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Flavonoids as fruit and vegetable intake biomarkers –

Development, validation and application of flavonoid biomarkers in nutritional research

Ph.D. thesis · 2011

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"As long as methodologic problems prevent us from seeing the truth we cannot rule out that truth

(Schatzkin et al. 2009)".

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Kirstine Suszkiewicz Krogholm, January 2011

Summary

Most validation studies show that the food frequency questionnaire (FFQ) is rather low in precision and accuracy, and there is an ongoing debate regarding the applicability of such self-reported data with regard to diet-disease relationships. However, no other method has so far been able to replace these dietary assessment questionnaires, and a thorough validation of the FFQ relative to one or two independent reference methods, such as objective biomarkers, is thus particularly important. If two independent reference methods are measured it is possible to use the method of triads for the validation, while calculation of the bivariate correlation coefficients is the common approach when using only one reference method. Back in 2002, a strictly controlled dietary intervention study indicated that the sum of 7 different flavonoid aglycones excreted in 24h urine samples potentially could be used as a biomarker of fruit and vegetable intake (Nielsen *et al.* 2002). The overall aim of the present Ph.D. thesis was to further develop and validate this potentially new fruit and vegetable biomarker and furthermore use it for the validation of self-reported dietary intake of fruits and vegetables in intervention and cohort studies.

The Ph.D. thesis contains four scientific papers. **Paper I** provides evidence that the sum of 7 flavonoids in 24h urine respond in a linear and sensitive manner to moderate increases in the intake of fruits and vegetables, and thus consolidates that the flavonoids are a valid biomarker of fruit and vegetable intakes. In **Paper I**, the urinary recovery of the 7 flavonoids in morning spot urine (i.e. all urine voids from midnight including the first morning void) was also found to respond to moderate increases in the intake of fruits and vegetables. However, the association was somewhat weaker than in 24h urine samples, indicating that the 24h urinary recovery of the 7 flavonoids is a stronger biomarker of the intake of fruit and vegetables than the urinary recovery of the 7 flavonoids in morning spot urine.

In **Paper II**, the biokinetic profiles of some of the most important dietary flavonoids are described (quercetin, naringenin and hesperetin), since there was a need for improved understanding of bioavailability and metabolism of the flavonoids included in the flavonoid biomarker assay originally developed by Nielsen *et al.* (2000). In **Paper I-II** we observed a high degree of inter-individual variation in the absorption and urinary recovery of the flavonoids, and this makes it very difficult to separate individuals according to intake by use of the flavonoid biomarker in urine. The intra-individual variation was on the contrary low, and **Paper II** therefore supports the assumption, that 24h urinary recovery of flavonoids has great potential as a compliance biomarker and as a biomarker of both controlled and uncontrolled changes in the intake level of fruits and vegetables achieved by an intervention.

In **Paper III** we use the flavonoid biomarker in 24h urine samples in a workplace-based intervention study providing free fruit or no free fruit to employees as validation of a 24h dietary recall. We found that the estimation of fruit intake by the 24h dietary recall was a valid estimate of the true effect of the free-fruit intervention programme,

due to highly significant correlations between 24h urinary recovery of flavonoids and the self-reported intake of fruit.

One of the aims of **Paper IV** was to use the method of triads (Kaaks 1997, Ocké & Kaaks 1997) to validate the intake of fruits, vegetables and beverages rich in flavonoids in a population-based cohort in Denmark (referred to as the `Inter99' cohort). The method of triads requires three different and independent measures of the variable of interest, and therefore, beside the FFQ, also the plasma concentration of carotenoids was measured in addition to the flavonoid biomarker. The second aim of **Paper IV** was to investigate whether the urinary recovery of flavonoids in morning spot urine could substitute the 24h urinary recovery of flavonoids as an alternative and more feasible biomarker of fruit and vegetable intake. Using the method of triads resulted in validity coefficients (VCs) for FFQ that were higher than the bivariate correlation coefficients between total fruit, juice, tea and vegetable intake estimated by FFQ and each of the biomarkers. This finding, of a statistically significant correlation between the Inter99 FFQ and two independent biomarkers indicates that the Inter99 FFQ provides a useful estimate of the overall dietary intake of fruits, juices, tea and vegetables in the Inter99 cohort. VCs for the FFQ ranged from 0.43-0.68 using 24h urine and from 0.35-0.64 using morning spot urine, indicating that the intake of fruits, juice, tea and vegetables was reflected by flavonoids both in morning spot and 24h urine. Collection of 24h urine is difficult and time consuming, and therefore morning spot urine may be a more convenient tool than 24h urine for validating the fruit and vegetable consumption in large population studies.

Summary in Danish

Adskillige valideringsstudier har vist, at fødevarefrekvensspørgeskemaer (FFQ) har lav præcision og akkuratesse, og det diskuteres til stadighed hvilken anvendelighed sådanne selvrapporterede data har med hensyn til at undersøge sammenhænge mellem kost og sygdom. Der er til dags dato dog ikke et bedre alternativ til de selvrapporterende metoder og derfor er en grundig validering af FFQ i forhold til én eller flere uafhængige referencemetoder, så som objektive biomarkører, meget aktuel og vigtig. Hvis to forskellige uafhængige referencemetoder er tilgængelige er det muligt at beregne triangulære validitets koefficienter (VCs) som et udtryk for det sande, men ukendte indtag. Hvis der kun indhentes information om én anden referencemetode beregnes normalt den bivariate korrelation mellem test og reference metode. I 2002 blev der publiceret et kostkontrolleret interventionsforsøg, der indikerede, at summen af 7 flavonoid aglyconer i 24t urin potentielt kunne anvendes som biomarkør for indtaget af frugt og grønt (Nielsen *et al.* 2002). Udgangspunktet for denne Ph.d. afhandling var at udvikle og validere denne potentielt nye frugt og grøntsags biomarkør yderligere og derefter at bruge den til validering af det selv-rapporterede indtag af frugt og grønt i interventions og kohorte studier.

Denne Ph.d. Afhandling er baseret på fire videnskabelige artikler. **Artikel I** beskriver, at kontrollerede stigninger i indtaget af frugt og grønt medfører en lineær og sensitiv stigning i udskillelsen af de 7 flavonoider i 24t urin og underbygger dermed anvendeligheden af flavonoider i 24t urin som biomarkør for indtaget af frugt og grønt. Udskillelsen af de 7 flavonoider i morgen spoturin (dvs. alt urin udskilt mellem kl. 24:00 til og med første vandladning om morgenen) responderede også signifikant med indtaget af frugt og grønt. Sammenhængen var dog svagere end i 24t urin og indikerer dermed, at udskillelsen af de 7 flavonoider i 24t urin er en stærkere biomarkør for indtaget af frugt og grønt end udskillelsen af de 7 flavonoider i morgen spoturin.

I **Artikel II** beskrives den biokinetiske profil af tre, fra kosten, vigtige flavonoider (quercetin, hesperetin og naringenin), da større forståelse af biotilgængeligheden og metabolismen af flavonoider er nødvendig for at kunne vurdere anvendeligheden af flavonoiderne som biomarkører. I **Artikel I** og **II** blev der observeret en høj inter-individuel variation i absorption og urinudskillelse af de undersøgte flavonoider, og det må derfor konkluderes, at udskillelsen af flavonoider i urin ikke er anvendelig som biomarkør på individniveau. Den intra-individuelle variation var til gengæld lav og dermed kan flavonoid biomarkører godt bruges til at validere en ændring i et individs indtag af frugt og grønt og til at inddele en større gruppe af individer i forhold til indtag af frugt og grønt.

