicylic acid from hydrolysed food samples. Acidified liquid sample after alkaline hydrolysis is introduced into A. Diethyl ether is added in A until flask B contains 50 mL. Heating is started after connecting the extractor with the condenser.

Chromatography

The HPLC system consisted of a Perkin-Elmer ISS-100 automatic injector, a Gynkotek 480 pump, a Lichrospher RP-18 (Merck) column (4 x 250 mm, 5 µm) protected by a Perisorb RP-18 (3.9 x 40 mm, 30-40 µm) precolumn both placed in a column oven set at 30°C, a fluorescence detector (Merck Hitachi F-1050) with excitation wavelength at 300 nm, and emission wavelength at 400 nm, and a post-column stainless steel reaction coil (0.5 x 5000 mm) immersed in a glycerol-filled bath set at 60°C. Acetylsalicylic acid and

salicylic acid were separated with an eluent consisting of methanol/water/phosphoric acid 85% (40/60/0.2), at a flow rate of 0.9 mL/min. Acetylsalicylic acid was hydrolysed to salicylic acid in the post-column reactor by addition of 0.15 mL/min of 1 M NaOH to the eluent using a Gilson minipuls-3 pump.

Results

The salicylates in foods are partly bound as esters or glycosides (16). We used alkaline hydrolysis plus ether extraction to liberate matrix-bound salicylates and to convert salicylate esters and glycosides to their parent salicylic acid. Values thus obtained were named "free-plus-bound salicylic acid". Acetylsalicylic acid is unstable in alkali. We therefore used a gentle extraction to detect acetylsalicylic acid; this technique also detects the extractable or "free" salicylic acid. Contents were measured with an optimized HPLC method with fluorescence detection.

Optimization of column and eluent

We tested a Novapak RP-18, 150 x 3.9 mm, 4 µm column (Waters Associates, Milford MA), a Lichrospher RP-18, 250 x 4 mm, 5 µm column (Merck), and a Inertsil ODS 2, 150 x 4.6 mm, 5 µm column (GL Sciences Inc.) with UV detection at 245 nm. Dissociation of salicylic acid (pK_a=3.0) is an important variable in its chromatographic behaviour. We therefore tested acetic acid and phosphoric acid as eluent acidifiers, taking care that the pH of the eluent remained between 2.2 and 2.4. Methanol/water and acetonitrile/water both with acetic acid or phosphoric acid were used.

The Inertsil column showed low plate numbers for both acetylsalicylic acid (N=3,200) and salicylic acid (N=5,700) with acetonitrile/water/phosphoric acid. Therefore it was not tested with any of the other eluents. Acetylsalicylic acid showed symmetric peaks on the Novapak and the Lichrospher column with all eluents, but salicylic acid showed variable degrees of peak tailing. The Lichrospher column was superior for both acetylsalicylic acid (N=11,200) and salicylic acid (N=11,500) with all mobile phases. Phosphoric acid was more compatible with the post-column reaction than acetic acid, because a lower amount of sodium hydroxide was required to neutralise it. We therefore chose methanol/water/phosphoric acid as a mobile phase and the Lichrospher as a column (Fig 4a).

Post-column hydrolysis

Acetylsalicylic acid can be made fluorescent by conversion to salicylic acid. Post-column hydrolysis conditions were essentially as described by Siebert and Bochner (15), but the use of 0.2% instead of 0.1% of phosphoric acid in the eluent and 1 M instead of 0.5 M NaOH resulted in somewhat better plate numbers.

Acetylsalicylic acid hydrolysis was complete at 60 ^{0}C using a coil of 0.5mm x 5m and addition of 0.15 mL/min of 1 M NaOH. Salicylic acid proved to be stable under these conditions. Excitation was at 300 nm and emission at 400 nm. The fluorescence of salicylic acid was about 8 times higher at alkaline than at acid pH. Phenolic compounds that possibly interfere in UV detection, such as cinnamic and hydroxybenzoic acids, showed little fluorescence under these conditions. The sensitivity of the fluorescence detection of salicylic acid was about a factor 1000 higher than that by ultraviolet light absorption at 235-245 nm.

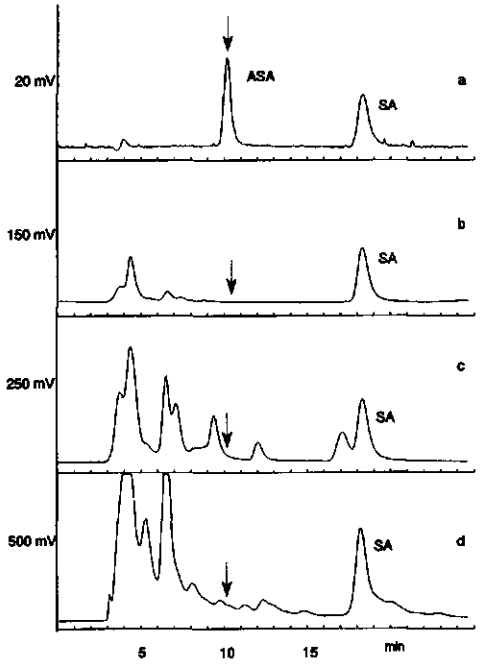


Figure 4: Typical chromatograms of acetylsalicylic acid and salicylic acid in foods.
Peaks: ASA, acetylsalicylic acid; SA, salicylic acid. Arrows indicate retention time of acetylsalicylic acid: a=standards, b=tea extract, c=wine, d=thyme. Detection: fluorescence after post-column hydrolysis of acetylsalicylic acid; excitation, 300 nm; emission, 400 nm; flow rate: eluent, 0.9 mL/min; sodium hydroxide 1 M, 0.15 mL/min.

Stability of acetylsalicylic acid and salicylic acid

The stability of acetylsalicylic acid in various extraction solvents was tested by comparing UV spectra of the solutions. The UV absorbance maximum in eluent of salicylic acid is 303 nm and that of acetylsalicylic acid is 275 nm. Acetylsalicylic acid hydrolysed rapidly to salicylic acid when dissolved in methanol. The stability was better in the presence of acetic acid plus methanol or acetonitrile (Table 1).

Salicylic acid was stable for more than 1 week in all solvents if kept in the dark, as judged by its unchanged UV-spectrum. We never observed any degradation of acetylsalicylic acid or salicylic acid in the HPLC elution pattern.

Sample extraction and hydrolysis

Free acetylsalicylic acid and salicylic acid

Acetylsalicylic acid added as a solution in acetonitrile to samples showed a recovery ranging from 95-98% (Table 2) and for added free salicylic acid from 92-102% (Table 3). Recoveries did not change after extracts had been stored for 24 h.

Table 1: Stability of acetylsalicylic acid in various solvents.[a]

solvent	time	loss (%)
methanol	4 h	7
methanol	1 d	22
methanol/1% acetic acid	5 d	2
acetonitrile/1% acetic acid	5 d	3
acetonitrile/water/acetic acid (25/75/5)	5 d	2
acetonitrile/water/H_3PO_4 (25/75/0.2)	5 d	3

[a] **All determinations are based on single sample analysis; concentrations during storage were 1 mg/mL, and during measurement 20 µg/mL, except for acetylsalicylic acid in water where we used a saturated solution.**

Table 2: Recovery of acetylsalicylic acid when added to various foods.[a]

	acetylsalicylic acid		
product	amount in food (mg/kg)	amount added (mg/kg)	recovery (%)
grape	<0.02	0.075	98
tomato	<0.02	0.077	98
thyme	<0.02	0.150	95

[a] **Means of duplicate analyses, except for thyme which was analyzed in quadruplicate.**

Free-plus-bound salicylic acid

We extended the ether extraction as used by Swain (14) to 12 h, because extraction was not complete after 5 h (Table 4). This was probably due to a less efficient construction of our extraction apparatus (Fig 3). After the first 12 h of extraction, a new flask with 50 mL of ether was placed on the extraction apparatus and the ether was refluxed for an additional 3 h. This extension of the extraction period added little to the salicylic acid yield: the gain was 0% for wine and 5% for thyme.

In the free-plus-bound method acetylsalicylic acid was completely hydrolysed to salicylic acid. Methylsalicylate, a flavourant, was also hydrolysed completely.

The mean free-plus-bound salicylic acid content of a sample of freeze-dried endives analyzed on 6 different days was 0.132 mg/kg; the coefficient of variation between days was 8%. Recovery of salicylic acid added as a solution in acetonitrile to samples in the free-plus-bound method ranged from 79-94% (Table 2).

Determination in foods

No acetylsalicylic acid was detected in any of the food samples (Table 5). Salicylic acid was frequently detected in vegetables and fruits though in concentrations of only 0-1 mg/kg. Some herbs and spices like thyme, rosemary and cinnamon contained amounts up to 28 mg/kg. The poor duplicates of the tomato sample (Table 4) may be due to the fact that the sample was not homogeneous; pits and peel were very difficult to grind.

Figure 4 shows some typical chromatograms of the separation of salicylic acid and acetylsalicylic acid in various foods.

Table 3: Recovery of salicylic acid when added to various foods.[a]

product	amount of free-plus-bound salicylic acid in food (mg/kg)	amount of salicylic acid added (mg/kg)	recovery (%)	
			free salicylic acid method	free-plus-bound salicylic acid method
brewed tea	0.500	0.400	99	88
grape	0.032	0.075	102	90
tomato	0.225	0.200	102	79
thyme	16.1	10.0	92	94

[a] **Means of duplicate analyses, except for thyme which was analyzed in quadruplicate; for methods see text.**

Table 4: Influence of extraction time on the amounts of salicylic acid extracted from different foods by the free and free-plus-bound methods.[a]

product	free salicylic acid (mg/kg)	free-plus-bound salicylic acid (mg/kg)	
		5 h	12 h[b]
grape	<0.02	0.020	0.028
tomato	0.05	0.236	0.276[c]
thyme	no determination	12.05	15.85

[a] **Means of duplicate determinations.**
[b] **After 5 h new solvent in heated flask; cf Materials and Methods.**
[c] **Poor duplicate, sample possibly not homogeneous.**

Table 5: Content of free acetylsalicylic acid and salicylic acid and of free-plus-bound salicylic acid in various foodstuffs.[a]

product	content in edible part of food (mg/kg)					
	this study			published values for salicylic acid		
	free acetylsalicylic acid	free salicylic acid	free-plus-bound salicylic acid	Swain (1985; ref 14)	Herrmann (1990, ref 16)	Robertson (1981; ref 13)
Vegetables and fruits:						
apples with peel	<0.02[b]	<0.02[b]	<0.02[b]	0.8-5.9 av 4	<1	0.04
blueberry jam	<0.02	0.55	0.67			
canned apricot	<0.02	0.13	0.1	14.2	<1	0.03
canned cherries	<0.02	0.27	0.36	3-27.8	<1-2	
cucumber with peel	<0.02	0.02	0.077	7.8[c]	<1-3	
grape	<0.02	<0.02	0.03	0.94-1.88		0.04
nectarine	<0.02	0.03	0.87	4.9		0.04
orange	<0.02	<0.02	<0.02	23.9	<1	0.07
strawberry	<0.02	0.03	0.65	13.6	0-2	0.04
tomato with peel	<0.02	0.11	0.36	1.3	<1-1	0.05
Herbs and spices:						
cinnamon	<0.2	6.6	23.8	152	10	
mild curry powder	<0.2	3.69	5.55	2180		
French mustard	<0.2	0.2	0.48	260[d]	19-39	
oregano	<0.2	11.8	19.9	660		
hot paprika powder	<0.2	1.73	2.98	2030		
black pepper	<0.2	1.18	3.05	62	3	
rosemary	<0.2	6.76	28.4	680	21	
thyme	<0.2	11.95	12.8[e]	1830	11	

product	content in edible part of food (mg/kg)					
	this study			published values for salicylic acid		
	free acetylsalicylic acid	free salicylic acid	free-plus-bound salicylic acid	Swain (1985; ref 14)	Herrmann (1990, ref 16)	Robertson (1981; ref 13)
Beverages:						
pilsner beer	<0.02	<0.02	<0.02	3[f]		
brewed coffee	<0.02	0.24	0.37	0-9[d]		
brewed tea	<0.02	0.33	0.42	30[g]		
red French Bordeaux wine	<0.02	0.65	0.71	9		
red Spanish Rioja wine	<0.02	0.68	0.70			
red Italian Chianti wine	<0.02	0.32	0.32			
red Californian wine	<0.02	0.26	0.28			
Miscellaneous:						
grape currant	<0.2	0.32	0.41	58		
honey	<0.2	0.59	0.66	25-112 av 63		
licorice	<0.2	1.25	1.62[e]	79.6-97.8		
peppermint	<0.2	<0.2	0.07	7.7-75.8 av 27.8		
grape raisins	<0.2	0.60	0.98	66.2-78		
tomato paste	<0.02	0.48	0.75	4.3-14.4 av 8.1		0.07

[a] All analyses were done in simplo, except when the content was >1.0 mg/kg for high-consumption foods or 10 mg/kg for low-consumption foods; samples were then reanalysed in duplicate.
[b] Detection limit. [c] Without peel. [d] Powder. [e] Mean of three analyses. [f] Not pilsner. [g] Bag.

Discussion

We found that foods previously reported to be important sources of salicylic acid and potential sources of acetylsalicylic acid (7,14), contained low amounts of salicylic acid and no acetylsalicylic acid. We used a sensitive and specific HPLC method with post-column hydrolysis of acetylsalicylic acid and fluorescence detection optimized for the determination in foodstuffs. For the acetylsalicylic acid content in foods, we used a fast extraction into a solvent in which acetylsalicylic acid was stable. The solubility of acetylsalicylic acid in the solvent was sufficient, so that any free acetylsalicylic acid present in the food sample would be extracted.

