Assessment of flavonoid and fatty acid intake by chemical analysis of biomarkers and of duplicate diets

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Stellingen

1. Gehaltes van flavonolen in plasma en urine zijn geschikte indicatoren om mensen met een lage en een hoge inneming van deze stoffen uit de voeding van elkaar te kunnen onderscheiden. *Dit proefschrift.*

2. De bestaande voedselfrequentiemethodes zijn niet geschikt om de inneming van flavonoïden nauwkeurig vast te stellen. Mogelijke verbanden tussen de inneming van flavonoïden, vastgesteld met deze methodes, en het voorkomen van ziekten zijn daarom moeilijk aan te tonen. *O.a. dit proefschrift.*

3. Rode wijn is geen goede bron van biobeschikbare flavonolen. Daarom kan inneming van deze stoffen met rode wijn *de Franse paradox* niet verklaren.
Dit proefschrift.

4. Het afwijzen van methodes voor het vaststellen van de voedselconsumptie alleen omdat ze te duur zijn, kan worden aangemerkt als zuinigheid die de wijsheid bedriegt.

5. Biologische indicatoren zullen een belangrijke methode zijn voor het meten van de voedselconsumptie in een toekomstig Europa zonder grenzen, omdat ze objectieve vergelijking tussen studies in verschillende landen beter mogelijk maken dan andere voedselconsumptiemethodes. O.a. *P. van 't Veer et al. Eur J Clin Nutr 1993; 47, Suppl2, S58-S63.*

6. Met uitzondering van de te onderzoeken voedingsstof heeft een goede proefvoeding niet alleen precies dezelfde samenstelling als de controlevoeding qua voedingsstoffen, maar ook qua voedingsmiddelen.

7. Naarmate experimenten beter zijn opgezet wordt de kans op het optreden van het placebo-effect groter.

8. Paramedici, zoals verloskundigen, fysiotherapeuten en diëtisten, zouden verplicht moeten worden hun therapieën op een wetenschappelijk verantwoorde wijze te evalueren, omdat van sommige behandelingsmethodes schadelijke neveneffecten niet uit te sluiten zijn.

9. Er zal meer aandacht moeten komen voor het ontwikkelen van aantrekkelijke gemakkelijk vol te houden diëten als therapie voor cholesterolverlaging, zodat het maximaal mogelijke effect kan worden gehaald. *D. Kromhout, Diet-heart issues in a pharmacological era. Lancet 1996;348:S20-S22.*

10. Korter werken is beter voor de economie.

11. Pas als niet alleen 's morgens evenveel vaders als moeders hun kinderen naar school brengen, maar ook 's middags ouders van beide sexen evenredig vertegenwoordigd zijn voor het halen van de kinderen zal de emancipatie van mannen en vrouwen een feit zijn.

12. Het belangrijkste resultaat verkregen bij het bereiken van het einde van de weg is het plezier waarmee je hem hebt afgelegd.

Stellingen behorend bij het proefschrift

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Jeanne H.M. de Vries
Wageningen, 6 februari 1998

Ieder mens wil leren

Ter nagedachtenis aan mijn vader

Abstract

Assessment of flavonoid and fatty acid intake by chemical analysis of biomarkers and of duplicate diets

PhD thesis, Jeanne H.M. de Vries, Division of Human Nutrition and Epidemiology, Wageningen Agricultural University, the Netherlands. February 6, 1998

Dietary intake is important to investigate the relationship between diet and the occurrence of disease. However, it is difficult to assess the intake of nutrients such as flavonoids, minor fatty acids and plant sterols because the data on these nutrients in food composition tables are insufficient or because the bioavailability of these nutrients differs between foods. The results of studies investigating the relationship of these nutrients to disease are inconsistent, perhaps because of errors in the methods used to assess nutrient intake. The aim of this thesis was to evaluate physically and chemically based methods of measuring the intake of flavonoids, fatty acids, sterols and energy.

We found differences of up to 80 mg per day in the intake of flavonols and flavones in subjects eating a variety of diets. The ratio of the within- to the between-subject variation in the intake of flavonols and flavones was lower than one, indicating that it is possible to study the relationship between flavonoid intake and disease. The food frequency questionnaire used in this study was suitable for classifying subjects by their flavonol intakes.

We also found that the bioavailability of the flavonol quercetin differs between the major dietary sources. The bioavailability of quercetin from red wine was 75% of that from onions and from tea 50% of that from onions. Therefore, flavonols from red wine can probably not explain the lower incidence of coronary heart disease in France compared to other western countries. Concentrations of quercetin in plasma can be used as biomarkers to distinguish between subjects with a low and with a high flavonol intake. This is possible because of a relatively small variation of plasma quercetin and a linear relationship between quercetin intake to its concentrations in plasma and excretions in urine.

Chemical analysis of food composites is a suitable method to assess the intake of a large number of fatty acids and sterols for subsamples of populations. We found large differences in the amount of these nutrients in the diets of middle-aged men living in 16 cohorts in seven countries. Three-day records are not suitable to measure individual energy intake, but they can be used to classify subjects by their energy intakes.

In conclusion, the most feasible method to assess flavonol intake in epidemiological studies appears to be a food frequency questionnaire specially devised to assess flavonol intake and validated in a sub-population by biomarkers of flavonol intake. In addition, chemical analysis of food composites is a good tool to assess specific fatty acids and sterols in the diet of a population. Finally, when using 3-day food records, even from a motivated, lean, well-educated population, underestimation of intakes has to be taken into account.

Contents

1

General introduction

Introduction

Diet plays an important role in the cause and prevention of various diseases [1-3]. Examples are the intake of fatty acids in relation to coronary heart disease [4,5], and the intake of vegetables and fruit in relation to the occurrence of cancer [6]. However, the outcomes of epidemiological studies sometimes conflict. As dietary assessment plays a crucial role in these studies, one of the major problems may be the imprecision of methods to assess dietary intake [7]. Imprecise methods show large variances in intake within subjects relative to actual variances in intake between subjects. Imprecise methods could cause subjects to be classified wrongly by their nutrient intakes. As a result, correlation coefficients or relative risks in relation to disease could be attenuated, and relationships that are actually present could not be demonstrated. Methods to assess dietary intake differ in concept and technical application. Thus, it is important that the measurement errors of each method are understood in order to choose the best methods for different purposes and target groups [7,8].

Methods of dietary assessment

Dietary assessment methods can roughly be divided into methods assessing current diet, consisting of records and 24 h recalls, and methods assessing habitual diet, consisting of dietary histories and food frequency questionnaires. The intake data derived by these methods, combined with analytical data from food composition tables or from chemical analysis, result in individual nutrient intakes. In addition to these methods, some biological indicators of dietary exposure have been developed [9].

For all methods it is important that the measurement error or variability of the method is not too large compared to the actual variability in intake between subjects. All methods suffer from measurement error, both systematic and random error, but the scopes of error differ between the various methods. Sources of error include underreporting of intake [10], incorrectly estimated portion sizes [11], and missing or inaccurate nutrient data in food composition tables.

In selecting a method it is important to know what type of information is required: is this information about individual or group intake, about foods or food groups, about all nutrients or only specific ones? Based on the literature, Beaton et al. [12] suggested distinguishing four approaches to analyses which each require a different type of information. These approaches are:

1) estimation of mean intakes of a group or comparison of mean intakes across categories of individuals (for example by ethnicity),
2) distributional analyses, for example to assess the proportion of individuals with inadequate or excessive intakes,
3) correlation and regression analyses to assess relations between intake levels and outcome measurements,
4) categorical analyses to categorize individuals by intervals of intake and by the occurrence of disease, as is common in epidemiological studies.

As Beaton indicated [12], systematic error is acceptable in all but the second approach as long as the only question is "Is there a relationship?" and not "What is the relationship?".

This thesis evaluates the measurement errors of several methods to assess the intake of flavonoids, fatty acids, sterols, and energy. These methods are food records, a food frequency questionnaire, biological indicators of intake and chemical analysis of food composites.

Assessment of flavonoid intake

Flavonoids are non-nutrients in plant foods. More than 4000 flavonoids have been determined until now [13]. Some flavonoids appear to have antioxidative properties [14], which could explain their beneficial effect on the occurrence of coronary heart disease. Flavonoids are also reported to have anticarcinogenic properties. Further, effects on the occurrence of cancer or cardiovascular disease of some flavonoid-rich foods such as onions [15], tea [16], and red wine [17] have been reported. However, it is uncertain whether flavonoids were responsible for these effects [16].

Flavonoids include flavonols, flavones, catechins, and anthocyanidins [13]. Flavonols are a major compound in the diet and they appear to be strong antioxidants *in vitro* [18]. Their major representatives in the diet are quercetin and kaempferol. The amount of another flavonol, isorhamnetin, in the diet is rather low, but it is formed in the body from quercetin. Flavonols in the diet are mainly derived from tea, onions, apples and red wine [19]. Some other types of vegetables or fruit, such as leek and berries, also contain high amounts of flavonols.

The intake of flavonols is in the range of most vitamins, but large differences between countries exist (Table 1.1). The intake of flavonols and flavones in the Netherlands is comparable to that in the United States [20] and in the United Kingdom [21]. However, the intake of flavonols and flavones in Finland is much lower [22]. Flavonol intake measured in the Seven Countries Study confirmed that differences in intake may be large. In the Netherlands [19], quercetin contributes about 70% and kaempferol 20% to the intake of flavonols and flavones.

The relation between the intake of flavonols and the occurrence of disease is sometimes conflicting. Some studies show an inverse relationship between intake and the occurrence of coronary heart disease [22-25], whereas others do not [20,21]. One of the major explanations could be that the methods used to assess flavonol intake were not suitable [26]. In studies that showed a relationship, flavonoid intake was assessed by a dietary history method or by sampling of food composites. In studies that showed no effects, flavonoid intake was assessed by a food frequency questionnaire. The use of standard portions, instead of estimated or weighed portions, in the food frequency questionnaires to estimate the amount of flavonol-rich foods could have been inaccurate. Also, foods important for flavonol intake might have been lacking in the questionnaires. However, a food frequency questionnaire is a more feasible method to assess flavonol intake than is a dietary history method: the number of foods in such a questionnaire could be small, as few foods contribute to flavonol intake.

It may however be questioned, whether recall and record methods are in any case suitable to assess flavonol intake. Flavonol intake assessed by these methods may cause

Table 1.1 *Summary of dietary assessment methods and flavonol and flavone intake in epidemiological prospective studies*

Population	number of subjects n	gender m/f	age y	dietary method*	flavonol and flavone intake mg/d
Zutphen[23];1993 (the Netherlands)	805	m	65-84	history	25.9 ± 14.5 (mean ± SD)
Finland[22];1996	5133	m/f	30-69	history	0 - 41.4 (range) 3.4 (median)
Health Professionals[20]; 1996 (USA)	34789	m	40-75	frequency	20.1 (mean) 7.1 - 40.0 (medians 1th - 5th quintile)
Caerphilly[21];1997 (UK)	1900	m	49-59	frequency	26.3 ± 12.5 (mean ± SD)
Zutphen[24];1996 (The Netherlands)	552	m	50-69	history	23.5 ± 7.6 (mean ± SD)
Seven Countries Study[25];1995	12763	m	40-59	total diet	2.6 - 68.2 (range)

* history = dietary history method; frequency = food frequency questionnaire; total diet = chemical analyses of food composites based on food records.

specific problems. One problem is that flavonols are concentrated in a few foods. Therefore wrongly estimated portion sizes of these foods could easily lead to a large discrepancy between estimated and actual flavonol intake. Another problem is that the bioavailability of flavonols differs between dietary sources. For instance, the bioavailability of quercetin from apples is one third of that from onions [27]. Finally, although many foods have already been analyzed for flavonol content, the flavonol composition of many foods, especially ready-to-eat foods, is unknown. These foods may contribute substantially to flavonol intake because they often contain onions. Also, there may be large variations in the flavonol content of the same foods [28].

A biochemical indicator for flavonol intake could possibly overcome some of the above problems. The principle of this method is that the nutrient of interest or a metabolite is measured in a body fluid or tissue, such as blood or urine samples, but also toenails, hair and depot fat. A biochemical indicator may be used to assess intake in epidemiological and nutritional studies and to validate other methods of dietary assessment [9,29,30]. A biochemical indicator should be sensitive to intake within the range of habitual consumption, reflect

long-term intake, and preferably is related linearly to intake [9,30]. A major disadvantage of biological indicators is that they do not characterize the diet. Also, the variability of the level of the marker within subjects, superimposed on laboratory errors, may seriously reduce markers' usefulness. Flavonols have been measured in the plasma and urine of subjects after consumption of single foods [27,31] by a method developed by Hollman [32]. Thus, concentrations of flavonols in plasma and excretion in urine might be used as biomarkers of flavonol intake.

Assessment of fatty acids and sterols

The intake of fatty acids and sterols is related to the risk of coronary heart disease [33-35] and to certain types of cancer [2]. Saturated fatty acids, trans fatty acids and cholesterol in the diet affect blood lipids, which increases the risk of coronary heart disease. Yet, unsaturated fatty acids have effects related to a lower risk of coronary heart disease [2]. However, not all types of fatty acids within a cluster of saturated or unsaturated fatty acids have the same effects [34,36-38]. For instance, the saturated fatty acids myristic and palmitic acid clearly increase blood lipids whereas stearic acid, which is also a saturated fatty acid, has no or little effect [37]. Also, trans-unsaturated fatty acids show adverse effects [39-43], whereas effects of cis-unsaturated fatty acids are thought to be beneficial. In addition, a high intake of *n*-3 fatty acids, a subgroup of the polyunsaturated fatty acids, may reduce coronary mortality, whereas *n*-6-fatty acids [44,45], another subgroup, show no effect. Finally, the association of intake with coronary heart disease may differ between the plant sterols [46]. Thus, to achieve more information about the effects of single fatty acids and sterols in relation to disease, they have to be determined separately in the diet. Otherwise, fatty acids and sterols with opposite effects are investigated in one score, and existing relationships will not become clear.

The major sources of fat and sterols in the diet are edible fats and oils, meat, dairy products, and commercial and fast food products. There is a large difference between these foods in the type of fat present. For example, the composition of fats may differ considerably between processed foods [47] because various fats and oils may be used in their manufacture.

Fatty acids and sterols in the diet have been measured by various dietary assessment methods. Food disappearance data have often been used but they are only indirectly related to actual intake. Also, record and recall methods are often recommended to assess fat intake, but they are not suitable to measure single fatty acids and sterols. The major reason is that these methods need food composition tables, and most tables have no data on specific fatty acids and sterols or the data are insufficient or inaccurate. Methods that do not need food composition tables are biological indicators and chemical analysis of food composites.

Biological indicators of fatty acids have already been developed and validated. Certain fatty acids in blood, urine or fat tissue may be used as indicators of intake [48-52]. However, they are not available for all important fatty acids or for sterols. Also, chemical analysis of food composites has already been successfully applied to assess the intake of

fatty acids [53]. This method chemically analyses fatty acids and sterols in a food composite. This composite consists of all food items which are collected, based on the known average dietary intake of the population at large or at risk [54]. An advantage of this method is that information is derived from real foods. Disadvantages are that the accuracy of intake data relies on that of reported data and that no information is available about the ranges in individual intake. Thus, chemical analysis of food composites successfully determines the presence of more single fatty acids and sterols than do methods needing food tables [53], but only for a group.

Assessment of energy intake

Energy intake is often assessed by record or recall methods. Using food records, which are often applied in studies, subjects record the type and portion size of all foods they eat. They estimate portion sizes or weigh the foods on a scale. Food records for several days have often been regarded as the most accurate method to determine dietary intake. However, studies performed during the last ten years have shown that this method also suffers severely from underreporting [10,55]. Obese subjects [56,57], women, and older persons in particular appear to underestimate their intake [58]. In addition, subjects need a certain grade of literacy to provide adequate self-reports by records. In line with this, non-obese adults in western countries have provided the best reports [10].

Aim of the thesis

There is increasing doubt about the validity of recall and record methods including food records. These methods are not precise enough to assess the intake of nutrients and non-nutrients lacking data in food composition tables, or whose bioavailability differs between the most important sources in the diet. Also, these methods are not precise enough to assess nutrients that have a large day-to-day variation. Methods that are possibly better qualified to assess the intake of these nutrients are biochemical indicators of intake and chemical analysis of food composites. The aim of this thesis was ***to evaluate physically and chemically based methods to assess the intake of certain nutrients lacking accurate data in food composition tables or nutrients whose bioavailability differs between foods***. To that end, we selected and evaluated methods to assess the intake of flavonoids, fatty acids, sterols, and energy. This thesis focused on the following sub-questions:

- How large are the within- and between-subject variations of flavonol intake as reported in food records or in a food frequency questionnaire by free-living subjects (Chapter 2)?
- Is it necessary to account for differences in the bioavailability of flavonols from tea, red wine and onions (Chapter 3 and 4)?
- Are flavonols in plasma and urine useful biomarkers of flavonol intake (Chapter 4 and 5)?
- Can chemical analysis of food composites identify differences in the intake of fatty acids and sterols between populations (Chapter 6)?
- Do food records provide valid estimations of energy intake in young, non-obese, and well-educated adults (Chapter 7).

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2

Consumption of quercetin and kaempferol in free-living subjects eating a variety of diets

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Abstract

Quercetin and related flavonoids are anticarcinogenic in rats, but little is known about human intakes. The intake of five major flavonols and flavones was calculated using 1-day dietary records of 17 volunteers from 14 countries, and using both 3-day records and a food frequency questionnaire of eight Dutch adults. Total consumption (± SD) was 27.6 ± 19.5 mg/d in the international subjects, 34.1 ± 31.2 mg/d in the Dutch adults according to 3-day records, and 41.9 ± 23.7 mg/d according to questionnaires. Quercetin contributed 68-73%, and kaempferol 22-29%, the major sources being tea and onions. A brief food frequency questionnaire may be a suitable method for ranking individuals by flavonol intake.

Introduction

Flavonoids, a group of polyphenolic compounds with antioxidant properties, are widely distributed in foods of plant origin such as vegetables, fruit, tea and wine [1,2]. It has been suggested that the consumption of flavonols and flavones, a subgroup of the flavonoids, would protect from cancer [3]. Furthermore, beneficial associations with the occurrence of coronary heart disease have been reported [4].

Within the subgroup of flavonols and flavones the flavonol quercetin is the major compound in foods, with smaller contributions from kaempferol, myricetin, and the flavones apigenin, and luteolin [5]. The main sources of flavonols and flavones are tea and onions [5]. The most important flavonol in onions is quercetin [1], whereas tea contains considerable amounts of both quercetin and kaempferol [2].

In order to study the relation of flavonoids in the diet with chronic diseases a method for ranking of individuals by flavonoid consumption is needed. For evaluation of dietary assessment methods information is needed about the main dietary sources and the within- and between-subject variations. For these purposes, we calculated the intake of the five flavonols and flavones from three existing datasets, obtained with 1-day records, 3-day records, and a food frequency questionnaire.

Methods

Subjects and design

Thirteen non-resident aliens and 4 Dutch subjects consuming non-traditional diets enroled in the "international" part of the study. These participants, 6 men and 11 women from 14 countries and 4 continents, ate a wide variety of diets (Table 2.1). Their mean age was 29 ± 7 (±SD), and their body mass index 21 ± 3 kg/m^2. Four female and 4 male Dutch subjects, aged 25 ± 9 y (mean ± SD), with a body mass index of 23 ± 5 kg/m^2 consumed a normal Dutch diet (Table 2.2). All subjects were healthy and gave their written informed consent for participation in the study. The protocol was approved by the Medical Ethics Committee of the Department of Human Nutrition.

Dietary assessment

The 17 "international" subjects weighed and recorded all foods and beverages, including herbs and spices, in a diary for 1 day. The 8 Dutch subjects recorded their foods and beverages in household measures for 2 weekdays and 1 weekend day, and filled out a 74-item food frequency questionnaire which specifically asked about the habitual consumption of flavonoid-rich foods such as vegetables, fruit, beverages, ready-to-eat meals containing onions, herbs, and spices. Trained dieticians checked the food records and the questionnaires.

The intake of 5 flavonols and flavones (quercetin, kaempferol, myricetin, luteolin, and apigenin) was calculated using published values for contents in vegetables, fruit and beverages [1,2], supplemented with values for other Dutch foods including herbs and spices, and some American and Italian foods, also determined at the RIKILT-DLO laboratory

(Hollman P.C.H, unpublished data), using an identical method [6]. We calculated energy and dietary fibre intakes using the computerized version of the Netherlands Nutrient Data Bank NEVO [7]. We assessed the between and within subject variations by analysis of variance using SAS procedure ANOVA [8].

Results

In the 17 international subjects, intake of energy was 9.0 ± 3.1 MJ (mean ± SD), and mean intake of dietary fibre was 1.7 g/MJ of energy. The lowest consumption of the 5 flavonols and flavones was 3.6 mg/d for the subject with a South-American diet, whose single source was a small amount of onions (Table 2.1). The highest value was 77 mg/d for the subject with a Scandinavian diet, who drank almost 2 litres of tea on the day records were kept. The between-subject coefficient of variation was 70%. The mean contribution of quercetin to the total flavonol plus flavone consumption was on average 73%, of kaempferol 22%,

Table 2.1. *Flavonol and flavone intake in subjects eating a variety of diets according to a 1-d weighed food record, including herbs and spices.*

Subject	Country of origin	Type of diet	Intake of flavonols plus flavones
			mg/day
1	USA	Macrobiotic	15.7
2	China	Chinese	5.1
3	Czechoslovakia	East-european	24.2
4	Ethiopia	African	52.9
4	Finland	Scandinavian	77.0
6	India	Asian	15.6
7	Indonesia	Asian	28.3
8	Italy	Mediterranean	15.3
9	Lithuania	East-european	13.2
10	Malaysia	Asian	12.7
11	Mexico	South American	3.6
12	The Netherlands	Lacto-ovo vegetarian	50.3
13	The Netherlands	Vegetarian	26.3
14	The Netherlands	"Prehistoric" *	26.8
15	The Netherlands	Western	47.7
16	Surinam	Surinam	35.4
17	Turkey	Middle-eastern	19.8
Mean ± sd			27.6 ± 19.5

* uncooked and unprocessed products, vegetables and fruit were typical of this diet

and of myricetin 4%. The intakes of luteolin and apigenin were almost at zero. Tea was the major source contributing 37% of flavonols and flavones, followed by onions 26%, vegetables 14%, fruits 22%, and red wine 1%.

In the 8 Dutch adults the daily energy intake was 10.6 ± 1.5 MJ, and the mean dietary fibre intake 2.2 g/MJ of energy according to the 3-d records. Flavonol plus flavone intake calculated from the food records ranged from 1 to 81 mg/d, and from the food frequency questionnaires from 9 to 75 mg/d (Table 2.2). The mean contribution of quercetin to the total flavone and flavonol consumption according to 3-d records and food frequency questionnaires was 68% for both, of kaempferol 28% and 29%, and of myricetin 4% and 2%, respectively. The contributions of luteolin and apigenin were again negligible.

Table 2.2. *Habitual flavonol and flavone consumption of 4 Dutch men and 4 Dutch women according to 3-d records and a food frequency questionnaire for assessment of flavonoid intake.*

Subject	Age	Sexe	Flavonoids 3-d records	Flavonoid questionnaire
			mg/day	*mg/day*
1	21	male	11	9
2	24	male	1	37
3	22	female	24	27
4	47	male	2	33
5	21	female	28	25
6	25	female	81	72
7	22	female	71	75
8	20	male	55	57
Mean ± sd			34.1 ± 31.2	41.9 ± 23.7

The within- and between-subject coefficients of variation calculated from the 3-d records were 63% and 84%, respectively. The ratio of the within-subject and between-subject components of variance was 0.57. The between-subject coefficient of variation calculated from the food frequency questionnaires was 56.5%. Tea contributed 47% to the total amount of the five flavonols and flavones according to 3-d records, and 28% according to the food frequency questionnaires, onions 26% and 34%, vegetables 20% and 27%, fruits 6% and 11%, and red wine 1% and 0%, respectively. The correlation coefficient between the 3-d records and the food frequency questionnaires in the full Dutch group was 0.85 (n=8).

Discussion

We found marked differences of up to 80 mg/d in flavonol and flavone intake between free-living subjects. Tea and onions were the most important sources for flavonoid consumption in both groups. The between-subject coefficient of variation was higher in the "international" than in the Dutch group. The variation within subjects in the Dutch subjects according to the 3-d records was large, and comparable with, for example, vitamin C in US women [9]. However, in contrast with many nutrients the ratio of within- and between subject variance was lower than 1 [9]. The relatively large between-subject variation gives a fine opportunity to study the relation between flavonoids and health.

The results of 1 or 3-day records were possibly not sufficient to represent the long-term average daily individual intake. The within-subject coefficient of variation of 63% suggests that the number of days needed to estimate the individual intake within 20% of the actual intake 95% of the time is 38 days [10]. The results of the food frequency questionnaire probably provided the best data as this method inquired after food intake over 30 days. The high correlation coefficient of this method with the 3-d records suggests that these methods are similar for ranking of individuals.