I **Artikel III** bruges flavonoid biomarkøreren i 24t urin til at validere indtaget af frugt hos medarbejdere, der har deltaget i en 'gratis-frugt på arbejdspladsen' intervention. Udskillelsen af flavonoider i 24t urin blev fundet at korrelere signifikant med indtaget af frugt estimeret ved 24t kosthistorisk interviews og indikerer dermed, at det anvendte 24t kosthistoriske interview giver et validt estimat af den sande effekt af 'gratis-frugt på arbejdspladsen' interventionen.

Ét af formålene med **Artikel IV** var at anvende den triangulære metode (Kaaks 1997, Ocké & Kaaks 1997) til at validere det FFQ selvrapporterede indtag af frugt, grønt og drikkevarer med et højt indhold af flavonoider i en populationsbaseret kohorte i Danmark (omtales som `Inter99´ kohorten). Det andet formål med studiet omtalt i **Artikel IV** var, at undersøge om udskillelsen af flavonoider i morgen spoturin kunne substituere 24t urinopsamling og dermed gøre flavonoid biomarkøren mere anvendelig i større epidemiologiske undersøgelser. Forudsætningen for den triangulære metode er tre forskellige og uafhængige bestemmelser af frugt- og grønt-indtaget, og derfor blev også plasma koncentrationen af carotenoider, ud over FFQ, målt i tillæg til flavonoid biomarkøren. Den triangulære metode resulterede i VCs for FFQ, der var højere end bivariate korrelationskoefficienter mellem det totale indtag af frugt, grøntsager, juice og te estimeret ved FFQ og de enkelte biomarkører. De statistiske signifikante VCs for Inter99 FFQ og 2 uafhængige biomarkører indikerer, at Inter99 FFQ giver et validt estimat af indtaget af frugt, juice, te og grøntsager i Inter99 kohorten. VCs for FFQ var mellem 0.43-0.68 ved brug af flavonoid biomarkøreren i 24t urin og mellem 0.35-0.64 ved brug af flavonoid biomarkøreren i morgen spoturin. Dette indikerer at flavonoid biomarkøreren var i stand til at afspejle indtaget af frugt og grøntsager, te og juice i både 24t urin og i morgen spoturin i Inter99 kohorten. Opsamling af 24t urin er svært og tidskrævende og derfor vil brugen af flavonoider i morgen spoturin være en mere praktisk metode i forhold til 24t urinopsamling i valideringen af selvrapporterede indtag af frugt og grønt i større populationsstudier.

List of papers included in the Ph.D. thesis

Paper I. Krogholm KS, Haraldsdóttir J, Knuthsen P, Rasmussen SE. Urinary total flavonoid excretion, but not 4-pyridoxic acid or potassium, can be used as a biomarker for the intake of fruits and vegetables. Journal of Nutrition, 134**:**445-451, 2004.

Paper II. Krogholm KS, Bredsdorff L, Knuthsen P, Haraldsdóttir J, Rasmussen SE. Relative bioavailability of the flavonoids quercetin, hesperetin and naringenin given simultaneously through diet. European Journal of Clinical Nutrition, 64:432-435, 2010.

Paper III. Krogholm KS, Bredsdorff L, Alinia S, Christensen T, Rasmussen SE, Dragsted LO. Free fruit at workplace increases total fruit intake: a validation study using 24h dietary recall and urinary flavonoid excretion. European Journal of Clinical Nutrition, 64: 1222-1228, 2010.

Paper IV. Krogholm KS, Bysted A, Brantsæter AL, Jakobsen J, Rasmussen SE, Kristoffersen L, Toft U. 24h and morning spot urine flavonoids and plasma carotenoids in the validation of self-reported intake of fruit and vegetable in the Inter99 cohort study, using the method of triads. Accepted for publication in British Journal of Nutrition on 23 December 2010 if revised.

List of papers related to biomarker research not included in the Ph.D. thesis

Rasmussen SE, Frederiksen H, **Krogholm KS,** Poulsen L. Dietary Proanthocyanidins. Occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. Molecular and Nutritional Food Research, 49: 159-174, 2005.

Kristensen M, **Krogholm KS,** Frederiksen H, Bügel S, Rasmussen SE. Urinary excretion of total isothiocyanates from cruciferous vegetables shows high dose-response relationship and may be a useful biomarker for isothiocyanate exposure. European Journal of Clinical Nutrition 46: 377-382, 2007.

Kristensen M, **Krogholm, KS,** Frederiksen H, Duus F, Cornett C, Bügel S, Rasmussen SE. Improved synthesis methods of standards used for quantitative determination of total isothiocyanates from broccoli in human urine. Journal of Chromatography B, 852: 229-234, 2007.

Alinia S, Lassen A, **Krogholm KS**, Christensen T, Hels O, Tetens I. A workplace feasibility study of the effect of a minimal fruit intervention on fruit and nutrient intake. Public Health and Nutrition, 8: 1-6, 2010.

1 Introduction and motivation

Risk reduction (RR) estimates from epidemiological prospective cohort and intervention studies are less impressive than initially observed in case–control studies with respect to the preventive effect of fruits and vegetables, including single food groups or specific bioactive compounds, against chronic diseases like cancers (World Cancer Research Fund 2007) and cardiovascular diseases (CVD) (Dauchet *et al.* 2009).

There may be several reasons for this. First, case-control studies are affected by recall and selection bias. Cases, in a case-control study, have been diagnosed with the disease before they report on their 'usual diet' and may thus be affected by their illness or treatment. Controls are intended to be a random sample of the whole population under study, but normally the response rate among potential controls is well below 100% and those who respond are rather 'health conscious'. Prospective studies are not affected by recall bias as is the case with case-control studies, because measurements are made before any disease is diagnosed, and prospective studies therefore offer a more useful methodological compromise for studying associations between fruit and vegetable intakes and chronic diseases (Dauchet *et al.* 2009).

Second, social and behavioural factors cannot always be measured precisely, or might not be appropriately considered in statistical analysis, and their confounding effect in epidemiological studies of fruit and vegetables might not be fully appreciated. Fore instance, fruit and vegetable intake is also a biomarker of a healthy lifestyle, high educational level and wealth, each of which are associated with beneficial effects on health (Padayatty & Levine 2008). Fruit and vegetable consumption might also simply be a surrogate for reduced intake of undesirable nutrients such as saturated fat.

Third, much of the epidemiological evidence relating the intake of fruits and vegetables to risks of chronic diseases rely on information gathered using various retrospective dietary assessment methods (i.e. FFQ, 24h dietary recalls and dietary history). In most cases, dietary assessment methods requires a systematic estimation of the frequency of consumption and the portion size of the foods consumed as well as more or less detailed information on the recipe ingredients, combination of foods consumed together, and cooking methods, which may affect the estimation of exposure to a particular dietary component of the food group of interest (Jenab *et al.* 2009). When these issues are coupled to the complexity and daily variation in dietary patterns, large number of possible confounders (e.g. age, smoking, social class and sex), and numerous reporting biases (e.g. social desirability bias and BMI-bias), it is of no surprise that all dietary assessment methods have significant limitations that could contribute to an underestimating of the relative risk (RR) between diet and disease and reduced statistical power of epidemiological studies.