None of the 30 products tested by us contained measurable amounts of acetylsalicylic acid. Swain (7) tested 56 foods with a qualitative thin layer chromatographic method and found acetylsalicylic acid in 14 and both acetylsalicylic acid and salicylate in 23. The identity of the acetylsalicylate spot on the thin layer plate was confirmed by co-elution of a standard and by the slow hydrolysis to salicylic acid that occurred on the thin layer plate. Swain extracted acetylsalicylic acid by alkaline extraction for 2 h (17). In our hands all acetylsalicylic acid was hydrolysed completely to salicylic acid under those conditions.

Swain *et al* (14) found salicylic acid contents ranging from 0 to 60 mg/kg in vegetables and fruits and from 0 to 2180 mg/kg in condiments. Other investigators found much lower contents: Robertson and Kermode (13) found salicylic acid in the range of 0.01-0.82 mg/kg in vegetables and fruits, and Herrmann (16), using a less sensitive method, found only traces of salicylic acid in vegetables and fruits. Herrmann (16) also determined the presence of salicylates in herbs and spices and found contents of 0-81 mg/kg (Table 5) and questioned the high values found by Swain (7,14). Differences might arise by differences in origin, processing or storage. Therefore, we measured salicylates in composite samples of 3 different origins or brands. Swain did find a high natural variation amounting to 10-fold differences. However, all our results are much lower than the lowest value given by Swain. This excludes natural variation as a possible explanation for these discrepancies. Discrepancies in salicylate contents between extraction methods may arise through differences in the extent of liberation of matrix-bound salicylates. However, though we used essentially the same extraction method as Swain we found much lower contents. Possibly the HPLC separation and UV detection of Swain was not specific enough and some other component co-eluted at the same time as salicylic acid.

Muller and Fugelsang (18) published salicylic acid contents in red wine of 11-21.5 mg/L. Again we believe that these values are too high (19). In 4 wine samples from different countries we found 0.26-0.71 mg/kg. This agrees with data of Robertson (20) of 0.04 mg/kg in grapes and 0.08 mg/kg in fermented juice.

Little free salicylic acid is present in fresh products because salicylate is bound as ester or as glycoside (12). In most of the processed products salicylic acid apparently was liberated by food processing.

The difference in the amounts of free salicylic acid and free-plus-bound salicylic acid for peppermint (Table 5) is probably due to methylsalicylate used as a flavourant.

Some herbs and spices contain rather high amounts of salicylic acid, but their consumption is low. Using the food intake data of the Dutch Food Consumption Survey (21) and our data of free-plus-bound salicylic acid, we estimate the intake of salicylates in the Netherlands 0-5 mg/d.

Our data suggest that the acetylsalicylic acid and the salicylic acid content in a normal mixed daily diet may be too low to produce measurable physiological effects *in vivo*.

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3.2 No aspirin in red wine

Muller and Fugelsang (1) suggest that wine is a good source of salicylic acid and that this may explain a preventive effect in cardiovascular diseases. We measured salicylic and acetylsalicylic acid in red Bordeaux wines (Rineau 1993, Lavergne 1993, and Mondetour 1992) using HPLC and a highly specific fluorescence detection method. The method was validated by varying extraction conditions, mainly extraction solvents and extraction time. In red wine we found 0.7 mg salicylic acid per litre and no acetylsalicylic acid (detection limit 0.025 mg/L). Thus, by contrast with Muller and Fugelsang (1) we found negligible amounts of (acetyl)salicylic acid in wine. Even if wine did contain larger amounts of salicylic acid this would not be expected to affect cardiovascular risk. Salicylic acid and dihydroxybenzoic acids do not affect thromboxane formation and platelet aggregation; this specifically requires acetylsalicylate (aspirin), which inactivates cyclo-oxygenase by irreversible acetylation (2).

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Chapter 4

Urinary salicylate excretion in subjects eating a variety of diets shows that amounts of bio-available salicylates in foods are low

Janssen PLTMK, Hollman PCH, Reichman E, Venema DP, van Staveren WA, Katan MB.
Urinary salicylate excretion in subjects eating a variety of diets shows that amounts of bio-available salicylates in foods are low.
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Abstract

Intake of acetylsalicylic acid reduces the risk of cardiovascular disease, and is associated with a decreased risk for colorectal cancer. Amounts of salicylates in foods are thus of interest, but data are scarce and controversial. We gave 58 μmol (10.5 mg) pure acetylsalicylic acid or 66 μmol (9.1 mg) salicylic acid to 6 volunteers and recovered 77-80% in 24-h urine samples. Thus, urinary excretion is a valid indicator for intake of free forms of (acetyl)salicylic acid. In order to estimate the bio-available salicylate contents of diets, we subsequently studied salicylate excretion in 17 volunteers from 14 countries and 4 continents who ate a wide variety of self-selected diets. Median 24-h urinary salicylate excretion was 10 μmol (range 6-12). Values increased with the fibre content of the diet ($r=0.73$), suggesting that vegetable foods are the main sources of salicylates. However, amounts of salicylates in a variety of diets are evidently low and probably insufficient to affect disease risk.

Introduction

Acetylsalicylic acid, aspirin, has a variety of biological effects. It is effective in the prevention of cardiovascular disease in doses as low as 30 mg/d (1-3). Intake has also been associated with a decreased risk for colorectal cancer (4-5). Feingold (6) suggested that the intake of dietary salicylates causes hyperactivity in children, but this could not be substantiated in properly controlled trials (7-8).

Plants contain natural salicylates, but data on the salicylate contents of foods are scarce and controversial (9-15; AR Swain, RH Lobley, AS Truswell, unpublished observations, 1985). Swain *et al* (13-14) suggested that a normal mixed diet provides 72-1448 μmol/d (10-200 mg/d) of total salicylates, and significant but unknown amounts of acetylsalicylate. In contrast, we (15-16) found total salicylate contents of only 0-0.7 μmol/100 g (0-0.1 mg/100 g) in vegetables and fruits, and 2-20 μmol/100 g (0.3-2.8 mg/100 g) in herbs and spices. We could not confirm the presence of acetylsalicylate in any of the 30 Dutch products studied; the limit of detection was 0.1 μmol/kg (0.02 mg/kg) for fresh, and 1.4 μmol/kg (0.2 mg/kg) for dry products. Uncertainties about concentrations of salicylates in the diet may arise because of a limited selection of foods or differences in analytical techniques; for instance liberation of salicylates from a plant matrix is notoriously difficult. Acetylsalicylate is excreted in urine mainly as various salicylates (17-18). Swain *et al* (13; AR Swain, RH Lobley, AS Truswell, unpublished observations, 1985) showed that this is also the case with other salicylates in foods.

We therefore assessed the validity of using excretion of salicylates in urine as a marker for salicylate intake. We then investigated the urinary excretion of salicylates in subjects with a wide range of dietary habits to estimate the bio-available salicylate contents of human diets.

Subjects and methods

Subjects

Six Dutch women working at our university participated in a preliminary study to check the validity of urinary salicylates as a marker of intake. These control subjects were aged 27±4 y and ate a normal mixed Western diet.

We next recruited volunteers from the town and surroundings of Wageningen. We sought nonresidents from foreign countries and Dutch subjects eating nontraditional diets to maximize the chances of encountering a wide range of salicylate intakes. All applicants had to speak English or Dutch. Twenty-nine volunteers responded to posters in university buildings, apartments, shops, ethnic restaurants, and community centres for foreigners. We excluded one applicant because she was pregnant, five because their dietary habits deviated too much from their native habits, one because she used medication, and another five at random. Six men and 11 women aged 29±7 y (mean±sd) with a mean body mass index (in kg/m^2) of 21±3 participated in the study. They habitually ate a wide variety of diets (Table 1). Thirteen subjects came from abroad and most of them were temporarily living in the Netherlands; four were Dutch.

All subjects were healthy on the basis of a medical questionnaire and negative checks for urinary protein and glucose. They did not smoke and had not used any regular or homoeopathic medication or dietary supplements for at least 1 mo before the study, except for 7 women who took oral contraceptives. We instructed subjects to use no dietary supplements or medication during the study; only the use of oral contraceptives was permitted. We supplied them with Naproxen (naproxenum; Pharmachemie BV, Haarlem, The Netherlands), which is a salicylate-free pain medication.

The protocol was approved by the Medical Ethics Committee of the Wageningen Agricultural University Department of Human Nutrition. We explained the protocol fully to the subjects who gave their written informed consent.

Methods

The 6 control subjects of the validation study participated in a randomized cross-over study for 3 d separated by washout periods of 14 d. They swallowed an indistinguishable capsule containing pure salicylic acid or acetylsalicylic acid, or nothing as a placebo together with a *para*-aminobenzoic acid capsule. On analysis, capsules contained 58±1 μmol (10.5±0.2 mg; n=3) acetylsalicylic acid, 66±2 μmol (9.1±0.2 mg; n=3) salicylic acid, or 78.2±0.6 mg *para*-aminobenzoic acid (n=3). The 17 subjects with unusual diets participated in the study for 1 d.

We visited the 6 subjects participating in the validation study and the 17 subjects with the different diets at their homes and explained the protocol. We provided them with bottles to sample urine and herbs and spices that they used in cooking. We also gave them a box containing dry ice, a household scale, a diary, and 3 capsules containing *para*-aminobenzoic acid which served to check the completeness of the 24-h urine samples (19).

The day after this visit participants swallowed the first capsule of *para*-aminobenzoic acid just before breakfast, the second before lunch, and the third before dinner. They collected urine for 24 h from the time they swallowed the first capsule. The volunteers collected each urine sample in a separate bottle containing 0.225 g of thimerosal (T5125; Sigma, Axel, The Netherlands) as a preservative. They wrote the times of urine collections on each bottle, and put filled bottles on dry ice immediately. The 6 subjects of the validation study and the 17 subjects eating their specific diets maintained their usual eating and drinking habits during the study day.

All subjects weighed and recorded in a diary all foods and drinks they consumed during the days of urine collection. They saved samples of all herbs and spices in amounts equal to those that they had used that day. They put dried herbs and spices in plastic bottles

and fresh ones on dry ice. Subjects recorded in the diary any signs of illness, medications used, and times at which they had swallowed the capsules.

A trained dietician (ER) checked the food records at the subjects' homes on day 2. She asked about possible deviations from dietary habits, adverse effects, illness, medications and visits to a dentist or doctor, and collected the urine samples and herbs and spices. We assigned random codes to the urine samples and stored them at -20 ^{0}C. We weighed the samples of herbs and spices.

We weighed the thawed 24-h urine samples and pooled them per subject. We took 10 mL aliquots of the 24-h pools, and stored the samples at -20 ^{0}C until analyzed.

We determined concentrations of total salicylates in the 24-h pools after hydrolysis with 5 M HCl for 2 h at 120 ^{0}C according to Swain (13). The residue of the ether-extract was taken up in 1.5 mL acetonitrile:water:acetic acid (25:75:5, by vol) and filtered through a 0.45 μm filter for organic solvents (Acrodisc CR PTFE; Gelman Sciences, Ann Arbor, MI). We injected 10 μL onto a Lichrospher 100 RP18 (Merck, Darmstadt, Germany) column (4.6x250 mm, 5-μm particle size) by using methanol:water:phosphoric acid 85% (40:60:0.2, by vol) as the mobile phase at a flow rate of 0.9 mL/min. The eluent was mixed with 0.15 mL 1 mol NaOH/L per min in a post-column stainless steel reaction coil (0.5 mm x 5 m) placed in a waterbath at 60 ^{0}C. Fluorescence was measured at 400 nm with a Merck Hitachi F-1050 (Tokyo) fluorescence detector with the excitation wavelength set at 300 nm. A urine control sample was included in each series of analyses; the relative SD of the between-run variation was 9%, and of the within-run variation 2%. The limit of detection was 0.1 μmol/kg (0.02 mg/kg) urine.

We measured *para*-aminobenzoic acid photometrically using fluorescamine (F9015; Sigma, Axel, The Netherlands) after hydrolysis of urine samples with 0.7 M HCl for 40 min at 100 ^{0}C (20).

Recoveries of salicylates in the validation study with salicylic acid and acetylsalicylic acid supplementation were corrected for salicylate excretion with placebo.

We calculated the intake of energy and nutrients of the 17 subjects eating unusual diets using the Netherlands Nutrient Data Bank NEVO (21), and calculated the daily intake of herbs and spices using the weights of the duplicate portions. We did not calculate the food intake of the 6 participants in the validation study.

To estimate the range of salicylate intake we calculated the intake of dietary salicylate of 2 subjects having the highest (nos. 6 and 14; Table 1) and 2 having the lowest (nos. 10 and 16; Table 1) excretion of salicylates using their food records and salicylate concentrations of foods as determined by Swain *et al* (13-14), Robertson and Kermode (11), Herrmann (10), and Janssen *et al* (15), and Venema *et al* (16).

Results

Two subjects (nos. 1 and 7; Table 1) were unwilling to take *para*-aminobenzoic acid; the other 15 and the 6 subjects of the validation study stated that they had swallowed all capsules of *para*-aminobenzoic acid. Mean recovery of *para*-aminobenzoic acid in the 24-h urine pools was 80±14% (mean±sd) in the 15 diet study subjects and 86±10% in the 6 validation study subjects.