We did not have flavonol values for all plant foods consumed by the subjects. However, we have lacked only the data of a few foods, for example, citrus fruits, which we do not expect to contribute significant amounts of flavonols and flavones [2]. Therefore we probably made only a small underestimation of actual flavonoid intake. The contribution of herbs and spices to flavonol and flavone intake was at most 3 mg/d.

The flavonol and flavone intake of all subjects was within the distribution of flavonoid intake in the Netherlands [5], although some subjects were at the highest intake range. Hertog [4,5] also found tea and onions to be the major sources of flavonol and flavone consumption. However, the contribution of tea that he found in elderly men (61%), and in Dutch adults (48%) was higher than in our study.

We conclude that there was a large variation in flavonol and flavone consumption in this study, but the within-subject variation was less than the between-subject variation. For ranking of subjects for flavonoid intake, a short food frequency questionnaire appears to be suitable. However, it needs to be tested in a larger study.

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3

Red wine is a poor source of bioavailable flavonols

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Abstract

Coronary mortality in France is low even though the intake of saturated fat is high. This so-called French paradox has been ascribed to a high consumption of red wine. Red wine is thought to be an important source of polyphenolic antioxidants, especially flavonols, and consumption of wine may therefore prevent LDL oxidation and atherosclerosis. We compared the bioavailability of flavonols from wine with that from onions and from tea. Twelve healthy men consumed 6 glasses of red wine, 50 g of fried onions or 3 cups of strong black tea, in random order. Concentrations of flavonols in plasma and their excretions in urine on red wine were lower than or similar to those on onions or tea. We conclude that red wine is a poor source of flavonols. Thus, intake of polyphenolic antioxidants from red wine cannot explain the French paradox, and red wine may not be superior to other alcoholic beverages in preventing heart disease.

Introduction

Mortality from coronary heart disease is low in France, even though saturated fat intake is high [1]. This so-called French paradox has been ascribed to a high consumption of red wine [2], the idea being that polyphenols from red wine prevent oxidation of low-density lipoproteins. Red wine contains polyphenols such as flavonols, catechins, resveratrol [3], anthocyanins, and proanthocyanidins. Polyphenols, and especially the flavonol quercetin are potent inhibitors of LDL oxidation *in-vitro* [4]. However, it is unknown to what extent quercetin and other flavonols are absorbed from wine in man. We now compared the bioavailability of flavonols from red wine with that from two other important sources, tea and onions.

Methods

The Medical Ethical Committee of the Division of Human Nutrition and Epidemiology approved the study. Twelve healthy male subjects (mean age 25 years, mean body mass index 22 kg/m^2) were thoroughly informed about the study, and they filled out and signed an informed consent form. The subjects consumed six 125-ml glasses of a red wine selected for its high flavonol content (Médoc Chateau Latour St. Bonnet 1993), or 50 grams of fried onions, or three 125-ml cups of extra-strong black tea daily during four days in random order with a wash-out of three days between treatments. Subjects followed a diet low in flavonols. The wine provided 14.2 ± 0.3 mg (± SD), the onions 15.9 ± 0.5 mg, and the tea 13.7 ± 1.6 mg of quercetin per day. We told the subjects to consume one-third of the wine or tea at lunch, one-third at dinner, and one-third between 10.00 and 12.00 pm; consumption of onions was divided between lunch and dinner. On day 4 blood was sampled after lunch, and urine was collected for 24 hours.

Table 3.1 *Effects of daily consumption of 750 ml red wine rich in the flavonoid quercetin, of 50 g of fried onions, and of 3 cups of black tea on concentrations of flavonols in plasma in 12 healthy men*

Treatment	Plasmaconcentration*		
	Quercetin	Kaempferol	Isorhamnetin
		µg/L	
Red wine	8 ± 3^a	1 ± 1^a	3 ± 2^a
Fried onions	16 ± 5^b	1 ± 0^a	2 ± 1^b
Black tea	8 ± 4^a	4 ± 2^b	1 ± 1^b

* $\bar{x}$ ± SD, average of two blood samples per subject

^{a,b} Values in the same column with different superscript are significantly different from each other ($p<0.05$)

Results

Quercetin levels in plasma and urine [5] after red wine were significantly higher than baseline, but lower than after onions, and almost similar to those after strong tea (Tables 3.1 and 3.2). Kaempferol after wine was the same as after onions, but lower than after tea; isorhamnetin was highest after wine.

Table 3.2 *Effects of daily consumption of 750 ml red wine rich in the flavonoid quercetin, of 50 g of fried onions, and of 3 cups of black tea on amounts excreted in 24-h urine in 12 healthy men*

Treatment	Urinary excretion		
	Quercetin	Kaempferol	Isorhamnetin
		μg/24hr	
Red wine	112 ± 33[a]	72 ± 25[a]	106 ± 58[b]
Fried onions	153 ± 66[a]	95 ± 139[a]	55 ± 26[b]
Black tea	76 ± 45[b]	202 ± 116[b]	29 ± 27[a]

[a,b] Values in the same column with different superscript are significantly different from each other ($p<0.05$)

Discussion

Thus some of the flavonols from red wine were absorbed. However, plasma concentrations of the sum of flavonols after 750 ml (6 glasses) of red wine were the same as after 3 cups of tea, and lower than after 50 g of onions. The wine and tea used here had about two times the quercetin content found normally. This means that consumption of one glass (125 ml) of red wine with an average content of quercetin [6] would produce plasma quercetin levels similar to those after consumption of ½ cup (125 ml) of average tea or 5 grams of onions. The French drink on average 3 glasses of wine per day [1], and the flavonol content in French wines is about half that in our study [6]. Therefore, daily intake of flavonols from wine in France - including the flavonol myricetin - does not exceed 10 mg per day. In the Netherlands the average daily intake of flavonols from tea is 11 mg and from onions 7 mg per day [6]; in the UK the intake from tea is 21 mg per day [7]. Therefore, the intake of bioavailable flavonols from wine in France is quite modest, and cannot explain the difference in coronary heart disease between France and other countries. Probably, other phenolics in wine also cannot explain the French paradox; the level of catechins in red wine [3] is lower than in tea [8], and the amount of resveratrol is probably too low to show anti-oxidant activity *in-vivo* [4]. It is uncertain whether anthocyanins and proanthocyanidins could play a role. Also no specific effect of red wine was apparent in a meta-analysis of the cardioprotective effect of alcohol [9]. Intake of flavonols and probably also other polyphenols substances from red wine cannot explain the French paradox.

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4

Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake

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Abstract

Flavonols are antioxidants that may reduce the risk of coronary heart disease. Two major flavonols in the diet are quercetin and kaempferol, and their main sources are tea and onions. We investigated whether plasma concentrations and urinary excretion of quercetin and kaempferol in humans could be used as biomarkers of intake. We provided 15 subjects with strong black tea (1600 ml/d) or fried onions (129 g/d) for 3 days each in random order, separated by a 4 day wash-out. The tea provided 49 mg quercetin and 27 mg kaempferol daily, and the onions 13 mg of quercetin, and no kaempferol. Flavonols from both foods were clearly absorbed. However, the excretion of unmodified quercetin was 0.5% of intake after tea, and 1.1% after onions. Thus, the absorption of quercetin from tea is half of that from onions. The onion supplementation period was repeated 7 - 14 d later, so as to estimate coefficients of variation within subjects when the same treatment is given twice. Coefficients of variation were 30% for quercetin in plasma and 42% in urine. The magnitude of these variations relative to actual variations of about 60% between free-living subjects indicate that levels of quercetin in plasma and urine are applicable as biomarkers of its intake. We conclude that flavonols in plasma and urine reflect flavonol intake, and that they could be applied as biomarkers to distinguish between high and low flavonol consumption in epidemiological studies.

Introduction

Flavonoids, a group of polyphenolic compounds, are widely present in vegetables, fruit, tea, and wine [1,2]. A main subgroup of the flavonoids are the flavonols, of which quercetin and kaempferol are the major representatives [3,4]. Onions are an important source of quercetin in the diet, and tea of both quercetin and kaempferol [1,2].

Beneficial effects of dietary flavonols in the diet on the risk of coronary heart disease have been suggested by epidemiological studies [5], but whether these components can prevent heart disease is still controversial [6]. Protective effects of flavonols against cancer have also been proposed [7], but epidemiological data hitherto fail to support this [8,9].

Further studies investigating the relation of flavonols with disease are needed. Such studies require an accurate method to assess intake of flavonols in free-living subjects. However, it is difficult to assess flavonol intake by common dietary record or recall methods. Such methods rely on self-report and their accuracy is therefore uncertain [10,11]. It is also difficult to assess true daily flavonol intake because flavonols are concentrated in a few foods [12,13]. Therefore, small errors in the assessment of the consumption of these few flavonol-rich foods result in large errors in estimated flavonol intake. Also, when record or recall methods are used, the intake of flavonols has to be calculated from the flavonol content of foods. The flavonol content of many foods is not known, and they are not commonly included in nutrient databases. Moreover, these methods do not take into account the bioavailability of flavonols which varies widely between foods [14]. For example, the percentage of quercetin absorbed from apples was only one-third of that from onions [15]. For many foods, tea for one, absorption has not even been reported yet. The use of biomarkers of intake can bypass most of these problems.

Like other dietary assessment methods, biomarkers suffer from measurement error. This error consists of random and systematic errors [16]. Random error includes within-subject fluctuations, metabolic between-subject differences, and imprecision in laboratory analyses. Random error can be determined by studying the reproducibility of the level of the marker at constant levels of intake. To determine this reproducibility the same treatment has to be given twice in the same individual. Systematic errors cannot be determined easily, but this error is possibly much smaller for biological measurements than for record or recall methods.

The objective of our study was to determine whether levels of flavonols in plasma and urine may be used as biomarkers of their intake. A second objective was to compare the bioavailability of flavonols from tea and onions. Therefore we measured concentrations of quercetin and kaempferol in plasma and excretions of these flavonols in urine after feeding volunteers tea or onions, and we repeated the treatment with onions to determine reproducibility.

Subjects and methods

Subjects

We obtained approval from the Ethics Committee of the Division of Human Nutrition and

Epidemiology. We recruited eight men and seven women via posters and local newspapers, and informed them about the aim of the study and possible discomforts that it could entail. The subjects gave their written informed consent. They were medically evaluated and considered healthy by a physician. The mean age of the subjects was 27.6 y (range 19 - 56 y), and their mean body mass index was 23.5 kg/m^2 (range 19.7 - 30.9 kg/m^2).

Design

The experiment lasted 3 weeks. The 3 treatments were provided in random order, and each treatment was given for 3 days on days 5 to 7 of each week (Figure 1.1). Days 1 to 4 served as a wash-out period. The tea treatment consisted of 1600 ml concentrated black tea and the onion treatment of 129 grams of fried onions, both divided over the day. The treatment period with onions was performed twice to assess reproducibility within subjects.

Day	1	2	3	4	5	6	7
Lithium 250 μmol/day							
Diet low in flavonols							
1600 ml tea or 129 grams of onions							
24-hour recall							
10 ml blood sample							
24-hour urine							

Figure 4.1 *Design of one study week. The experiment consisted of 3 study weeks; each subject took tea in 1 week and onions in the two other weeks, in random order*

On days 4 to 7 of each treatment week (Figure 4.1) the subjects followed a low-quercetin diet. For this purpose they were given a list of vegetables and fruits containing > 15 mg quercetin/kg and of beverages containing > 4 mg quercetin/L [1,2] and were instructed not to consume any of these. On day 7 of each week blood was sampled and urine was collected for 24 hours. On the morning of day 4 of the second week of treatment, blood was sampled to determine baseline values for plasma quercetin. At this time point the subjects had followed the quercetin-low diet for 1 day, and they had not yet started consuming the second series of supplements. For the baseline value of kaempferol in plasma the concentration after consumption of onions was used as a surrogate, because onions do not contain kaempferol [1]. Eight months after the experiment we repeated one treatment week in 8 of the 15 subjects without providing supplements so as to collect baseline values for urine. During all treatment weeks subjects recorded all deviations from the guidelines in a diary. Medications were not allowed except for oral contraceptives and paracetamol.

Supplements and diets

Tea was made 3 times per day. One litre of boiling water was poured on 4 tea bags (Pickwick, Douwe Egberts) containing 16 grams of black tea leaves, which is twice the quantity for normal use. The tea was allowed to brew for five minutes. The tea supplements provided on average 49 ± 4 mg (± SD, 9 samples) quercetin and 27 ± 2 mg kaempferol per day, as determined by HPLC [17] in duplicate. Prior to the study, eighteen 1 kg portions of yellow onions from a single batch were fried with 68 grams of margarine per kg for nine minutes. All fried onions were mixed, weighed out in portions of 43 grams in plastic pots, and frozen at - 20 °C until supplementation. Just before consumption, the onion supplements were heated in a microwave oven for 1 minute and served with a slice of bread. The onion supplements contained 13 ± 1 mg (mean ± SD; n=4) quercetin [17]; the content did not diminish during 3 weeks storage in a freezer or after heating in a microwave oven (399 mg/kg after frying versus 396 mg/kg after subsequent freezing and reheating). Between 8:00 and 9:00 am, 12:00 and 1:00 pm, and again between 4:00 and 5:00 pm, subjects consumed the onion supplement under our supervision or received the tea supplement in a thermos jug.

We checked compliance to the low quercetin diet by 24 hour recalls. Quercetin and kaempferol intakes were calculated using our published values for contents in vegetables, fruit and beverages [1,2].

Collection of blood and urine samples

Venous blood samples were taken between noon and 1:00 pm as described [18]. Urine samples were collected by each subject in 500 ml and 1000 ml bottles which contained thymol dissolved in isopropanol as a preservative. The first urine voiding after rising in the morning was discarded and all subsequent urines until the next morning, including the first urine after rising, were collected. The urine bottles were immediately put into polystyrene boxes containing dry ice. Within 1 to 5 days the urines were thawed at 40° C, pooled per subject per day, and aliquots were stored at - 40 °C until analyses. Completeness of urine was checked by assessment of recovery in urine of 2.0 mg of lithium (235 μmol) as lithium chloride dissolved in 10 ml water taken by the subjects every morning. On days 5, 6, and 7 they took this under our supervision. The dose of 2 mg/d of lithium is 1% of that considered safe for chronic use in patients with bipolar disorders [19]. The recovery in urine of orally ingested lithium is about 95% [20,21].

Analytical methods

Quercetin, kaempferol and their conjugates in plasma or urine were simultaneously extracted and hydrolysed to the aglycone using 2 M HCl in aqueous methanol [15], and determined by HPLC with fluorescence detection [22]. The limit of detection was 2 μg/L for quercetin, and 0.6 μg/L for kaempferol.

Lithium was analysed in a separate undiluted acidified urine sample by atomic absorption spectrophotometry [23].

Statistical analysis

Data are reported as mean ± SD. To achieve normality we converted the amounts of quercetin analyzed in urine to ^{10}log values. We determined within- and between-subject coefficients of variation for quercetin levels in plasma and urine after the two treatments with onions using the SAS procedure VARCOMP. We calculated confidence intervals for Pearson correlation coefficients using Fisher's Z transformation.

Results

Variability and reproducibility

Reproducibility on the 2 treatments with onions was similar for quercetin in plasma and in urine. Between the first and the second treatment with the same intake from onions the coefficients of variation within subjects were 20% for plasma concentrations, and 22% for urinary excretion. The coefficient of variation between subjects given identical treatments was lower for plasma than for urine; it was 23% for concentrations in plasma and 36% for excretions in urine. The correlation between the concentrations of quercetin in plasma and the amount excreted in urine, taken as the averages for each individual after tea and onions,

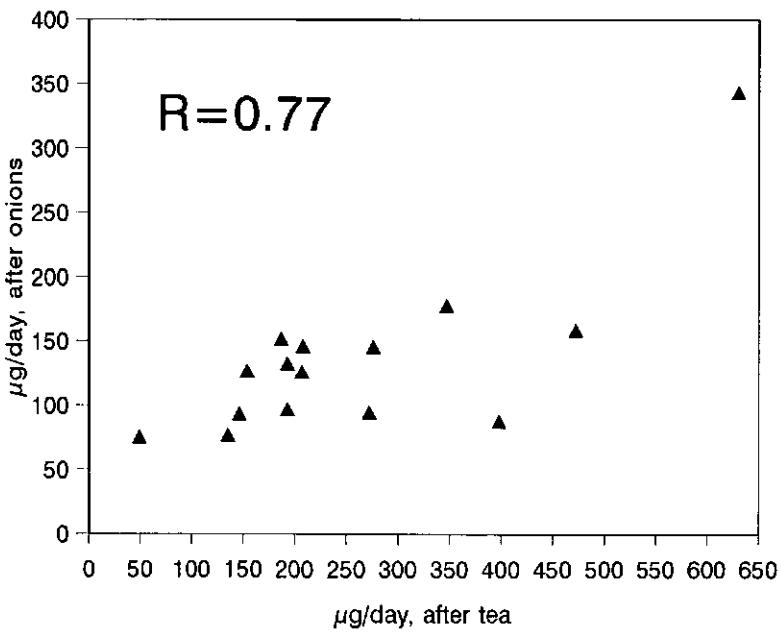

Figure 4.2 *Correlation between the excretions of quercetin in 24-hour urine after consumption of tea and after that of onions in 15 subjects. The subjects consumed 1600 ml tea providing 49 mg, and 129 grams of onions providing 13 mg of quercetin per day during three days for each treatment. Quercetin excretions after onions were the average of two treatments.*

was 0.46. It was higher than that for kaempferol after tea, which was 0.19. However, when we dropped one outlying value the correlation for kaempferol increased to 0.43. The individual excretions of quercetin after consumption of tea and after onions correlated well. The Pearson correlation coefficient was 0.77 (Figure 4.2).

Table 4.1 *Effect of the consumption of tea or onions on levels of quercetin in plasma and 24-hour urine in 15 subjects. Each subject received 1600 ml strong tea or 129 grams of fried onions for 3 days.*

Supplement		Quercetin in plasma	Quercetin in urine	
Type	Amount of quercetin		Absolute amount	Relative amount
	mg/day[1]	μg/L[1]	μg/day[1]	% of intake
Baseline	0	7 ± 4	30 ± 11[2]	
Tea	49 ± 4	29 ± 8	258 ± 150	0.5 ± 0.3
Onions				
Period 1[3]	13 ± 1	22 ± 5	140 ± 66	1.1 ± 0.5
Period 2[3]	13 ± 1	22 ± 7	131 ± 72	1.0 ± 0.6
Average	13 ± 1	22 ± 5	135 ± 66	1.1 ± 0.5

Values are means ± SD, [1] expressed as aglycone, [2] determined in 8 of 15 subjects
[3] the onion supplement was repeated once to assess variability

Quercetin and kaempferol in plasma and urine

The concentrations of quercetin in plasma after consumption of tea and after onions, and of kaempferol after tea were increased in all subjects. The concentration of quercetin in plasma was on average 4 times higher after tea and 3 times higher after onions than at baseline (Table 4.1). The concentration of kaempferol in plasma after tea was on average more than 6 times higher than the surrogate baseline concentration after onions (Table 4.2). The amount of quercetin excreted in urine after consumption of tea was 8 times and after onions 4 times higher than that after the diet low in quercetin (Table 4.1). However, if both were expressed as a percentage of the amount consumed (Table 4.1, Figure 4.3) the relative excretion of quercetin after onions was twice as high as that after tea. Differences in total urinary excretion of quercetin between treatment and baseline were on average 230 ± 158 μg/d after tea and 124 ± 85 μg/d after onions. The amount of kaempferol excreted in urine after tea was 25 times higher than at baseline, and 7 times higher than after onions (Table 4.2). If expressed as a percentage of intake, the relative excretion of kaempferol after tea consumption was twice that of quercetin. Differences in excretion of kaempferol between treatment and baseline were 586 ± 197 μg/d after tea, and 74 ± 68 μg/d after onions.

Compliance with diets and completeness of urine collection

The subjects consumed all their supplements, except for one subject who did not drink all her tea on the third day of the tea period. The average intake of quercetin plus kaempferol from the background diets as determined by 24 h recalls was low. It was on average 2 ± 2 mg/d for the baseline period (n=8) and 2 ± 2 mg/d for all treatment periods combined (n=3x15).

Lithium recoveries were 87 ± 13% (± SD;n=8) in the baseline period, 93 ± 11% (n=15) after the tea, 97 ± 9% (n=15) after the first, and 96 ± 6% (n=15) after the second treatment with onions.

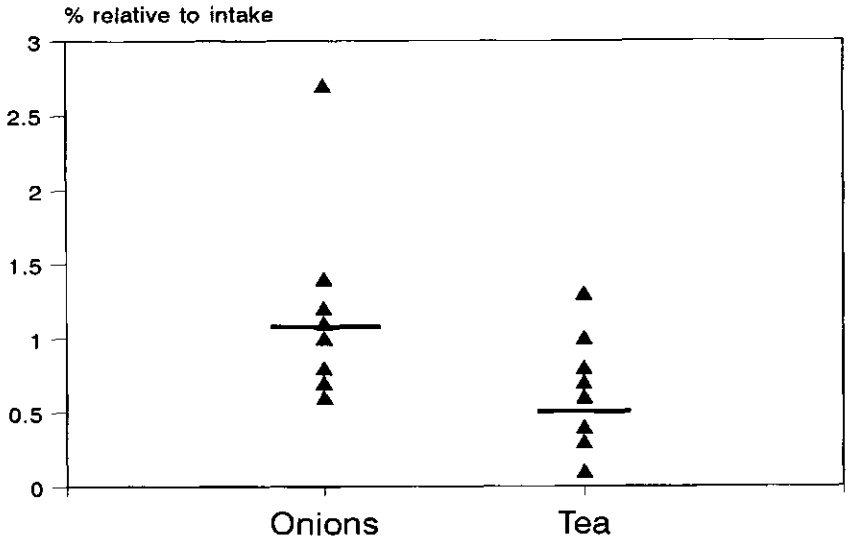

Figure 4.3 *The amount of quercetin excreted in 24-hour urine after consumption of tea and after that of onions as a percentage of the amount consumed per subject. Quercetin excretions after onions are the averages of two treatments.*

Discussion

Biomarkers of intake

The first objective of our study was to determine whether levels of flavonols in plasma and urine may be used as biomarkers of their intake. Our results suggest that plasma concentrations of quercetin and kaempferol and their excretions in urine can indeed be used as markers of their intake. A prerequisite of a biomarker is that differences in consumption can be discriminated [16]. In our study, the consumption of quercetin and kaempferol from tea and onions increased their plasma concentrations and urinary excretion 3 to 25-fold. A low quercetin intake - as from the background diet - resulted in low levels of quercetin, and an average intake of quercetin - as after onions, which provided 13 mg or 80% of the average daily Dutch consumption of quercetin - elevated plasma and urine levels of

quercetin clearly in all subjects compared to baseline. However, a low intake of kaempferol, as after onions which do not contain kaempferol, did not result in low excretion of kaempferol (Table 4.2). This may be explained by consumption of kaempferol-rich vegetables from the background diet by some subjects. A high intake of quercetin and kaempferol - as after tea in our study - resulted in the highest plasma and urine levels of both flavonols. Thus, our study shows that levels of quercetin and kaempferol in plasma and urine reflected levels of intake.

Table 4.2 *Effect of the consumption of tea or onions on levels of kaempferol in plasma and 24 hour urine in 15 subjects. Each subject received 1600 ml strong tea or 129 grams of fried onions for 3 days.*

Supplement		Kaempferol in plasma	Kaempferol in urine	
Type	Amount of kaempferol		Absolute amount	Relative amount
	mg/day	*μg/L*	*μg/day*	*% of intake*
Baseline	0	not determined	26 ± 7[1]	
Tea	27 ± 2	15 ± 5	668 ± 360	2.5 ± 1.3
Onions				
Period 1[2]	< 2	3 ± 4	123 ± 157	
Period 2[2]	< 2	1 ± 1	63 ± 27	
Average	< 2	2 ± 2	93 ± 75	

Values are mean ± SD.

[1] determined in 8 of 15 subjects

[2] the onions supplement was repeated once to assess variability

Another requirement for a biomarker is a certain degree of precision with which intake can be assessed. To evaluate this, the coefficient of variation of a single estimate in a single subject has to be determined. The coefficient of variation for quercetin in plasma or urine includes random biological fluctuations within subjects, and variations between subjects due to differences in absorption and metabolism between subjects. The correlation of 0.77 between urinary excretions of quercetin after onions and those after tea showed that there are indeed consistent differences in flavonoid metabolism between subjects (Figure 4.2): subjects with a low or high excretion after onions showed a low or a high excretion after tea, respectively.

For plasma, the coefficient of variation between 2 measurements on the same intake of quercetin from onions was 20% within subjects. The coefficient of variation between subjects was 23%. Thus, the total observed coefficient of variation of quercetin in plasma

sampled once at the same intake can be estimated as $\sqrt{(20^2 + 23^2)}$= 30%. A similar calculation yields a total coefficient of variation of 42% for the measurement of quercetin in urine. Whether this measurement error has to be considered as large or small can only be evaluated in relation with the actual variation in intake found between free-living subjects. From various studies [5,13,24,25] it can be estimated that the coefficient of variation for true differences in intake between subjects in the population is about 60%. This suggests that the random error in our proposed biomarkers is modest relative to the expected variations due to true variation in intake. We will illustrate the degree of precision of quercetin in plasma and urine as biomarkers of their intake for two hypothetical examples with practical applications.