Fourth, the limited variability of dietary intake within a cohort or intervention group combined with the imprecise dietary measurement estimates may underestimate the association between intake of fruit and vegetables and risk of chronic disease. For instance, in a large (n=20649) prospective study with an average follow-up of 9.5 y it was estimated that

the difference between the lowest and highest quartile of vitamin C concentration was about one daily serving of fruit and vegetable, perhaps an amount too small for a FFQ to detect precisely (Myint *et al.* 2008).

Fifth, for most potential mechanisms by which fruits and vegetables might reduce disease risk, some types of fruit and vegetables would be expected to be much more strongly associated with risks than others, and such associations might be missed in examinations of disease risk in relation to total fruit and vegetable consumption. There could also be effects of particular identified or unidentified bioactive compounds in certain fruits and vegetables, as fruit and vegetables have very varied composition. Bioactive compounds are essential and non-essential compounds that occur in nature, are part of the food chain, and can be shown to have an effect on human health (Biersalski *et al.* 2009). So far, interest has especially gathered around three groups of bioactive compounds: Water soluble compounds (e.g. vitamins and polyphenols), dietary fibers and plant sterols. Flavonoids belong to the group of water soluble compounds, and the effect of flavonoids on mechanisms related to chronic diseases have been investigated in numerous *in vitro* cell cultured systems and in animal models. Current knowledge from *in vitro* and animal studies on mechanisms by which dietary flavonoids may play a role in preventing degenerative pathologies was recently reviewed by Crozier and co-workers (2009). Potential modes of actions include;

1. Suppression of nuclear factor kappa B (NF-κB) transcription factor activation by curcumin, resveratrol, ellagic acid, and (-)epigallo catechin-3-*O*-gallate. NF-κB regulates the expression of cytokines, inducible nitric oxide (NO) synthase, cyclooxygenase 2, growth factors and inhibitors of apoptosis, and pathological dysregulation of NF-κB is associated with inflammatory disease and cancer.

2. Suppression of Activator Protein-1 (AP-1) transcription factor by several phenolic compounds such as green tea flavan-3-ols, quercetin, *trans*-resveratrol and curcumin. AP-1 activation is linked to growth regulation, cell transformation, inflammation, and innate immune response and has been implicated in regulation of genes involved in apoptosis and cell proliferation.

3. Anthocyanins, gallic acid, curcumin and *trans*-resveratrol have been shown to activate Phase II antioxidant and detoxifying enzymes, and are thus considered as potential candidates for preventing tumour development.

4. Green tea flavan-3-ols have been found to suppress tumorigenesis through induction of the antioxidant-response element and the Mitogen Activated Protein Kinase (MAPK) in several chemical-induced animal carcinogenesis models in a dose- and time-dependent manner. (+)-Catechin and quercetin have been found to exhibit cardiovascular protection through suppressing Plasminogen Activator Ihibitor-1 (PAI-1) expression in human

coronary artery endothelial cells *in* vitro through activating the MAPK signalling pathways (Croizer *et al.* 2009).

Many of these investigations, however, lack any physiological significance because of the high doses used, typically of parent compounds, such as quercetin, rather than their conjugated mammalian metabolites or microbial degradation products. Consequently, observation on beneficial effects of single compounds made in *in vitro* studies are not reproduced in randomised controlled supplementation trials with single, purified, well defined, common dietary flavonoids and effects on recognised or suspected cancer and CVD risk factors (Croizer *et al*. 2009). When discussing the potential health beneficial effects of flavonoids it is also important to consider their potential harmful effects. A few dietary flavonoids are under suspicion for having toxic or cancerous effects in humans. Coumarin is a flavonoid found in herbs and species with hepatotoxic and carcinogenic properties (Abraham *et al.* 2010) and the isoflavones have been shown both to have cancer promoting and cancer protecting effects in animal studies (Mortensen *et al.* 2009). Epigallocatechin gallate mainly from green tea is suspected of being liver toxic (EFSA working group on botanicals and botanical preparations).

Some clinical intervention studies have shown that foods such as cocoa, tea, purple grape juice (rich in flavan-3-ols and flavonols), and soy (rich in isoflavones) have biological effects within the cardiovascular system. It has been suggested, that flavan-3-ols and flavonols are associated with improved endothelial function via improving the NO signal pathway and decreasing platelet function (both *in* vivo and *in* vitro) and may have a role in blood pressure control—all toward promoting cardiovascular health (Erdman *et al.* 2007).

In observational epidemiological studies investigating the association between dietary intake of flavonoids and risk of disease the determination of dietary flavonoid intakes are most often limited by the accuracy of both the food composition data and the dietary intake data. Urinary biomarkers may be more useful for comparing the individual's intake or exposure to flavonoids rather than estimates of intake based on food composition databases and food intake data, and would allow for more detailed population-based studies to be conducted.

If future research activities regarding diet-disease relations should be more informative, measurements of dietary intake and exposure of total fruit and vegetables, individual types of fruit and vegetables and bioactive compounds require improvements. One approach could be an improvement of dietary assessment tools by the development and validation of new innovative methods for dietary assessment. For instance internet based dietary assessment or use of digital cameras, cellular telephones and personal digital assistants are currently being developed and validated (Dowell & Welch 2006, Subar *et al.* 2007, Wang *et al.* 2006). Another approach could be the use of new statistical methodologies that combine information from different sources to adjust for measurement errors or to provide validity checks. It is for those reasons that more and more studies are beginning to measure biomarkers of fruit and/or vegetable intake in their assessment of the disease risk associations

to this important food group as an additional or substitute estimate of dietary intake or exposure (Jenab *et al.* 2009). However there is still a large need of a larger variety of dietary biomarkers to be developed and validated to reflect wider aspects of diet.

The work included in the present Ph.D. thesis took its offset in a strictly controlled dietary intervention study, measuring the 24h urinary recovery of 7 flavonoid aglycones (quercetin, naringenin, phloretin, hesperetin, kaempferol, isorhamnetin and tamarixetin) following six weeks consumption of diets either low (349-686 g/d) or high (725-1441 g/d) in fruits, vegetables and berries (Nielsen *et al.* 2002). The urinary recovery of the 7 flavonoids was clearly higher after the high fruit and vegetable diet as compared with the low fruit and vegetable diet, thus indicating the potential usefulness of urinary flavonoids as biomarkers of fruit and vegetable intake. The focus of this PhD thesis is the further development and validation of this potentially new fruit and vegetable biomarker and its applicability to provide validity check on self-reported dietary assessments of fruit and vegetable intake in free-living populations.