Table 1: Origin and diet of 17 subjects eating a variety of self-selected diets who participated in the study of urinary salicylate excretion.

subject	country of origin	diet		
		Type of diet	typical foods consumed	foods excluded
1	United States	Macrobiotic	Whole grains, vegetables, fruit, sea weeds, soy products	Meat, dairy products, eggs, coffee, black tea
2	China	Chinese	Meat, plant products, spices	Alcoholic beverages
3	Czechoslovakia	East European	Meat, potatoes, vegetables, tea, bread	
4	Ethiopia	African	Bread, fish, chicken, spices	Other sea foods, pork, turkey, alcoholic beverages
5	Finland	Scandinavian	Rye bread, knäckebröd, dairy products	Coffee, meat, alcoholic beverages
6	India	Asian	Rice, vegetables, pulses, spices	Meat, fish, alcoholic beverages
7	Indonesia	Asian	Rice, meat, soy products	Pork
8	Italy	Mediterranean	Pasta, fruit, olives and olive oil, herbs	Alcoholic beverages
9	Lithuania	East European	Meat, eggs, potatoes, vegetables	
10	Malaysia	Asian	Rice, meat, fish, vegetables, fruit, herbs, spices	Pork (products), alcoholic beverages
11	Mexico	South American	Tortilla, fruit, vegetables, meat, cheese, hot sauce	Sea foods, milk
12	Netherlands	Lactoovovegetarian	Plant products	Animal products, dairy, eggs
13	Netherlands	Vegetarian	Plant products, dairy products	Meat, fish
14	Netherlands	"Prehistoric"	Uncooked and unprocessed products, fruit, vegetables	Cooked foods
15	Netherlands	Western	Bread, dairy products, meat, potatoes, vegetables	
16	Suriname	Surinamese	Rice, vegetables, grains, pulses, soy products, spices	Beef
17	Turkey	Middle Eastern	Rice, vegetables, meat, tea, pulses	Pork

Mean recovery of administered acetylsalicylic or salicylic acid in 24-h urine of the 6 validation study subjects was 80±18% for salicylic acid and 77±10% for acetylsalicylic acid (Fig 1). Median salicylate excretion was 10 µmol/24 h, with a range of 6-12 µmol/24 h (1.4 mg/24 h, range: 0.8-1.6 mg/24 h) for the 6 subjects (Fig 1) on the day they took no salicylic or acetylsalicylic acid.

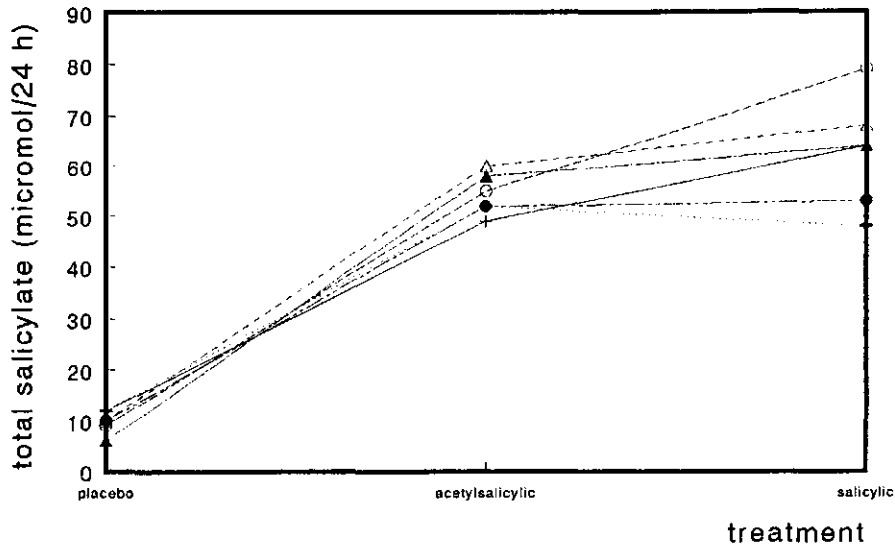

Fig 1: Urinary excretion of salicylates in 6 healthy volunteers participating in a randomized validation study lasting 3 d, separated by washout periods of 14 d. On each urine collection day subjects swallowed a capsule containing 66±2 µmol (9.1±0.2 mg) of pure salicylic or 58±1 µmol (10.5±0.2 mg) of acetylsalicylic acid, or nothing as placebo. Each symbol represents a different subject. To convert salicylate values to mg/24 h, multiply by 0.13812.

In the 17 subjects with nontraditional diets, energy intake was 9.0±3.1 MJ (range: 3.8-16.9 MJ), of which 15±4% (range: 10-24%) was provided by proteins, 51±12% (range: 31-71%) by carbohydrates, and 33±10% (range: 13-47%) by fats; only 2 of the 17 subjects consumed alcohol (nos. 3 and 13; Table 1). Median intake of vegetable proteins was 7% of total energy intake (range: 4-12%), and intake of dietary fibre was 1.7 g/MJ (range: 0.6-5.7 g/MJ) (Fig 2).

Median excretion of salicylates in urine for the 17 subjects eating unusual diets was 10 µmol/24 h (1.4 mg/24 h), with a range of 4-34 µmol/24 h (0.5-4.7 mg/24 h; Fig 2). Salicylate excretion correlated positively with intake of dietary fibre ($r=0.73$, $p<0.01$) and with intake of protein from vegetable sources ($r=0.42$, $p=0.10$).

Calculations of the intake of dietary salicylates of 4 subjects (nos. 6, 10, 14, and 16; Table 1) by using salicylate contents of foods as determined by Swain *et al* (13,14) produced intake values that were 2 to 18-fold higher than the amounts excreted in urine (Table 2). Calculating the intake of dietary salicylate using salicylate contents of foods as determined by us (15,16) or others (10,11), revealed lower values (Table 2).

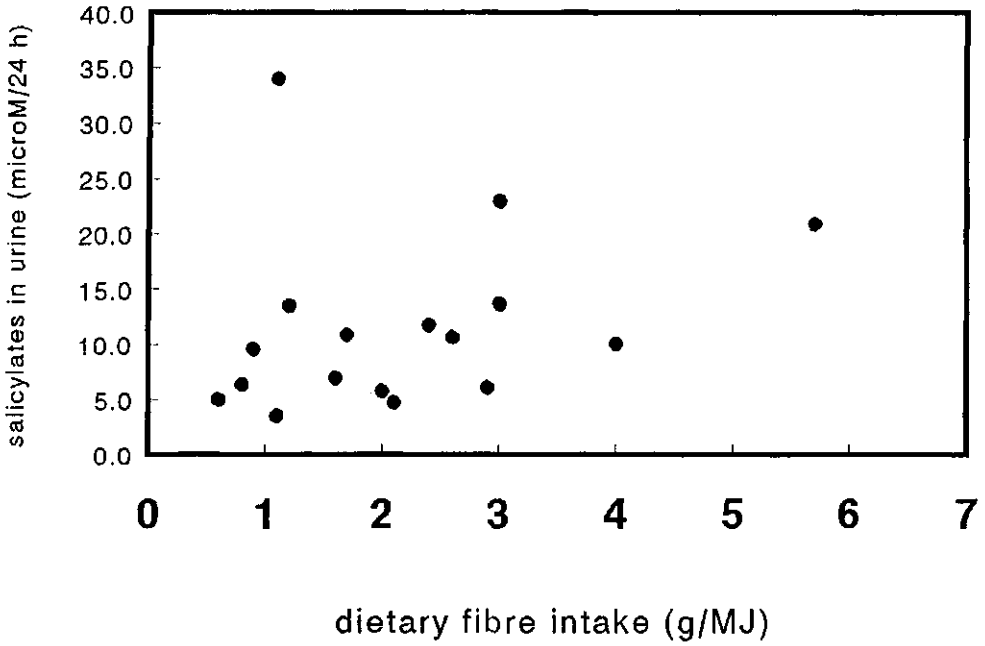

Fig 2: Urinary excretion of salicylates versus intake of dietary fibre in 17 healthy volunteers eating a variety of self-selected diets. To convert salicylate values to mg/24 h, multiply by 0.13812.

Table 2: Excretion of salicylates in urine of 4 healthy volunteers eating an Indian (no. 6), Malaysian (no. 10), "prehistoric" (no. 14), or Surinamese (no. 16) diet, respectively. [1]

subject	urinary salicylates (μmol/24 h)	estimated intake of dietary salicylates (μmol/24 h)[2]			
		Swain *et al* (13-14)	Robertson and Kermode (11)	Herrmann (10)	Janssen *et al* (15-16)
6	34	62	0	1	2
10	4	54	0	1	2
14	23	211	1	5	4
16	5	83	1	3	3

[1] **Subjects weighed and recorded all foods and drinks consumed during the urine-collection day in a diary and sampled equal amounts of the herbs and spices they used that day.**

[2] **We calculated the intake of dietary salicylates using the food records, the weights of the herbs and spices consumed, and salicylate contents of foods as determined by Swain *et al* (13-14), Robertson and Kermode (11), Herrmann (10), and Janssen *et al* (15-16). To convert salicylate values to mg/24 h, multiply by 0.13812.**

Discussion

We found that subjects eating self-selected diets high in a variety of plant foods excreted only a few milligrams of salicylates per day (Fig 1-2). When we gave subjects small amounts of pure acetylsalicylic or salicylic acid we recovered about 80% in urine (Fig 1).

Our assay only measures salicylates and its conjugates. However, a small fraction of the salicylates is metabolized to gentisic acid, which would escape detection (18). Thus urinary excretion is a valid marker for intake, even at low amounts. Our recoveries of *para*-aminobenzoic acid indicate that the 24-h urine samples were nearly complete.

Our results are at variance with those of Swain *et al* (13; AR Swain, RH Lobley, AS Truswell, unpublished observations, 1985). They reported urinary salicylate excretions of 825±43 μmol/24 h (114±6 mg/24 h; mean±sem, n=25) after consumption of a diet providing ostensibly 637 μmol salicylates/d (88 mg/d) for 2 d; salicylate excretions were 268±29 μmol/24 h (37±4 mg/24 h, n=28) after a diet providing 0 mg salicylates/d for 2 d (13). Thus, Swain *et al* (AR Swain, RH Lobley, AS Truswell, unpublished observations, 1985) recovered 86% (n=24) of dietary salicylates in urine.

Swain *et al* (13-14) also reported 10-100-fold higher concentrations of total salicylates in foods than we (15-16) and others (9-12) did. A plausible interpretation of these discrepancies is that the assays used by Swain *et al* lacked specificity, and that substances other than salicylates were included in the salicylate figures. Estimated dietary salicylate intake data for the four subjects in table 2, using the data of Herrmann (10), Robertson and Kermode (11), and ourselves (15-16) may be somewhat underestimated due to the limited number of foods analyzed for salicylate, compared to the number of foods Swain *et al* analyzed (13-14). The correlation between salicylate excretion and dietary intake of fibre or vegetable protein that we found confirmed that plant foods are the major source of dietary salicylates. Therefore, we think the possible underestimation cannot be large because salicylate concentrations of the most important salicylate sources -plant foods- have been given by Robertson and Kermode (11), Herrmann (10) and ourselves (15-16). If we correct our salicylate excretion data using a salicylate recovery of 100%, we estimate that even pure vegetable diets provided less than 43 μmol (6 mg) salicylates/d. Even if most of this were in the form of acetylsalicylate, which is highly unlikely (15-16), these intakes are probably still too low to affect risk for coronary heart disease or colon cancer.

At the same time, our data suggest that worries about adverse effects of dietary salicylates in children are unfounded. The data on salicylates in the Dutch Food Intolerance Databank (22) overestimate dietary salicylate intake because these data are derived almost entirely from Swain *et al* (13-14). Even if dietary salicylates affect behaviour, which is doubtful, our results suggest that amounts of salicylate in foods are so low that diet may be ignored as a source.

We found that salicylate excretion in urine is a valid indicator for the intake of salicylates and that daily excretion of salicylates in urine is very low in subjects eating a variety of self-selected diets. We conclude that the amount of dietary (acetyl)salicylate is probably too low to affect disease risk.

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Chapter 5

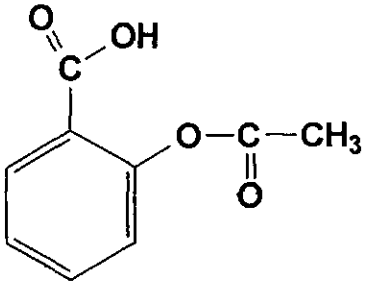
Salicylates in diets: a review

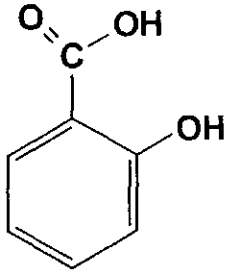
Janssen PLTMK, Hollman PCH, Venema DP, van Staveren WA, Katan MB.
Salicylates in foods.
Nutr Rev *(in press)*.

Acetylsalicylic acid or 2-acetoxybenzoic acid (Fig 1) is the formal name of a drug commonly known as aspirin. Acetylsalicylic acid is effective as an anti-thrombotic drug in doses as low as 30 mg/d (1-3) Intake of acetylsalicylic acid is also associated with a decreased risk for colon cancer (4-5). Foods are thought to contain acetyl- and other salicylates (6-7), and Ingster *et al* suggested that intake of salicylates in foods might have contributed to the decline in cardiovascular disease mortality in the USA (7). If acetylsalicylic acid were present in foods, a diet rich in acetylsalicylic acid might have anti-thrombotic and possibly anti-carcinogenic effects (6).

Adverse effects of salicylates have also been described. Hypersensitivity may occur after medication containing salicylates, but improvement by a salicylate-free diet is highly unlikely (8). Feingold (9) suggested that elimination of artificial food colours and flavours, as well as of foods containing natural salicylates, improves behavioral disturbance of children. Although this could not be substantiated in properly controlled trials (10-11), belief in the efficacy of treatment with salicylate-free diets is firmly held (12). Contents of salicylates in foods are thus of interest.

Data on salicylate contents of foods are scarce and contradictive (Table 1) (13-19). Salicylic acid (Fig 1) and other salicylates, mainly methyl esters and glucosides, have been isolated from a variety of plants (20). These salicylates can be hydrolysed to the parent salicylic acid (19). Consequently, food contents of total salicylic acid published include these to various extents. Robertson and Herrmann found salicylate contents of 0.03-3 mg/kg in fruits and vegetables, and 3-39 mg/kg in herbs and spices (15-18). Salicylate values reported by Swain *et al* were much higher: 0.8-23.9 mg/kg for fruits and vegetables, and 62-2180 mg/kg for herbs and spices (13-14). Muller and Fugelsang reported salicylic acid concentrations ranging from 11.0-21.5 mg/L in Californian and other red and white wines. Concentrations in red wines were higher than in white (21). Venema *et al* (19) found salicylate contents comparable to data of Robertson and Herrmann (15-18): 0.03-0.87 mg/kg in fruits and vegetables, and 0.48-28.4 in herbs and spices. They also did not confirm the high contents in wine (22) reported by Muller and Fugelsang (21).