The first example addresses assessment of the compliance of a subject in an intervention study with a diet that provides 50 mg of quercetin per day. The interval in which plasma quercetin of one of the subjects is predicted to lie with a probability of 95% is $\mu \pm 1.96 \times \sigma$, where μ denotes the expected mean level and σ the standard deviation of the measurement [26]. After an intake of 13 mg of quercetin per day the average concentration of quercetin in plasma in our subjects was 22 μg/L with a coefficient of variation of 30%. If we assume (de Vries et al, unpublished observations) that the levels of quercetin in plasma and urine increase linearly with the dose then the expected level in plasma of a subject with an intake of 50 mg per day is 22 * 50/13 = 85 μg/L. If the coefficient of variation is the same at various levels then the standard deviation will be 30% of 85 μg/L or 25.5 μg/L and the 95% confidence interval will range from 85 - 1.96 * 25.5 = 35 μg/L to 85 + 1.96 * 25.5 = 135 μg/L. Thus, there is reason to doubt dietary compliance of the subject if a single plasma quercetin is less than 35 μg/L. Better precision can be achieved by taking more plasma samples: for a mean of two measurements the lower boundary point for compliance would be 50 μg/L.

Bioavailability

The second example addresses the correlation coefficient between quercetin intake and a continuous variable e.g. the oxidizability of LDL. For this we estimate the attenuation factor of the correlation coefficient caused by measurement error. The attenuation factor is the ratio of the observed correlation to the "true" correlation. The factor is calculated as $\sqrt{(1/(1+\sigma^2_a/(n*\sigma^2_b))}$, in which σ^2_a denotes the variance of the measurement, σ^2_b the true variance of intake between subjects, and n the number of measurements [26,27]. From our data we estimate that the attenuation factor for a single measurement of quercetin in plasma or urine is $\sqrt{(1/(1+30\%/1x60\%)}$ = 0.82 for plasma and $\sqrt{(1/1+43\%/1x60\%)}$ = 0.76 for urine. This implies that when the true correlation between quercetin intake and for example lagtime of LDL-oxidation is 0.5, the observed correlation will be 0.4. The attenuation factors we found for the quercetin markers are similar to those for intake measurements of energy and various nutrients by the cross-check dietary history method [28].

The second objective of our study was to determine whether the bioavailability of flavonols from tea is similar to that of onions. Our results show that quercetin and kaempferol from tea are absorbed, but that the bioavailability of quercetin from tea is less than that from onions. Plasma concentrations of quercetin were 30% and urinary excretions

100% higher after tea than after onions, but the intake from tea was about fourfold higher than from onions. When adjusted for intake - as shown by the urinary excretion relative to the amount consumed (Table 4.1) - the absorption from tea was about half that of onions. The reason could be that the absorption of a higher dose is relatively lower, but it is more likely that the difference in type of quercetin conjugate present in the two foods plays a role. The absorption of quercetin-rutinoside, the major quercetin compound in tea, is probably less than that of quercetin-glucoside, the major compound in onions.

This is in agreement with the differences in absorption we found between these two compounds in previous studies [14]. In ileostomy patients, quercetin in 13 h urine collections was 0.3% of intake after onions and 0.1% after quercetin-rutinoside [14]. The excretions in the present study were higher, which could be explained by enhanced absorption due to a lower dose or to bacterial activity in the colon which is not present in ileostomy patients. In a previous study in healthy volunteers [15], the relative excretions were 1.4% after an onion rich, and 0.4% after a breakfast rich in quercetin-rutinoside. Thus, in all studies the relative excretion of quercetin in urine after onions was higher than after quercetin-rutinoside or after tea, which confirms that quercetin from onions is better absorbed.

Incomplete collection of urine cannot explain the lower absorption of quercetin observed after tea. The average recoveries of lithium were in agreement with the expected values of 95%. Deviations in sampling by some subjects probably decreased the excretions of quercetin after tea, but after correction for incomplete collection the excretion of quercetin relative to intake after tea became 0.6% instead of 0.5%. Also, exclusion of the data from one subject who became ill during the experiment did not affect the results.

The excretion of unchanged kaempferol in urine was 2.5% of the amount consumed and that of quercetin only 1%. Therefore kaempferol was absorbed better or metabolised to a lesser extent than quercetin.

Utility of the biomarkers

Epidemiological studies that investigate the relation between intake and disease require measurements of long-term intake [16]. Flavonol markers probably do not reflect long-term flavonol intake. Quercetin and kaempferol accumulate in plasma after repeated intake of onions, apples, and tea [15], but the elimination half-life of some 20 h [15] indicates that a steady-state concentration in plasma is reached after about 4 days and that plasma concentrations would reflect intake of only the last three days. Thus, repeated measurements in time may be needed to obtain an estimate that represents the long-term intake. That would also reduce the error associated with variations in the interval between blood-sampling and the most recent intake of flavonol-rich foods. Because of these short-term variations we may have missed the quercetin peak concentrations in plasma for onions as well for tea [15].

Whether the use of a biomarker is feasible also depends on the costs of its analytic measurements and its stability in stored samples. A method for chemical determination of quercetin and kaempferol in plasma was developed by Hollman [22], and can now be conducted economically. The maximum decrease in quercetin content of stored plasma

samples after one year was about 10% (Hollman; unpublished data). We expect the same for plasma kaempferol. Therefore a long interval between sample collection and chemical analyses would not be a problem [16].

Plasma concentrations and urinary excretion of quercetin and kaempferol may also be regarded as markers of flavonol status in the body, rather than of flavonol intake. Therefore, these levels - when assessing the relation between flavonols and disease - may be more directly related with the outcome of disease than intake data. In this way, differences in absorption between subjects and bioavailability between dietary sources of flavonols are taken into account.

Conclusion

We conclude that differences in flavonol intake can be discriminated by the levels of quercetin and kaempferol in plasma and in urine. The reproducibility of quercetin levels in plasma and urine suggest that these levels can be used as biomarkers to determine quercetin intake in epidemiological studies. We also conclude that flavonols from tea are absorbed but that the bioavailability of quercetin from tea is only half of that from onions.

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5

A linear relationship between flavonol intake and flavonol levels in plasma and in urine: implications for their use as biomarkers of intake

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Submitted for publication

Abstract

Flavonols are a major subgroup of the flavonoids, with quercetin and kaempferol as the main representatives. Intake of these flavonols may affect health, but it is difficult to assess flavonol intake by common record or recall methods. These problems might be overcome by use of biomarkers of flavonol intake. Dietary flavonols appear in plasma and urine after consumption of flavonol-rich foods. However, the dose-response relationship is as yet unknown. Also, no data are available about plasma and urine levels after prolonged intake of a diet high in flavonols. We therefore asked 9 subjects to consume a high-flavonol or a low-flavonol diet for 4 weeks each, in a cross-over design. The high-flavonol diet provided 160 mg of flavonols per day. Levels of flavonols in plasma and urine increased 10- to 40-fold on the high-flavonol diet. Plasma levels of flavonols on this diet were stable from day 8 until the end of the diet period. The coefficient of variation of repeated measurements within subjects was 69% for plasma quercetin and 53% for kaempferol. We combined the results obtained after the high-flavonol diet with those obtained after lower intakes in two previous studies. Intakes of flavonols up to 132 mg/d were related linearly with their concentrations in plasma and excretions in urine. Based on this relationship and the within-subject variation of the flavonol levels, we conclude that flavonols in plasma and probably also in urine can be used as biomarkers to distinguish individuals with a low from individuals with a high habitual flavonol intake. These results may facilitate studies on the relation between flavonol intake and occurrence of disease.

Introduction

Flavonols are a subgroup of the flavonoids, with quercetin and kaempferol as their major representatives in the diet. Flavonols are thought to have beneficial health effects [1]. However, studies that investigated the relationship of flavonol intake with the occurrence of coronary heart disease or cancer [2-7] yielded conflicting outcomes [8]. One reason for this could have been that the recall or record methods used in these studies caused errors in the estimation of flavonols [8]. Such errors could have attenuated the relative risks observed [9].

It is difficult to assess flavonol intake accurately because flavonols are concentrated in a few foods: tea, onions, apples and red wine account for more than 80% of intake [3]. This leads to large day-to-day variations in intake [9]. Also, errors in the estimation of portion sizes of flavonol-rich foods might easily cause individuals to be classified into the wrong category of flavonol intakes. A second problem is that the bioavailability of flavonols differs between foods. For instance, the bioavailability of quercetin from apples was only one-third [10], and that from tea and red wine one-half of that from onions. Thus, recall and record methods are probably not the most appropriate methods to measure flavonol intake and exposure to flavonols.

Biomarkers of flavonol intake may overcome some of these problems. Levels of flavonols in plasma or urine are potentially valid biomarkers of flavonol intake. In a previous study, we found that quercetin and kaempferol levels in plasma and urine reflected quercetin and kaempferol intakes from tea or onions consumed for two days. Moreover, the measurement error of quercetin levels in plasma and urine was small enough to allow their use as biomarkers of quercetin intake. It is likely that levels of flavonols in plasma are superior to intake levels for estimating the exposure of target tissues to flavonols in the body. This strengthens the case for relating levels of flavonols in body fluids directly to the outcomes of disease.

However, flavonol levels after prolonged or high intakes are as yet unknown, and so is the shape of the dose-response curve. This information is needed to assess the sensitivity of levels of flavonols to the amount consumed and to assess the variability of the flavonol levels. These factors are important to evaluate the suitability of flavonol levels as biomarkers of intake. A linear relation between the intake of a nutrient and its biomarker improves the suitability of the biomarker to discriminate individuals by their intakes [11,12]. Unfortunately, the intake of nutrients is rarely related linearly with their levels in plasma. These levels often show a plateau effect at higher intakes [11]. An example of such a non-linear relation is that between vitamin C intake and its level in plasma [12]. Another potential problem is variability. A large variability of a biomarker within subjects and between days indicates that [11] multiple measurements of the biomarker are required. The objective of this study was to evaluate the suitability of flavonols in plasma and urine as biomarkers of dietary intake and exposure. To that end, we determined flavonols in plasma repeatedly and in urine once after a high dose consumed during a prolonged period.

Methods

Subjects

We recruited subjects via posters and advertisements in regional papers, and selected nine subjects who smoked at least ten cigarettes per day. We selected smokers because another aim of this study - the results of which are not presented here - was to determine the effect of flavonols on LDL oxidation. The subjects were healthy and had never undergone gastro-intestinal surgery. They were thoroughly informed about the study, and they filled out and signed an informed consent form. The Medical Ethical Committee of the Division of Human Nutrition and Epidemiology approved the study. The mean age (± SD) of the subjects was 33 ± 17 y and their mean body mass index was 23 ± 4 kg/m^2.

Protocol

The study lasted 52 days and consisted of a test and control period of 26 days each, in a cross-over design. During the whole study the subjects followed a background diet low in flavonols. This diet was also restricted in vitamin C because high vitamin C intakes might have interfered with the sub-study on LDL oxidation. The subjects received flavonol-rich supplements in the test period, but received no supplements in the control period.

The subjects were not allowed to use medications except for oral contraceptives and paracetamol. They recorded all deviations from the guidelines in a diary, and they recorded their food intake in each period for 3 days. According to these records, flavonol intake from the background diet was 1 ± 1 mg/d.

Supplements

On each day of the high-flavonol period the subjects consumed four types of flavonol-rich supplements. These were 160 grams of fried onions, 220 grams of applesauce enriched with 20 grams of freeze-dried apple-peels, 6 cups of tea, and 1 piece of apple. The onions and the freeze-dried apple peels were prepared prior to the study. Yellow onions from a single batch were fried for nine minutes, mixed, weighed out in portions of 160 grams in aluminium trays, and stored at -20° C. Jonagold apples were peeled and the peels were cut and freeze-dried: one kg of apple peels yielded 186 grams of freeze-dried powder. This powder was added to applesauce (Jonker Fris). To prepare one cup of tea, subjects added boiling water to 0.5 g of tea-extract. They consumed the piece of apple (Jonagold) with the peel.

The subjects came to our department three times a week to collect the supplements. They consumed the supplements distributed over the day at a time they preferred, except on days that blood was sampled. On these days, the subjects were asked to consume the applesauce, the apple, and three cups of tea before noon, and the onions and the remaining three cups of tea after blood sampling. In this way, short-term effects of consumption on plasma concentrations were standardized.

We took duplicate samples of the supplements and analyzed them for quercetin, kaempferol, and isorhamnetin as described [13]. The total intake of quercetin (± SD) was 132 ± 4, of kaempferol 22 ± 2, and of isorhamnetin 6 ± 0 mg/d. Onions contributed the largest

amount of quercetin (Figure 5.1), and tea the largest amount of kaempferol. Onions and apples contributed equally to the intake of isorhamnetin.

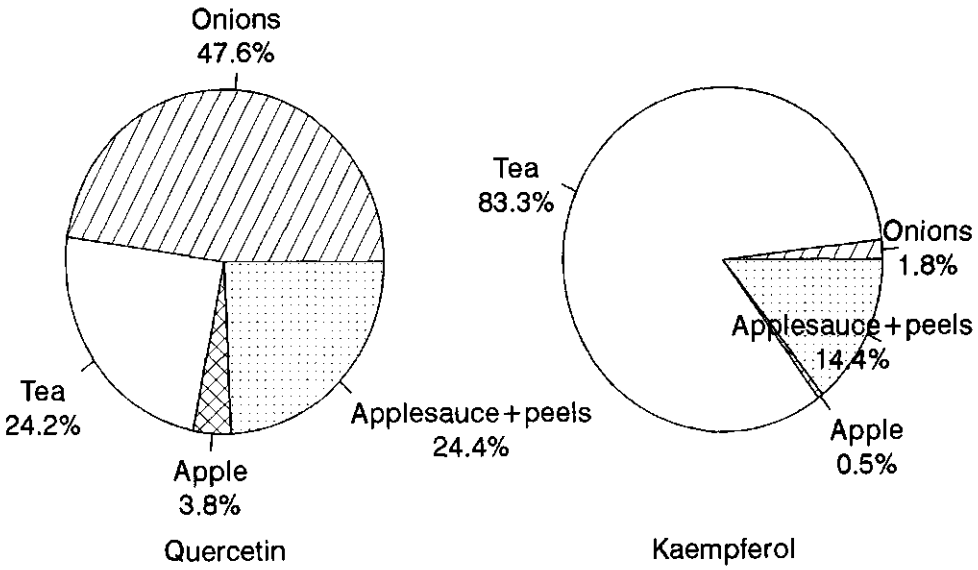


Figure 5.1 *Contribution of the various supplements to the intake of quercetin and kaempferol on the high-flavonol diet. The total intake of quercetin on this diet was 132 mg and of kaempferol 22 mg per day.*

Sampling of blood and urine

Blood and urine were sampled during each period, and plasma concentrations and urinary excretion of flavonols were analyzed after the study. Venous blood samples were taken as described [10] between 14.00 and 15.00 pm on days 8, 15, 25, and 26 of each period. Urine samples were collected on day 25 of each period by the subjects in bottles that contained thymol dissolved in isopropanol as a preservative. The first urine voiding after rising in the morning was discarded. All subsequent urines until the first urine after rising the next morning were collected and immediately put on dry ice. Within one to five days the urines were thawed at 40° C, pooled per subject per day, and aliquots were stored at -40°C until analyses. Completeness of urine collection was checked by assessment of recovery in urine of 250 μmol of lithium. It was taken by the subjects as 10.6 mg of lithium chloride dissolved in 10 ml water from day 19 until day 26 of each period. This dose is 1% of that considered safe for chronic use in patients with bipolar disorders. The recovery in urine of orally ingested lithium is about 95% [14,15]. The average lithium recovery in our study was higher; it was 105 ± 5% (n=8) in the control, and 99 ± 11% (n=9) in the test period. Eleven out of 17 measured values of lithium in urine were within 10% of the expected value of 95%. Five values indicated that too much and one that too little urine had been collected. These urine values were corrected for lithium recovery.

Analytical methods

Quercetin, kaempferol, and isorhamnetin were simultaneously extracted and hydrolysed to the aglycone using 2 M HCl in aqueous methanol [16], and determined by HPLC with fluorescence detection [17]. The limit of detection was 2 μg/L for quercetin in plasma and 3 μg/L for that in urine, and 1 μg/L for kaempferol and isorhamnetin in plasma as well as urine. Lithium was analyzed in a separate undiluted acidified urine sample by atomic absorption spectrophotometry [18].

Statistical analyses

We performed statistical analyses with SAS [19]. We calculated the mean of the four plasma concentrations of flavonols on the low flavonol diet and the mean on the high flavonol diet for each subject, and the average difference between these for the nine subjects. We investigated the variability of flavonol concentrations in plasma between the four blood samples by analysis of variance (PROC ANOVA). After converting the amounts of flavonols in plasma levels to ^{10}log values, we used PROC VARCOMP to determine the within- and between-subject variances as components of total variability in plasma. In addition, we determined Pearson correlation coefficients; we calculated their confidence intervals using Fisher's Z transformation.

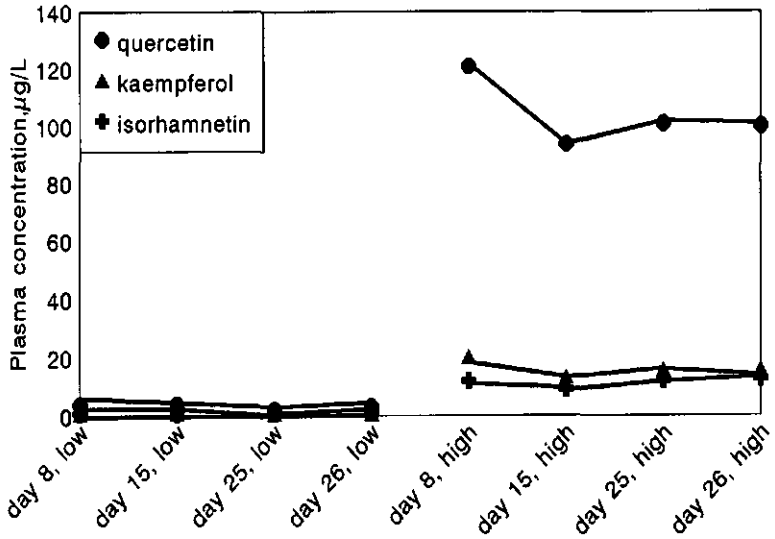
We used additional data from two previous studies to determine how the intake of quercetin and kaempferol was related with their levels in blood and urine. In the first study 15 men and women consumed black tea providing 49 mg quercetin and 27 mg kaempferol, or fried onions providing 13 mg of quercetin and no kaempferol, both for two days. In the second study, 12 men consumed red wine, fried onions, or black tea providing about 15 mg of quercetin each. In addition the wine provided 1.5 mg kaempferol and the tea 9 mg of kaempferol. The wine, the onions, and the tea were each taken for 4 days. We determined the regression equations and the 95% confidence intervals (CI) for the regression coefficients of doses of flavonols on responses of plasma or urine levels using the combined results of the previous studies and the present study.

Results

Levels after a prolonged high intake

Both the concentrations of flavonols in plasma and the excretions in urine were clearly higher on the high-flavonol than on the low-flavonol diet (Figure 5.2). These levels increased 30- to 40-fold for quercetin, 20- to 25-fold for kaempferol, and 10- to 15-fold for isorhamnetin. The differences in plasma concenteations between the high-flavonol and low-flavonol diet were 96 ± 50 μg/L for quercetin, 15 ± 7 μg/L for kaempferol, and 10 ± 8 μg/L for isorhamnetin (means ± SD;n=9);Figure 5.2A). The differences in 24h-urinary excretions of flavonols between the two diets were 921 ± 400 μg/d for quercetin, 434 ± 145 μg/d for kaempferol, and 217 ± 124 μg/d for isorhamnetin (Figure 5.2B). Urinary excretion as a percentage of the amount consumed was on average 0.7 ± 3% for quercetin, 2.1 ± 0.8% for kaempferol, and 4.0 ± 2.4% for isorhamnetin.

2A. Plasma concentrations on the low versus the high flavonol diet



2B. Urinary excretions on the low versus the high-flavonol diet

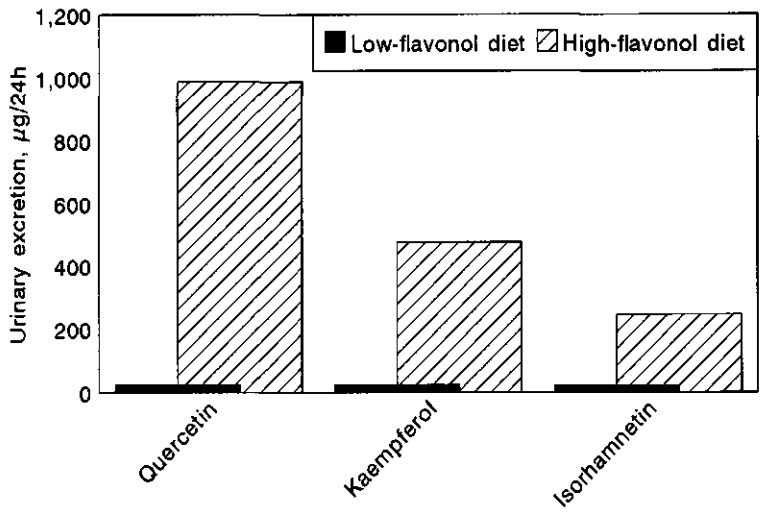


Figure 5.2 *The effects of the intake of 132 mg quercetin, 22 mg of kaempferol, and 6 mg of isorhamnetin on concentrations of these flavonols in plasma (2A) and excretions in 24-hour urine (2B) of nine volunteers. Each diet period lasted 26 days.*

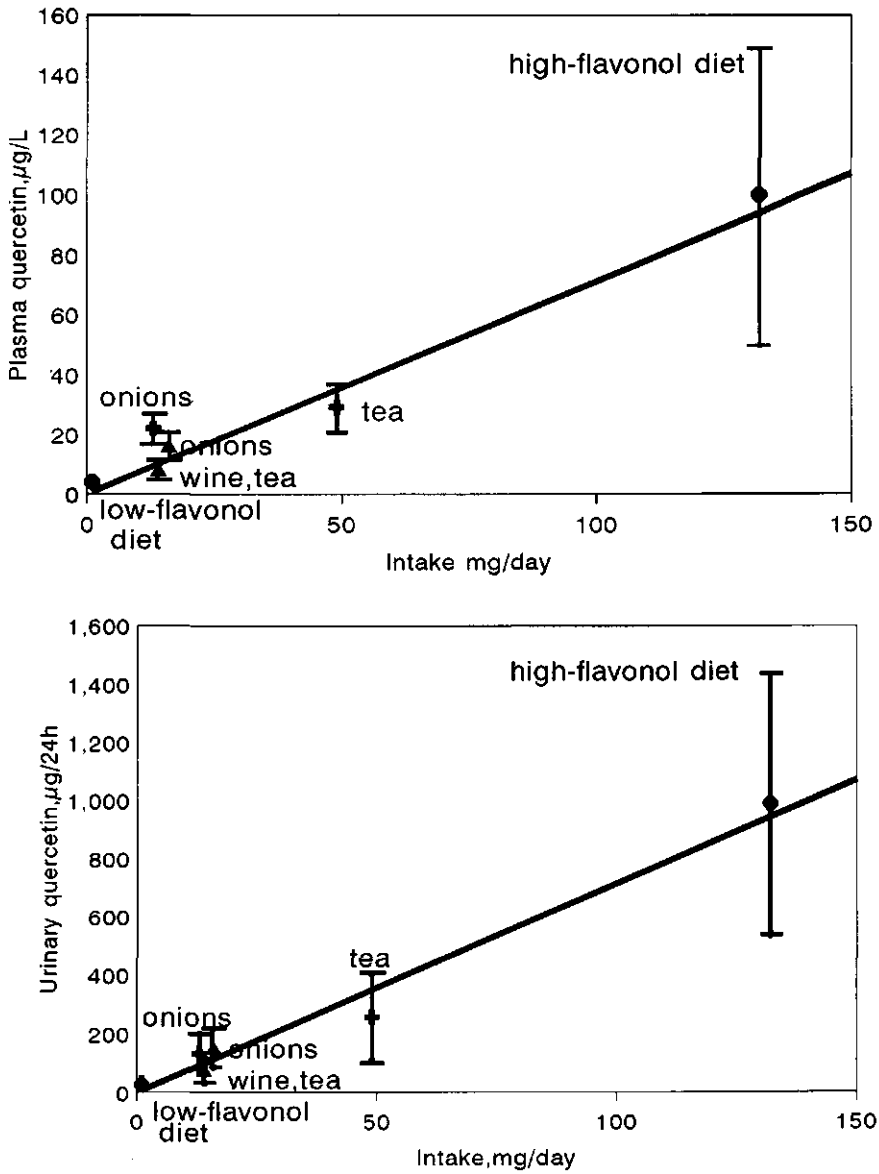


Figure 5.3 *The dose-response relationship between quercetin intake and mean levels in plasma and urine across studies. Data are from two previous studies that investigated the effects of flavonol intakes from single flavonol-rich foods, and from the present study, which investigated the effect of a high flavonol intake from a combination of flavonol-rich foods. Each point represents the mean and SD of 12 (▲), 15 (+), or 9 subjects (●)*

Dose-response relationship

Flavonol intake was related linearly with flavonol concentration in plasma and with excretion in urine (Figures 5.3 and 5.4). Figure 5.3 shows the mean plasma levels and urinary excretions of quercetin in the present and previous studies as a function of intake. Figure 5.4 shows the same data for kaempferol. The equations and 95% confidence intervals describing the relations of plasma and urinary quercetin and kaempferol with the amounts consumed are presented in Table 5.1.