2 Aims

The overall aim of the present Ph.D. thesis was to further develop and validate the urinary recovery of flavonoids as a potentially new fruit and vegetable biomarker and furthermore use it for the validation of self-reported dietary intake of fruits and vegetables in intervention and cohort studies. First, it was investigated how the flavonoid biomarker in both 24h and morning spot urine samples would respond to different controlled doses of fruits and vegetables (**Paper I**). Secondly, a biokinetic intervention study was performed in order to describe the relative bioavailability of some of the flavonoids included in the flavonoid biomarker assay, and intra- and inter-individual variations in the urinary recovery of the flavonoids were studied (**Paper I-II**). In **Paper III- IV** the flavonoid biomarker was used for the validation of self-reported dietary intake of fruits and vegetables by calculation of bivariate correlation coefficients (**Paper III-IV**), by using the method of triads (**Paper IV**), and by calculation % grossly misclassified subjects (**Paper IV**). **Paper IV** furthermore estimated the validity of the flavonoid biomarker in 24h urine samples versus spot urine samples in relation to the food frequency questionnaire applied in the Inter99 cohort by using the method of triads. The specific research questions addressed in this Ph.D. thesis are the following;

- Does the urinary recovery of 7 flavonoids in 24h and morning spot urine samples respond in a linear and sensitive manner to moderate increases in the intake of fruits and vegetables? (**Paper I**)
- Is the relative bioavailability and urinary recovery value of quercetin, hesperetin, naringenin similar when they are provided simultaneously and in equal amounts through diet? (**Paper II**)
- Is the 24h dietary recall used in the `free fruit at workplace intervention´ a valid tool for estimation of the fruit intake? (**Paper III**)
- Is the FFQ used in the Inter99 cohort a valid tool for measuring the intake of fruit, vegetables and beverages rich in flavonoids? (**Paper IV**)
- Can the urinary recovery of flavonoids in morning spot urine substitute the 24h urinary recovery of flavonoids as an alternative and more feasible biomarker of fruit and vegetable intake? (**Paper IV**)
- Is the performance of the flavonoid biomarker improved by the inclusion of eriodictyol, apigenin, daidzein, genistein and enterolactone in the biomarker assay? (**Paper III-IV**)

3 Difficulties with dietary assessment methods

Assessment of dietary intake is difficult and the choice of assessment method may influence the results. As an example, the mean self-reported intake of fruits and vegetables in the European Prospective Investigation into Cancer and Nutrition (EPIC) study was estimated to be 486 g/d when using a 16d weighed food record and 824 g/d when estimated by a 130-items semi-quantitative FFQ (Bingham *et al.* 2008). Obviously, the choice of dietary assessment method plays a key role in the observed magnitude and the direction of the association between fruit and vegetable intake and risk of diseases. The choice of dietary assessment method usually differs according to the study objective, available resources, the population under study and the design of the epidemiological study (Bingham & Nelson 1991). The classic methods to measure food and nutrient intake (estimated and weighed food records, 24h dietary recalls, dietary history and FFQ, see **Table 1**) have instrument-specific advantages and disadvantages, and are subject to different types of biases, and both random and systematic errors.

Table 1 Commonly used dietary assessment methods to estimate the food consumption of individuals and groups (Gibson, 1990)

Dietary assessment methods	Procedures
24h dietary recall	Subjects or caretaker recall food intake of previous 24h in an interview. Quantities estimated in household measures using food models as memory aids and/or to assist the quantifying portion size. Nutrient intake calculated using food composition data.
Estimated food record	Record of all food and beverages as eaten over periods from 1-7 days. Quantities estimated in household measures. Nutrient intake calculated using food composition data.
Weighed food record	All food consumed over a defined period is weighed by the subject, caretaker, or an assistant. Food samples may be saved individually for nutrient analysis. Alternatively, nutrient intake is calculated using food composition data.
Dietary history	Interview method consisting of a 24h dietary recall of actual intake, plus information on overall usual eating pattern, followed by a FFQ to verify and clarify initial data. Usual portion size recorded in household measures. Nutrient intake calculated using food composition data.
Food frequency questionnaire (FFQ)	Uses comprehensive list or list of specific food items to record intakes over a given period (day, week, month, and year). Questionnaire can be semi-quantitative (FFQs) when subjects are asked to quantify usual portion size of food items, with or without the use of food models. Nutrient intake is calculated using food composition data.

Random error is the result of fluctuations around a true value because of sampling variability. It can occur during data collection, coding, transfer, or analysis. Examples of random error include: poorly worded questions, a misunderstanding in interpreting an individual answer from a particular respondent, or a typographical error during coding. Random errors affect measurements in a transient, inconsistent manner and it is impossible to correct for random errors. Precision in epidemiological variables is a measure of random error, and precision is inversely related to random errors, so that reducing random error will increase precision. Confidence intervals are calculated to demonstrate the precision

of RR estimates. The narrower the confidence interval, the more precise the RR estimate. There are two basic ways to reduce random errors in an epidemiological study. The first way is to increase the sample size of the study, and the second is to reduce the variability in measurements in the study. This might be accomplished by using a more precise dietary assessment method or dietary biomarker of intake, or by increasing the number of measurements.

A systematic error or bias occurs when there is a difference between the true value (in the population) and the observed value (in the study) from any cause other than sampling variability. An example of systematic error is a mistake in coding that affects all responses for that particular question. The validity of a study is dependent on the degree of systematic error, and is usually separated into internal validity and external validity. Internal validity is dependent on the amount of error in measurements, including intake and/or exposure, disease, and the associations between these variables. Good internal validity implies a lack of errors in measurements and suggests that interferences may be related to the subjects under study. External validity relates to the process of generalizing the findings of the study to the population from which the sample was drawn. This requires an understanding of which conditions are relevant (or irrelevant) to the generalization. Internal validity is clearly a prerequisite for external validity (Taylor, 1999).

One of the main sources of errors in dietary assessment is misreporting, comprising both under- and over-reporting of energy or specific food groups or individual food items. Common sources of misreporting in dietary assessment include following subject specific biases;

1. Social desirability bias, where certain respondents report intake in a manner consistent with perceived social norms rather than actual intake: for example are fruit and vegetable intakes often systematically over-reported, due to social approval bias (Gibson 1990, Kristal *et al.* 2000, Miller *et al.* 2008).

2. BMI-related bias, where underreporting is related to obesity: for instance, it was found by Tooze *et al.* (2004) that BMI and perceived body size were significantly higher for the male energy under-reporters and that perceived body size and percentage of energy from fat were significantly higher and lower, respectively, for the energy under-reporters than for the accurate female reporters.

3. Age and sex-related bias, where a higher proportion of low-energy reporters have been found among women and older subjects several times according to a recent review by Poslusna *et al.* (2009).
4. Socio-economic status and educational-bias, where lower socio-economic class and lower level of education have been found to be predictors of underreporting (Poslusna *et al.* 2009).
5. Smoking and dieting-bias, where a higher prevalence of energy under-reporters is found among smokers and people on a diet (Poslusna *et al.* 2009).

6. Interviewer-bias, where different interviewers probe for information to varying degrees, intentionally omit certain questions and/or record responses incorrectly. This kind of bias can be random across days and subjects and systematic for a specific interviewer, or exist as an interaction between certain interviewers and respondents only (Anderson & Hrboticky 1986).

7. Respondent memory laps-bias, when individuals are asked to recall their diet in the very recent past, their episodic memory is reasonably accurate. However, after only a few days memory of diet erodes, possibly to a higher extend for some respondents than others, and recall of past diet is constructed from general knowledge about foods, most probably based on individual's beliefs and hopes about one's characteristic diet (Rumpler *et al.* 2008).

8. Incorrect estimation of portion size-bias, when the respondent may be unable to accurately quantify the portion of food consumed, or the perceived average serving may differ form the standard average serving (Poslusna *et al.* 2009).