ACETYLSALICYLIC ACID **SALICYLIC ACID**

Fig 1: Structure of acetylsalicylic acid and salicylic acid.

Table 1: Content of salicylic acid and acetylsalicylic acid in foods according to various authors (13-19).

	content in edible part of food (number of foods analyzed)[a]					
	salicylic acid (mg/kg)				acetylsalicylic acid (mg/kg; + =present, - =absent)	
product	Swain[b] (333)	Herrmann[c] (84)	Robertson[d] (44)	Venema[e] (30)	Swain[f] (56)	Venema[g] (30)
fruits, vegetables:						
apples with peel	0.8-5.9	<1	0.04	<0.02	+	<0.02
apricot, canned	14.2	<1	0.03	0.13		<0.02
cucumber with peel	7.8[h]	<1-3		0.08	+	<0.02
orange	23.9	<1	0.07	<0.02	+	<0.02
herbs, spices:						
cinnamon	152	10		23.80	+	<0.2
curry powder, mild	2180			5.55		<0.2
thyme	1830	11		12.8		<0.2
beverages:						
tea, brewed	30[i]			0.42	+	<0.02
wine, red	9			0.28-0.71	-	<0.02
other:						
honey	25-112			0.66	-	<0.2

[a] To convert values for salicylic acid from mg/kg to mmol/kg divide by 138.12; to convert values for acetylsalicylic acid from mg/kg to mmol/kg divide by 180.15; [b-g] Methods used and their detection limits: [b] HPLC with UV detection; [c] thin layer chromatography preceded by hydrolysis for fruits and vegetables, detection limit 1 mg/kg; gas chromatography for herbs and spices, detection limit 1 mg/kg; [d] spectrofluorimetry; [e,g] HPLC with fluorescence detection, detection limit 0.02 mg/kg for fresh and 0.2 mg/kg for dried products; [f] thin layer chromatography; [h] without peel; [i] bag.

Acetylsalicylate contents of foods are important to study their possible anti-thrombotic effects (1-3,6-7). Data on acetylsalicylate contents in foods are lacking. Qualitative analyses showed the presence of acetylsalicylate in 37 out of 56 foods studied by Swain (13). Acetylsalicylic acid is spontaneously hydrolysed to salicylic acid in aqueous solutions (23). Venema *et al* therefore developed a mild extraction method for acetylsalicylic acid in foods which prevented hydrolysis. Subsequently, they determined acetylsalicylate quantitatively in 30 foods previously reported to contain salicylates. Acetylsalicylate contents were lower than the limit of detection (0.02-0.2 mg/kg) in all foods studied (19,22).

Differences in salicylate contents found in foods by various authors may be caused by differences in origin, processing, storage, or by differences in analytical methods. Swain (13-14) found a high natural variation amounting to 10-fold differences. Venema *et al* reported that this variation was much smaller (19). This excludes natural variation as a possible explanation for these discrepancies. Discrepancies in salicylate contents between extraction methods may arise through differences in the extent of liberation of matrix-bound salicylates. However, though Venema *et al* (19) used essentially the same extraction method as Swain (13-14), salicylate contents found were much lower (Table 1). There is reason to believe that HPLC separation and UV-detection as described by Swain *et al* was not specific enough, and that some other components may have co-eluted with salicylic acid. In contrast, Venema *et al* (19,22) used fluorescence detection thus gaining specificity over UV detection.

It was recently shown that urinary excretion is a valid indicator of the intake of pure salicylic and acetylsalicylic acid (24). Thus, urinary salicylate excretion might be an indicator of the amounts of bio-available salicylates in the diet. Swain reported mean salicylate excretion (±SEM) of 114±6 mg/24 h (825±43 μmol/24 h) in urines of healthy volunteers consuming a diet providing 88 mg/d (637 μmol) of salicylates for 2 d according to their food analyses. Mean salicylate excretions were 37±4 mg/d (268±29 μmol/24 h) after a 2-d diet providing no salicylates (13-14). In contrast Janssen *et al* (24) found a median salicylate excretion in urine of only 1.4 mg/24 h, with a range of 0.8-1.6 mg/24 h (median 10 μmol, range 6-12) in 18 healthy subjects eating a wide variety of diets. The high values found by Swain *et al* (13-14) may be explained by co-elution of various compounds at the retention time of salicylate, as the HPLC-column used probably was too short and the flow may have been too high to obtain adequate separation.

Based on the urinary salicylate excretion data of the volunteers eating a wide variety of diets, their food records, and Venema's salicylate contents of foods (19,22,24), it was estimated that even pure vegetable diets provide less than 6 mg (43 μmol) of salicylates daily. If most of this were in the form of acetylsalicylate -which is unlikely (19,22)-, these intakes are probably still too low to affect disease risk. Ingster *et al* (7) proposed an increase in dietary salicylates in the US with time since 1960 reaching 90 mg/d/person in 1960 and 125 mg/d/person in 1970. Those calculations, however, were based on salicylate contents published by Swain *et al* (14), which in our view overestimate dietary intake. We feel that true intakes of dietary salicylates in the US population are closer to 0-6 mg/d, and that the estimates of Ingster *et al* (7) are too high by almost two orders of magnitude.

It is concluded that a normal mixed diet provides only 0-6 mg of salicylates daily, and no or almost no acetylsalicylic acid. Those amounts are probably too low to affect disease risk. At the same time, worries about adverse effects of dietary salicylates on the

behaviour of children are probably unfounded.

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Chapter 6

Effects of the flavonoids quercetin and apigenin on haemostasis in healthy volunteers: results from an *in vitro* and a dietary supplement study

Janssen PLTMK, Mensink RP, Cox FJJ,
Harryvan JL, Hovenier R, Hollman PCH, Katan MB.
Effects of the flavonoids quercetin and apigenin on haemostasis in healthy volunteers: results from an *in vitro* and a dietary supplement study.
(submitted)

Abstract

Intake of dietary flavonols and flavones is inversely associated with risk for cardiovascular disease. This might be due to effects on haemostasis, because flavonoids have been reported to inhibit platelet aggregation *in vitro*. We found that 2.5 μmol/L of the flavone apigenin inhibited collagen- and ADP-induced aggregation in platelet-rich plasma and washed platelets by about 26%; the flavonols quercetin and quercetin-3-glucoside had no effect. To test this *in vivo* we fed 18 healthy volunteers onions providing 114 mg/d of quercetin glucosides, parsley providing 84 mg/d of apigenin glycosides -both expressed as aglycones-, or a placebo for 7 d each in a randomized cross-over experiment of 3x2 weeks. No effects were found on platelet aggregation, thromboxane B_2 production, factor VII and other haemostatic parameters. We conclude that anti-aggregatory effects of flavonoids seen *in vitro* are due to concentrations that cannot be attained *in vivo*. Effects of dietary flavonols and flavones on cardiovascular risk are possibly not mediated by haemostatic parameters.

Introduction

Flavonoids are polyphenolic compounds that occur ubiquitously in plant foods. Flavonols and flavones are subclasses of flavonoids (Fig 1) (1-4). Average daily intake with the Dutch diet was (5) for flavonols 16 mg of quercetin, 4 mg of kaempferol, and 1 mg of myricetin, and of flavones 1 mg of apigenin and 1 mg of luteolin. The intake of those 5 dietary flavonoids was associated with a reduced risk for coronary heart disease or stroke in several (6-9), though not all (10-11) studies.

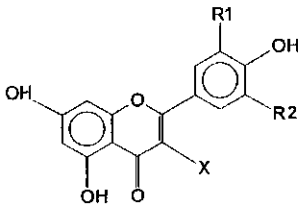

Fig 1: Structure of flavonols and flavones. Flavonols: X=OH; quercetin: R_1=OH, R_2=H; kaempferol: R_1=H, R_2=H; myricetin: R_1=OH, R_2=OH. Flavones: X=H; apigenin: R_1=H, R_2=H; luteolin: R_1=OH, R_2=H.

Two types of mechanisms have been proposed to explain this protective effect, namely inhibition of LDL-oxidation or inhibition of platelet aggregation (12,13). Results obtained by incubation of human platelets or animal cells with isolated flavonoids suggest that flavonoids inhibit platelet aggregation -probably by inhibition of cyclo-oxygenase activity-. Conflicting observations on the effects of flavonoids on *in vitro* platelet aggregation have been found (14-21). It has been stated that flavones are strong and flavonols moderate inhibitors of cyclo-oxygenase, and that flavonoid glycosides are less effective inhibitors than their aglycones --the sugar-free part of the flavonoid molecule-- (14,21). The latter is relevant because flavonoids in foods are present mainly as glycosides (1), and we showed earlier that absorption of flavonoid glucosides is more effective than absorption of aglycones (22). Hollman *et al* found peak plasma quercetin concentrations in humans of 0.6 μM (196 ng/mL) after consumption of 64.2 mg of quercetin from onions (23). Flavonoid concentrations used in *in vitro* studies ranged between 10 and 1000 μM

(14-21), which is thus 10 to 1000 times higher than plasma levels reached after oral intake. Data on plasma concentration of apigenin in humans are absent. There are thus several reasons why the effect of flavonoids on platelets *in vivo* might differ from those observed *in vitro*.

Flavonoids may also affect the activity and/or the concentration of plasma coagulation or fibrinolysis factors (24-26). The formation of a thrombus in atherosclerotic arteries gives rise to the clinical state of acute coronary events, and is closely controlled by the haemostatic system, including coagulation and fibrinolysis (27,28). Several studies showed that plasma fibrinogen concentration is an independent risk factor of ischemic heart disease (29-31). Factor VII and plasminogen activity were associated with ischemic heart disease risk (30) and plasma PAI-1 activity was associated with increased risks of myocardial (re)infarction (31,32).

We now carried out both an *in vitro* study and a dietary supplement study in healthy volunteers. In the *in vitro* study we investigated whether a test tube addition of flavonoids in the estimated physiological range (0-2.5 μM) inhibited *in vitro* platelet aggregation, and we included unphysiologically high concentrations of flavonoids (>2.5 μM) to enable comparisons with published studies. In the dietary study we examined whether administration of foods rich in flavonoids affects platelet aggregation, coagulation and fibrinolysis in healthy volunteers.

Subjects and methods

Both protocols were approved by the Medical Ethics Committee of the Department of Human Nutrition, and were fully explained to the participants, who gave their written informed consent. Participants were all non-smokers.

In vitro study

Subjects

Four men from the Department of Human Biology, Maastricht University, aged 24, 29, 35, and 47 yrs, respectively, served as blood donors for the *in vitro* study. All were healthy, according to a medical questionnaire, and ate an ordinary western diet. None of the blood donors used any medication from 2 weeks preceding until the end of the study.

Methods

We investigated the effects on platelet aggregation of 6 concentrations (0, 0.25, 2.5, 25, 250, and 2500 μM) of pure quercetin-3-glucoside (Apin Chemicals LTD, Abingdon Oxon, United Kingdom), quercetin aglycone -i.e. quercetin without a sugar moiety- (Quercetin Dihydrate, C. I.nr. 75670, Fluka Chemika AG, CH-9470, Buchs, Germany), apigenin aglycone (nr 10798, Fluka Chemika, Meppel, The Netherlands), and catechin ((+)-catechin hydrate, Fluka Chemika, Meppel, the Netherlands). It was not possible to study *in vitro* effects of apigenin-glycosides, as these are not commercially available. Effects were studied both in platelet-rich plasma and in washed platelets to exclude possible effects of plasma factors on aggregation. Effects were not studied in whole blood as this is only stable during a short period.

Indomethacin (I-7278; Sigma Chemie, St Louise Mo. 63178, USA), a specific inhibitor of the enzyme cyclo-oxygenase, was used as a positive control. Effects of final

Indomethacin concentrations of 0, 0.1, 1, 10, 100, and 1000 μM on *in vitro* platelet aggregation in platelet-rich plasma and washed platelets were tested using citrated blood of one of the donors.

Aggregations in platelet-rich plasma were stimulated with final concentrations of 2 μg of collagen/mL (Collagen Horm, München, Germany) or 2.5 μM adenosine 5'-diphosphate (ADP, A6521 Sigma Chemical, St Louis, USA). Aggregations in washed platelets were induced by 2.5 μg/mL collagen or 18 μM ADP.

As polyphenols are known to bind proteins, catechin, a flavonoid with a high protein binding capacity (33) but hardly any effect on platelet aggregation or prostaglandin synthesis (14,20,34), served as a control for aspecific effects.

Each donor gave blood 4 times within a period of 1-6 wks. Effects of apigenin, quercetin, quercetin-3-glucoside, and catechin were tested in random order for all 4 donors. Effects of the 6 different concentrations of each compound were tested on the same day in random order.