Table 5.1 *Equations describing the relation of levels of flavonols in plasma and urine (Y) with their intake (X)*

Flavonol levels	Number of observations	Regression equation	95% Confidence interval for the regression coefficient
Plasma quercetin μg/L	36	$Y = 2.2 + 0.7\ X^{*}$	0.6;0.9
Urinary quercetin μg/24h	36	$Y = 3.4 + 7.2\ X^{*}$	5.9;8.6
Plasma kaempferol μg/L	36	$Y = 0.4 + 0.6\ X^{\dagger}$	0.4;0.8
Urinary kaempferol μg/24h	36	$Y = 24.7 + 22.4\ X^{\dagger}$	17.5;27.3

* X = intake of quercetin (mg/d)

† X = intake of kaempferol (mg/d)

Variability

Concentrations of quercetin and kaempferol in plasma differed little between the four blood samples obtained on the high-flavonol diet. No significant differences ($p<0.05$) between concentrations of these samples were found. Therefore, concentrations of total flavonols in plasma were stable with time. The coefficient of variation within subjects of the levels in the four blood samples obtained on the high-flavonol diet was 32% for quercetin, and 53% for kaempferol. The coefficient of variation between subjects was 61% for quercetin, and 18% for kaempferol.

The correlation between plasma concentrations and urinary excretions was better for quercetin than for kaempferol: the Pearson correlation coefficient between the average plasma concentrations on days 25 and 26 on the high-flavonol diet and the excretion in urine on day 25 was 0.66 (CI 0.01;0.92) for quercetin, and 0.32 (CI -0.44;0.81) for kaempferol. Plasma concentrations of quercetin appeared to be related to those of isorhamnetin. The correlation coefficient between these plasma concentrations was 0.78 (CI 0.24;0.95).

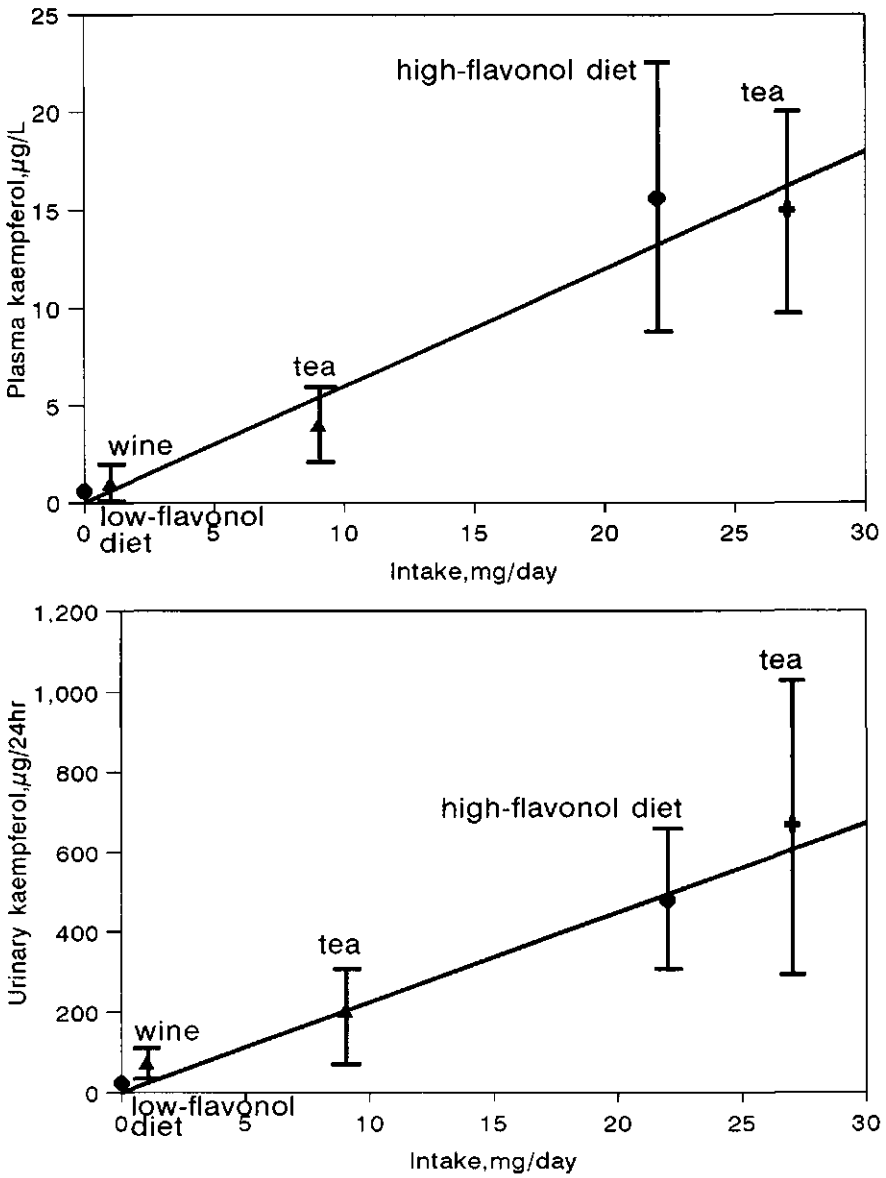


Figure 5.4 *The dose-response relationship between kaempferol intake and levels in plasma and urine. Data are from two previous studies investigating the effects of low-flavonol intakes from single flavonol-rich foods, and from the present study investigating the effect of high-flavonol intakes from multiple flavonol-rich foods. Each point represents the mean and SD of 12 (▲), 15 (+) or 9 subjects (●)*

Discussion

The objective of this study was to evaluate the suitability of flavonol concentrations in plasma and flavonol excretions in urine as biomarkers of flavonol intake. The major conclusion of our study is that the concentration of quercetin in plasma can be used to distinguish individuals with a low from those with a high intake of quercetin. We base this conclusion on an evaluation of flavonol levels with respect to two factors. These factors are their sensitivity to intake, and their variability. We will discuss both factors in more detail below.

Sensitivity to intake

An important prerequisite of a biomarker is that it is sensitive to intake. In addition to this, it is important to know the shape of the relationship between intake of the nutrient and the level of the biomarker.

Concentrations of flavonols in plasma and excretions in urine appeared to be sensitive to their intake. This was shown by the fact that levels of quercetin and kaempferol were clearly higher on the high-flavonol than on the low-flavonol diet. In addition, flavonol excretions in urine on the high-flavonol diet agreed well with those observed previously [10], after consumption of single foods, if type and dose of food were taken into account. As urinary excretion predicts bioavailability of flavonols [10], this implies that the bioavailability was not influenced by the dose or by prolonged consumption. We will illustrate this with the following calculation. In the high-flavonol diet, onions contributed 63 mg, tea 32 mg, and apples 37 mg to quercetin intake. In studies of single foods, urinary excretion of quercetin was 1.0% of the amount consumed from onions, 0.5% of the amount consumed from tea, and 0.3% of the amount consumed from apples [10,20]. The predicted excretion on the high-flavonol diet therefore is 1.0% of 63 mg + 0.5% of 32 mg + 0.3% of 37 mg = 0.90 mg/d, which equals 0.7% of the total intake of 132 mg. This value is identical to that observed. For kaempferol, it was also clear that bioavailability was not influenced by the dose (Figure 5.4B): a constant percentage was excreted over a wide range of intakes. These findings point to a linear relationship between intake of flavonols and their excretion in urine. This was confirmed by the combined results of the present study and the two previous studies. These showed that intake of flavonols was related linearly with their concentrations in plasma and their excretions in urine within the range of normal intakes (Figure 5.3 and 5.4). Small deviations from the regression lines for quercetin could be explained by differences in bioavailability between the foods: levels after onions were above and after tea below the regression lines. Thus, levels of flavonols in plasma and urine were sensitive to intake within the habitual range of consumption [3,21].

Variability

The variability of a biomarker should not be too large, because variability leads to misclassification when individuals are classified by their intake [9]. Information about variability can be obtained by observing the levels of biomarker in time, and by calculating

the coefficient of variation within subjects after repeated measurements.

Plasma levels of flavonols stabilized within one week on the high-flavonol diet. This was evidenced by the fact that plasma concentrations of flavonols did not change beyond day 8. The level of quercetin on day 8 on the high flavonol diet was somewhat higher than that on day 15, but this was probably by chance. Stable plasma levels beyond day 8 agree with the half-live of 24 h with which quercetin is eliminated from the body [10]. A half-live of 24 h indicates that after repeated intakes of quercetin-rich foods quercetin in plasma reaches 87.5% of its plateau level after 3 days. Thus, it is to be expected that plateau levels of quercetin in plasma and probably also of kaempferol are reached between three and seven days after the start of repeated consumption.

Variability of the level of a biomarker is caused by within-subject variations, metabolic between-subject variations, and imprecision in the laboratory. We determined these components of variability for quercetin from the four repeated measurements in plasma on the high-flavonol diet. The within-subject coefficient of variation for quercetin was 32%, and the between-subject coefficient of variation was 61 %. These coefficients of variation include imprecision in the laboratory. Therefore, the total coefficient of variation for a single measurement of quercetin in plasma between individuals with the same intake was $\sqrt{(32^2 + 61^2)}$= 69%. This coefficient of variation is larger than that of 30% which we found for plasma quercetin in a previous study after a lower dose of quercetin from onions [20]. When we left the data of one subject with the lowest urinary excretion out of the analysis, the coefficient of variation decreased to 52%. This subject probably did not consume all the supplements, because his excretion was 0.3% lower than the lowest level seen in a previous study [20]. Nevertheless, the value of 52% is still higher than the value of 30% found before. This larger variation may be explained by the higher dose, the longer period, an intake from more than one food source, or by chance. Also, heavy smoking might have increased differences in metabolism between subjects in this study [22]. For subsequent calculations of practical consequences we use the variation coefficient of 52%.

Applicability of plasma quercetin in epidemiological studies

Biomarkers of intake can be used to classify individuals into categories of intake. Therefore, we calculated what differences in quercetin intake might be discriminated when plasma quercetin is used as a biomarker. For these calculations we used the formula $b=(Z_p\sigma/\sqrt{n})$ and a probability of misclassification of 0.05 [23]. In this formula, b denotes the distance between the true mean flavonol intake for an individual and the boundary point between a higher and a lower class of intake, Z_p the percentage point of the standard normal distribution, σ the measurement error, and n the number of measurements. We calculated the measurement error of the plasma quercetin, σ, for an average intake of 16 mg/d [3]. The equation: plasma quercetin = 2.2 + (0.7 x quercetin intake) (Table 5.1) then predicts a plasma level of quercetin of 13 μg/L. 52% of 13 μg is 7 μg/L; thus σ= 7 μg/L. This value of 7 μg/L when included in the formula results in a difference in flavonol intake of 14 mg per day. Thus, when the upper boundary point of a lower quantile differs 14 mg or more from the lower boundary point of a higher quantile, the probability for an indivual to be

misclassified from the lower quantile to the higher quantile or conversely by one measurement of plasma quercetin is < 0.05. According to recent studies [3,5,6,24] differences observed between the lowest and the highest quantile of quercetin intake vary between 20 and 30 mg/d; therefore subjects with a low or with a high flavonol intake could be easily distinguished by one measurement of quercetin in plasma. In the Health Professional Study [6] the difference between the medians of flavonol intake of the third and fifth quintile was 18 mg/d of quercetin. In the Zutphen Study [2], the highest intake of the lowest tertile differed 11 mg/d from the lowest intake of the highest tertile. Thus, these two studies indicate that one or two plasma samples are sufficient to distinguish individuals at least by low and high flavonol intakes.

In cross-cultural studies, differences in quercetin intake between individuals might be larger than in cross-sectional studies. In the Seven-Countries Study [4] the difference in quercetin intake between the cohort with the lowest intake (West-Finland) and the highest intake (Ushibuka, Japan) was 33 mg/d. In a group of subjects from a variety of countries [21], we found differences in intake of more than 50 mg/d. Thus, in these studies a larger number of quantiles could be distinguished than in one population.

Levels of other flavonols

In addition to quercetin in plasma, quercetin in urine and kaempferol in plasma or urine could probably also be used as biomarkers of intake. As excretion of quercetin in urine agreed reasonably well with concentration in plasma urinary (Figure 5.3) excretion may also be a suitable biomarker of quercetin intake. However, we were not able to determine the within-subject variation because measurements in urine had not been repeated. It was also not possible to assess the suitability of habitual kaempferol in plasma or in urine as biomarkers of their intake, because variation of kaempferol intake between free-living subjects was not known. Also, the role of isorhamnetin is not yet clear. Levels of isorhamnetin may be used in addition to those of quercetin to determine quercetin intake, as it is formed from quercetin in the body. This relationship was confirmed by a correlation coefficient of 0.78 between plasma levels of quercetin and isorhamnetin.

Conclusions

We found that intake of flavonols is related linearly with their levels in plasma and urine. Because of this relation, and because variability of plasma levels is limited, plasma quercetin can be used to classify individuals into categories of quercetin intakes. Therefore, flavonol biomarkers may be a valuable tool to assess flavonol intake in epidemiological studies.

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6

The fatty acid and sterol content of food composites of middle-aged men in seven countries

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Abstract

Specific fatty acids and sterols in food composites from seven countries were analyzed. In the 1960s, groups of 8 to 49 men from 16 cohorts, ages 40 - 59 y and living in the United States, Finland, the Netherlands, Italy, Greece, the former Yugoslavia, or Japan recorded their food intake. In 1987, we collected food composites representing the average food intake per cohort sample in the 1960s. The foods were transported to the Netherlands, pooled, and centrally analyzed for energy, total fat, 42 fatty acids, cholesterol, and four plant sterols. The fat content ranged from 12% of total daily energy in the Tanushimaru, Japan, cohort to 50% in the US cohort sample, and the polyunsaturated to saturated fat ratio from 0.17 in the East-Finland cohort to 1.2 in Tanushimaru. The amount of *trans* fatty acids with 16 or 18 carbon atoms varied between 0.2 g/day in Corfu, Greece and 8.6 g/day in Zutphen, Netherlands, and that of α-linolenic acid between 0.8 g/day in Rome and 2.5 g/day in east Finland. The sum of eicosapentaenoic (EPA) and docosahexanoic acid (DHA) ranged from 0.1 (U.S. railroad) to 2.0 g/day (Ushibuka, Japan), and phytosterols from 170 (US railroad) to 358 mg/day (Corfu, Greece). Thus the intake of various fatty acids and sterols with potential relevance for coronary heart disease occurrence varied 10-fold or more between cohorts. Our data can be used to generate new hypotheses about the causes of differences in incidence of diseases between countries.

Introduction

Fatty acids and sterols in the diet play a major role in the etiology of coronary heart disease [1]. Therefore, it is important to know the amounts and types of fatty acids and sterols that are present in the diets of populations, and to know which foods are the main contributors. However, food tables lack reliable data on the content of specific fatty acids, such as *trans* fatty acids [2], long chain polyunsaturated (*n*-3) fatty acids, and plant sterols. As a consequence, the content of these nutrients in diets cannot be calculated from food balance sheets or dietary surveys, using nutrient data bases.

At the end of the 1950s, the Seven Countries Study was designed to study the relationships between diet and the occurrence of chronic diseases [3]. The food intake was recorded at that time by random samples of men, ages 40 - 59 years, who were part of a total of 16 cohorts in the United States, Finland, the Netherlands, Greece, Italy, the former Yugoslavia, and Japan. Duplicate portions were collected in 1960, and protein, total fat, and overall fatty acid composition were analyzed. However, a large number of nutrients and other components in the diet which could be important with regard to the occurrence of diseases were not determined. In 1987 we therefore decided to sample equivalent food composites, representing the average reported food intake of the men in the 1960s [4,5], and to analyze these chemically for a large number of nutrients and nonnutrients.

One of our aims was to obtain detailed information on the fatty acid and sterol composition of the diets, and we therefore determined the mean content of 42 fatty acids and four sterols in the collected food composites in a central laboratory. We present here the results of these chemical analyses, which can be used to investigate relationships between the composition of diets in the 1960s and the disease outcome in later years.

Methods

Subjects

Sixteen groups each containing 502 to 2571 men, ages 40-59 years, were selected in the seven countries. Random samples of 8 to 49 men in each cohort (Table 6.1), recorded their food intake, mostly during seven days at the baseline survey in the 1960s [4-6].

The US participants were railroad employees. One Finnish cohort lived in east Finland, and the other in west Finland. In the Netherlands men living in the commercial town of Zutphen, in the eastern part of the country, participated. Italy contributed three cohorts: Montegiorgio and Crevalcore, both rural villages, and railroad personnel in Rome. Greece contributed two: one on Crete and one on the island of Corfu. Five cohorts were from the former Yugoslavia: two from Croatia included inhabitants of six towns on the Dalmatian Coast and of Dalj, a small town in Slavonia. The other three cohorts consisted of farmers from the village of Velika-Krsna, workers in a large cooperation in Zrenjanin, and university professors in Belgrade in Serbia, respectively. Japan had two cohorts: farmers from the village of Tanushimaru and fishermen from the village of Ushibuka.

Table 6.1 *Characteristics of food records reported by men ages 40 to 59 years in 16 cohorts in seven countries in the 1960s*

Cohort	Country	Year	Number of subjects	Number of recording days
US railroad	United States	1960-62	30	1
East-Finland[a]	Finland	1959	30	7
West-Finland[a]	Finland	1959	30	7
Zutphen	Netherlands	1960	45	7
Crevalcore[a]	Italy	1960	29	7
Montegiorgio[a]	Italy	1960	35	7
Rome railroad	Italy	1969	49	7
Corfu[ab]	Greece	1961-63	37	7
Crete[ab]	Greece	1960-65	31	7
Dalmatia	Croatia	1960	24	7
Slavonia[a]	Croatia	1960	24	7
Belgrade[a]	Serbia	1964	41	7
Velika-Krsna[a]	Serbia	1963	21	7
Zrenjanin[a]	Serbia	1963	40	7
Tanushimaru[a]	Japan	1964	24	7
Ushibuka[a]	Japan	1971	8	4

Note The full cohorts contained 502 to 2571 men, of whom a random sample or 8-49 recorded their food intake [a] Food recorded as amounts of raw products [b] Original records no longer available

Food records

Between 1959 and 1964 samples of participants from 13 cohorts recorded and weighed all they ate and drank for 7 days and a sample of U.S. railroad employees did the same for 1 day. The participants from Rome recorded their food intake in 1969 for 7 days and those from Ushibuka in 1971 for 4 days. Thirteen cohorts reported the foods as weight of edible parts of raw products, and three cohorts as prepared foods. The two Greek cohorts recorded their intakes in different seasons. As the original 7-day records of the Greek cohorts were no longer available, we reconstructed the diets using a publication of Keys [5], and information provided by the Greek dietician involved in the original study. We derived missing information on some foods, especially fruits and vegetables, from food balance sheets of Greece from the period 1961-65 [7].

Food composites

In 1986, the food intake data were recoded in a standardized way by one dietician. We converted amounts of prepared into amounts of raw foods, taking into account loss of water and loss and gain of fat during cooking. For each cohort sample, we averaged the daily

consumption of each food across persons and collected this amount.

Two Dutch dieticians assisted by local researchers and co-workers from the Seven Countries Study bought all foods between April and October 1987 in the same regions in which the men were living during the baseline survey in the 1960s. In 10 of the 16 locations they bought the duplicates in the same season (± 2 months) during which the food intakes had been recorded. For the United States, Belgrade, Zrenjanin, and Tanushimaru the purchasing of the foods took place in a different season.

The fat compositions of foods have changed between the 1960s and 1987 [8], and therefore we had to select the food items carefully to approximate the composition of the foods as consumed by the men in the 1960s as near as possible. For this purpose, we used food composition tables, and data from the literature [4]. The major food groups contributing to the fat composition of the diets were meat, dairy products, edible fats and pastries. For meat, we often chose the fatter variants (Table 6.2). We reduced the quantity of products to be bought by combining foods. We substituted foods consumed in amounts of less than 10 g per day by foods consumed in higher amounts, with a similar nutrient composition. In Table 6.2, we show the substitutions made for meat. If a food was no longer available locally, we also used a substitute with a comparable composition. Detailed information on the food sampling has been described in a Dutch report [9]. The maximum number of different foods bought per cohort was 80. The dieticians vacuum-packed all foods in plastic bags, and sent them to the Netherlands in cooled boxes by air express.

The average period between acquiring the foods and their first workup in the laboratory was 2 days (range 1-4 days). Inedible parts were removed so as to produce the edible part as recorded and consumed in the 1960s. For example, we only peeled apples if that is how they were consumed originally. Thereafter, the foods were weighed out, and homogenized at the Department of Human Nutrition, Agricultural University Wageningen. Foods were divided into subsamples before homogenizing. Homogeneous foods present in relative big amounts were blended together into a main sample after adding of t-BHQ (500 mg/kg). Nonhomogeneous foods or products present in small amounts were blended into a subsample first. The sizes of these subsamples were scaled up 5-50 times from the original amount. After that a proportional amount of the subsample separate was added to a part of the main sample. Foods containing vitamin C were also blended in separate subsamples containing 2% oxalic-acid. If a sample was too dry to homogenize, a known amount of demineralized water was added. After homogenization, the final combined samples were stored at -20°C.

Chemical analyses

We determined the amount of total fat according to Folch *et al.* [10]. The fatty acids were assayed after saponification of the Folch extract and derivatization to methyl esters [11]. Fatty acids were analyzed by three different methods, on a Chrompack 438AS gas chromatograph equipped with a flame ionization detector. For determination of C8, C10, C12, C14, C14:1, C15, C16, C16:1, C17, C17:1, C18, C18:1, C18:2, C18:3(n-6), C18:3(n-3), C20, C20:1, and C20:2, the column was a Durabond-225 fused silica, length x internal diameter 15m x

Table 6.2 *Types of meat including substitutions made for the types of meat consumed in small amounts or not available locally, sampled for food composites of 16 cohorts in seven countries*

Types of meat per cohort	Amount	Including	Types of meat per cohort	Amount	Including	Types of meat	Amount	Including
US cohort			***Zutphen***			***Rome***		
pork[a]	43	minced pork	beef liver	2	kidney, pork liver	beef liver	2	
fat pork	15	fat bacon x 2.5	minced beef	21	fat beef	mutton	6	
fat beef	9		pork[a]	30		hambone	5	
lean beef	17		fat bacon	13	lean bacon	beef[a]	147	fat veal
chicken	46	turkey,pheasant	lean beef	35		sausage	14	salami
beef[a]	122		uncooked ham	5	frankfurter,sliced meat	chicken	44	
frankfurter	5	luncheonmeat	smoke-dried beef	2		rabbit	21	horse mat
ham	15	bacon	luncheon-meat	4	liver sausage,bacon,pâté	tripe	3	other intestines
salami	2		saveloy	4	smoked sausage	lamb	8	
East-Finland			bacon	1		veal	21	cooked veal
beef liver	4		***Crevalcore***			lean beef	5	
lean beef	19	veal[a]	veal liver	2	chicken liver	duck	4	
fat mutton	14	fat pork	pork	9	sausage,drippings	gammon	11	
lean mutton	10		beef	29		mortadella	5	bacon
beef[a]	11		rabbit	8		***Corfu***		
fat beef	7	canned beef	duck	2		beef	17	
pork[a]	10		chicken	36	pigeon	chicken	18	
fat pork	11		veal	30		***Crete***		
lean pork	6		horse meat	12	lean pork	mutton	14	
teemakkara	20		salami	9		goat	7	
canned pork	3	bacon (x1.8)	mortadella	8		chicken	14	
smoked ham	2		bacon	6		***Belgrade***		
West-Finland			gammon	5		pork liver	4	
beef liver	1		***Montegiorgio***			dripplings	6	
lean beef[a]	17		veal liver	1		sausage	22	
pork[a]	10		sausage	15	pork[a]	pork[a]	40	smoked meat
fat pork	13	canned pork	goose	5	duck	chicken	14	turkey,goose
lean pork	5	fat beef	beef[a]	4	mutton	veal[a]	7	lamb[a]
teemakkara	24	berliini-makkara	chicken	12	pigeon	beef[a]	46	
suomimakkara	8		veal[a]	17	lean veal	pork brains	3	pork intestines
ryynimakkara	6		rabbit	6		bacon	10	
salted pork	6	saveloy	tripe	6	other intestines	smoked ham	4	bacon,liver
smoked ham	1		salami	9		salami	11	frankfurter
			mortadella	3	bacon,canned meat,ham	canned ham	7	gammon

Table 2 - *continued*

Types of meat per cohort	Amount	Including	Types of meat per cohort	Amount	Including	Types of meat	Amount	Including
Dalmatia			***Velika-Krsna***			***Tanushimaru***		
lamb liver	2		roasted piglet	22	pork*,smoked meat	beef liver	2	
smoked pork ham	20	sausage,hot-dog	roasted mutton	5	smoked meat	fat pork	3	fat beef, 25%
smoked *mutton*	5		*chicken*	2	pigeon	chicken	3	75% ham
lamb*	27		lamb*	3	roasted lamb	***Ushibuka***		
beef*	28	smoked bacon	smoked bacon	38		fat pork	3	
fileki	6		***Zrenjanin***			chicken	5	
veal	9	chicken	pork liver	2	duck and chicken liver			
bacon	20	salami,gammon	pork*	47	fat pork,50% mutton			
Slavonia			duck	14	50% mutton			
pork liver	3	beef, veal liver	sausage	25	sausage,dripplings (x2)			
pork*	59	sausage	chicken	38	pigeon			
dripplings	7		veal*	8				
chicken	24	*pigeon*	*intestines*	3	pork brains			
veal*	4		beef*	10	rabbit,smoked beef			
lamb*	10		smoked bacon	36				
beef*	16		smoked ham	5	gammon			
salami	3		salami	5				
bacon	58	blood pudding	bacon	15	frankfurter			
gammon	4							

* average fat content

0.54mm. The flow rate of the carrier gas hydrogen was 7.5 ml/min. The oven temperature was programmed from 100 to 180°C at a rate of 10°C/min, then to 225°C at 4°C/min, which was maintained for a further 2.75 min before cooling. The injector temperature was 220°C, and 0.5 µl was injected directly into a modified liner. The detector temperature was 250°C, the hydrogen flow for the detector was reduced to 17.5 ml/min, and the flow rate of the make-up gas nitrogen was 25 ml/min.