9. Fear of negative evaluation-bias, where respondent is worried about being perceived in an unfavorable way by others or about doing the "wrong" things: for example, a high fear of negative evaluation were associated with higher odds of energy underreporting in women (Tooze *et al.* 2004).
10. Respondent burden-bias, where respondent lower their calorie intake due to the cumbersome work with weighing the food prior to eating (Willett, 1998).

Weighed food records offer the advantages over FFQ, dietary history and 24h dietary recall, that they are not affected by laps of memory-bias and incorrect estimation of portion size-bias. Therefore weighed food records are probably the most accurate self-reporting method to measure food intake, and is considered the "gold standard" in dietary assessment. However, even weighed food records, have their drawbacks. Studies have shown that subjects, when doing a weighed food record, in general lower their calorie intake due to the cumbersome work with weighing the food prior to eating (respondent burden-bias). Furthermore, keeping a dietary record requires a literate, motivated and cooperative population (Willett 1998).

The most practical and economical method for collection of comprehensive dietary data in epidemiological studies is the FFQ (Subar *et al.* 2001). However, every new or adjusted FFQ has to be validated to get an expression of the degree to which it is an accurate measure in the target population. The approach taken in most studies is to examine the concordance of FFQ with another more precise dietary reference method such as multiple 24h dietary recalls or food records using measurement error models to estimate bivariate correlations between food intake measured by FFQs and the 'truth'. However, FFQ, 24h dietary recalls, dietary history and food records have many of the same sources of errors and therefore they could be correlated and might not be independent. The use of biomarkers as the

reference method therefore represents a valuable and independent method for validation of self-reported intake data and the value of this approach will be discussed more thoroughly in the next section of this Ph.D. thesis. In addition, biomarkers may also be very useful in cohort studies when dietary intake has not been measured, when interesting food items are not included in dietary assessment, or when some important information are lacking (e.g., a distinction between the intake of orange and apple juice or if apples are eaten with or without peel). In section 5 current candidate biomarkers of fruit and vegetable intake will be presented and discussed.

4 Approaches for validating dietary assessment methods

As mentioned in the previous section, every new or adjusted dietary assessment method has to be validated to get an expression of the degree to which it is an accurate measure of intake in the target population. The dietary assessment method being validated is in such circumstances normally referred to as the test method, and the method against which the test method is being compared and validated is called the reference method. Ideally the reference method should reflect the subjects true intake, and an attempt of this has been done by the direct observation of people eating during the study period. However, this strategy is very time consuming and furthermore presents some practical difficulties (Poslusna *et al.* 2009). In practice, perfect measurements of intake are generally not available and true intake must therefore be considered as a value of a variable that exists but cannot be observed without error (Kaaks, 1997).

A validation study is typically done in a representative sub-sample of the study population and there are several ways of estimating the validity of a dietary assessment method including the following;

1. Comparison of replicate measurements collected at different periods throughout the year
2. Validation of dietary assessment methods against other more superior or well known dietary assessment methods
3. Validation of dietary assessment methods against a validated biomarker
4. Validation of dietary assessment methods against actual controlled intake
5. Validation of dietary assessment methods against a more superior dietary assessment method and a validated biomarker (triad method)
6. Validation of dietary assessment methods against two independent validated concentration biomarkers (triad method)

The most common way of assessing validity of dietary assessment is still by using the traditional model of validation using only two methods (e.g. FFQ *versus* biomarkers, food records or multiple 24h dietary recalls). Studies comparing FFQ with multiple 24h dietary recall or food records generally show bivariate correlations in the order of 0.4-0.7. If biomarkers of intake also correlate to a similar degree with the FFQ method one may conclude that the FFQ method is sufficiently valid for etiologic studies according to Drs. Willett, Block and Byers (Block 2001b, Byers 2001, Willett 2001). However, high correlation between two self-reported dietary assessment methods does not necessarily indicate validity, since the errors are similar in both methods, and this can result in distorted measures of validity. Recognition of this problem has increased the interest in developing dietary biomarkers, to validate dietary intake. The capacity to demonstrate a correlation between the estimated intake and the biomarker provides almost unquestionable qualitative documentation of validity (Willett 1998, Byers 2001).

Kaaks and co-workers describe an approach to measure validity using biomarkers (Kaaks, 1997). They recommend that when determining the validity coefficient (VC) (i.e. the correlation between dietary assessment data and the subjects true, but unknown, habitual intakes) of a given dietary assessment method, at least two additional measurements of dietary intake are necessary. I.e. biomarker measurements and weighed food records or multiple 24h dietary recalls. Weighed food records and multiple 24h dietary recalls are suggested as reference methods since they are generally assumed, on average, to have no, or relatively small, constant or proportional scaling bias (Kaaks 1997). This triangular validation approach, known as the method of triads, uses the correlation between each of the three methods to calculate the VC (see **Figure 1**). The major assumption of the method of triads is that the correlations between the three measurements are explained by the fact that they are all linearly correlated with 'true', but unknown, intake and that their errors are independent. However, since questionnaires, 24h dietary recalls and food records may have common sources of errors, the errors may be positively correlated. Consequently, the bivariate correlation coefficient between the questionnaire and the food record could be overestimated. For that reason it is suggested by Ocke and Kaaks (1997) that the VCs calculated by the method of triads should be used as the upper limit of the true VC. The precision of the VC estimates is generally expressed by the 95% confidence interval and this is not simply an attempt to estimate the coefficient without bias. The technique most often used to calculate the 95% confidence intervals for the VC is the bootstrap technique. Bootstrapping is a non-parametric re-sampling technique that uses the original data to generate hundreds of samples with the same 'n' as the original sample. Thus, the lower the generated bivariable correlation between studied variables are, the greater the 95% confidence interval (Slater *et al.* 2010).

Despite being costly and laborious, the inclusion of two dietary biomarkers, instead of only one, may show advantages due to the three independent variable errors generated, and this strategy has been applied in **Paper IV** and in Brantsæter *et al.* 2007 for self-reported FFQ estimates of fruit and vegetable intake. In **Paper IV** the urinary flavonoid biomarker and the plasma concentration of carotenoids were used as reference methods, and VCs were calculated using FFQ intakes of fruits and vegetables. Furthermore, the method of triads is used to validate the flavonoid biomarker in morning spot urine. In **Paper III** we used the traditional approach for validation, comparison of a 24h dietary recall and biomarkers.

Since dietary measurements and biomarkers are usually utilized as categorical variables in epidemiological studies, another approach for evaluating dietary questionnaires is to divide both questionnaire and biomarker into quintiles to examine their joint classification (Willett *et al.* 1985). However, when measuring many different variables it becomes very burdensome. An advantage of the method of triads is that it requires no special software for latent variable analysis. It can even be applied with a simple pocket calculator, starting from sample correlations between the three different types of measurements (Ocke & kaaks, 1997). Because the scale of the dietary measurement and the biomarker is usually not in the same unit it is not possible to describe the relationship between two measures as a simple regression equation (Willett 1998). It is the same case for the Bland-Altman plot (or differences plot)

where the differences between two measurements (y-axis) are plotted against the mean of the two methods (x-axis).