After the participants had fasted during the night free-flowing venous blood was sampled without stasis (Strauss Kanüle, 1.2 mm Syringe, Luer, Wächterbach, Germany) with the subject in supine position. The first 3 mL of blood was discarded. Blood was collected into tubes prefilled with sodium-citrate solution (final concentration 10.9 mM, pH 7.3; Merck BV, Amsterdam, The Netherlands). Platelet-rich plasma was prepared by centrifugation at 150-160g for 15 min at room temperature, and diluted with autologous platelet-poor plasma to a final concentration of $185*10^9$ platelets/L. Washed platelets were prepared by mixing 5.8 mL tri-sodium-citrate 80 mM, citric acid 52 mM, and glucose 183 mM Acid Citrate Dextrose (ACD) with 29.2 mL of blood; this was centrifugated for 15 min at 160 g, and platelet-rich plasma was removed. Subsequently 25 mL of the platelet-rich plasma was mixed with 1 mL ACD, centrifugated for 15 min at 610 g, and the platelet pellet was resuspended in 2 mL Hepes buffer (pH 6.6; NaCl 136 mM, KCl 2.7 mM, Hepes 10 mM, $MgCl_2.6H_2O$ 2 mM, glucose 1 mg/mL, BSA 1 mg/mL), Hepes buffer was added to a total volume of 30 mL, and 1 mL ACD/15 mL was added. The suspension was centrifuged at 610 g for 15 min, and the platelet pellet was resuspended in Hepes buffer (pH 7.45). Glucose and BSA were added to the buffer just prior to use.

After the first blood donation optimal doses of the stimuli collagen and ADP were determined per person using 5 μL blank solvent dimethylsulfoxide (DMSO, Fluka Chemie, Meppel, the Netherlands) instead of a flavonoid solution, under conditions as described below. The optimal stimulus doses, defined as the final concentrations leading to a maximal aggregation of 65%, were for the aggregations in platelet-rich plasma of the 4 donors 2.0, 4.0, 2.0, and 4.0 μg collagen/mL, and 9.0, 5.0, 2.5, 2.5 μM ADP. The doses for aggregation in washed platelets of the 4 donors were 7.5, 7.5, 2.5, 7.5 μg collagen/mL, and 18.0, 18.0, 18.0, 6.0 μM ADP, respectively. These doses were used throughout.

For each aggregation measurement 400 μL of the diluted platelet-rich plasma or washed-platelets were incubated in an aggregometer at 37^0 C, at 1000 rpm (Chronolog Corporation, Havertown, PA, USA). Flavonoids were dissolved in DMSO, and 5 μL were added to the platelets to produce final concentrations of 0, 0.25, 2.5, 25, 250, and 2500 μM. Ten μL of collagen suspension or ADP were added exactly 10 min after addition of the flavonoid. We added fibrinogen (final concentration 0.5 mg/mL; fraction 1, type 4, bovine plasma, Sigma Chemie, Brunschwig, Amsterdam, the Netherlands) during the ADP-induced aggregation of washed platelets. The change in percentage of transmitted light was monitored continuously during 7 min. Light transmitted was set at 100% for platelet-poor

plasma and Hepes buffer, and 0% for platelet-rich plasma and the washed platelet suspension.

Aggregation measurements were completed within 2 h after blood sampling. We found in earlier studies (unpublished observations) that platelets were stable during this period. Platelets were always handled in plastic material and at room temperature. Maximal aggregation was calculated for all measurements. For ADP-induced aggregations maximal aggregation of the first wave was used as outcome variable. Maximal aggregation on 5 μL of solvent DMSO using optimal individual stimulus concentrations was chosen as 100%. Mean maximal aggregation values for each flavonoid concentration was calculated by taking the mean value of the 4 donors.

Dietary supplement study

Subjects

Ten men and 12 women were recruited through announcements in the University newspaper and posters in student dormitories. One man was excluded because of elevated alanine-amino and gamma-glutamyl transferase. One woman withdrew on her own accord. Nine men and 9 women were selected at random from the remaining 20 subjects. One man dropped out during the first week of the study, due to personal reasons unrelated to the study. A women who had participated in the screening replaced him; she started at day 7 of the study. All 18 volunteers successfully completed the study. Participants were healthy based on a medical questionnaire. All had normal values for urinary protein and glucose, haematocrit, haemoglobin, white blood cell and platelet count, mean red cell volume, plasma alanine-amino transferase, plasma gamma-glutamyl transferase, serum creatinine, prothrombin and activated partial thromboplastin time. Mean age (±sd) was 25±8 years, and body mass index was 22±1 kg/m^2.

Methods

Design: We investigated the effects of daily consumption of flavonoid-rich dietary supplements on parameters for haemostasis in a randomised placebo-controlled multiple cross-over study (fig 2). All subjects participated simultaneously. Participants consumed supplements daily during weeks 2, 4, and 6. Week 1 served as run-in, and weeks 3 and 5 as wash-out periods. Supplements were given in random order.

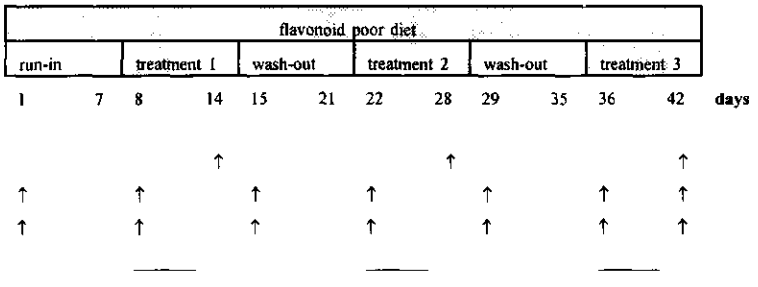

Fig 2: Design of the randomized placebo-controlled cross-over study of 3x2 wks. Ten women and 8 men consumed 400 g bouillon daily with 220 g of onions, 4.9 g of parsley, or nothing as a placebo for 7 d. All treatments were given to every subject in random order.

Supplements: Supplements consisted of 400 g of bouillon, to which was added either 220 g of cooked yellow onions (Favorit MSP, class II 60-80 mm, Luctor, Dronten, The Netherlands), 4.9 g of dried Western-European parsley (Verstegen Specerijen, Rotterdam, The Netherlands), or nothing as a placebo. Onions, parsley, and bouillon powder (Maggi Bouillonkorrels, Nestlé Foodservice Catering, Amsterdam, The Netherlands) were bought in one batch just before the study, and stored in the dark at 4 ^{0}C. The onions were thinly peeled 1 week before the study started, cut into pieces of 8x8x8 mm (Robot-Coupe S.A., Montceau-en-Bourgogne, France), heated in portions of 600 g in a microwave oven for 5 min at 800 W or 7 min at 500 W, mixed, and heated for another 4 min at 800 W, or 5 min at 500 W. Portions of 220 g were weighed out, and stored at -20^{0}C. Onions were thawed in the dark at room temperature overnight before consumption. Before the study started the parsley was mixed, weighed out in portions of 4.9 g, and stored in the dark at room temperature until consumption. Bouillon was prepared each day before consumption using 16.7 g of bouillon powder per kg of boiling water; portions of 400 g were weighed out, and stored in the dark at 4^{0}C. Duplicate portions of the supplements were prepared daily, and stored at -20 ^{0}C until analyses (35). The onion supplement contained 114±3 mg (377±10 μmol; n=15, mean±sd) and the placebo 0.015±0.004 mg (0.05±0.01 μmol; n=6) of quercetin. The parsley supplement contained 84±6 mg (n=15) apigenin.

In wks 2, 4, and 6 subjects came to the department daily in fasting status between 7.30-9.00 a.m. on working days, and 8.00-10.00 on weekend days to consume their supplements. Parsley and onions were mixed with the bouillon just prior to consumption. The onion-soup was heated for 6 min at 800 W, or for 7 min at 500 W; the parsley-soup and placebo bouillon were heated for 4.5 min at 500 W, or 3.5 min at 800 W. Participants were not allowed to eat or drink anything, except (mineral)water until 2 h after consumption of their supplements.

Food consumption, physical activity and medication: Participants were urged not to consume any fruits and vegetables containing more than 15 mg quercetin or apigenin per kg and any beverages containing more than 4 mg/L (i.e. apples, endive, beans, broccoli, celery, cherries, cloves, grapes, leek, onions, parsley, tea, tomato, wine) (35-38), or any fatty fish. Subjects were asked to maintain their normal eating and drinking habits and physical activity levels during the study. They were instructed to avoid all regular and homoeopathic medicines, and vitamin and mineral supplements from 1 month preceding the study until the end of the study. Use of oral contraceptives was permitted. Participants were supplied with Paracetamol which could be used for pain relief. Subjects were urged to record health complaints, medications used, and any deviations from their normal physical activity and dietary habits in a diary. We determined body weights of the participants, and checked the diaries weekly; food intake was measured 3 times (wks 2, 4, and 6) using 24-h recalls (39) (fig 2). The habitual intake of energy of the subjects was on average 9.3±2.7 MJ/d (2214±642 kcal/d, mean±sd), of which 31±9% was provided by fat, 14±3% by protein, 53±10% by carbohydrate, and 1±3% by alcohol, with no changes during the study. Mean body weight decreased by 0.3±1.4 kg during the study. Subjects did not consume any fatty fish. There was no evidence from the diaries of changes in physical activity patterns or any deviations that might have affected the results. One subject took iron tablets (Ferrofumaraat, 3 tablets of 200 mg daily) from day 33 until the end of the study because of low hemoglobin values. Except for the Paracetamol supplied by us no medication was used. All participants denied having used acetylsalicylic acid from 1 month preceding until

the end of the study.

Blood sampling: Blood samples were taken on days 14, 28, and 42 (fig 2) as described above. About 25 mL of blood were drawn, at 90 minutes after the participant had consumed the supplement. The participant was lying down for 20 min before, until the end of venepuncture. Blood was drawn into tubes containing a final concentration of 10.9 mM sodium-citrate. Samples for coagulation and fibrinolysis measurements were put on ice immediately.

Analyses: Immediately after venepuncture one mL of citrated blood was incubated in a prewarmed aggregometer (37 ^{0}C) and stirred at 1000 rpm. Exactly 5 min later 10 µL of collagen suspension (final concentration 2 µg/mL) was added and whole blood aggregation using an impedance method was recorded for 10 min on a computerized system. Maximal aggregation was measured to study effects of the supplements on platelet aggregation under most physiological conditions.

Platelet-rich plasma containing $250*10^9$ platelets/L was prepared as described earlier (40). Some diluted platelet rich plasma was frozen in fluid nitrogen, and stored at -80 ^{0}C for determination of flavonoid concentrations. HPLC separation was used combined with fluorescence detection for determination of quercetin (Hollman *et al.*, unpublished observations), and with UV detection for apigenin concentrations (35).

For each aggregation 400 µL platelet-rich plasma was incubated in a prewarmed (37 ^{0}C) aggregometer and stirred at 1000 rpm. Exactly 5 min later 10 µL of collagen (final concentration 2.0 µg/mL) or ADP (final concentration 1.5 and 3.0 µM) were added to induce aggregation. The change in percentage of transmitted light was monitored continuously during 10 min and maximal aggregation was calculated as described for the *in vitro* study.

Maximally stimulated thromboxane B_2 production in platelet-rich plasma was measured as described earlier (40) as a specific measure of active cyclo-oxygenase present. All samples of one subject were analyzed within one run.

Immediately after blood sampling plasma for measurements of coagulation and fibrinolysis parameters was separated by centrifugation at 1500 g for 20 min at 4^{0}C . It was divided into aliquots, snap-frozen in liquid nitrogen, and stored at -80^{0}C. Factor VII and plasminogen activity were determined using a laser-nephelometric centrifugal ACL-200 analyzer (Instrument Laboratory, Milano, Italy). Factor VII clotting time was determined in a standard one-stage assay: plasma samples and factor VII-deficient plasma were mixed, the clotting process was initiated, and clotting time was measured using PT-Fibrinogen and thromboplastin from Instrumentation Laboratory (IJsselstein, The Netherlands), and Factor VII from Organon Technica (Oss, The Netherlands). Plasminogen activity was determined according to the test manufacturer's instructions using COATEST antiplasmine and Streptokinase from Chromogenix (Amsterdam, The Netherlands). Blood for a normal plasma pool was donated by 40 healthy volunteers. Factor VII and plasminogen results were expressed in percentages relative to values for this pool. Standards for factor VII and plasminogen measurements were derived from BIOPOOL (Haemostasis Reference Plasma, Kordia, Leiden, The Netherlands). Plasma fibrinogen concentrations were determined using a STA II coagulation Analyser (STA-fibrinogen, Diagnostica Stago, Boehringer, Mannheim, Germany): a fixed surplus of thrombin was added to diluted platelet-poor plasma samples,

and the clotting time was measured of a series of dilutions of a human plasma pool with a known fibrinogen concentration. Standards for fibrinogen measurements were derived from Boehringer (STA-Preciclot I & II, Boehringer, Mannheim, Germany). PAI-activity was measured using a Chromogenic Assay (Spectrolyse/pL PAI, Biopool, Umea, Sweden). Plasminogen and factor VII measurements were done in duplicate; fibrinogen and PAI-1 measurements were done in simplo.

Statistics: We checked the data for normality using residual analysis (41). Effects of onions and parsley were analyzed using the General Linear Models of the Statistical Analyses System (α=0.05) (42), with subject and treatment as class variables. A period term was introduced into the model to check for time effects, and a treatment-by-period interaction term to check for carry-over effects. Subsequently 95%-confidence intervals were calculated for effects of onions and parsley.

Results

In vitro study

Indomethacin 1 μmol/L inhibited maximal collagen-induced aggregation in human platelet-rich plasma by 67%, and in washed platelets by 74%; 1000 μmol/L inhibited aggregation in platelet-rich plasma by 98%, and in washed platelets by 82%. ADP-induced aggregation was hardly affected. Catechin inhibited collagen- and ADP-induced aggregation in platelet-rich plasma only at a concentration of 2500 μM (Fig 3-4). Results in washed platelets were similar to results found in platelet-rich plasma (data not shown).