Trans fatty acids were determined by gas chromatography in our laboratory, and these results were checked by gas chromatography, and Fourier transform infrared spectrometry in the research laboratory of the Raisio group at Raisio, Finland. The *trans* fatty acid content of the food composite of Zutphen could not be analyzed in Finland due to solvent in the sample, and therefore it was re-analyzed by Fourier transform infrared spectrometry in our own laboratory together with the food composites of east and west Finland, and Velika-Krsna. The geometric and positional isomers of C16:1, C18:1, and C18:2 fatty acid methyl esters were measured using a CP-Sil88 fused silica 50-m x 0.25-mm column (Chrompack, the Netherlands). The oven temperature was programmed from 160°C to 220°C at a rate of 2°C/min plus 5 min at 220°C. The carrier gas was hydrogen, and the inlet pressure was 130 kPA. The injector was a splitter with a split ratio of 1:60, and a volume of 0.5 µl was injected at a temperature of 250°C. Fourier transform infrared spectrometry in Wageningen was done using a Biorad Digilab Division, Type FTS-7 infrared spectrophotometer. The preparation of fatty acid methyl esters for gas chromatography in Finland was done according to Bannon et al [12], and for the infrared spectrometry method according to IUPAC 2.301. Duplicate esters of the samples were analyzed as neat solutions according to Sleeter and Matlock [13]. For gas chromatography in Finland a Hewlett-Packard 5890 II was used, and the IR spectrophotometer was a Perkin-Elmer 16PC, Type FTIR.

The fatty acids C18:4(*n*-3), C22, C22:1(*n*-9), C20:3(*n*-3), C20:4(*n*-6), C24, C20:5(*n*-3), C24:1, C22:3(*n*-3), C22:4(*n*-6), C22:5(*n*-3), and C22:6(*n*-3) were assessed separately in a third analysis, again using a CP-Sil88 fused silica 50-m x 0.25-mm column. The initial oven temperature was 160°C for 4 min, followed by a rise of 25°C/min to 215°C, which was kept for a further 12 min before cooling down. The injector was a Splitter with a split ratio of 1:15, and the injected volume was 1.5 µl. Injector and detector temperature were 250°C.

We extracted the unsaponifiable part of the lipid [14] and determined cholesterol, campesterol, stigmasterol and ß-sitosterol by gas chromatography using a Chrompack-Packard model 439, column CP-Si119/cb (25 m x 0.25 mm). The oven temperature was programmed from 240 to 265°C at a rate of 1.25 °C/min, then to 285°C by 5°C/min, which was maintained for a further 8 minutes before cooling down. The carrier gas hydrogen had an inlet pressure of 105 kPa. The injector was a splitter, and the injected volume 1 µl with a split ratio of 1:30. The temperature of injection was 300°C and of detection 305°C. The flow rate of the make-up gas nitrogen was 25 ml/min. We determined response factors by injection of pure compounds with 5α-cholestane as internal standard. For campesterol we used the factor for cholesterol. Chromatograms for fatty acids and for sterols were recorded by a personal computer equipped with PCI-GC software version 4.0 (Chrompack).

We tested the recovery of *n*-3 fatty acids by adding herring of known EPA and DHA content to 1766 grams of the food composite of Zutphen. Recovery was 106 ± 5% for C20:5 and 98 ± 6% for C22:6 after addition of 11 g, and 93 ± 5% for C20:5 and 88 ± 4 % for C22:6 after addition of 25 g of herring.
The conversion factor used for fat to fatty acids was 0.956. We determined protein [14] and alcohol [15] and calculated carbohydrates as the sum of analyzed values of galactose, glucose, saccharose, lactose, maltose and starch. Atwater factors for the calculation of energy were 17 kJ (4 kcal) for protein, 37 kJ (9 kcal) for fat, 16 kJ (3.75 kcal) for carbohydrates, and 29 kJ (7 kcal) for alcohol.

Calculation by use of food composition tables
We compared the analyzed values for the food composites from the United States, Finland, the Netherlands, and Italy with values calculated using food composition tables. We used the Dutch computerized nutrient data bank [16] for foods with a nutrient composition similar to that of Dutch foods and for foods containing no fat or sterols, and the food composition table of the specific countries [17-19] for the remainder.

Table 6.3 *Total energy and fat content and major classes of fatty acids, determined by chemical analysis of food composites reflecting the average recorded food intake of samples of middle-aged men living in 16 cohorts in seven countries in the 1960s*

Cohort	Energy	Total fat		SFA	MUFA	PUFA	P/S
	MJ/d	*g/d*	*% of energy*		*g/d*		*ratio*
US railroad, USA	9.6	131	50	55	49	21	0.4
East-Finland	14.4	166	42	89	55	15	0.2
West-Finland	14.7	142	36	73	49	14	0.2
Zutphen, Netherlands	11.3	137	45	61	50	20	0.3
Crevalcore, Italy	15.2	155	38	54	73	21	0.4
Montegiorgio, Italy	12.1	101	31	31	50	15	0.5
Rome railroad, Italy	11.0	96	32	28	54	10	0.4
Corfu, Greece	11.3	105	34	22	63	15	0.7
Crete, Greece	11.8	131	41	28	84	13	0.5
Dalmatia, Croatian	15.8	148	35	40	78	24	0.6
Slavonia, Croatian	15.5	179	43	69	81	22	0.3
Belgrade, Serbia	11.4	143	46	57	54	26	0.4
Velika-Krsna, Serbia	14.3	115	30	52	42	16	0.3
Zrenjanin, Serbia	13.4	149	41	55	65	23	0.4
Tanushimaru, Japan	10.0	33	12	10	10	12	1.2
Ushibuka, Japan	10.2	46	17	14	17	13	0.9

* SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; P/S ratio = ratio of polyunsaturated to saturated fatty acids.

Results

Energy and total fat

The energy content varied from 9.6 MJ/day in the diets of the US railroad employees to 15.8 MJ/day in the Croatian cohort Dalmatia, and the fat content in the food composites from 12% of total energy in the Japanese farmers in Tanushimaru to 50% in the USA (Table 6.3). The ratio of polyunsaturated to saturated fatty acids (P/S) varied from 0.2 for east and west Finland to 0.9 and 1.2 for Ushibuka and Tanushimaru in Japan, respectively (Table 6.3).

Saturated fatty acids

The saturated fatty acid content was low (<10% of energy) in the food composites of Greece and Japan, the Italian cohorts Montegiorgio and Rome, and the Croatian cohort Dalmatia. We found high levels of saturated fatty acids (>15% of energy) for east and west Finland, the Netherlands, the United States, the Serbian cohorts Belgrade and Zrenjanin, and the Croatian cohort Slavonia.

Table 6.4 *Saturated fatty acids in food composites from 16 cohorts of middle-aged men in seven countries, representing the average food intake in the 1960s, sampled in 1987*

Cohort	C8:0	C10:0	C12:0	C14:0	C15:0	C16:0	C17:0	C18:0	C20:0	C22:0	C24:0
					grams per day						
US railroad, USA	0.5	1.3	1.8	5.8	0.7	30	0.7	14	0.3	0.3	0.1
East-Finland	1.3	3.3	4.3	14	1.6	41	0.9	22	0.4	0.3	0.1
West-Finland	0.4	2.1	3.4	11	1.1	35	0.7	18	0.3	0.4	0.2
Zutphen, Netherlands	0.6	1.3	2.2	8.0	0.8	30	0.6	15	1.0	1.1	0.5
Crevalcore, Italy	0.5	0.8	1.1	4.2	0.5	31	0.5	15	0.5	0.4	0.2
Montegiorgio, Italy	0.1	0.3	0.3	1.3	0.1	19	0.3	9.4	0.3	0.2	0.1
Rome railroad, Italy	0.2	0.4	0.7	2.4	0.3	17	0.3	6.2	0.3	0.2	0.1
Corfu, Greece	0.2	0.4	0.3	1.1	0.2	16	0.1	3.1	0.4	0.2	0.1
Crete, Greece	0.3	0.7	0.5	1.8	0.3	18	0.2	5.6	0.6	0.3	0.1
Dalmatia, Croatian	0.3	0.5	0.5	2.5	0.3	24	0.4	10	0.5	0.3	0.2
Slavonia, Croatian	0.2	0.5	0.6	3.7	0.5	42	0.5	20	0.2	0.2	0.1
Belgrade, Serbia	0.7	1.2	1.3	5.6	0.8	31	0.7	15	0.4	0.4	0.2
Velika-Krsna, Serbia	0.9	1.3	1.5	5.8	0.8	29	0.5	12	0.1	0.2	0.1
Zrenjanin, Serbia	0.1	0.3	0.4	2.6	0.2	33	0.5	17	0.4	0.2	0.1
Tanushimaru, Japan	0.1	0.0	0.1	0.8	0.1	6.5	0.1	2.0	0.1	0.1	0.1
Ushibuka, Japan	0.0	0.0	0.2	1.0	0.1	9.2	0.2	3.0	0.1	0.1	0.1

The levels of the medium chain fatty acids (MCT) caprylic (C8:0) and capric acid (C10:0) were high in the Finnish samples (Table 6.4). The contribution of lauric (12:0), myristic (14:0), and palmitic acid (16:0) varied from 65% of saturated fatty acids for Zrenjanin in Serbia to 79% for Corfu and Crete (Table 6.4). Stearic acid content ranged

from 20% (Tanushimaru, Japan) to 32% (Zrenjanin, Serbia) of saturated fatty acids. The highest amount of saturated fatty acids of more than 20 carbon atoms was 2.5 g/day in Zutphen (Table 6.4).

Monounsaturated fatty acids

The range of mono-unsaturated fatty acids varied from 9.8 g/day in Tanushimaru, Japan, to 84 g/day on the Greek isle of Crete. The levels of monounsaturated fat in the food composites of Greece exceeded 20% of energy.

Sixty to ninety percent of monounsaturated fat (Table 6.5) in the food composites occurred as oleic acid [*cis* C18:1,(*n*-9)]. The absolute amount varied from 8.0 g/day in Tanushimaru, Japan, cohort to 82 g/day in Crete. The second major monounsaturated fatty acid was palmitoleic acid [*cis* C16:1,(*n*-7)] with a highest consumption of about 4 g/day in Slavonia, Croatia. The maximum intake of both erucic [C22:1,(*n*-9)] and cetoleic acid [C22:1,(*n*-11)] was 2.9 g/day in Zutphen (Table 6.5).

Total intake of very long chain fatty acids (saturated and unsaturated fatty acids with more than 20 carbon atoms) was about 14 g/day in Zutphen, and 2-5 g/day in the other cohorts (Tables 6.4, 6.5, and 6.6).

Table 6.5 *cis-Monounsaturated fatty acids in food composites from 16 cohorts of middle-aged men in seven countries, representing the average food intake in the 1960s, sampled in 1987*

Cohort	C14:1	C16:1		C17:1	C18:1[a]			C20:1	C22:1	C24:1
		total	(*n*-7)*cis*	(*n*-7)*cis*	total	(*n*-9)*cis*	(*n*-7)*cis*			
					grams per day					
US railroad, USA	0.8	3.0	3.0	0.4	44	37	2.6	0.4	0.1	0.0
East-Finland	1.3	3.4	3.0	0.0	49	42	2.4	0.6	0.2	0.1
West-Finland	1.1	2.8	2.5	0.3	44	38	2.4	0.7	0.3	0.0
Zutphen, Netherlands	0.6	4.3	1.7	0.3	37	29	2.2	4.3	2.9	0.2
Crevalcore, Italy	0.4	2.7	2.3	0.0	68	62	4.6	1.0	0.3	0.0
Montegiorgio, Italy	0.2	1.7	1.4	0.0	47	42	3.3	0.9	0.4	0.0
Rome railroad, Italy	0.4	1.6	1.4	0.2	51	47	2.8	0.8	0.3	0.0
Corfu, Greece	0.1	1.4	1.2	0.1	61	56	4.9	0.3	0.1	0.0
Crete, Greece	0.2	1.3	1.1	0.2	82	77	4.7	0.4	0.1	0.0
Dalmatia, Croatian	0.3	2.8	1.9	0.2	73	67	4.6	1.3	0.2	0.1
Slavonia, Croatian	0.4	5.3	4.1	0.0	73	64	7.0	2.0	0.3	0.1
Belgrade, Serbia	0.6	3.0	2.8	0.4	49	43	4.0	1.1	0.1	0.0
Velika-Krsna, Serbia	0.6	2.6	2.2	0.2	38	33	2.8	0.7	0.3	0.0
Zrenjanin, Serbia	0.3	3.8	3.0	0.4	59	52	5.5	1.4	0.3	0.0
Tanushimaru, Japan	0.1	0.8	0.6	0.0	8.0	6.9	0.9	0.0	0.8	0.2
Ushibuka, Japan	0.2	1.6	1.3	0.0	15	13	1.8	0.0	0.3	0.1

[a] No C18:1(n-12)*cis*

Trans fatty acids

The amount of *trans* fatty acids with 16 and 18 carbon atoms as determined by gas chromatography in Wageningen varied from 0.2 grams or 0.1 % of energy in Corfu, Greece to 8.6 grams or 2.8 % of energy in Zutphen, the Netherlands (Table 6.6). The highest levels, absolute as well as relative to energy intake, were found in the food composites of the Dutch, U.S., and Finnish cohorts, and the lowest amounts in those of Greece, Italy, and Japan. The main contribution to *trans* fatty acids was formed by *trans* isomers of C18:1 (Figure 6.1). The food composite of Zutphen contained the highest amount of *trans* isomers of palmitoleic acid (16:1), while *trans*-16:1 was not present in the food composites of Italy, Greece, Croatia, and Zrenjanin, Serbia. The values measured in Finland by gas chromatography were on average 0.4 ± 0.9 g/day lower than in Wageningen. The value for Zutphen, Netherlands was 3.5 g/day higher while differences for other cohorts were less than 1 g/day. The total amount of *trans* fatty acids as assessed by infrared spectrometry was in general higher than the amounts of 16:1, 18:1 and 18:2 *trans* fatty acids assessed by gas chromatography (Figure 6.2). We found a large difference between the methods for the Zutphen cohort.

Table 6.6 *C16 and C18 trans fatty acids, as determined by gas chromatography, and total trans fatty acids as determined by infrared spectrometry in food composites from 16 cohorts of middle-aged men in seven countries, representing the average food intake in the 1960s, sampled in 1987*

Cohort	C16:1 (n-7)*trans*	C18:1(n-9) to C18:(n-12) *trans*	C18:1 (n-7)*trans*	C18:2 *trans,trans*	C18:2 *trans,cis*	C18:2 *cis,trans*	Sum of *trans* C16:1,C18:1 C18:2	Total *trans* fatty acids (infrared)
				grams/day				
US railroad, USA	0.3	3.9	0.0	0.3	0.2	0.4	5.0	6.0
East-Finland	0.8	4.9	0.0	0.6	0.4	0.4	7.1	9.0[b]
West-Finland	0.6	3.6	0.0	0.2	0.0	0.1	4.5	7.6[b]
Zutphen, Netherlands	2.6	2.4	3.1	0.0	0.3	0.3	8.6	26
Crevalcore, Italy	0.0	1.1	0.0	0.0	0.0	0.0	1.1	1.8
Montegiorgio, Italy	0.0	0.1	0.3	0.0	0.0	0.1	0.6	1.1
Rome railroad, Italy	0.0	0.7	0.0	0.0	0.0	0.0	0.7	1.6
Corfu, Greece	0.0	0.2	0.0	0.0	0.0	0.0	0.2	0.3
Crete, Greece	0.0	0.4	0.0	0.0	0.0	0.0	0.4	0.6
Dalmatia, Croatian	0.0	1.1	0.0	0.0	0.0	0.0	1.1	2.3
Slavonia, Croatian	0.0	1.2	0.0	0.0	0.0	0.0	1.2	2.1
Belgrade, Serbia	0.3	0.0	2.0	0.0	0.0	0.0	2.3	3.0
Velika-Krsna, Serbia	0.1	2.2	0.0	0.0	0.0	0.0	2.3	3.8[b]
Zrenjanin, Serbia	0.0	1.3	0.0	0.0	0.0	0.0	1.3	1.4
Tanushimaru, Japan	0.1	0.0	0.1	0.0	0.0	0.0	0.2	0.4
Ushibuka, Japan	0.0	0.0	0.3	0.0	0.0	0.0	0.3	0.4

[a] determined in the research laboratory of the RAISIO group, Raisio, Finland; value for Zutphen determined in the Wageningen laboratory [b] average value of determinations in Raisio, Finland and in Wageningen

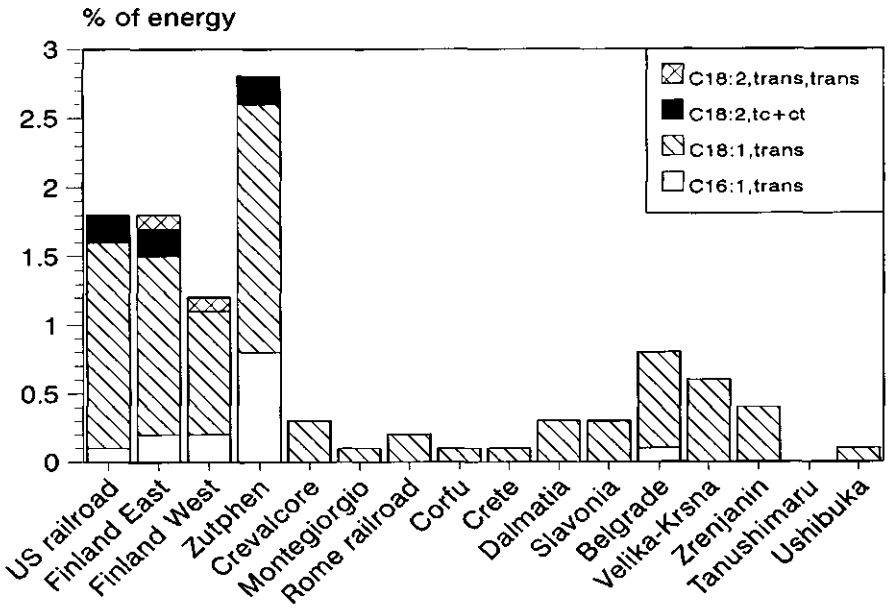

Figure 6.1 *The amount of C16:1, C18:1, and C18:2 trans fatty acids according to gas chromatography in food composites from 16 cohorts of middle-aged men in seven countries, representing the average food intake in the 1960s, sampled in 1987*

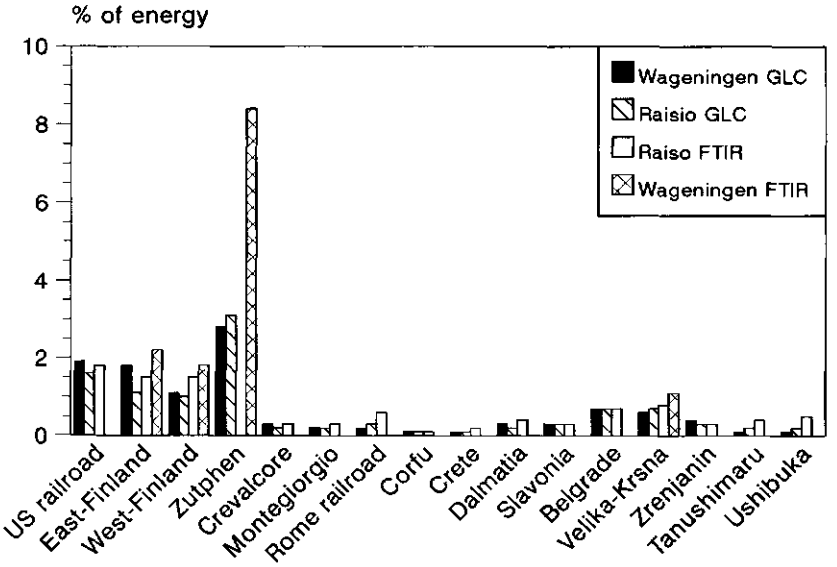

Figure 6.2 *Sum of C16:1, C18:1, and C18:2 trans fatty acids according to gas chromatography in Wageningen and Raisio, Finland, and total trans fatty acids including C20 and C22 isomers according to infrared spectrometry in Raisio, Finland, in food composites from 16 cohorts and according to infrared spectrometry in Wageningen from 4 cohorts of middle-aged men in seven countries, representing the average food intake in the 1960s, sampled in 1987*

Polyunsaturated fatty acids

The polyunsaturated fatty acid fraction (Table 6.7) consisted for 54% to 85% of linoleic acid [18:2,(*n*-6)]. The intake of linoleic acid was high in the Serbian cohorts of Zrenjanin and Belgrade and in the United States, and low in Finland. As for the other (*n*-6) polyunsaturates the amount of γ-linolenic acid [18:3,(*n*-6)] was at the most 0.29 grams, and arachidonic acid [C20:4,(*n*-6)] 0.5 g per day. The content of the major *n*-3 (ω-3) polyunsaturated fatty acid, α-linolenic acid, varied from 0.79 grams in the diet of Rome railroad workers to 2.5 grams per day in east Finland. The food composites of Japan, Dalmatia, east Finland, and Corfu contained the highest, and those of the United States, Rome, and Velika-Krsna the lowest levels of eicosapentaenoic [EPA; C20:5(*n*-3) and docosahexanoic acid [DHA; C22:6,(*n*-3); see Figure 6.3). The ratio of linoleic to α-linolenic acid ranged from 3.2 in east Finland to 15 in Velika-Krsna (Table 6.7). T he (*n*-3) fatty acids have critical roles in membrane structure and are precursors of eicosanoids in the body. A number of studies have shown that the consumption of foods containing EPA and DHA is associated with decreased risk of coronary heart disease [20]. α-Linolenic acid is elongated in the body to EPA and DHA, and the efficiency of this conversion is estimated to be about 20% [21]. Therefore, 1 g of dietary α-linolenic acid is not equivalent physiologically to 1 g of EPA or DHA but to much less. In order to allow comparison of the total intake of (*n*-3) fatty acids across cohorts independent of source we multiplied α-linolenic acid intake by 0.2 and combined these values with intakes of EPA and DHA (Figure 6.3). The total intake of (*n*-3) fatty acids calculated in this way was highest in Ushibuka, Dalmatia, Tanushimaru, and east Finland (Figure 6.3).