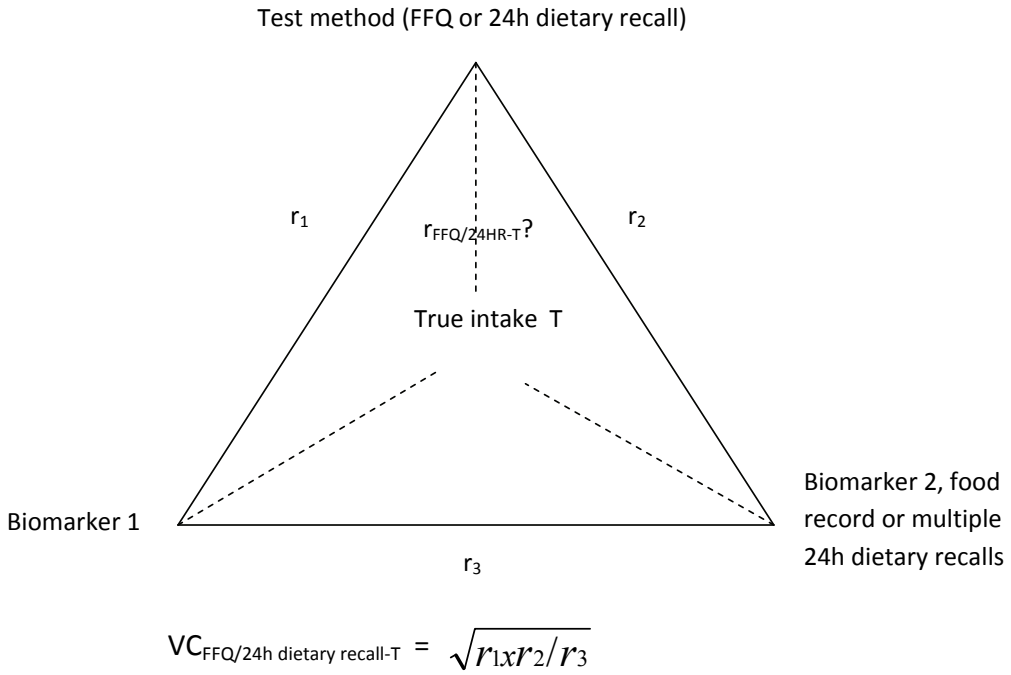

Figure 1 The method of triads for estimation of validity coefficients (VCs) between the test method (e.g. FFQ or 24h dietary recall) and the 'true' intake (T) where r_1, r_2 and r_3 are the correlation coefficients between the FFQ and each of the reference methods and between the two reference methods (adopted from Ocké & Kaaks, 1997)

5 Dietary intake biomarkers of fruits and vegetables

A biomarker to validate dietary data can be defined as; '*any biochemical index in an easily accessible sample that gives a predictive response to a given dietary component*' (Bingham 1984). The main assumption behind dietary biomarkers is that they are objective measures and are independent of all the biases and errors associated with study subjects and dietary assessment methods (Jenab *et al.* 2009). It is important that the biological sample collections are unannounced, or at least that the purpose of urine or blood sampling is unknown to the participants. Otherwise biomarkers may be affected by some of the same biases as self-reported dietary assessment methods are.

In general, dietary biomarkers can be divided into several classes including the recovery, predictive, concentration and replacement biomarkers (see **Table 2**). Existing biomarkers of fruits and vegetables belong to the class of concentration biomarkers and they cannot be translated into absolute levels of intake. However, their concentrations do correlate with intake of fruit and vegetable, although the strength of the correlations are often lower than 0.6 (Jenab *et al.* 2009).

Table 2 The different classes of dietary biomarkers

Biomarker class	Description	Application	Example	Expected strength of correlation
Recovery biomarker	Sensitive, time-dependent and show dose-response relationship between dietary intake and recovery (excretion)	As reference measurements to assess validity/accuracy of diet assessment methods	Doubled labeled water, 24h urinary nitrogen or potassium	>0.8
Predictive biomarker	Sensitive, time-dependent and show dose-response relationship with intake level, but the distinction is that their overall recovery are lower than for the recovery biomarker	As reference measurements to assess validity/accuracy of dietary assessment methods	24h urinary sucrose and fructose as biomarker of intake of sugars	
Concentration biomarker	Replacements biomarkers are closely related to concentration biomarkers and refer specifically to compounds for which information in food composition databases are unsatisfactory or unavailable	Asses correlations with estimates of dietary intake and estimations of diet-disease risk associations (as a substitute or complimentary to dietary assessments)	Blood vitamins, blood lipids, urinary polyphenols and electrolytes	<0.6
Replacement biomarker			Some phytoestrogenes, salt and metabonomic factors	

In order to determine if a given biochemical index can be used as a biomarker of fruit and vegetable intake a number of factors must be evaluated. The development of an accurate and reproducible analytical method is the first key step, and it is also important that the biofluid of interest is easy to collect and can be stored for a long period of time without degradation of the biomarker. Information on the kinetic profile is also important in order to

establish what timeframe the biomarker covers. Ideally, a biomarker should have a linear response to increasing intake of fruits and vegetables, and remain unaffected by commonly used supplements (e.g. multi-vitamin and mineral-tablets and vitamin C), food additives (e.g. beta-carotene as colouring agent or vitamin C as an antioxidant), drugs or liver and/or renal impairment. Finally, if a biomarker is intended to reflect one whole food group, such as fruits and vegetables, the biomarker should reflect a variety of fruits and vegetables (Padayatty & Levine 2008).

Existing biomarkers of fruit and vegetables are not 'ideal', but they are functional and have found wide spread applicability in modern nutritional epidemiology. Current validated biomarkers of dietary intake of fruits and vegetables, regarded as one whole food group, include vitamin C, carotenoids and polyphenols, including the flavonoids. Furthermore, some food-specific biomarkers also do exist. For instance, a highly significant dose-response relationship between intake of cruciferous vegetables and isothiocyanate equivalent recovery in human urine has been demonstrated (Kristensen *et al.* 2007, Kristensen *et al.* 2007b). In **Paper III-IV,** significant correlations were found between the intake of citrus fruits and juices and the urinary recovery of citrus specific flavanones (hesperetin, naringenin and eriodictyol), and intake of apples significantly correlated with the 24h urinary recovery of phloretin. Regarding the carotenoids, the plasma concentration of β-cryptoxanthin has been found to correlate significantly with the estimated intake of orange fruit, and α- and β-carotene were significantly correlated with intake of carrots (**Paper IV)**. The association between less specific fruit and vegetable constituents have also been investigated (e.g. folic acid, potassium and 4-pyridoxic acid, Tucker *et al.* (1996), Broekmans *et al*. (2000), Brevik *et al.* (2005a), **Paper I**), but they cannot compete with the flavonoids, carotenoids and vitamin C.

In the following three sections (5.1 – 5.3) a more thorough description of the carotenoids, vitamin C and flavonoids, as dietary concentration biomarkers of fruit and vegetable intake, will be reviewed, with special focus on the flavonoids.

5.1 Carotenoids

Carotenoids are tetraterpenoid organic pigments that are naturally occurring in the chloroplasts and chromoplasts of plants and some other photosynthetic organisms. They are lipid soluble and the average consumption has been estimated to be 4.8 mg/d in Denmark with carrots and tomatoes as the dominating dietary sources (Leth *et al.* 2000). The carotenoids reside in the body for a longer period of time than flavonoids, approximately 1-3 months (Burri *et al.* 2001), and are therefore considered to represent a long- or intermediate term biomarker of fruit and vegetable intake. FFQ usually reflect the average dietary intake during the previous months or year, and thus, FFQ and the carotenoid biomarker more or less reflect the same period of time.