Apigenin in a concentration of 2.5 μM inhibited collagen-induced aggregation in platelet-rich plasma by 26% (SEM 17%), and ADP-induced aggregation by 19% (SEM 17%). Apigenin 25 μM inhibited collagen-induced aggregation by 31%, whereas 2500 μM inhibited aggregation by 91%. Apigenin 25 μM inhibited ADP-induced aggregation in platelet-rich plasma by 31, and 2500 μM inhibited aggregation by 83%. In platelet-rich plasma 2500 μM of the flavonol quercetin inhibited collagen- and ADP-induced aggregation by about 95%, whereas lower concentrations were ineffective. Concentrations of 0.25 and 2.5 μM of the flavonol-glucoside quercetin-3-glucoside did not affect platelet aggregation; concentrations of 25, 250 and 2500 μM decreased collagen-induced platelet aggregation, but ADP-induced aggregation was not affected. Effects on aggregation in washed platelets were comparable with those in platelet rich plasma (Fig 3-4).

Dietary supplement study

No adverse reactions to the supplements were reported, although some people had difficulties in consuming the amount of onions at once. One subject consumed only part of the onion supplement at one occasion; the left-over contained 29 mg of quercetin. Subjects consumed negligible amounts of flavonol- or flavon-rich products during the study.

Concentrations of quercetin in platelet-rich plasma were 1.48±0.39 μM (447±117 ng/mL) 90 min after consumption of onions, and 0.02±0.01 μM (5±4 ng/mL) after placebo. Contents of apigenin in platelet-rich plasma were all below the limit of detection of 1.1 μM or 330 ng/mL.

Daily treatment with 220 g of cooked onions or 4.9 g of dried parsley for 7 d did not affect collagen-induced platelet aggregation in whole blood or platelet-rich plasma,

ADP-induced platelet aggregation in platelet-rich plasma, thromboxane B_2 production, platelet number, factor VII, plasminogen and PAI-I activity, or fibrinogen concentration (Table 1). There were no treatment sequence or time effects.

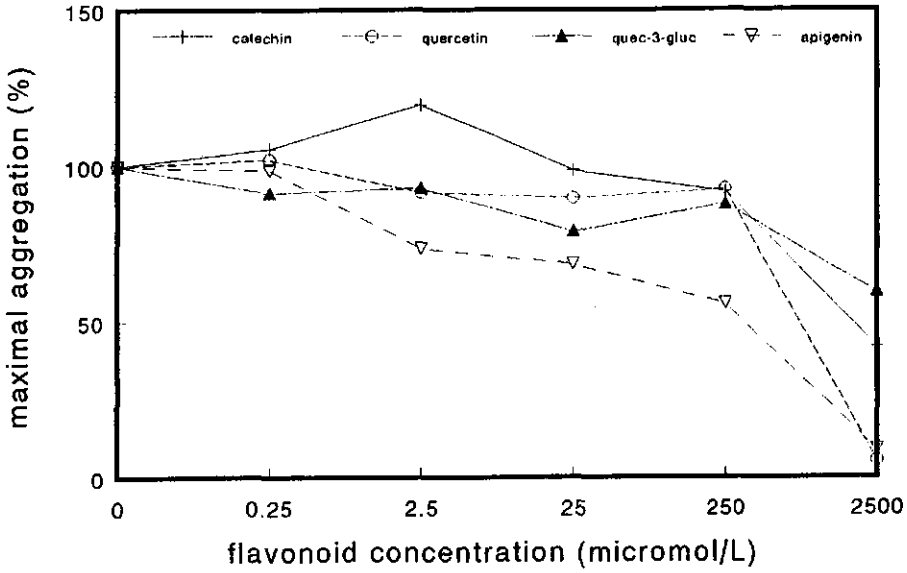

Fig 3: Relation between flavonoid concentration added to platelet-rich plasma and maximal *in vitro* platelet aggregation (%) induced with collagen (n=4).

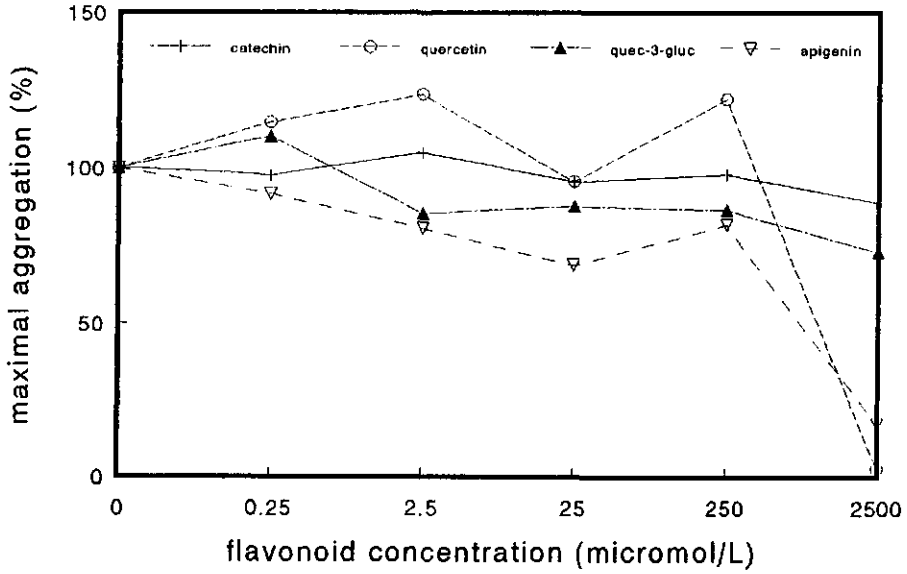

Fig 4: Relation between flavonoid concentration added to platelet-rich plasma and maximal *in vitro* platelet aggregation (%) induced with adenosine-diphosphate (n=4).

Table 1: Mean values (±sd) of haemostatic parameters in 18 healthy volunteers after consumption of placebo, and effects of consumption of 220 g of onions or 4.9 g of parsley daily for 7 d (95% confidence intervals).

			effect (treatment-placebo)			
			onions▲		parsley▼	
variables	*n*	after placebo#	mean±sd	95% c.i.	mean±sd	95% c.i.
factor VII activity (%)	18	92±25	-4±13	-10, 2	-1±2	-2, 0
fibrinogen (g/L)	18	2.4±0.6	0.1±0.7	-0.2, 0.4	0.2±0.6	-0.1, 0.5
plasminogen activity (%)	17	87±19	1±8	-3, 5	0±6	-3, 3
PAI-1 activity (IU/mL)	17	4.35±3.54	-0.90±3.17[a]	-2.66, 0.86	0.31±3.97[d]	-1.73, 2.35
maximally stimulated thromboxane B_2 production in platelet rich plasma (nmol/10^{11} platelets)	18	2502±593	-122±438	-340, 96	-131±503	-381, 119
maximal collagen-induced aggregation (Ohm) in whole blood (final 2 µg/mL)	18	14±4	-0.6±5.2	-3.2, 2.0	-0.1±5.7	-2.9, 2.7
maximal collagen-induced aggregation (%) in platelet-rich plasma (final 2 µg/mL	13	84±13	-6.4±14.5	-15.2, 2.4	-10.5±27.4	-27.1, 6.1
maximal ADP-induced aggregation (%)[b] in platelet rich plasma (final 1.5 µM)	18	23±8	7±20[c]	-4, 18	6±19[d]	-4, 16
maximal ADP-induced aggregation (%)[b] in platelet rich plasma (final 3 µM)	16	57±28	-8±22[a]	-20, 4	-6±26[a]	-20, 8
platelet number (*10^9/L)	18	235±47	0±28	-14, 14	9±25	-3, 21

[a] n=15; [b] first wave; [c] n=16; [d] n=17; # quercetin concentration in platelet-rich plasma 0.02±0.01 µM; ▲ quercetin concentration in platelet-rich plasma 1.48±0.39 µM; ▼ apigenin concentration in platelet-rich plasma were lower than the limit of detection (1.1 µM.).

Discussion

In vitro study

The *in vitro* study showed that a test tube addition of 2.5 μmol/L of apigenin inhibits collagen- and ADP-induced aggregation in human platelets, whereas quercetin and quercetin-3-glucoside did not. Flavonoid concentrations of 25 to 2500 μmol/L, inhibited platelet aggregation *in vitro*. The dietary supplement study, however, showed that administration of large amounts of foods rich in apigenin or quercetin-glucosides did not affect platelet aggregation or other haemostatic parameters in healthy volunteers.

We carried out highly standardized aggregation measurements in both studies. Published *in vitro* studies were not always strictly standardized as to platelet number and optimal stimulus concentration for each donor. Also, the experimental conditions varied from one study to another, which probably explains the large variety in outcome (14-21).

To check the validity of the *in vitro* aggregation measurements, Indomethacin was used as a positive and catechin as a negative control. As expected Indomethacin -a specific inhibitor of cyclo-oxygenase (43)- inhibited *in vitro* platelet aggregation induced by collagen, but not by ADP. We therefore conclude that our assay was able to detect specific inhibiting effects on cyclo-oxygenase activity in both platelet-rich plasma and washed platelets. The *in vitro* study showed that concentrations of 0.25 and 2.5 μM of catechin - which has a high affinity for proteins- did not affect collagen- or ADP-induced platelet aggregation, although unphysiologically high concentrations (25-2500 μM) did (Fig 3,4). The latter may be an a-specific inhibiting effect, as flavonoids readily bind to proteins (34). Thus, it is not very likely that a-specific protein-binding explains the results for apigenin at physiological concentrations.

Dietary supplement study

Values of the haemostatic parameters measured in the dietary supplement study after treatment with placebo were in the normal range for healthy volunteers (Table 1). Data from the diaries, body weights, and 24-h recalls did not reveal any confounding effects. All 18 subjects ate the supplements under our supervision. Plasma quercetin values after treatment with placebo indicated negligible dietary quercetin contents in the background diets. Plasma quercetin concentration after treatment with the onion supplement indicate that quercetin was absorbed. We bought all onions, parsley and bouillon before the study to exclude differences between batches. We stored all onion supplements at -20^0 C, and the parsley in dark at room temperature to diminish changes in composition of the onions. We prepared all supplements in a standardized way (see Methods section) to prevent differences in composition of the supplements during the study.

Comparison with results of earlier studies

Gryglewski and coworkers reported stimulation of cyclo-oxygenase activity by addition of quercetin or rutin to ram seminal vesicle microsomes (17). In contrast, results of other structure-function studies suggest that flavones such as apigenin are strong inhibitors, and flavonols such as quercetin are moderate inhibitors of cylo-oxygenase. Those studies also suggested that glycosylated compounds are less potent inhibitors of cyclo-oxygenase than their aglycones (14,21). A major drawback of *in vitro* studies done earlier is that only the effects of unphysiologically high flavonoid contents (>10 μM) were studied (14-21). It was recently shown that plasma peak quercetin concentrations in humans were

0.6 μM after consumption of 64 mg quercetin from onions (23). Although no data on apigenin absorption are available our data show that plasma peak apigenin concentrations in humans range between 0 and 1.1 μM. We found that the flavon apigenin did inhibit collagen-induced aggregation *in vitro* only at concentrations of 2.5 μM or higher both in platelet-rich plasma and in washed platelets, whereas quercetin and its glucoside -the form in which it appears in foods and in which it is probably absorbed (22)- did not affect collagen- or ADP-induced aggregation. The quercetin concentration in platelet-rich plasma after onion consumption was comparable with data reported earlier (23). Apigenin concentration in platelet-rich plasma could not be quantified because of the high limit of detection (330 ng/mL, 1.1 μM). Thus it is unclear whether apigenin was absorbed. It is unlikely that apigenin absorption was inhibited by dietary proteins as the subjects were not allowed to eat or drink anything until 2 h after consumption of the supplements, and as the supplements contained hardly any protein. The plasma concentration of quercetin suggests that these measures were adequate. It is possible that plasma peak values and elimination curves of apigenin are different from quercetin (23) and that blood sampling at a different time point would reveal apigenin concentrations higher than the detection limit. In any case, if apigenin is absorbed, the prolonged supplementation would ensure interaction with the blood components. Epidemiological studies showed that the inverse association between dietary flavonoid intake and coronary heart disease risk was strongest with quercetin (9). Besides average daily dietary intake of quercetin was high (16 mg), compared to intake of apigenin (1 mg) in the Netherlands (5).

In accordance with our *in vitro* data, no effect of consumption of apigenin- or quercetin-rich foods was found on collagen-induced aggregation in whole blood and platelet-rich plasma, on ADP-induced aggregation in platelet-rich plasma, on thromboxane production, and on other haemostatic variables (Table 1). This indicates that daily consumption of large amounts of quercetin- or apigenin-rich foods may not be effective as inhibitors of cyclo-oxygenase activity or platelet aggregation in human volunteers.

We think that the treatment periods of the dietary supplement study were long enough to detect possible effects on blood platelet function. Blood platelets have a mean life time of 7-10 days; therefore treatment during 7 d with very high daily doses of flavonoids must be sufficient to find effects on platelet aggregation and thromboxane B_2 production. We earlier showed in similar volunteers that even 3 mg/d of acetylsalicylic acid inhibited maximally stimulated platelet thromboxane production by 39±8% (40). Meade *et al* (30) showed that mean fibrinogen concentration in women who died from ischemic heart disease was 3.38 g/L (95% confidence interval 3.11 to 3.66), whereas the concentration in survivors was 3.31 g/L (95% confidence interval 3.25 to 3.37). Contents of clotting factor VII as percentage of standard were 120% (95% confidence interval 108 to 132%) in subjects with ischemic heart disease, and 113% (95% confidence interval 110 to 115%) in survivors (30). Hamsten *et al* showed (31) in a prospective study that plasma PAI-1 concentrations are positively associated with increased risk of myocardial infarction among survivors of a first myocardial infarct: PAI-1 concentrations of patients with reinfarction were 1.6 times the values of patients with uneventful courses. In conclusion we think that our study group was large enough to detect relevant effects on thromboxane production and platelet aggregation in our study (Table 1). We might have missed small biologically relevant effects (29-32) of dietary flavonoids on the coagulation and fibrinolytic parameters measured (Table 1); this should be examined in a larger study population.