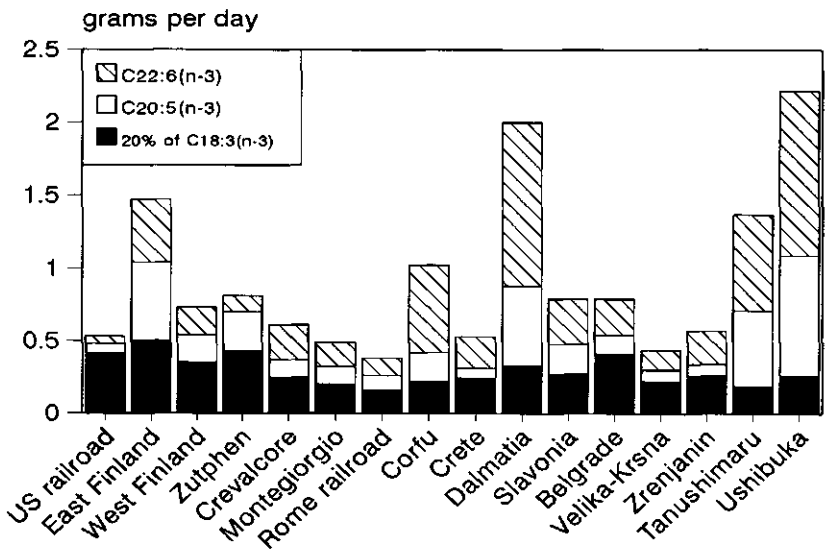

Figure 6.3 *The amount of the major (n-3) fatty acids in food composites from 16 cohorts of middle-aged men in seven countries, representing the average food intake in the 1960s, sampled in 1987, including 20% of the intake of C18:3 (n-3), as a measure for C18:3(n-3) converted into longer (n-3) fatty acids in the body. Values for C18:3 were divided by 5 because only about one-fifth of dietary C18:3 is elongated to EPA of DHA* [21]

Table 6.7 *Polyunsaturated fatty acids in food composites from 16 cohorts of middle-aged men in seven countries, representing the average food intake in the 1960s, sampled in 1987*

Cohort	C18:3 (*n*-3)	C18:4 (*n*-3)	C20:3 (*n*-3)	C20:5 (*n*-3)	C22:3 (*n*-3)	C22:5 (*n*-3)	C22:6 (*n*-3)	C18:2 (*n*-6)	C18:3 (*n*-6)	C20:4 (*n*-6)	C22:4 (*n*-6)	C20:2	C22:2	C18:2(*n*-6)/ C18:3(*n*-3)
	grams per day													*ratio*
US railroad, USA	2.1	0.3	0.0	0.1	0.0	0.1	0.0	17	0.2	0.4	0.1	0.0	0.0	8.3
East-Finland	2.5	0.6	0.1	0.5	0.0	0.2	0.4	8.0	0.3	0.4	0.1	0.0	0.0	3.2
West-Finland	1.7	0.6	0.1	0.2	0.0	0.1	0.2	8.7	0.1	0.4	0.0	0.0	0.0	5.0
Zutphen, Netherlands	2.2	0.2	0.0	0.3	0.1	0.1	0.1	12	0.1	0.5	0.1	1.5	1.2	5.8
Crevalcore, Italy	1.2	0.6	0.1	0.1	0.0	0.1	0.2	17	0.0	0.4	0.1	0.4	0.0	13
Montegiorgio, Italy	1.0	0.0	0.1	0.1	0.2	0.1	0.2	12	0.0	0.2	0.1	0.6	0.0	12
Rome railroad, Italy	0.8	0.0	0.0	0.1	0.1	0.1	0.1	8.1	0.0	0.3	0.0	0.2	0.0	10
Corfu, Greece	1.1	0.1	0.0	0.2	0.0	0.0	0.6	13	0.0	0.1	0.0	0.0	0.0	12
Crete, Greece	1.2	0.0	0.0	0.1	0.0	0.0	0.2	11	0.0	0.2	0.0	0.0	0.0	9.2
Dalmatia, Croatian	1.6	0.9	0.1	0.6	0.1	0.2	1.1	17	0.0	0.3	0.1	0.0	0.0	10
Slavonia, Croatian	1.4	1.5	0.2	0.2	0.2	0.2	0.3	16	0.0	0.5	0.1	0.0	0.0	12
Belgrade, Serbia	2.0	0.6	0.1	0.1	0.1	0.1	0.2	22	0.1	0.3	0.1	0.0	0.0	11
Velika-Krsna, Serbia	1.1	0.7	0.1	0.1	0.0	0.1	0.1	13	0.0	0.3	0.1	0.0	0.0	11
Zrenjanin, Serbia	1.3	0.7	0.1	0.1	0.0	0.2	0.2	19	0.0	0.4	0.1	0.0	0.0	15
Tanushimaru, Japan	1.0	1.0	0.3	0.5	0.2	0.1	0.7	8.0	0.0	0.1	0.0	0.0	0.0	8.4
Ushibuka, Japan	1.3	0.8	0.3	0.8	0.1	0.3	1.1	8.2	0.1	0.1	0.1	0.0	0.0	6.3

Sterols

The amount of cholesterol varied from 170 mg per day in Tanushimaru to 612 mg in Slavonia (Table 6.8). Intakes of plant sterols were lowest in the United States, and highest in Greece. The amounts of campesterol varied from 41 (Slavonia) to 63 mg per day (east Finland), of stigmasterol from 9 (Finland and Rome) to 30 mg per day (Tanushimaru), and of sitosterol from 113 (U.S. railroad) to 281 mg per day (Corfu).

Table 6.8 *Cholesterol and plant sterols in food composites from 16 cohorts of middle-aged men in seven countries, representing the average food intake in the 1960s, sampled in 1987*

Cohort	Cholesterol	Campesterol	Stigmasterol	Sitosterol	Sum of phytosterols	Sum of unknown compounds[a]
			mg per day			
US railroad, USA	582	39	19	113	170	33
East-Finland	537	63	9	159	232	122
West-Finland	492	60	9	149	218	96
Zutphen, Netherlands	476	60	18	146	224	80
Crevalcore, Italy	585	36	12	172	220	93
Montegiorgio, Italy	356	36	16	166	218	108
Rome railroad, Italy	409	33	9	136	179	91
Corfu, Greece	141	60	17	281	358	279
Crete, Greece	211	57	16	252	325	261
Dalamatia, Croatian	388	44	12	215	272	131
Slavonia, Croatian	612	41	24	151	215	89
Belgrade, Serbia	396	59	14	181	254	68
Velika-Krsna, Serbia	359	62	12	181	255	73
Zrenjanin, Serbia	311	54	15	172	242	85
Tanushimaru, Japan	170	44	30	134	208	71
Ushibuka, Japan	403	60	24	137	221	80

[a] refers to all peaks in the non-saponifiable lipid extract that eluted after the cholesterol peak on gas chromatography

Sources of visible fats

The visible fats as reported in the food records (Table 6.9) contributed from 9.7 to 72% of the total amount of fat. The main source of visible fats in the U.S. and Finnish samples in the 1960s was butter. In the Dutch sample Zutphen, margarine was more important than butter. Lard consumption was highest in the villages from Italy and the former Yugoslavia. The only visible fat eaten in the Greek cohorts was olive oil which was also an important fat source in Montegiorgio, Rome, and Dalmatia. In Belgrade the major visible fat was sunflower oil. The Japanese used small amounts of rapeseed (Tanushimaru) or peanut oil (Ushibuka).

Comparison with food composition tables

The largest difference between the calculated and chemically determined amount of total fat was 9% of energy or 40 g per day for Crevalcore (Table 6.10). For the other cohorts the

Table 6.9 *Type and amount of visible fats reported in food records in the 1960s by middle-aged men living in 16 cohorts in seven countries*

Cohort	Butter	Margarine	Lard	Olive oil	Sunflower oil	Other
			g/day			
US railroad, USA	26	3.9				3.1[a]
East-Finland	89	7.7				
West-Finland	66	6.8				
Zutphen, Netherlands	21	56				1.9[b]
Crevalcore, Italy	13	6.5	32	4.3	2.9	
Montegiorgio, Italy			40	40		
Rome railroad, Italy	3.6		1.8	43		
Corfu, Greece				75		
Crete, Greece				95		
Dalmatia, Croatia			16	72		
Slavonia, Croatia			62	7.8		
Belgrade, Serbia	18		9.0		28	
Velika-Krsna, Serbia			24		5.2	
Zrenjanin, Serbia			44		12	
Tanushimaru, Japan						3.2[c]
Ushibuka, Japan						7.3[b]

[a] soy oil
[b] peanut oil
[c] rapeseed oil

difference varied from 0.1 to 3% of energy. The largest difference for saturated fat was 3.5%, for monounsaturated fatty acids 4%, and for polyunsaturated fat 1.6% of energy. The difference between calculated and chemically determined cholesterol was at the most 6 mg/MJ or 21 mg/day (Table 6.10).

Discussion

We found large differences in the amounts of total fat, fatty acids, and sterols between the seven countries, and between cohorts in the same country. The difference between the highest and the lowest value for *trans* fatty acids in food composites was 8 g per day (2.8% of energy) for those *trans* fatty acids detectable by gas chromatography, and 25 g per day (8.3% of

energy) for the total amount of *trans* fatty acids as detected by infrared spectrometry. The sum of the long-chain *n*-3 fatty acids, EPA and DHA, between the highest and the lowest value, differed by almost 2 g per day. Amounts of fish providing these or smaller amounts of EPA and DHA are associated with marked differences in coronary heart disease incidence. Most differences can be explained by the differences in food intake, for example, higher intakes of hydrogenated fats and milk resulted in higher *trans* fatty acids levels, and higher fish consumption in a higher EPA plus DHA content of the food composites.

Two important questions are: (i) whether chemical analysis of food composites based on food records and composed 30 years after food recording provided valid data, and (ii) to what extent the results of samples may be extrapolated to the full cohorts and to their respective countries.

Table 6.10 *Comparison of the energy and nutrient content of food composites representing the average food intake in the 1960s, sampled in 1987 of the U.S., Finnish, Dutch, and Italian samples, according to calculation from food tables (Calc.) and to chemical analysis (Anal.)*

Cohort	Energy		Fat		Saturated fatty acids		Mono unsaturated fatty acids		Poly unsaturated fatty acids		Cholesterol	
	MJ		*% of energy*								*Mg/MJ*	
	Calc.	Anal.	Calc.	Anal.	Calc.	Anal.	Calc.	Anal.	Calc.	Anal.	Calc.	Anal.
US railroad	10.2	9.6	49	50	20	21	18	19	6.5	8.1	55	61
Finland East	14.6	14.7	39	42	20	22	11	14	2.1	3.8	34	36
Finland West	14.3	14.4	36	36	17	19	10	13	2.3	3.5	34	34
Zutphen	12.0	11.3	46	45	18	20	17	16	5.1	6.6	36	42
Crevalcore	14.6	15.2	29	38	9.7	13	14	18	3.6	5.0	33	38
Montegiorgio	12.6	12.1	28	31	8.6	9.6	13	15	5.0	4.5	25	29
Rome railroad	12.9	11.0	32	32	10	11	17	21	3.6	3.9	36	37

Food records

We selected the amounts and types of foods for composing the food composites on the basis of food records from the 1960s. Weighed food records give an average underestimation of energy intake of some 10% [22], but they still remain the most accurate method of dietary assessment [23]. Body mass index, age, and gender appear to be the major factors associated with underreporting [24-26], although some studies did not find an effect of obesity [27,28]. However, obese men in the Zutphen cohort reported a lower energy and fat intake than their lean counterparts [29]. Therefore, the energy and fat content of the food composites of Rome, Belgrade, Crevalcore and US railroad, where the men with the highest relative body weights lived (body mass index > 25), might have been underestimated more than in the other cohorts. Nevertheless, the proportional contribution of fat, fatty acids, and sterols might be still

reasonably correct, so that the data of these cohorts are still valuable. Gender and age range did not differ between the cohorts.

The reconstructed data of Greece appear to be useful, as they result in a total fat composition for Crete and Corfu that agrees with that reported earlier [30]. Although we derived the food composite of the USA from only 1-day records, it can still be considered representative for the food intake of the cohort, because food records of 30 men were used. It is uncertain if the eight Ushibuka men were representative for the men in their cohort, but as the total fat level of the food composite agrees with other data of Japanese dietary surveys [8,30], they probably were.

We do not expect that seasonal variation had a large effect on the results of our study. Seven day surveys covered seasons of the year, and the intake of energy-yielding nutrients was relatively constant throughout the year [5].

Food sampling

Most foods to be collected for the food composites were available. We acquired them within five months and sampled the foods for all cohorts in the same way. All food composites were analyzed in one laboratory. Although the relative position of the cohorts in the distribution of different foods between 1961-65 and 1975-77 was maintained [6], foods probably have changed in their total fat, fatty acid and sterol composition between the 1960s and the 1980s [8]. Meat and dairy products with the same total fat content as recorded by the men were largely still available. We only selected margarines of the stick form made from the same hydrogenated oils as in the 1960s. The main edible fat was margarine only in Zutphen, and as we periodically monitor the fat composition of Dutch margarines [31], it was possible to choose a margarine with a highly similar fatty acid composition in 1987.

In the 1960s, chemical analysis for determination of a limited number of nutrients of equivalent composites provided the amount of saturated, monounsaturated and polyunsaturated fatty acids for 13 of the 16 cohorts [4,5]. The correlation coefficients between these determinations and those found in our study for the same cohorts were 0.92 ($p<0.001$) for saturated fatty acids, 0.95 ($p<0.0001$) for monounsaturated fatty acids, and 0.65 ($p<0.02$) for polyunsaturated fatty acids. Therefore, we apparently succeeded in selecting the right foods, and composing representative food composites.

Chemical analysis

We were not able to assess butyric (C4:0) and caproic (C6:0) acids, due to workup of the samples and the method used for gas chromatography. Butyric and caproic acid contribute about 5% of total fatty acids in dairy products [32]. We calculated that as a result we could have missed at most about 3 g of fat per day in the cohorts with a high consumption of dairy products.

We considered the methods used well validated for food composites with more than 5% of fatty acid methyl esters. We used internal quality control samples, and within and between variations of determinations of these samples were respectively 1.4 and 1.7% for total fat (mean 18 g, n = 20), 1.4 and 2.0% for C16:0 (mean 34 g, n = 64), 2.9 and 3.6% for C18:0

(mean 5.2 g, n = 64), and 1.8 and 2.4% for C18:2 (mean 15 g, n = 64).

Comparisons between *trans* fatty acids levels between assessment by gas chromatography and Fourier transform infrared spectrometry, performed in the two laboratories showed good agreement, except for the Zutphen cohort which contains C20 en C22 polyunsaturated fatty acids in the *trans* configuration.

The recovery test for detection of (*n*-3) fatty acids showed that very small amounts of C20:5(*n*-3) and C22:6(*n*-3) could be determined accurately in the food composites. From all this it appears that our data are valid estimates of the fatty acid and sterol composition of the diets.

Calculation by use of food composition tables

The results of calculation by use of food composition tables agreed well with those of chemical analyses, except for the high fat content of the Crevalcore composite. This difference may be caused by a decrease of fat content of foods from the sixties to the eighties, although we did not find differences between calculated and analyzed fat content of this magnitude for Montegiorgio and Rome, for which we used the same food composition tables as for Crevalcore [16,18]. The total fat content assessed in the 1960s corresponds with the lower calculated level. This could point to selections of food with a fat content too low for this cohort. The calculations indicate that the difference in fat content was distributed evenly over the different fatty acids.

Fatty acid composition

Medium-chain fatty acids and stearic acid do not raise plasma LDL [33-37]. We found small differences in medium- chain fatty acid intake between cohorts, but stearic acid was 10 times higher in Finland and Slavonia than in Japan (Table 6.4). Palmitic (C16:0), myristic (C14:0), and lauric acid (C12:0) clearly raise LDL cholesterol, and therefore are considered atherogenic [1,36,38]. Finland had the highest intakes of all three of these fatty acids, due to a high intake of butter and milk, and Tanushimaru, Japan had the lowest.

Oleic acid in the diet shows beneficial effects on the lipoprotein risk profile for coronary heart disease [37,39]. Cohorts in Greece, Italy, and the former Yugoslavia, with a high consumption of olive oil, showed the highest intake of oleic acid. Long-chain monoenoic fatty acids with 20 and 22 carbon atoms occur in margarines containing partially hydrogenated marine oils. That was confirmed for the Zutphen sample, in which these fatty acids contributed 1% of energy. Erucic acid (C22:1) may cause cardiac lipidosis in rats [32]. The effects in humans are not clear, but toxicity is likely to be much less in a mixed diet [32]. A regulation in the European Community limits the erucic acid in foods to 5% of fat content. The highest amount of this fatty acid in our study, 0.9% of energy or 2% of fat content in the sample of Zutphen, was below this level. The erucic acid in Tanushimaru (Table 6.5) probably came from rapeseed oil.

Trans unsaturated isomers of 18:1 and 18:2 fatty acids are formed during partial hydrogenation of oils rich in linoleic and oleic acid. There is increasing evidence that these

trans fatty acids raise LDL, and reduce high-density lipoprotein cholesterol levels [40-42]. Sources for *trans* fatty acids are dairy products, meat, and margarines, cooking fats, and shortenings made with partially hydrogenated oils [2,32,43]. The data for C16 and C18 *trans* fatty acids assessed by gas chromatography agreed well between the Finnish and the Wageningen laboratory (cf. Methods). We found higher values by using infrared spectrometry. The gas chromatography values could be an underestimation due to a partial overlap of C18:1 *cis* and C18:1 *trans* positional isomers, and because they do not include *trans* fatty acids of 20 carbon atoms and more. The high values for total *trans* fatty acids for Zutphen (Figure 6.2) can be explained by a high consumption of margarines with hydrogenated fish oils. These oils contain large amounts of *trans* isomers with 20 or 22 C atoms which can be detected by infrared spectrometry but not by gas chromatography. The high amount of fatty acids with more than 20 carbon atoms in the Zutphen sample (14 g/day) confirmed the presence of hardened fish oils. Also, the high C16:1 (*n*-7) *trans* value for Zutphen probably originated from hydrogenated fish oils in margarines.

Aitchison [44] determined the mean daily intake of *trans* fatty acids by duplicate portion analysis for 11 and van den Reek [45] for 8 American women. Total *trans* fatty acids relative to energy agreed well with our value for the U.S. cohort: Aitchison found 1.8 g/1000 kcal, van Reek 2.1 g/1000 kcal, and we found 2.2 g/1000 kcal. The average daily intake of *trans* fatty acid for the USA was estimated as 8.1 g/day (about 3.9 g/1000 kcal) by Hunter [46], and as 13 g/day (about 6.3 g/1000 kcal) by Enig [47], as opposed to 5.0 g/day (2.2/1000 kcal) for our sample. The estimates of Hunter [46] and Enig et al. were based on disappearance and market share data, respectively, which may overestimate actual intake. Also, *trans* fatty acid intake could have risen between the 1960s and the 1980s as margarines replaced butter in the diet; margarines free from *trans* were available at that time in Europe [48], but not in the United States.

The *trans* fatty acid level determined in a representative Finnish diet in 1987 [49] was 1.7 g/day or 0.7 g/1000 kcal, while the Finnish value in our composites was 5.8 g/d or 1.7 g/1000 kcal. The difference could be partly explained by a decreased fat intake since the 1960s. Probably, it is even smaller in Finland nowadays, because the intake of fat has declined further, and all margarines and most vegetable fat blends have been reformulated and are very low in *trans* fatty acid content (Dr. Ingmar Wester, Raisio personal communication).

The amounts of *trans* fatty acids in the duplicate samples of a Dutch market basket study [50,51] was estimated to be 10.0 g/day or 3.2/1000 kcal, which agreed well with our value for the Dutch cohort Zutphen (8.6 g/day or 3.1 g/1000 kcal). Although both values may not be considered as representative for the Dutch population, they indicate that the intake of *trans* fatty acids in the Netherlands remained the same between the 1960s and the 1980s.

The (*n*-6) and (*n*-3)-fatty acids are essential for the formation of membranes, and are precursors of eicosanoids which have a variety of functions [52,53]. The presence of (*n*-3) polyunsaturated fatty acids in the diet may reduce the risk of coronary heart disease [54]. Fish oils are a rich source of (*n*-3) fatty acids and have a high content of fatty acids with 20 or more carbon atoms. This was reflected by high contents of these fatty acids in the food

composites of east Finland, the isle of Corfu in Greece, Dalmatia on the Yugoslavian coast, and Japan, which had a high fish consumption. We found differences in the intake of the (*n*-3) polyunsaturated fatty acids, EPA and DHA, of almost 2 g per day. Some studies suggest that the ratio between (*n*-6) and (*n*-3) polyunsaturated fatty acids is important in the prevention of coronary heart disease [8,55]. Experts recommend a ratio of linoleic to α-linolenic acid in the diet between 5:1 and 10:1 [8]. Only 6 of the 16 cohorts met this advice (Table 6.5); in the other 10 cohorts the ratio was lower for 1 and higher for 9. A problem with the use of this ratio is that α-linolenic acid is not equivalent to longer chain (*n*-3) fatty acids because only some 10-20% is elongated. Therefore indexes or recommendations for (*n*-3) fatty acid should probably take into account the lower "biological value" of α-linolenic acid relative to EPA and DHA; we have attempted to correct intake figures in this way in Figure 6.3.

The values for (*n*-3) and (*n*-6) polyunsaturated fatty acids in our study agreed rather well with those from earlier Finnish and Dutch studies [49,50], and corresponded with fish consumption levels of the cohorts [6].

Sterols

Dietary cholesterol is present in animal foods and is seen by some as an independent risk factor for coronary heart disease (Stamler and Shekelle 1988). A cholesterol intake of less than 300 mg per day is recommended [8]. We found high cholesterol intakes (> 475 mg/day) in the U.S. railroad workers, east Finland, west Finland, Zutphen, Crevalcore, and Slavonia; these were generally due to a high intake of milk fat, eggs and meat. Sterols in plants, phytosterols, are poorly absorbed, and interfere with the absorption of cholesterol. Large intakes may lower LDL cholesterol levels [8]. A high consumption of vegetable oils and a relative high intake of bread contributed to the high content of plant sterols in the Greek duplicates [56]. Our results confirm previous values for plant sterol intakes of 160-360 mg per day, amounts which probably have little effect on plasma cholesterol [57].

Visible fats

Visible fats were the most important fat source in the diets. Margarine and butter were major contributors to the high saturated and *trans* fatty acid content of the Finnish, Dutch and American diets, and olive oil to the mono-unsaturated fatty acid content of the Greek, Italian, and former Yugoslavian diet. A high consumption of lard (e.g. Slavonia) led to high intakes of total and saturated fat, but not of *trans* fatty acids. For the Japanese cohorts visible fats were not an important contribution to fat intake. The amounts and types of visible fats correspond with the fatty acid and sterol composition of the diets, which confirms the accuracy of our data.

Conclusion

We conclude that the assessment of fatty acids and sterols in the diets by chemical analysis of equivalent food composite portions is an adequate tool for estimating the average intake of subsamples of populations. However, the results of one or two cohorts do not necessarily reflect the average intake of a country.

We found large differences in the amount of total fat, fatty acids and sterols between cohorts, which reflected the variations in food consumption patterns. Chemical analysis of equivalent food composite portions allows assessment of more fatty acids and sterols than when using food tables, so that information on the intake of less known fatty acids and sterols can become available.

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Underestimation of energy intake by 3-day records compared with energy intake to maintain body weight in 269 non-obese adults

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Abstract

We assessed how accurately participants in dietary trials reported their free-living energy intake. We compared self-reported energy intake, calculated from 3-day food records, with actual intakes needed to maintain body weight during controlled trials lasting 6-9 weeks. In 269 free-living healthy male (n = 119) and female (n = 150) adults with mean body weights close to ideal values ($\bar{x}$ ± SD body mass index in kg/m^2, 22.1 ± 2.4), energy intake reported in food records was 1.2 ± 1.6 MJ/d (277 ± 378 kcal/d) lower than actual energy requirements during the experiments. The relative bias was significantly smaller (P = 0.01) for men (-8.0 ± 13.4%) than for women (-12.2 ± 13.7%). Body mass index, daily energy intake, and age were not significantly related to the extent of underestimation. We conclude that food records systematically underestimate energy needs in young, non-obese well-educated adults.

Introduction

Food-intake data are used in epidemiological research and for assessing nutrient requirements. There are several methods to estimate dietary intake. However, the validity of most methods is uncertain. In general, recall and record methods result in much lower energy intakes than do doubly labeled water estimates of energy expenditure or supervised feeding to maintain body weight [1,2].

Some characteristics of subjects who provide good records or of severe underreporters have been described. In literate and highly motivated subjects, self-report by dietary records yielded good agreement between mean energy intake and mean energy expenditure [1]. It appears that self-reports are not representative of energy requirements in obese subjects [3-6]. It has also been suggested that the degree of underreporting increases with the level of energy intake [2,7]. An explanation for this could be that individuals tend to report intakes that are closer to perceived norms than to actual intake [7].

The aim of our study was to determine whether young, lean, highly educated adults would provide valid estimations of their energy intake. Therefore, we compared self-reported energy intakes from 3-day dietary records with the actual amount of energy required to maintain stable body weights in dietary experiments. In these studies the feeding of diets with known energy contents was supervised and this provided us an almost "golden standard" for determining energy intake.

Subjects and methods

The experiments and diets

Actual energy needs were defined as the amount needed to maintain weight during controlled trials. Data were obtained from six dietary trials each with a duration of 6-9 weeks, which were conducted between 1986 and 1993; key aspects are summarized in Table 7.1. We performed experiments 2 and 3 in October and November, and the others between January and April. Approval for all studies was obtained from the Ethics Committee of the Department of Human Nutrition and Human Epidemiology.