More than 40 carotenoids have been identified, but six of them are found in humans at higher levels than the rest and have therefore received most attention from

researchers. These are α- and β-carotene, lycopene, lutein, zeaxanthin and β-cryptoxanthin (Garcia *et al.* 2010). The chemical structure of the most abundantly consumed carotenoid in the Danish population, β-carotene, can be seen in **Figure 2**.

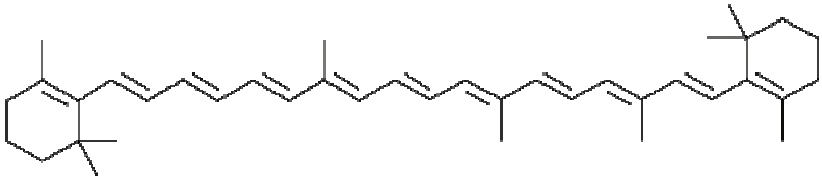

Figure 2 Chemical structure of β-carotene

Different fruits and vegetables contain different amounts of carotenoids, and the content may differ even for the same type of fruit or vegetable depending on external factors such as size, growing and harvesting conditions, and degree of maturity, processing, storage and cooking (Willet 1998). The intestinal absorption of the carotenoids can range from less than 10 % to more than 50 % depending on source and type of fruits and vegetables (Reboul *et al.* 2006). Host factors such as BMI, gender, smoking habits, physical activity and dietary factors such as fat, protein and fiber content may furthermore affect the carotenoid plasma response (Yeum & Russel 2002, Garcia *et al.* 2010). The intake of dietary supplements, fortified foods and foods where carotenoids are used for food colouring may also complicate the use of carotenoids as a biomarker of intake of fruits and vegetables. Different populations, sub-groups of populations and individuals may accordingly have different carotenoid profiles in their plasma. For instance, it has recently been shown that the carotenoids may be stronger correlated to dietary intake in normal weight than obese subjects (Vioque *et al.* 2007). Carotenoids do not appear to be under homeostatic control (IARC Working Group on the Evaluation of Cancer Preventive Strategies 1998), and are thus, in theory, capable of reflecting the whole spectrum of dietary intake.

Despite the above mentioned factors affecting the final plasma concentration of different carotenoids, data from controlled feeding studies suggest that plasma carotenoids may be potential biomarkers of fruit and vegetable intake (Brown *et al*. 1989, Bowen *et al.* 1993, Le Marchand *et al.* 1994, Martini *et al.* 1995, Yeum *et al.* 1996, Maskarinec *et al.* 1998, Brokmanns *et al.* 2000, Brevik *et al.* 2005b). In observational studies for validation of fruit and vegetable intake and compliance to trial the correlation between plasma carotenoids have furthermore been found to correlate moderately with self-reported intake of fruits and vegetables $r_s \approx 0.1\text{-}0.6$ (Micozzi *et al.* 1992, Byers *et al.* 1993, Campbell *et al.* 1994, Kristal *et al.* 2000, Michaud *et al.* 1998, Drewnowski *et al.* 1997, Polsinelli *et al.* 1998, Tucker *et al.* 1999, van Kappel, Block *et al*. 2001, El Sohemy *et al*. 2002, Al-Delaimy *et al.* 2005, Mikkelsen *et al.* 2007, Brantsæter *et al.* 2007, Toft *et al.* 2008, **Paper IV**).

5.2 Vitamin C

Vitamin C or L-ascorbic acid is an essential nutrient for humans and certain other animal species (see **Figure 3**). Average daily vitamin C intake has been estimated to be 108 mg for adults between 17-75 years in Denmark (Pedersen *et al.* 2010). More than 90% of the total vitamin C intake in Denmark has been estimated to originate from fruits and vegetables, making vitamin C a rather specific biomarker of fruit and vegetable intake (Trolle *et al.* 1998).

The primary sources of dietary vitamin C are fruits and to some extend vegetables, and vitamin C has been used many times as a biomarker of fruit and vegetable intake (Le Marchand *et al.* 1994, Drewnowski *et al.* 1997, Ness *et al.* 1999, Block *et al.* 2001, Bogers *et al.* 2004, Macdonald *et al.* 2009). Previously reported correlation values have been ranging from 0.12 to 0.64. The average plasma elimination half life of vitamin C is inversely related to dosage and body pool, being about 16-20 days in healthy, non-smoking men (Shils 1999), thus reflecting long- or intermediate-term intake of fruits and vegetables. However, the use of vitamin C as an intake biomarker has its limitations. Investigations with pure vitamin C have shown that there is only a steep sigmoidal dose-response relationship at intakes between 30-100 mg vitamin C. At 100 mg the concentration of vitamin C in fasting plasma is approximately 60 µmol/L, while at 200 mg fasting plasma concentration is only raised to 70 µmol/L and does barely increase with higher doses (Levine *et al.* 1999). A study of 271 middle-aged men and women showed that plasma vitamin C was 68.2 µmol/L for intakes of 2.5 portions of fruits and vegetables, 81.3 µmol/L for intakes of 3-4.5 portions, and 78.2 µmol/L for intakes of > 5 portions/d (Cappuccio *et al.* 2003). In a study by Harding *et al.* (2008) a similar plateau for plasma vitamin C was observed, although at a higher concentration (95 µmol/L for 5-7.5 portions, and 91 µmol/L for 7.5 portions). Likewise, MacDonald and co-workers recently found that plasma vitamin C increased with increasing intakes of fruits and vegetables up to 500 g/d, but with no further increase for intakes above 500 g/d (MacDonald *et al*. 2009). As for the carotenoids, the vitamin C content in plant foods varies with growth conditions and is highly affected by food processing, storage and cooking. Vitamin C is also widely used in fortification of foods and beverages and is taken by many individuals as a supplement (Hallund *et al.* 2007).

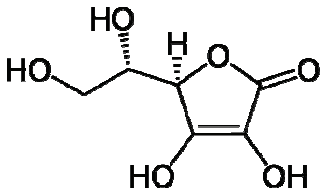

Figure 3 Chemical structure of L-ascorbic acid

5.3 Flavonoids

5.3.1 Classification and structure of flavonoids

Flavonoids are polyphenolic compounds comprising 15 carbons, with two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle (C_6-C_3-C_6) (see **Figure 4**). Flavonoids are found throughout the Plant Kingdom and in particular in the epidermis of leaves and in the skin of fruits. Based on the variation in the type of heterocycle involved, flavonoids are divided into different sub-classes: anthocyanidins, flavan-3-ols, flavanones, flavones, flavonols, isoflavones, dihydroflavonols, flavan-3,4-diols, coumarins, chalcones, dihydrochalcones and aurones. The basic C_6-C_3-C_6 flavonoid skeleton can have numerous substituents (e.g. hydroxyl, acetyl, methoxy and methyl groups) and the majority of the flavonoids exist naturally as glycosides e.g., conjugated to one or several different sugar moieties such as glucose, rhamnose and galactose (Crozier *et al.* 2009).