Results of our supplement study agree with results from the dietary supplement study of Srivastava *et al* (44). They found no effects of consumption of 70 g of raw onions daily for 7 d on platelet thromboxane production in 5 healthy volunteers: the mean effect of onion consumption on thromboxane production was 95 pmol/mL serum (sd=756, n=5). These authors could however have missed biologically significant effects due to the small study sample, the low dose, and the large variation in the outcome variable. We did not find any effects in a larger group consuming 220 g of onions daily.

Hertog *et al* showed that average daily intake with the Dutch diet was 16 mg/d of quercetin and 1 mg/d of apigenin (5). In our dietary supplement study the participants consumed very high doses of flavonoids: 114 mg/d of quercetin and 84 mg/d of apigenin. We think consumption of higher doses is impractical in a normal mixed diet.

Conclusions

Suggestions for an anti-aggregatory effect of flavonoids are based on the *in vitro* use of concentrations that cannot be attained *in vivo* by dietary consumption. Reported effects of dietary flavonoids on coronary heart disease risk are possibly not mediated through collagen- or ADP-induced platelet aggregation or cyclo-oxygenase activity. We cannot exclude small biologically relevant effects of dietary flavonoids on known risk indicators for coronary heart disease from the coagulation cascade or fibrinolytic system. The latter should be examined in a larger population.

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Chapter 7

Consumption of ginger (*Zingiber Officinale Roscoe*) does not affect *ex vivo* platelet thromboxane production in humans

Janssen PLTMK, Meyboom S, van Staveren WA, de Vegt F, Katan MB.
Consumption of ginger (*Zingiber Officinale Roscoe*) does not affect *ex vivo* platelet thromboxane production in humans.
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Abstract

Ginger (*Zingiber Officinale Roscoe*) has been claimed to exert an anti-thrombotic effect in humans as ginger extracts inhibit cyclo-oxygenase activity of platelets *in vitro*. Effects of ginger consumption on *ex vivo* platelet function, however, are contradictory. We therefore investigated whether daily consumption of raw or cooked ginger decreases platelet cyclo-oxygenase activity as assessed by *ex vivo* maximally stimulated platelet thromboxane B_2 production. We carried out a randomized placebo-controlled cross-over study of 3x2 weeks. Eighteen healthy volunteers aged 22±3 y (mean±sd) participated in the study; there were no drop-outs. Subjects consumed 15 g of raw ginger root, 40 g of cooked stem ginger, or placebo daily for 2 weeks each. We took fasted venous blood samples and measured thromboxane B_2 production in maximally stimulated platelet-rich plasma at days 12 and 14 of each treatment period. Mean decrease in thromboxane production relative to placebo was 1±9% for ginger root, and -1±8% for stem ginger, with no effect of treatment order (p=0.984). We cannot confirm the putative anti-thrombotic activity of ginger in humans.

Introduction

We are interested in natural aspirin-like factors in foods as such factors may partly explain the relatively low incidence of cardiovascular disease in oriental countries (1-3).

It has been suggested that ginger exerts an anti-thrombotic activity (4), because ginger extracts inhibit platelet aggregation and thromboxane B_2 production *in vitro* (5,6). However data on effects of ginger consumption on blood platelet function, and specifically on cyclo-oxygenase activity, are scarce and contradictive (7-10).

We therefore performed a study investigating the effect of daily consumption of raw ginger root or cooked stem ginger on platelet cyclo-oxygenase activity as assessed by maximally stimulated *ex vivo* platelet thromboxane production in healthy volunteers.

Subjects and methods

Subjects

We recruited 24 subjects through announcements in the University newspaper and posters in student dormitories. We excluded one subject because of elevated values for serum creatinine and alanine amino transferase, and one because of a marginally low value for mean red cell volume. We selected 9 men and 9 women at random from the remaining 22 subjects; all 18 volunteers completed the study.

Participants were healthy based on a medical questionnaire, none suffered from urinary protein or glucose, or high blood pressure. All had normal values for haematocrit, haemoglobin, mean red cell volume, erythrocyte sedimentation rate, plasma alanine amino transferase, gamma-glutamyl transferase, and creatinine, platelet count, thrombin time, prothrombin time, and activated partial thromboplastin time. The volunteers did not smoke and did not use any regular or homoeopathic medication. Mean age (±sd) was 22±3 years and body mass index was 22±2 kg/m^2.

We urged the participants not to consume any products containing ginger, or any fatty fish, and to maintain their normal eating and drinking habits and physical activity levels during the study. We instructed subjects to avoid all regular and homoeopathic medicines from 1 month preceding the study until the end of the study. We supplied them

with paracetamol which could be used as a pain medication. We urged subjects to record times of consumption of the supplements, health complaints, medications used, and any deviations from their normal physical activity and dietary habits in a diary.

The protocol was approved by the Medical Ethics Committee of the Department of Human Nutrition and was fully explained to the participants, who gave their written informed consent.

Methods

We investigated the effects of daily consumption of ginger root and stem ginger relative to placebo on *ex vivo* maximally stimulated platelet thromboxane production. We carried out a randomised multiple cross-over study of 3 consecutive 2-week periods during which each participant consumed all supplements in different order. All subjects participated simultaneously.

Supplements consisted of 125 g of vanilla custard (Coberco, Arnhem, The Netherlands), containing either 15 g of Brazilian ginger root (Toko Rinus, Nijmegen, The Netherlands), 40 g of stem ginger (Ambition, Polak Import, Rotterdam, The Netherlands), or no ginger as a placebo. The custard served to mask the pungent taste of ginger. Stem ginger and ginger root originated from two separate batches. We bought vanilla custard at a local supermarket every few days and preserved all products in the dark at 4^0 C. We prepared all supplements in a similar way during the whole study.

We prepared supplements with raw ginger each working day: we thinly peeled ginger roots, cut them into small pieces (Magimix, Micave BV, Utrecht, The Netherlands), and weighed out portions of 15 g. We prepared the placebo and stem ginger supplements 3 times a week. We rinsed the stem ginger, cut it into pieces, and divided it into portions of 40 g. We weighed out portions of 125 g of vanilla custard.

Participants came to the Department on working days to consume their supplements of raw ginger, and took their supplements home for the weekends on Fridays. We handed out the placebo and stem ginger supplements 3 times a week. We supplied all supplements in closed boxes containing cooling elements, and instructed all subjects to keep their supplements in a refrigerator and to mix the custard with the ginger immediately prior to consumption.

We weekly determined body weights of the participants using a digital scale (Berkel ED 60-T, Rotterdam, The Netherlands), checked the diary and measured food intake using a 24-h recall (11-12).

Venous blood was sampled using a butterfly needle system (Becton Dickenson, Meylan, France) after an overnight fast on days 12 and 14 of each treatment period while the participant was lying down. We discarded the first 3 mL of blood to prevent activation, and drew the next 18 mL slowly into 3.8% sodium-citrate tubes 1:10 *v/v* (Sarstedt, Etten-Leur, The Netherlands). We prepared platelet-rich plasma by centrifugation (Sigma, Osterode, Germany) at room temperature for 10 min at 200 *g*, removed the platelet-rich plasma, prepared platelet-poor plasma by centrifuging the residual blood for 15 min at 2000*g*, and normalized the platelet-rich plasma to $250*10^9$ platelets/L by adding autologous platelet-poor plasma. We counted platelets before and after dilution (Coulter, Coulter Corporation, Miami, FL) and stimulated 450 μL of normalized platelet-rich plasma with arachidonic acid (final concentration 1.5 mM) (Bio Data Corporation, Horsham, USA) in an aggregometer (37^0 C, 900 rpm; Payton Aggregation Module, Salm en Kipp, Breukelen,

The Netherlands). We then took out 50 µL of the aggregate exactly 10 min after the addition of arachidonic acid, added it to 950 µL buffer containing 9 g/L NaCl, 0.01 mol/L EDTA, 3 g/L bovine gamma-globulin, 0.005% Triton-X-100, and 0.05% sodium-azide in 50 mM phosphate buffer, pH 6.8 (NEN Research Products, Du Pont, Boston, MA), immediately submerged the samples in liquid nitrogen, and stored them at -80 °C until analysis. We measured thromboxane B_2 production in duplicate using a thromboxane B_2 [^{125}I] RIA kit (NEN Research Products, Du Pont, Boston, MA). We analyzed all samples from a particular subject within one run. Within-person variation over a 2-day period was 12% after placebo, 10% after raw and 8% after cooked ginger treatment.

Plasma of 2 healthy volunteers who had not used any medication for at least 1 month served as quality control for thromboxane measurements. We once sampled venous blood from these volunteers, and stimulated *ex vivo* platelet thromboxane production in platelet-rich plasma as described above. We stored aliquots of aggregates, and measured thromboxane B_2 production at every radio-immuno assay. Within-assay variation for thromboxane measurements was 9%; between-run variation was 9%.

We checked the data for normality using residual analysis (13). We averaged the 2 values of thromboxane production obtained for each subject on days 12 and 14 of each treatment period, and analyzed differences in thromboxane production, body weight, and intake of energy and macronutrients between treatments using the General Linear Models of the Statistical Analyses System (14) with subject and treatment as class variables; we used $p<0.05$ to indicate significant difference. We introduced a period term into the model to check for time effects, and a treatment-by-period interaction term to check for carry-over effects. Values in the text are means±sd.

Results

Daily treatment with 15 g of ginger root or 40 g of stem ginger for 14 d did not affect maximum *ex vivo* platelet thromboxane production ($p=0.616$). Mean thromboxane B_2 production was 2994±566 nmol/10^{11} platelets after treatment with raw ginger, 3044±546 after cooked ginger, and 3045±609 after placebo. The average effects on platelet thromboxane B_2 production relative to placebo were -1±9% (±sd) on raw ginger, and 1±8% on cooked ginger. There was neither a treatment sequence ($p=0.984$), nor a time effect ($p=0.932$).

The habitual diet of the subjects as measured by 24-h recall supplied on average 11±5 MJ/d (2629±1195 kcal/d, mean±sd), of which 32±9% was fat, 13±3% protein, 53±8% carbohydrate, and 2±3% alcohol. Mean body weight increased by 0.3±1.1 kg during the study. There were no changes in intake of macronutrients ($p=0.12$ for fat; 0.10 for carbohydrate; 0.73 for protein; 0.84 for alcohol) and energy ($p=0.23$) between treatment periods. Subjects did not consume any fatty fish. All participants consumed the raw ginger supplements under our supervision on working days and reported that they had consumed all other supplements at home. There was no evidence from the diaries of changes in physical activity patterns or any deviation that might have affected the results.

No adverse reactions to the supplements were reported. One subject took 7 tablets of paracetamol spread over 3 consecutive days because of toothache, and another took 2 tablets/d for 2 consecutive days because of a headache. One subject took 2x20 mg of temazepam during treatment with stem ginger, and 4x20 mg during treatment with ginger

root. Temazepam is a sedative and was taken because of a sleeping disorder unrelated to the study. All participants denied having used acetylsalicylic acid from 1 month preceding the study until the end of the study.

Discussion

We found that daily consumption of large amounts of ginger did not affect *ex vivo* platelet thromboxane production in healthy volunteers. Data from the diaries as well as data on food consumption did not reveal any confounding effects of medication, physical activity levels, or dietary patterns. We bought all ginger at once before the study to exclude differences between batches, stored the products in the dark at 4^0 C to diminish changes in composition of the ginger, and prepared the supplements in a standardized way (see Subjects and methods section) to prevent differences in composition of the supplements during the study. The compounds known to affect *in vitro* platelet activity (gingerols, shogaols, zingerone) generally exist in the fleshy centre of the rhizome (15); we used these parts of the ginger in the raw ginger supplements. We earlier showed in similar volunteers that even as little as 3 mg/d of acetylsalicylic acid decreased maximally stimulated *ex vivo* thromboxane B_2 production by 39±8% (16); we could thus calculate that the power of this study was 90% to pick up an effect of 6% or more.

Our data disagree with results of Dorso (7) and Verma *et al.* (10). Dorso (7) described a total inhibition of stimulated *ex vivo* platelet aggregation after consumption of large amounts of ginger-grapefruit marmalade; this study was done in only one subject, and the result may be due to chance. Verma *et al* (10) showed that consumption of butter increased platelet aggregation induced by adenosine diphosphate (ADP) and epinephrine, and that this increase was neutralized by simultaneously administering 5 g of powdered ginger for 7 d. Our results are in accordance with *ex vivo* results of Lumb (8) and Srivastava (9). Lumb (8) found no effects of consumption of 2 g of dried ginger on bleeding time and whole blood platelet aggregation. Platelet aggregation and bleeding time measurements, however, are much less specific for determination of platelet cyclo-oxygenase activity than measurement of platelet thromboxane production; results of Verma *et al* (10) may indicate that ginger consumption affects secretion in platelets (17). Lumb (8) could have missed possible long-term effects of ginger consumption, as he studied effects of only a single dose. It is highly unlikely that we missed possible long-term effects, as blood platelets have a mean life-time of 7-10 d. This indicates that our daily treatment with high doses of ginger during 14 d must have been long enough to trace effects on platelet thromboxane production. Srivastava (9) found no significant effect of consumption of 5 g/d of raw ginger for 7 d on serum thromboxane production in 7 healthy volunteers, but the power of Srivastava's study was too low to pick up relevant effects due to the large variation in the outcome variable.

We fed the participants 15 g of raw ginger root or 40 g of stem ginger. These are very high doses. Consumption of higher doses is impractical because of the pungent taste. If any inhibitors of cyclo-oxygenase activity are present in ginger they are probably not absorbed or are rendered ineffective during first-pass metabolism in the body. We think it is unlikely that daily consumption of raw or cooked ginger affects platelet cyclo-oxygenase activity. However, it remains worthwhile to search for other possible aspirin-like agents in foods.

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Chapter 8

Conclusions

In this chapter the answers to the questions listed in ***Chapter 1*** will be given, conclusions will be drawn and suggestions for future research will be given.