The experimental diets varied in total fat content and fatty acid composition. Vitamins and mineral contents of the diets met the recommended dietary allowances of the Dutch Food and Nutrition Council [8]. The diets were made available at 30 energy levels ranging from 5.5 MJ to 20 MJ (1315 to 4782 kcal) per day, in increments of 0.5 MJ (120 kcal). We supplied each individual with a diet that met his or her energy requirement, as judged by stable body weight during the trial. The diets consisted of conventional solid foods. On weekdays volunteers consumed their hot meals at the Department. All other food was supplied daily as a package and consumed at home. We provided about 90 % of total daily energy. Each day participants selected the remaining 5-13 % of energy from a list of foodstuffs low in fat and cholesterol. These free-choice items provided an amount of energy fixed for each energy level. We allowed subjects unrestricted consumption of coffee and tea without milk and sugar, and of water, herbs and spices, lemon juice, vinegar, and non-energy soft drinks. The subjects kept a diary in which they recorded their daily choice of

free-choice items and any deviation from the guidelines.

During the experiments we contacted subjects on every weekday and advised them about the diets and the protocol if necessary. We urged subjects not to change their smoking habits and physical activity. They were asked to record any relevant change in their diaries. Other details have been described [9-14].

Table 7.1 *Overview of the dietary trials*

Study and year	Duration	Fat content of diets	Number of subjects: Total (n=351)	Number of subjects: Included in analysis (n=269)
	wk	*% of energy*		
1. 1986[9]	8	22 or 39	57	45
2. 1987[10]	7.5	37	60	48
3. 1988[11]	9	39	59	48
4. 1990[12]	9	41	56	48
5. 1992[13]	9	39	59	45
6. 1993[14]	6	40	60	35

Subjects

We recruited volunteers via local newspapers, via posters, and by approaching former participants. For the six trials combined a total of 441 persons were screened. Because 48 of them participated more than once these subjects generated 503 data points. The subjects lived in or around Wageningen, a small college-town in the center of the Netherlands.

We supplied ample oral and written information about the aim of the study and the burden involved in participating. We measured blood pressure, urinary glucose and protein, obtained a written medical history and asked subjects to record their food intake for 3 days. Eight (1.6%) subjects were excluded because of a history of atherosclerotic disease, hypertension, anaemia or other medical conditions as judged by an independent physician, or because their alcohol intake exceeded 10% of daily energy. We excluded 25 (5.0%) applicants because they disliked foods that were essential parts of the study diets (eg, dairy products or olive oil). Another 25 (5.0%) withdrew for personal reasons before the trials started. Because men and older persons were underrepresented we admitted most of them, as well as their partners. Because only 60 subjects could be admitted to each trial, we excluded another 94 (18.7%) eligible persons by random lottery. A total of 351 (69.8%) subjects were enrolled in the six experiments. They gave their written informed consent for

participation.

For the present analysis 9 subjects (2.6% of the participants) were left out because their food records were missing or because they withdrew before the end of the trial. We omitted results of another 11 (3.1 %) subjects because they had gained or lost more than 2.0 kg body weight between day 14 and the last week of the study. Forty-eight subjects participated in more than one trial. We used the data from their first trial and dropped the 62 observations (17.7%) from their subsequent trials, because of possible bias from multiple participation. This left 269 (76.6%) observations. Characteristics of the subjects are presented in Table 7.2.

Table 7.2 *Subject characteristics*

	All (n = 269)	Men (n = 119)	Women (n = 150)
Age (y)	25.7 ± 9.6[1]	25.4 ± 9.3	25.9 ± 9.8
Age > 30 y (*n*)	43	17	26
Height (cm)	177.1 ± 9.4	184.5 ± 6.6	171.1 ± 6.6
Weight at start of trial	69.5 ± 9.6	75.4 ± 8.2	64.8 ± 7.8
BMI[2]	22.1 ± 2.4	22.2 ± 2.5	22.1 ± 2.4
BMI > 25.0 (*n*)	34	14	20

[1] $\bar{x}$ ± SD
[2] In kg/m^2

The subjects were highly educated: in study 5 two subjects had completed primary school only, all others had completed secondary school, most of whom were attending college. In experiments 5 and 6, 55-60 % of the subjects were students, and 30-35% were employed; the remaining subjects were housewives or unemployed. It is likely that the characteristics of subjects in experiments 1-4 were similar. Fifty-eight (21.6%) participants were lacto-ovovegetarians or disliked some kinds of meat. These subjects received diets without meat, which we compensated for by providing cheese, marmalade, oil, and eggs so as to achieve the same energy and macronutrient composition as the corresponding test diet with meat. Forty-seven women (31% of all women) used oral contraceptives. The participants received no financial reward except for the free food.

Food records

Two to three months before each trial the volunteers recorded their food intakes for two weekdays and one weekend day, using scales weighing the nearest gram (study 1-3) or

estimating quantities in household measures (study 4-6). Recording days were chosen at random and only changed if a serious deviation from habitual food intake could be expected (eg, major festivities). This occurred for only one or two subjects per experiment.

Dieticians employed for the assessment of dietary intake followed a course in food-consumption techniques developed at our Department (Blauw et al, unpublished observations) or were thoroughly instructed. During the first visit of the subject to our Department the dietician provided diaries for recording food intake plus oral and written instructions. The importance of keeping accurate records was explained; the subjects were told that the results would be used for determining their energy intake level during the experiment and that they were not allowed to gain or lose weight. Additional instruction was given about the use of scales to participants of studies 1-3 and about recording the amounts of foods in household measures to the participants of studies 4-6.

At the second visit of the subject to our Department the dietician checked whether the food records had been filled out properly, obtained additional information about poorly defined dishes and recipes and unclear items or amounts, and displayed examples of cups, glasses and spoons to improve the estimation of household measures. The volunteers also spread a slice of bread with their habitual amount of margarine and the portion size for coding food records was corrected if necessary.

The dieticians estimated other portion sizes by using a table of common household units. One dietician (JdV) checked and supervised the subsequent coding of the records and data-entry procedures. The energy content was calculated using the Netherlands Nutrient Data Base 1985 (14). Additional data on food composition were obtained by chemical analysis or from the food industry.

Energy intake during the experiments

At the start of the trial each subject was assigned a 10% higher energy intake than the value from the food records at the start of the trial because an underestimation was expected from the literature and from previous experience. Body weights were measured twice weekly before lunch with subjects wearing no shoes or heavy clothing such as jackets and sweaters. If body weight tended to change by > 0.2 kg, we adjusted the energy intake by raising or lowering it in steps of 0.5 MJ/day so as to maintain a stable body weight, ie, < 2.0 kg between day 14 and the end of the experiment.

We calculated the energy content of the experimental diets using the same data base as for the food records (14) and used this for the comparisons with self-reported energy-intake. In addition, each day we collected duplicate portions of each experimental diet for an imaginary participant with an energy intake of 10 MJ/day (2391 kcal, studies 1-3) or 11 MJ/day (2630 kcal, studies 4-6). Duplicates were stored at -20° C, pooled, and chemically analyzed for protein [15], total fat [16], fatty acid composition [17], dietary fiber [18], and cholesterol [19]. Available carbohydrate was calculated by the difference between total energy content and the amount of energy derived from protein and fat. The energy content calculated from these analyses plus the determined contribution of the energy intake from the consumed free-choice items yielded a check of the energy content of the diets as

calculated from the food table.

The mean decrease in body weight of all subjects was 0.32 ± 0.65 kg between days 1 and 7, 0.15 ± 0.53 kg between days 8 and 14, and 0.05 ± 0.91 kg between day 14 and the last week of the experiments. Seventy-five (28%) subjects lost or gained > 1.0 kg but < 2.0 kg between day 14 and the end of the experiment. We defined actual individual energy intakes as the mean energy level between day 14 and the end of the trial.

Dietary adherence was confirmed by the individual changes in the fatty acid composition of serum cholesteryl esters or erythrocytes, which were consistent with the differences in the dietary fatty acid composition in experiments 2, 3, 4 and 5 [10-13]. Anonymous questionnaires on compliance were filled out in studies 5 and 6. Inspection of these questionnaires and the diaries did not reveal deviations from the protocol that might have affected the results. It was estimated that illegal foods supplied at most 0.02 MJ or 5 kcal per day (study 5 and 6).

Statistical analysis

The null hypothesis was that the energy intake estimated from the 3 day food records before the study would not differ from the average energy need from day 14 until the end of the study. This was analyzed by a paired t-test with use of the Statistical Analysis System [20]. Differences between subgroups were tested by unpaired t-tests [20].

Results

Self-reported energy intakes versus calculated energy needs

Pretrial energy intake as reported in the 3-day records was on average 10.6 ± 3.0 MJ/day (2533 kcal/d ± 717). There was no difference between the three recording days; mean reported energy intake was 10.7 ± 3.4 on the first day, 10.6 ± 3.5 on the second day, and 10.4 ± 3.6 MJ on the third day. Actual intakes between day 14 and the end of the trial - when body weights were stable - averaged 11.7 ± 2.6 MJ/day (2796 ± 621 kcal/day). Mean self-reported energy intake was 10.4% lower than the energy need during the experiments (Table 7.3). The Pearson correlation coefficient between the recorded amount of energy and actual energy requirement was 0.85 for all subjects, 0.70 for the men, and 0.73 for the women (P<0.0001).

The absolute differences were similar for men (-1.1 MJ/day; 95% CI -1.4 to -0.8 MJ/d) and women (-1.2 MJ/day; 95% CI -1.4 to -1.0 MJ/d), but when expressed as a percentage of energy intake during the experiments the food records underestimated energy needs more in women (- 12.2%; 95% CI, -14.4 to -10.0%) than in men (- 8.0%; 95% CI, -10.4 to -5.6%, P for difference between men and women = 0.01). The underestimation in the 43 subjects aged 31 - 67 years was 10.7% versus 8.5% for the younger adults (P = 0.33). The 34 participants with a body mass index (BMI, in kg/m^2) > 25 kg/m^2 underestimated their energy intake by -12.0% as compared with -10.1% for the 235 subjects with a body mass index ≤ 25 (P = 0.53). Men with a body mass index of > 25 (n = 14) underestimated their energy intake by 15.0%, and men with lower BMIs (n = 105) by 7.1% (P = 0.06). In women the underestimation was 12.6 % for the higher BMI-group

Table 7.3 *Preexperimental daily energy intake reported in 3-d records vs actual energy intake required to maintain weight during controlled trials in non-obese adults*

	All subjects (n=269)	Men (n=119)	Women (n=150)
Reported intake			
(MJ)	10.6 ± 3.0[1]	12.8 ± 2.6	8.8. ± 2.0
(kcal)	2533	3070	2089
Required intake			
(MJ)	11.7 ± 2.6	14.0 ± 1.9	9.9 ± 1.4
(kcal)	2796	3338	2373
Difference[2]			
(MJ)	- 1.2 ± 1.58	- 1.1 ± 1.84	- 1.2 ± 1.34
(kcal)	- 277	- 268	- 283
Percent difference (%)	- 10.4 ± 13.7	- 8.0 ± 13.4	- 12.2 ± 13.7

[1] $\bar{x}$ ± SD.
[2] All differences are significantly different from zero, $P < 0.05$.

(BMI > 25, n = 20) and 9.9% for the lower BMI-group (BMI ≤ 25.0, n = 130; P = 0.54).The degree of underestimation was not correlated with absolute energy intake in both sexes combined (r = 0.00, P = 0.92) or in men (r = 0.00, P = 0.93), and weakly correlated in women (r = 0.15, P = 0.07). We found no association between misestimation and the change in body weight between day 14 and the end of the trial (r=-0.06, p=0.33).

The average underestimation was - 8.8 ± 14.9% in studies 1-3, when foodstuffs were weighed, and - 12.1 ± 12.0 in studies 4-6, when the amounts of food were estimated (P = 0.05). When all data of the 48 participants who participated twice or more were included (adding 62 observations, n=331) the underestimation was - 8.6% ± 13.3 for men and - 12.9% ± 14.1 for women versus - 8.0 % for men and - 12.2 % for women in the initial data set (n=269).

The chemical analysis of duplicate portions showed that the energy content of the provided diets was on average 0.2 MJ or 48 kcal/d (range - 0.1 to 0.5 MJ) lower than we calculated using the food table. Duplicate portions collected for a lower (8.0 MJ or 1913 kcal) and a higher (14.0 MJ or 3348 kcal) energy level in experiment 3 yielded similar results.

Table 7.4 shows that the mean underestimations differed between experiments. The average underestimation of 10.8% in experiments 2 and 3, which took place in autumn, was not significantly different from the 10.1% in experiments 1, 4, 5, and 6, which were performed in the spring.

Table 7.4 *Recorded minus actual energy intake per study*[1]

Study	Percent difference
1 (n=45)	- 4.6 ± 7.1
2 (n=48)	- 14.3 ± 9.6
3 (n=48)	- 7.2 ± 21.8
4 (n=48)	- 13.0 ± 12.1
5 (n=45)	- 13.1 ± 11.4
6 (n=35)	- 9.4 ± 12.8

[1] $\bar{x}$ ± SD. Pre-experimental energy intake in studies 1-3 was recorded by weighed portions and in studies 4-6 by estimated portions. Studies 2 and 3 were executed in autumn and studies 1, 4, 5, and 6 in spring.

Discussion

The subjects in our study undoubtedly underestimated their energy requirements when they recorded their food intake for 3 days. Although a self-reported energy intake of 10% less than actual energy intake seems small, the consequences for the interpretation of nutrition survey data are substantial.

The precision of the method proved to be high, as indicated by the strong association between reported and actual energy intake. Note that this association might be slightly inflated because the dietary records were used to calculate the energy needs during the trials. However misestimation was not associated with the change in body weight, which suggests that energy need as determined during the trials was independent of the over- or underestimation of energy reported in the dietary records. Thus, inflation - if any - could not have been substantial. The degree of underestimation was weakly associated with BMI in men, but not with BMI in women, age, or absolute level of energy intake. However, the relative underestimations were higher for women than for men.

It is conceivable that subjects intentionally underreported their food intake because they wanted to lose weight during the experiments. However, this is not likely because they knew that weight loss was not allowed during the trials and that their energy intake would be adjusted. Also, they were not obese. The participants were well-educated and the applicants with excess alcohol intakes were excluded. For these reasons the subjects in our study would be expected to provide accurate records [1].

The reported energy intakes of our subjects agreed with the results of the Dutch National Food Consumption Survey [21] for which a 2-day record was used. For women (n=1611) in the same age range, the reported energy intake was 9.0 MJ (2151 kcal) vs 8.7 MJ (2080 kcal) in our study and for men (n=1608), aged 16 to 49 years, 12.3 MJ (3940 kcal) vs 12.8 MJ (3059 kcal) observed here.

The differences between energy content of the diets as assessed by chemical analysis

and the calculated value would imply an average energy intake during the experiments of 11.5 MJ/d instead of 11.7 MJ/d. The difference between analyzed and calculated values was probably due to a slight overestimation of the energy content of foods in the nutrient database. In that case a correction should also be applied to the database used for evaluating the food records, and the degree of underreporting would remain unchanged. If the difference between calculated and analyzed energy contents of the experimental diets was due to smaller amounts of food being provided than planned for them, the degree of underreporting would become 7.8% instead of 10.4%. However, this is less likely to have happened because all foods were weighed out on high-quality scales which were periodically recalibrated. The average weight loss between day 14 and the final week of the experiment was 50 g, or 1.2 g/day. This implies that as a group our subjects were very close to energy balance and that the subjects' actual energy requirements were even slightly higher than calculated here.

The deviations from the protocol recorded in the diaries and filled out in the anonymous questionnaires would implicate that the subjects' energy intake was only 0.02 MJ/d (5 kcal/d) higher than determined. If subjects did underreport their extra consumptions, then the discrepancy between reported and actual energy intake would increase and the conclusions of this study would be reinforced. Although 2 or 3 months elapsed between the recording of food intake and the start of the trial, there is no plausible reason, why subjects should have increased their energy expenditure. The degree of underreporting was the same for experiments performed in autumn, with food records obtained in July and August, as for experiments performed in January - April with food records obtained in November and December. Thus seasonal fluctuations in activity do not explain our findings. The differences in underestimation between experiments as shown in Table 7.4 could be due to the use of different recording methods, because dietary records by estimated portions provide in general less accurate estimations than by weighed amounts of foods.

Comparison with other studies

The mean energy needs of our subjects were approximately the same as those in a number of other studies [1,2]. The men in our study consumed on average 14 MJ/day (3346 kcal) and the women 9.9 MJ (2379 kcal), whereas others [1,2,22,23] found on average 13.5 MJ/d (3260 kcal) for men and 9.5 MJ/d (2400 kcal) for women.

The subjects in our trials underestimated their energy intake by about 10%. Mertz et al.[2] found an underreporting of 18% in a group of free-living volunteers when comparing energy intake recorded during ≥ 7 days with energy intake needed to maintain body weight. Livingstone et al. [23] calculated the difference between self-reported energy intake and energy requirements as measured by the doubly labeled water method for 31 free-living men and women. They found an underestimation of 19% for men and of 18% for women [23]. Hallfrisch et al. [24] compared energy intake needed to maintain body weight in metabolic studies and found underestimations of 19% for the men and 37% for the women. Lissner et al. found [25] that women underestimated their energy intake by 24% when comparing self-

reported energy intake by 3-5 day records with measured energy intake in a metabolic unit.

Perhaps the high education level of the subjects in our study could be an explanation for the smaller underestimation observed here. Schoeller et al.[26] reviewed three studies [3,6,27] representing 79 non-athletic individuals from developed countries. These studies applied the doubly labeled water technique to determine actual energy requirements. Self-reported energy intake was on average 24% lower than determined energy need for men and 29% for women. However, obese subjects and adolescents were overrepresented in these studies, which could explain part of the large differences between reported and actual energy intake [27]. In some other studies, reviewed by Black et al. [1], the reported energy intake agreed with the energy requirements assessed by the doubly labeled water method, but the number of subjects was small.

Subgroups and characteristics

The women in our studies showed a larger relative degree of underreporting than the men. A gender-specific difference between reported and actual energy intake was also described by Schoeller et al. [26]. They found the largest bias in men with low energy needs and in women with relatively high energy needs. At most we could confirm this for women, in whom we found a weak, non-significant positive association between the level of energy intake and the degree of underestimation.

Underestimation is generally smaller in lean people than in obese [26]. We could confirm this for the men but not for the women. However, few of our participants were obese. In our study the bias between reported and determined energy intake was similar for subjects older than 30 years and subjects 18-30 years of age. Still, age might affect the accuracy of food records. In adolescents differences between age groups have been reported [1]. Also, Jϕrgensen [28] reported that older age groups provided more complete 7-day food records than younger age groups.

Conclusions

Our study adds to the growing body of evidence that dietary records or surveys underestimate food intake. However, the precision of the method is high, as indicated by the high correlation between self-reported energy intake and actual energy need. Therefore records appear suitable for ranking well-educated, lean subjects by energy intake. However, for epidemiological studies and for assessing nutritional requirements of individuals one has to reckon with underestimation not only in obese but also in non-obese subjects.

One way to improve the results of food records is an upward adjustment of the individual energy intakes with the mean expected underestimation of the group. The number of subjects classified within 5% of their actual energy need increased from 43 or 16% to 95 or 35% when self-reported energy intakes were raised by 11%. However, one has to be cautious in adjusting individual food records because underreporting is not consistent among individuals. Although many suggestions have been made as to the causes of underestimation the characteristics of severe underreporters are still not clear.

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8

General discussion

We found that biological indicators of dietary intake and chemical analysis of food composites are appropriate methods to assess the intake of specific nutrients examined in the present research. Flavonols in plasma and urine can be used as biological indicators to distinguish between individuals with a low and with a high flavonol intake. A food frequency questionnaire and a 3-day food record are appropriate methods to classify subjects by their flavonol intake. However, to assess exposure of the body to flavonols, the intake of flavonols has to be corrected for the specific bioavailability of the foods consumed. This appears to be necessary because flavonol-rich foods such as tea, red wine, and onions differ in the bioavailability of flavonols. We also found that chemical analysis of food composites is an appropriate method to classify populations by intake of fatty acids and sterols. This method can be used to assess the intake both of much more specific fatty acids, such as *trans* or *n*-3 fatty acids, and of sterols than can be done by recall and record methods. Finally, we found that food records are not as accurate a method that as they are often considered to be. However, they are an appropriate method to categorize individuals by energy intake.

Variation of flavonol intake in free-living subjects

We used both a 1-day and 3-day food record and a food frequency questionnaire to assess variations in flavonol intake of free-living subjects (Chapter 3). We found differences in intake of up to 80 mg/day.

The methods we used to assess flavonol intake had not been validated before. The 3-day food record showed a large within-subject variation; therefore this method is probably not appropriate to assess the habitual flavonol intakes of individuals. However, the ratio of within- to between-subject variations was lower than one, which enables to study the relation between flavonol intake and disease. Moreover, flavonol intake determined by records correlated well with that determined by the food frequency questionnaire. Thus, the 3-day food record and the food frequency questionnaire are appropriate to rank individuals by habitual flavonol intake and to estimate the mean flavonol intake of a group.

The average flavonol intake of the subjects in our study was higher than that in epidemiological prospective studies [1-5]. This could be due to a higher intake of flavonol-rich foods in our study. Also, the methods we used could have been more appropriate to assess flavonol intake than those used in the epidemiologic studies. For instance, the food frequency questionnaire was specially developed to estimate flavonol intake, and all important foods, also ready-to-eat foods containing onions, had been included. Thus, this method could have elicited reports of more foods contributing to flavonol intake.

It would be worthwhile to develop this food frequency questionnaire further. This would require regular updates of the listed foods and additional analyses of the flavonol composition of foods. To convert intake data from the questionnaire into flavonol intakes, we used the flavonol contents of Dutch foods from Hertog et al [6,7]. They selected foods for flavonol analyses based on food intake data of the first Dutch National Food Consumption Survey performed in 1988 [8]. However, intake of foods has changed in the Netherlands since

that time. This was shown by the differences in intake between the first and the second Dutch National Food Consumption Survey performed in 1992 [9]. For instance, the intake of ready-to-eat foods increased by 32% in this period [9]. Also, new foods important for flavonol intake, such as processed foods or new varieties of foods, have probably been included in the Dutch diet in this period. Thus, analysis of the flavonol composition of these foods is needed. After revision, the food frequency questionnaire should be validated in a larger population than in our study.

Bioavailability of flavonols

We found that the bioavailability of quercetin from tea was one-half of that from onions (Chapter 4 en 5), and bioavailability of quercetin from red wine was between that of onions and tea. We based these conclusions on the results of urinary excretions relative to the amount of quercetin consumed. These appear to reflect plasma levels [10]. In previous studies [10,11], we also found differences in absorption and bioavailability between different foods. Hollman suggested that the type of quercetin glycoside in foods plays a role [10] in absorption. Quercetin glucoside, the glycoside present in onions, would be better absorbed than quercetin rutinoside, present in tea. Similarly, the different types of quercetin glycosides present in red wine would result in an intermediate rate of absorption. In conclusion, the bioavailability of flavonols differs between the major dietary sources. Therefore, the intake of flavonols cannot be considered as a measure for flavonol exposure in the body [12]. Thus, flavonol intake has to be corrected for the bioavailability of the foods from which it is derived.

The bioavailability of other major sources in the diet, mainly vegetables and fruit, are not known yet. Hollman [10] suggested estimating the bioavailability of flavonols in foods by determining the types of quercetin glycosides that they contain. When data of flavonol intake are corrected for bioavailability, outcomes of epidemiologic studies could be more consistent and probably better interpreted. For example, in some studies consumption of red wine was related to the occurrence of coronary heart disease, whereas in others it was not [13-18]. Therefore, some researchers [19-21] suggested that consumption of red wine could explain the so-called French paradox: a high consumption of saturated fat intake in France while the occurrence of coronary heart disease is low. We showed (Chapter 4) that the bioavailability of flavonols from red wine, compared to that from other sources, was not high enough, to explain the French paradox by consumption of flavonols from red wine.

Assessment of flavonol intake by biological indicators

We found that levels of quercetin and kaempferol in plasma and urine were sensitive to their intakes from onions, tea and a diet of several flavonol-rich foods (Chapter 5 and 6). We also found that flavonol intake was related linearly to their levels in plasma and urine within the range of normal intake (Chapter 6).

The use of flavonol markers is limited in practice because levels of flavonol in plasma or urine reflect only the intake of 3 or 4 days. This is a relative short period of intake for the purpose of most studies, for instance to investigate the relationship to chronic

disease. Therefore, measurements of flavonols in plasma or urine probably have to be repeated during a longer period. Further studies are required to determine how many of these measurements are needed and at what time intervals they have to be performed.