Acetylsalicylic acid, haemostasis and thrombosis

We showed that even 3 mg/d of acetylsalicylic acid (the amount previously thought to be present in a normal mixed Western diet) decreased *ex vivo* thromboxane B_2 formation in healthy subjects (***Chapter 2***). Based on these results we concluded that quantitative data on acetylsalicylate in foods and their possible anti-thrombotic effects deserved closer investigation.

The total salicylate contents we found were, however, only 0-1 mg/kg in vegetables and fruits, and 3-28 mg/kg in herbs and spices. Acetylsalicylate contents were lower than the limit of detection (0.02 mg/kg for fresh products and 0.2 mg/kg for dried products) in all foods studied (***Chapter 3***).

We found that urinary excretion was a valid indicator for the intake of pure acetylsalicylic and salicylic acid. Median 24-h salicylate excretion in subjects eating a wide variety of diets was low: 1.4 mg (range 0.8-1.6; median 10 μmol, range 6-12). Values increased with the fibre content of the diet, suggesting that vegetable foods are the main sources of bio-available dietary salicylates (***Chapter 4***).

Based on the food analyses (***Chapter 3***) and the urinary excretion data (***Chapter 4***) we estimate that even pure vegetable diets provide less than 6 mg/d (43 μmol/d) of total salicylates. If most of this were in the form of acetylsalicylate, which is highly unlikely, these intakes are probably too low to affect coronary heart disease risk (1-2). It was recently suggested that dietary salicylate intake might have contributed to the decline in cardiovascular disease mortality in the USA (3). Those calculations were however based on salicylate contents published by Swain *et al* (4-5). We think (***Chapter 3-5***) Swain *et al*'s data highly overestimate dietary salicylate intake. On the other hand, our data (***Chapter 3-5***) suggest that worries about the adverse effects of dietary salicylates on, for example, the behaviour of children may be unfounded (6) (***Chapter 5***).

Flavonoids, haemostasis and thrombosis

Concentrations of 2.5 μM of the flavone apigenin inhibited collagen- and ADP-induced platelet aggregation *in vitro*, whereas the flavonols quercetin and quercetin-3-glucoside had no effect. We found no effects of daily consumption of large amounts of onions (quercetin-) or parsley (apigenin-rich) on platelet aggregation, thromboxane production, or other haemostatic parameters in healthy subjects.

We conclude that claims for the anti-aggregatory effects of flavonoids (7-8) are based on the *in vitro* use of concentrations (9-11) that cannot be attained *in vivo* (12). Our *in vitro* and *ex vivo* findings suggest that reported effects of dietary flavonoids on coronary vascular disease risk are probably not mediated through collagen- or ADP-induced platelet aggregation or cyclo-oxygenase activity. We might have missed small biologically relevant effects on known risk indicators for coronary heart disease from the coagulation cascade or the fibrinolytic system. This should be examined in a larger population (***Chapter 6***).

Ginger, haemostasis and thrombosis

In our study consumption of raw or cooked ginger did not affect *ex vivo* platelet thromboxane production in healthy volunteers. The test used did not confirm the putative anti-thrombotic effect of ginger consumption in humans (13-15).

We think consumption of higher doses than we used in our study is impractical because of the pungent taste. If any inhibitors of cyclo-oxygenase activity are present in ginger, they are probably not absorbed or are rendered ineffective during the first pass metabolism in the body (***Chapter 7***).

Conclusions

The main question of this thesis was: ***Do dietary non-nutrients (salicylates, flavonoids, ginger-components) affect haemostatic parameters in humans?*** Our answer to this question is: we estimate (based on the salicylate analyses in foods and our salicylate excretion data) that the contents of acetylsalicylate in diets is nil or negligible, which is probably too low to affect coronary heart disease risk. Besides, the tests used did not confirm the putative anti-thrombotic effects of consumption of ginger and flavonoid-rich foods in healthy subjects.

There is reason to believe that the separation and detection methods Swain and coworkers used (4-5) were not specific enough, and that some other components may have co-eluted with salicylic acid, leading to an overestimation of dietary salicylate intake. The salicylate contents we found, using a highly specific and sensitive method, were 10-100 times lower and comparable to values found by Robertson and Herrmann (16-19). Although Robertson and Herrmann published their salicylate data during 1981-1990 in peer-reviewed international journals, only the data of Swain *et al* (4-5) were mentioned in a recent review on the salicylate contents of foods and their putative effects on health (20). We conclude from this that the belief in the presence and effects of foods high in salicylates is firmly held, but unjustly so. A possible explanation for this strong belief in the results of Swain *et al* is that their data were published in the Journal of the American Dietetic Association (4), which may be frequently read by dieticians, nutritionists, and physicians. In contrast to this, the data of Herrmann and Robertson were published in journals with main topics on food analyses and food composition (16-19). It is likely that these journals are mainly read by food scientists or analytical chemists. Furthermore, one of Herrmann's articles (17) was written not in English but in German, which might have diminished the accessability of the information.

Based on our findings we also conclude that worries about the adverse effects of dietary salicylates on, for example, the behaviour of children may be unlikely (6) (***Chapter 3-5***).

Suggestions

Our data on the salicylate contents of foods should be added to salicylate databanks and used in advice on diet and hyperactivity. I suggest that researchers publish their data in peer-reviewed English journals, frequently read by the people they want to reach to increase the chance that their message reaches the proper audience.

Information on the metabolism of flavonoids in humans needs further investigation

to further understanding their possible effects on health (7-8). Possible effects of dietary flavonoids on coagulation or fibrinolytic parameters should be examined in a larger study. Claims for anti-aggregatory effects of flavonoids (7-11) are based on the *in vitro* use of concentrations that cannot be attained *in vivo* (***Chapter 6***) (12). Therefore we suggest that studies on dietary effects on human health should not be limited to *in vitro* experiments only, but also carried out in humans as much as possible. We recommend the use of physiological conditions, especially of *in vitro* studies to increase the generalizability of the results. In addition we would like to stress the importance of standardization of analyses of haemostatic parameters to increase the comparability of studies. The latter is extremely important as analyses of haemostatic parameters are prone to interferences and as a golden standard for measuring haemostasis is absent yet.

As coronary heart disease is the most important cause of death and disease in the Western world (21), it remains worthwhile to search for possible anti-thrombotic compounds in foods.

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Samenvatting

Hart- en vaatziekten, zoals een hartaanval en beroerte, zijn een groot probleem voor de gezondheid in welvarende landen. Het dagelijks slikken van kleine hoeveelheden acetylsalicylzuur, de werkzame stof in aspirine, helpt hart- en vaatziekten te voorkómen. Bloedplaatjes, gespecialiseerde bloedcellen, spelen hierbij een belangrijke rol. Ze helpen bij de bloedstolling, dus het afsluiten en herstel van wondjes. Als bloedplaatjes echter te veel aan elkaar, of aan de wand van een bloedvat plakken, kunnen ongewenste bloedpropjes ontstaan. Dit kan leiden tot verstoppingen van het bloedvat en dus tot hart- en vaatziekten. Acetylsalicylzuur remt het cyclo-oxygenase in de bloedplaatjes. De bloedplaatjes maken dan minder thromboxaan en plakken daardoor minder aaneen. Acetylsalicylzuur voorkomt zo verstoppingen in een bloedvat en daarmee het ontstaan van hart- en vaatziekten.

Aspirine-achtige stoffen komen mogelijk ook van nature in voedingsmiddelen voor. Een voeding rijk aan deze stoffen zou dan kunnen helpen om hart- en vaatziekten te voorkómen. Australische onderzoekers schatten dat een normale Westerse voeding per dag 3 mg acetylsalicylzuur bevat, maar exacte hoeveelheden waren onbekend (***Hoofdstuk 1***). Wij vonden dat de thromboxaanproductie door bloedplaatjes van gezonde mensen daalde na het slikken van 3 mg acetylsalicylzuur per dag (***Hoofdstuk 2***). Dit kan erop wijzen dat zelfs een kleine hoeveelheid aspirine helpt om hart- en vaatziekten tegen te gaan. Wij bepaalden daarom de hoeveelheid acetylsalicylzuur in Nederlandse voedingsmiddelen. Ook werd de hoeveelheid salicylzuur, de moeder-verbinding van aspirine, gemeten. Salicylzuur zou mogelijk hyperactiviteit of overgevoeligheid veroorzaken. We vonden geen acetylsalicylzuur in de onderzochte voedingsmiddelen. De hoeveelheid salicylzuur was laag: 10-100 keer lager dan de Australiërs vonden. Hun methode was echter minder specifiek en nauwkeurig (***Hoofdstuk 3***). Vervolgens slikten 6 mensen 10 mg acetyl- of salicylzuur. We vonden hiervan 80% terug in urine, verzameld gedurende 24 uur. De hoeveelheid in urine lijkt dus een goede maat voor de inname. We vonden dat mensen met uiteenlopende voedingsgewoonten slechts een paar mg salicylzuur per 24 uur uitplasten (***Hoofdstuk 4***). Dit wijst erop dat een mens via voeding hooguit enkele mg acetyl- of salicylzuur per dag binnenkrijgt, waarschijnlijk te weinig om de gezondheid te beïnvloeden (***Hoofdstuk 5***).

Er waren aanwijzingen dat flavonoïden, natuurlijke stoffen in plantaardige voedingsmiddelen, beschermen tegen hart- en vaatziekten door de thromboxaan-vorming en de klontering van bloedplaatjes te remmen. We namen bloed af bij gezonde mensen en voegden hieraan flavonoïden toe in concentraties zoals ze ook in het lichaam voorkomen. Het flavonoïd apigenine ging het aan elkaar plakken van bloedplaatjes tegen, het flavonoïd quercetine had geen effect. Vervolgens aten 18 gezonde mensen per dag 220 g ui of 4,9 g gedroogde peterselie. Ui bevat veel quercetine en peterselie veel apigenine. We vonden geen effecten op het aan elkaar plakken van de bloedplaatjes en de thromboxaan-vorming (***Hoofdstuk 6***). Flavonoïden beïnvloeden dus mogelijk niet de activiteit van bloedplaatjes.

Gember vermindert mogelijk ook de activiteit van bloedplaatjes. Wij vonden echter geen effect op de thromboxaan-vorming bij 18 gezonde vrijwilligers die dagelijks grote hoeveelheden rauwe of gekookte gember aten gedurende 2 weken (***Hoofdstuk 7***).

Wij concluderen (***Hoofdstuk 8***) dat de hoeveelheid (acetyl)salicylzuur in voeding waarschijnlijk te laag is om hart- en vaatziekten te voorkomen of om hyperactiviteit en overgevoeligheid te veroorzaken. We konden niet aantonen dat het dagelijks eten van veel gember, ui en peterselie eenzelfde beschermend effect heeft als acetylsalicylzuur.

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Publications

Publications: peer-reviewed

1. Janssen PLTMK, Hollman PCH, Venema DP, van Staveren WA, Katan MB. Salicylates in foods (Nutr Rev, in press) (this thesis).
2. Janssen PLTMK, Katan MB, van Staveren WA, Hollman PCH, Venema DP. Acetylsalicylate and salicylates in foods. Abstract (Cancer Letters, in press).
3. de Vries JHM, Janssen PLTMK, Hollman PCH, van Staveren WA, Katan MB. The consumption of quercetin and kaempferol in free-living subjects eating a variety of diets. (Cancer Letters, in press).
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Curriculum Vitae

Petronella Louise Theodora Maria Karin Janssen was born in Meerlo, the Netherlands on June 19, 1969. She passed secondary school, Gymnasium B, at the "Boschveld College" in Venray in 1987. That same year she started studying "Human Nutrition" at the Wageningen Agricultural University. She obtained the MSc-degree in Human Nutrition in March 1993, with main topics Human Nutrition, Physiology, and Clinical Nutrition. In 1993 she obtained the Wageningen "Scriptie-prijs" for her doctoral thesis in Physiology entitled "Effect of marginal iodine deficiency on thyroid function and deiodinase activities in rats." Since March 1993 she has been working as a PhD student at the Department of Human Nutrition. Her project was entitled "Possible presence of inhibitors of cyclo-oxygenase in foods and the effects on platelet aggregation and platelet thromboxane production in man." In July 1994, she attended the Annual New England Epidemiology Summer Program, a 3-week course at Tufts University, Boston, USA, and she was officially registered as "Epidemioloog A" by the Netherlands Epidemiology Society in 1995.

ASPIRINE

Tekst/Muziek: Dirk Witte©

Als je op de tafel kijkt,
Van den dokter, van den dokter,
Wat daar zoo voor deftigs prijkt;
Als je op de tafel kijkt,
Is het eerste wat we zien:
Aspirine, aspirien!
Aspirine voor je beenen,
Aspirine voor je buik,
Tegen blaren op je teenen,
En als je je pols verstuikt.
Aspirine voor je armen,
Voor je nek en voor je darmen,
De soldaatjes, één voor één,
Gaan met aspirine heen.

's Morgens staan ze kwart voor acht
Voor den dokter, voor den dokter,
Alles wat maar moet op wacht,
Staat al klaar om kwart voor acht;
Negen krijgen van de tien:
Aspirine, aspirien!
Aspirine voor de goeierds
Na een slapeloozen nacht,
Aspirine voor de knoeierds,
Die verlangen: "vrij van wacht!"
Aspirine alleen kan baten
Voor of'cieren en voor soldaten,
Voor fourier en voor sergeant,
En voor 't paard van d'Adjudant.

Als we weer eens burger zijn,
Gaan we nooit meer naar den dokter,
Zelf genezen w'alle pijn.
Als we eerst maar burger zijn
Koopen w'elk een pond of tien
Aspirine, aspirien!
Aspirine voor je oudje,
Aspirine voor je hond,
Aspirine voor je vrouwtje,
Als er weer een kleintje komt.
Aspirine zal niet hind'ren
Voor je kanarie en voor je kind'ren;
Dokters kan ik niet meer zien:
Ik zweer trouw aan d'aspirien.

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