The size of the measurement error of plasma quercetin allows individuals with a low flavonol intake to be distinguished from those with a high flavonol intake (Chapter 5 and 6). However, we found a rather large difference in measurement error (about 30%) between our two studies. An important source of error is the between-subject variation in metabolism. The inconsistency between the two studies could be explained by differences in responsiveness to flavonols, dietary compliance, or smoking habits between subjects. In free-living subjects some additional factors such as age, disease, short-time effects of flavonol intake, and intake of drugs or supplements may increase the error. Thus, the use of plasma quercetin to assess quercetin intake appears to be promising. However, its variability has to be determined in free-living subjects to make the suitability of plasma quercetin as a biological indicator of quercetin intake clearer.

We do not have sufficient information about the variability of plasma kaempferol and urinary quercetin and kaempferol to evaluate their utility. We do not have information about the actual variation in kaempferol intake between subjects, or about the variations of quercetin and kaempferol in urine within subjects. However, we expect that both plasma kaempferol and urinary quercetin and kaempferol will also be suitable to determine low and high flavonol intakes. This expectation is based on their sensitivity to intake and, for urinary markers, also on their reasonably high correlation with plasma flavonols.

Flavonols are metabolized to a large extent. As long as they reflect intake, the extent of metabolism is not a problem for using flavonols as markers of flavonol intake. Quercetin in the body is partly metabolized to isorhamnetin, as was confirmed by the relationship between this flavonol and quercetin in our study (Chapter 6). However, the metabolism of flavonols is largely unknown. Therefore, it is not clear whether flavonols in plasma reflect flavonol status in the body and whether they could be directly related to the occurrence of disease. Finally, it may also be interesting to investigate whether a combination of flavonol markers, for example in a ratio, would improve prediction of intake.

Assessment of the intake of fatty acids

Sampling of food composites appeared to be a good method to use for categorizing populations by intake of fatty acids and sterols. We found clear differences in the consumption of total fat, specific fatty acids and sterols between the 16 cohorts. These data, which represent intakes of fatty acids and sterols in the Sixties, are useful to investigate the relationship of intake of these nutrients to the occurrence of disease in the Eighties and the Nineties.

We sampled food composites 25 years after food records were sampled. In general, it is preferable to sample foods at the time that intake data are acquired, making it easier to obtain foods with the same composition as consumed. In the Sixties, duplicate portions of recorded food intakes were sampled, but not analyzed for specific nutrients such as trans

fatty acids and *n*-3 fatty acids. The health effects of these nutrients and methods for their chemical analysis only became known in the Eighties. Therefore, one of the most important prerequisites of our study was to sample foods with the same fat content as consumed 25 years before. The high correlations between the fatty acid composition of the food composites in our study with that of duplicate portions collected in the Sixties confirmed that we succeeded in sampling similar foods.

Another important prerequisite for sampling of food composites is that reported intakes have to be sufficiently accurate. The accuracy of food composites is ultimately as good as the food intake data on which they are based. For our study we needed intake data on the population level. The number of food records present for most cohorts was sufficient to meet this purpose. In addition, measurement error of the food records had to be similar for all cohorts to enable appropriate categorizing by their intake of fatty acids and sterols. In our study, we had some questions about the reports of the Belgrade cohort which included a higher amount of obese subjects reporting relatively lower energy intakes [22]. For all other cohorts we could expect that measurement error of food records was similar.

Sampling of food composites is not a suitable method to determine the intake of all nutrients. For example nutrients whose content in foods changes during storage or preparation cannot be determined accurately. The reason is that raw foods are sampled and that cooking practices are not taken into account. This is not a serious problem to determine fatty acids and sterols because they are rather stable. However, differences in cooking practices between cohorts, for example in preparing meat, could slightly have affected the results. In addition, it is only possible to determine nutrients for which a valid method for chemical analysis in foods is available. In our study we had to account for differences between trans fatty acids determined by gas chromatography and by Fourier transform infrared spectrometry. In general, the first method underestimates and the second method overestimates the content of trans fatty acids. We accounted for this when interpreting the results.

Assessment of energy intake by food records

On the average, young subjects underestimated energy intake reported by 3-day records by 10% as compared with energy needs to maintain body weights (Chapter 2). The method in these subjects proved to be reasonably precise: the correlation coefficient between self-reported energy intake and energy needs during the experiments was 0.70 for the men and 0.73 for the women.

The reference method in our study, energy needs during supervised feeding, can be regarded as an almost golden and independent standard because body weights of the subjects were stable [23,24], and because energy needs of the first two weeks of the experiment were not used for analyses. Because the subjects were non-obese, well-educated and motivated to provide good estimates, they could be expected to give the best possible reports [25]. This was confirmed by the size of underestimation which was much smaller than in most other studies [23]. Thus, in general it may be expected that self-reports by 3-day records will be underestimated by at least 10%.

Although a discrepancy of 10% is rather small, it may have consequences for the outcome of epidemiological studies. The reason is that underestimation of energy intake is not consistent in all subjects. In our study an underestimation of 10% affected only a small subset of the sample. This makes it difficult to estimate the actual energy intake of individuals. However, classification of subjects by their intake is possible because the method appeared to be reasonably precise. Thus, as long as the actual energy intake of individuals is not required, and the only purpose of the study is to investigate whether there is a relationship between the intake and the occurrence of disease, and not what the relationship is (Beaton, see also the introduction of this thesis), a 3-d food record will be an appropriate tool to determine energy intake in individuals.

Conclusion

In this thesis we evaluated methods to determine the intake of flavonoids, fatty acids, sterols, and energy for different purposes. It is clear that no one dietary assessment method suits all purposes. We found that quercetin in plasma is a biological indicator of flavonol intake and can be used to distinguish between subjects with a low and with a high intake. A food frequency questionnaire could also be used to classify subjects by their flavonol intake. Thus, the most feasible and accurate method to determine the intake of flavonoids in future epidemiological studies will probably be an adjusted and improved food frequency questionnaire validated in a substudy by levels of flavonols in plasma. To measure exposure of the body to flavonols, contents of flavonols in foods should be corrected for their bioavailability because bioavailability differs between the major sources in the diet.
The bioavailability of quercetin from tea and red wine is respectively 50% and 75% of that from onions. In addition, we found that sampling of food composites was a successful method to distinguish the intake of more than forty fatty acids and sterols between 16 cohorts in seven countries. Finally, we found that three-day food records underestimate energy intake at least by 10%, but that they can be applied successfully to categorize healthy adults by their energy intake. Thus, the goal of a study defines which dietary assessment method is to be selected. If the limitations of the methods are accounted for, assessment of dietary intake can be a successful enterprise.

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Summary

Diet is clearly related to the occurrence of disease. To investigate this relationship, it is important that dietary intake can be assessed accurately. Several methods are available to assess intake, but they all have their specific measurement errors. It is important to comprehend these errors, so the most appropriate method for the goal of the study can be selected. In view of this, common recall and record methods should not be selected to assess the intake of nutrients lacking accurate data in food composition tables. In addition, these methods are not appropriate to assess nutrients whose bioavailability differs between the most important sources in the diet. For these nutrients, such as flavonoids, minor fatty acids and plant sterols, a biological marker of intake or chemical analysis of food composites could be a more appropriate method of measurement. In this thesis, we focused on physically and chemically based methods to assess the intake of flavonoids, fatty acids, sterols and energy.

Flavonols and flavones are a subgroup of the flavonoids. Quercetin and kaempferol are main representatives of the flavonols. Several studies showed that flavonoids are inversely related to the occurrence of coronary heart disease. However, other studies showed no relationship. One reason for this inconsistency could be that the assessment of flavonoid intake was too imprecise. Therefore, we investigated how the intake of flavonoids, especially of flavonols, could be assessed accurately.

We first investigated the average intake of flavonols and flavones in the diet of two groups of free-living subjects by using food records and a food frequency questionnaire (Chapter 2). One group consisted of thirteen non-resident aliens and four Dutch volunteers who all ate a wide variety of diets. The other group consisted of eight Dutch volunteers who ate normal Dutch diets. We found marked differences of up to 80 mg/day in flavonol and flavone intake between these subjects. The variation of intake of these flavonoids within subjects was relatively small compared to variations between subjects, giving a fine opportunity to study the relation between flavonoids and health. However, the within-subject variation indicated that food had to be recorded for 38 days to assess individual habitual flavonoid intake; results of 3-day food records are probably not representative of habitual intake. The food frequency questionnaire seems to be more suitable, because it reflects a period of 30 days of intake. Nevertheless, the results of this method correlated well with those derived by the 3-day food records. Thus, both methods may be suitable to classify subjects by flavonoid intake.

We also investigated whether it was necessary to account for the bioavailability of flavonols from different flavonol-rich foods (Chapter 3). In a first study, we compared the bioavailability of the intake of 15 mg of quercetin from red wine, black tea, and fried onions. Concentrations of quercetin in plasma and excretions in urine after wine were significantly higher than after baseline. The bioavailability of quercetin from red wine was 75% of that from onions and that of tea 50% of that from onions. Consumption of one glass of wine with an average quercetin content would produce plasma quercetin levels similar to those after consumption of half a cup of average tea or 5 grams of onions. Therefore, the intake of bioavailable flavonols from wine is quite modest and cannot explain the so-called French paradox: the difference in coronary heart disease between France and other western

countries. We concluded that red wine is a poor source of bioavailable flavonols compared to tea and onions. In a following study, described in Chapter 4, it was confirmed that the bioavailability of quercetin from tea was smaller than that from onions. In this study, we fed 15 volunteers tea or onions and then measured both concentrations of quercetin and kaempferol in plasma and excretions of these flavonols in urine. Similar to quercetin, kaempferol from tea was also absorbed.

We also examined whether levels of flavonols in plasma and urine may be used as biomarkers of intake. To that end, we repeated the treatment with onions to measure the within-subject variation of quercetin in plasma and urine. This variation has to be relatively small compared to the between-subject variation of flavonol intake in free-living subjects. We found ratios of the within-subject to the between-subject components of variance of lower than one. Therefore, we concluded that concentrations of quercetin in plasma and its excretions in urine can be used in epidemiological studies as biomarkers to measure intake.

In a subsequent study (Chapter 5), we further investigated the suitability of flavonols in plasma and urine as biomarkers of dietary intake. In that study, we measured flavonols in plasma and urine after a high dose of flavonols consumed by nine subjects during four weeks. In addition, we combined the results with those of the studies described in Chapter 3 and Chapter 4. The intake of flavonols appears to be related linearly to their concentrations in plasma and excretions in urine. Moreover, the variability of quercetin in plasma allows its use as a biological marker to distinguish individuals with a low or with a high intake of quercetin. We did not have enough data on the variability of urinary quercetin, plasma kaempferol, or urinary kaempferol to draw conclusions about their utility as biomarkers.

In another study, we assessed the intake of fatty acids and sterols of middle-aged men living in 16 cohorts in seven countries (Chapter 6). The objective of this study was to obtain detailed information on the fatty acid and plant sterol composition of the diets. Therefore, we determined the mean content of 42 fatty acids and four sterols in food composites. Foods for these composites were collected in the regions where the men lived in the Sixties. Chemical analyses of the composites were performed in a central laboratory. We found large differences in the amounts of total fat, fatty acids, and sterols between the seven countries and also between cohorts in the same country. These differences reflected the variations in food consumption patterns. The results of these chemical analyses can be used to investigate relationships between the composition of diets in the 1960s and the occurrence of disease in later years. We concluded that assessing fatty acids and sterols in diets by chemical analysis of equivalent food composite portions is an adequate tool to estimate the average intake of subsamples of populations. This method allows assessment of more fatty acids and sterols than do methods needing food tables.

Finally, we also investigated the accuracy of food records to assess energy intake (Chapter 7). To that end, we compared self-reported energy intake, calculated from 3-day records of 269 adults, with actual energy intake needed to maintain body weight during controlled trials. The subjects were young, non-obese, and highly educated adults, and could therefore be expected to provide valid estimations of their energy intake. The consumption

of diets with known energy contents was supervised. This provided us with an almost "golden standard" for determining energy intake. On the average the subjects in our study underestimated their energy requirements by 10%. Although a self-reported energy intake of 10% less than actual energy intake seems small, the consequences for the interpretation of nutritional survey data are substantial. The method proved to be reasonably precise, as indicated by the correlation coefficient of 0.70 between reported and actual energy intake for men as well as for women. Thus, a 3-day food record can be used to classify subjects by their energy intake, but it is not an appropriate method to assess individual energy intake.

Studies described in this thesis show that biological markers of intake or chemical analysis of food composites can be good tools to assess nutrients lacking in food composition tables. Also, biological markers of intake can be good tools to assess nutrients whose bioavailability differs between the most important dietary sources. By these methods the intake of flavonoids, fatty acids, and sterols can be measured successfully within certain limitations. In addition, a food frequency questionnaire specially devised to measure flavonoid intake and validated by biomarkers of intake appears to be a feasible method to assess the intake of flavonoids in epidemiological studies. However, when using food records even from lean, well-educated adults underestimation of intakes has to be taken into account. The conclusion of this thesis is that the intake of flavonoids, fatty acids, sterols and energy can be assessed successfully if the method is selected carefully in regard to the specific properties of these food components and the aim of the study.

Samenvatting

Voeding speelt een belangrijke rol bij het ontstaan van ziekten. Om de relatie tussen voeding en ziekten te kunnen onderzoeken is het belangrijk dat de inneming van voedsel nauwkeurig kan worden vastgesteld. Verschillende methoden zijn beschikbaar om de inneming te bepalen, maar allen hebben specifieke meetfouten. Het is belangrijk om deze fouten te kennen, zodat er in onderzoeksontwerp en interpretatie van resultaten rekening mee kan worden gehouden. In het onderzoeksontwerp zal men zorgvuldig de meest geschikte methode voor het doel van de studie moeten kiezen. Zo kunnen de gewone opschrijf- en navraagmethoden beter niet worden gekozen om de inneming van voedingsstoffen vast te stellen, waarvan geen gegevens in voedingsmiddelentabellen beschikbaar zijn. Ook zijn deze methoden minder geschikt voor het vaststellen van voedingsstoffen, waarvoor de biobeschikbaarheid tussen voedingsmiddelen verschillend of niet bekend is. Voor deze voedingsstoffen zou een biologische merker of chemische analyse van 24-uursvoedingen een geschiktere methode kunnen zijn. Voorbeelden van dit soort voedingsstoffen zijn flavonoïden, specifieke vetzuren en plantensterolen. In dit proefschrift richtten we ons met name op het vaststellen van de inneming van deze voedingsstoffen. Ook onderzochten we of met de drie-daagse opschrijfmethode de energie-inneming nauwkeurig kan worden vastgesteld.

Flavonolen en flavonen zijn subgroepen van de flavonoïden. Quercetine en kaempferol zijn de belangrijkste vertegenwoordigers van de flavonolen. Uit verschillende studies kwam naar voren dat flavonolen en flavonen een inverse relatie met het voorkomen van hart-en vaatziekten hebben. Echter in sommige studies kon geen verband worden aangetoond. Een mogelijke reden zou kunnen zijn dat de inneming van flavonoïden niet nauwkeurig genoeg kon worden vastgesteld. Daarom onderzochten we methoden om de inneming van flavonoïden, en met name van flavonolen, in de voeding te bepalen.

Eerst onderzochten we de gemiddelde inneming van flavonolen en flavonen in de voeding van twee groepen "vrijlevende" personen met behulp van de opschrijf- en voedselfrequentiemethoden (Hoofdstuk 2). Een groep bestond uit 13 buitenlandse en vier Nederlandse deelnemers met een zeer gevarieerd voedingspatroon. De andere groep bestond uit acht Nederlandse vrijwilligers met een normale Nederlandse voeding. We vonden verschillen in inneming van flavonolen en flavonen tussen deze deelnemers tot 80 mg per dag. Deze verschillen waren groter dan die gevonden werden in epidemiologische studies. De binnen-persoonsvariatie in de inneming van deze flavonoïden was klein, in verhouding tot de tussenpersoonsvariatie. Hieruit blijkt dat de spreiding tussen personen groot genoeg is om de relatie van flavonoïdeninneming met het ontstaan van ziekten te bestuderen. Uit de binnenpersoonsvariatie kon berekend worden dat voor het vaststellen van de individuele flavonoïdeninneming de voeding ongeveer 38 dagen moet worden genoteerd. De drie-daagse opschrijfmethode lijkt hiervoor dus niet geschikt. De referentieperiode van de voedselfrequentielijst was echter 30 dagen. Niettemin vonden we een goede relatie tussen de uitkomsten van de voedselfrequentelijst en die van de drie-daagse opschrijfmethode. Daarom lijken beide methoden geschikt om personen te classificeren naar hun flavonoïdeninneming. Om te onderzoek of de voedselfrequentielijst ook geschikt is om de individuele flavonoïdeninneming vast te stellen zal deze echter eerst in een grotere groep

deelnemers moeten worden gevalideerd.

Ook onderzochten we of het nodig was om rekening te houden met verschillen in biobeschikbaarheid van flavonolen tussen de bronnen in de voeding. In een eerste studie vergeleken we de biobeschikbaarheid van de inneming van 15 mg quercetine tussen rode wijn, zwarte thee en gebakken uien (Hoofdstuk 3). Concentraties van quercetine in plasma en uitscheiding in de urine na consumptie van rode wijn bleken significant hoger dan de basiswaarden. De biobeschikbaarheid van quercetine uit rode wijn was echter 75% en uit thee 50% van die uit uien. De consumptie van één glas rode wijn met een gemiddelde hoeveelheid quercetine zou in quercetinegehalten in plasma resulteren, die hetzelfde zijn als na een halve kop thee of na vijf gram uien. Daarom is de hoeveelheid flavonolen in rode wijn, beschikbaar voor het lichaam, vrij beperkt. Flavonolinneming uit rode wijn kan daarom niet de Franse paradox, het verschil in hart- en vaatziekten tussen Frankrijk en andere landen, verklaren. We concludeerden dan ook dat rode wijn in vergelijking met thee en uien een slechte bron is van flavonolen voor het lichaam. In een tweede studie naar biobeschikbaarheid, beschreven in Hoofdstuk 4, werd de slechtere beschikbaarheid van flavonolen uit thee bevestigd. In deze studie, bepaalden we concentraties van quercetine en kaempferol in plasma en de uitscheiding van deze flavonolen in de urine van 15 vrijwilligers die uien of thee kregen. Behalve quercetine, bleek ook kaempferol uit thee te worden geabsorbeerd. Een ander doel van deze studie was om te onderzoeken of flavonolen in plasma en urine kunnen worden gebruikt als biologische merkers voor hun inneming. Daartoe gaven we dezelfde hoeveelheid uien twee keer teneinde de binnen-persoonsvariatie van quercetine in plasma en urine vast te stellen. Op basis van de gevonden variaties concludeerden we dat concentraties van quercetine in plasma en de uitscheiding van deze stof in urine gebruikt kunnen worden om de inneming in epidemiologische studies te bepalen.

We onderzochten de geschiktheid van flavonolen in plasma en urine als biologische merkers voor de inneming verder in een volgende studie (Hoofdstuk 5). In deze studie, bepaalden we flavonolen in plasma en urine na een hoge dosis flavonolen ingenomen door negen proefpersonen gedurende vier weken. Daarna combineerden we de uitkomsten van deze studie met die van twee andere studies, beschreven in Hoofdstuk 3 en 4. Er bleek een lineair verband te bestaan tussen de inneming van flavonolen met de concentraties van deze stoffen in plasma en hun uitscheiding in de urine. Ook bleek dat de variabiliteit van quercetine in plasma het mogelijk maakt om deze concentraties te gebruiken om onderscheid te maken tussen personen met een lage en hoge inneming. We hadden niet genoeg gegevens over de variabiliteit van quercetine in urine en van kaempferol in plasma en urine om ook hun bruikbaarheid als biologische merker vast te stellen.

In een andere studie onderzochten we in hoeverre het verschil in inneming van vetzuren en sterolen tussen mannen afkomstig uit 16 regio's kon worden bepaald met behulp van directe analyse van 24-uursvoedingen. Om hierover gedetailleerde informatie te krijgen bepaalden we de gemiddelde samenstelling van 42 vetzuren en vier sterolen in deze 24-uursvoedingen. We verzamelden de voedingsmiddelen hiervoor in de 16 regio's verdeeld over zeven landen waar de mannen in de jaren 60 woonden. De samengestelde 24-

uursvoedingen werden in een centraal laboratorium geanalyseerd. We vonden grote verschillen in de hoeveelheid totaal vet, vetzuren en sterolen tussen de 7 landen en ook tussen de cohorten in hetzelfde land. Deze verschillen gaven een goede afspiegeling van het verschil in voedingspatroon. De resultaten van deze chemische analyses kunnen worden gebruikt om de relaties tussen de samenstelling van de voedingen in de jaren 60 en het ontstaan van ziekten in latere jaren te onderzoeken. We concludeerden dat het vaststellen van vetzuren en sterolen in de voedingen door chemische analyse van 24-uursvoedingen een geschikte methode is om de gemiddelde inneming van subgroepen in de populatie vast te stellen. Met deze methode kunnen meer vetzuren en sterolen worden vastgesteld dan met het gebruik van voedingsmiddelentabellen. Tenslotte onderzochten we de nauwkeurigheid van de drie-daagse opschrijfmethode voor het vaststellen van de energie-inneming. Daartoe vergeleken we de energie-inneming volgens de drie-daagse opschrijfmethode gerapporteerd door 269 deelnemers met hun energiebehoefte tijdens gecontroleerde voedingsproeven. Hun lichaamsgewicht werd daarbij constant gehouden. De deelnemers waren jong, niet-obees en hoog-opgeleid. Daarom mocht worden verwacht dat ze nauwkeurig zouden rapporteren. De verstrekkingen van de voedingen tijdens de proeven werden goed gecontroleerd. We beschikten daardoor over een bijna gouden standaard om de energie-inneming vast te stellen. De deelnemers bleken hun energie-inneming gemiddeld 10% te onderschatten. Hoewel een onderschatting van 10% niet veel is, lijkt de consequentie voor de interpretatie van uitkomsten van voedingsonderzoek substantieel. De precisie van de methode bleek redelijk goed te zijn, zoals bleek uit de correlatie van 0.70 tussen gerapporteerde en werkelijke energie-inneming zowel voor mannen als voor vrouwen. Daarom lijkt de drie-daagse opschrijfmethode geschikt om deelnemers te classificeren naar inneming, maar niet om de werkelijke inneming van individuen te schatten.

De studies over de inneming van voedingsstoffen beschreven in dit proefschrift tonen aan dat biologische merkers voor het vaststellen van de inneming en chemische analyse van 24-uursvoedingen goede methoden kunnen zijn voor het vaststellen van voedingsstoffen, die niet opgenomen zijn in voedingsmiddelentabellen. Biologisch merkers kunnen ook een goede methode zijn voor het vaststellen van voedingsstoffen voor welke de biobeschikbaarheid tussen verschillende voedingsmiddelen verschilt. Deze methoden blijken geschikt om de inneming van flavonoïden, vetzuren en sterolen te meten. Daarnaast zou ook een voedselfrequentielijst een goede methode kunnen zijn om de inneming van flavonoïden vast te stellen. De drie-daagse opschrijfmethode bleek echter niet zo nauwkeurig als vaak wel wordt gedacht. De resultaten toonden aan dat zelfs een groep niet-dikke, goed-opgeleide deelnemers hun energie-inneming met 10% onderschatten. Deze methode is daarom niet geschikt om de individuele energie-inneming vast te stellen, maar wel om personen te classificeren naar hun energie-inneming. De conclusie van dit proefschrift is dat de inneming van flavonoïden, vetzuren, sterolen en energie met succes kan worden vastgesteld. De methoden moeten echter zorgvuldig worden gekozen met betrekking tot de specifieke eigenschappen van deze voedselcomponenten en het doel van het onderzoek.

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Over de auteur

Jeanne de Vries werd op 16 december 1955 te Son en Breugel geboren. Zij behaalde het Atheneum-B diploma in 1974 aan het van der Puttlyceum te Eindhoven en het diëtetiek-diploma in 1978 aan de diëtistenopleiding "de Schutse" te Nijmegen. In hetzelfde jaar werd zij als diëtist op projectbasis aangesteld aan de Vakgroep Humane Voeding van de Landbouwhogeschool te Wageningen. Zij werkte mee aan de uitvoering van gecontroleerde voedingsproeven en van voedselconsumptie-onderzoek, aanvankelijk als uitvoerend, later ook als coördinerend en leidinggevend diëtist. Van 1979 tot 1987 gaf zij daarnaast leerling-ziekenverzorgenden van de VERA-school te Arnhem les in voedings- en dieetleer. In de periode van 1988 tot 1992 sloot ze met goed gevolg enkele cursussen aan de Open Universiteit af, waaronder Statistiek en Methoden & Technieken. In de loop der jaren verzorgde zij ook zelf cursussen, trainingen en colleges op het gebied van voeding en gezondheid. Buiten haar werkzaamheden om vervulde zij een aantal bestuurlijke functies. Sinds 1995 is zij redactielid van het Informatorium voor Voeding en Diëtetiek. Jeanne de Vries begon in 1993, voor een deel naast haar reguliere werkzaamheden, aan het promotie-onderzoek dat in dit proefschrift wordt beschreven.

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