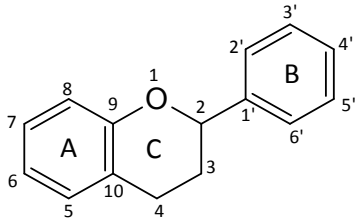

Figure 4 Flavonoid basal structure

5.3.2 Dietary intake and major sources of flavonoids

The intake of polyphenols, including flavonoids, are usually determined using dietary assessment methods such as 24h dietary recalls, dietary history and FFQ (see **Table 3**). This food intake is then translated into quantitative information regarding the specific flavonoids of interest using food composition databases. Such methods rely on self-reporting of dietary patterns by the study participants, and consequently their accuracy is often uncertain. In particular, food perceived as being healthy, such as fruits and vegetables which represent the main dietary sources of flavonoids, are often being over-reported (Kristal *et al.* 2000, Miller *et al.* 2008). Furthermore, the flavonoid composition in plant foods varies significantly with external factors such as plant variety, degree of ripeness at time of harvest, UV-exposure and food processing related factors. In addition, as many polyphenolic compounds possess anti-microbial activity in plants, their concentration in plant food would be expected to be higher if plants had experienced bacterial or viral infection during growth.

This is, for instance, observed in tomatoes grown under organic conditions, where the polyphenol content has been reported to be higher than in non-organically grown varieties (Caris-Veyrat *et al*. 2004). Other studies find no differences in polyphenol content between conventional and organically grown tomatoes (Juroszek *et al.* 2009). The considerable difficulties in assessing flavonoid intakes using traditional dietary assessment methods highlight the need for biomarker of intake which may circumvent these limitations. For instance, Mennen and co-workers have found a positive correlation between 10 dietary polyphenolic compounds in morning spot urine samples, in free-living subjects, indicating that these are potentially useful biomarkers of polyphenol exposure (Mennen *et al*. 2006). A linear relationship between oral dose of strawberry anthocyanins and urinary recovery has also recently been reported (Hollands *et al.* 2008). However, as mentioned in the previous section, the urinary concentration of flavonoids are correlated with factors other than the intake and may not necessarily be better than traditional dietary assessment methods to evaluate intake of flavonoids.

As can be seen in **Table 3**, several studies have provided data concerning the intake of various classes of polyphenols. The flavonols is the subclass of flavonoids most extensively studied, and average consumption of these substances has been estimated at ≈ 20-26 mg/d in the US, Denmark and Holland (Hertog *et al.* 1993, Justesen *et al.* 1997, Sampson *et al.* 2002). The intake of flavanones has been found similar or higher, with a mean consumption corresponding to 28-41 mg/d (Ovaskainen *et al.* 2008, Zamora-Ros *et al.* 2010). Anthocyanidin consumption has been studied in Finland, and was 47 mg/d on average (Ovaskainen *et al.* 2008).

Major dietary sources of flavonoids vary among different populations, as can be seen in **Table 3**. For instance, the high consumption of berries in Finland results in a high consumption of anthocyanidins, and the high consumption of oranges in Spain results in a relatively higher consumption of flavanones than in the northern part of Europe. Over the past few years, several comprehensive online databases on polyphenol content in foods have been released, and this have improved the quality of the calculated estimates of total intake of polyphenols (Kiely *et al.* 2010, Neveu *et al.* 2010, USDA database for the flavonoid content in selected foods). This has made the estimates higher than previously reported (Chun *et al*. 2007, Zamora-Ros *et al.* 2010), (see **Table 3**).

Table 3 Estimated intake of flavonoids in several countries

Country	Sample size, (age)[1]	Dietary assessment method	Mean dietary intake (mg/d)	Main dietary source	Flavonoid subclass	References
Denmark	Danish household consumption survey, number of participants and age not reported	Dietary history, quantification of flavonoids in a variety of commonly consumed foodstuffs	26	Tea, onion, apple, citrus fruit and juice	3 flavanones, 1 flavone, 2 flavonols	Justesen *et al*. 1997
Finland	10054 MW, (>25 y)	Dietary history, 1999 release of the Finnish FCD updated with values obtained from studies conducted in the Netherlands	24	Orange, apple, grapefruit, onion, white cabbage, berries, juice	3 flavanones, 2 flavones, 4 flavonols	Knekt *et al.* 2002
	1095 W, 912 M	48h dietary recall, 1999 release of the Finnish FCD updated with 2000-2007 data for anthocyanidins, phenolic acid and other flavonoids	47	Berries	Anthocyanidins	Ovaskainen *et al.* 2008
			33	Apple, orange, unspecified fruit, tea	Flavonols, flavanones, flavones	
			863	Coffee, cereal (rye bread), berries, fruit	Total polyphenols	
The Nether-lands	4112 MW (>19 y)	Dietary history, 1985 release of the Dutch FCD updated with 1993 data for flavonols, flavones and catechins	23	Tea, onion, apple	Q, K, M, A, L	Hertog *et al.* 1993
	738 M (65-84 y)		26	Tea, onion, apple	Q, K, M, A, L	Hertog *et al.* 1994
	728 M (65-84 y)		72	Tea, apple	Catechins	Arts *et al.* 2001
	115 W (29-78y)	3d dietary record, National standard Tables of food composition from 1982	17	Onion	1 flavone, 4 flavonols	Arai *et al.* 2000
US	37886 M 78886 W	FFQ, FCD contained values from the analyzed foods, previously published values from Dutch foods, and imputed values	20-22	Onion, tea, apple	Q, K, My, A, I	Sampson et al. 2002

(continues)

Table 3 (continued)

Country	Sample size (age)[1]	Dietary assessment method	Mean estimated dietary intake (mg/d)	Main dietary source	Flavonoid subclass	References
US	2908 (>19y)	24h dietary recall, The USDA Isoflavone database	≈1	Not reported	Isoflavones	Chun *et al*. 2009
US	40683 (35-64y)	24h dietary recall, The USDA database completed with the US food consumption data from the 1999-2002 National Health and Nutrition Examination Survey	190	Tea , citrus fruit juice, grain food, wine, legume	Flavan-3-ols, flavanones, flavonols	Chun *et al*. 2007
Japan	115 W (29-78y)	3d dietary record, National Standard Tables of food composition from 1982	47	Tofu	2 isoflavones	Arai *et al.* 2000
Spain	40683 MW (34-64y)	Dietary history, Expanded USDA database for flavonoid, isoflavone, and proanthocyanidin content	313	Total fruit, apple, orange, pear, peach, bean, red wine	Proantho-cyanidins, flavanones, flavan-3-ols, flavonols, anthocyanidins	Zamora-Ros *et al.* 2010
	40683 MW (34-64y)		41	Citrus fruit and juice	H, N E	
			16	Onion, lettuce, wine, apple	I, K, My, Q	
			3	Wine, orange, lettuce, pepper	A, L	
			0		D, Ge, Gl	

[1]M, men; W, women; y, year; FCD, food composition database; H, hesperetin; N, naringenin; E, eriodictyol; I, isorhamnetin; K, kaempferol; My, myricetin; Q, quercetin; A, apigenin; L, luteolin; D, daidzein; Ge, genistein; Gl, glycetein; USDA, The US Department of Agriculture databases include information about 385, 128, and 205 items for flavonoids, isoflavones, and proanthocyanidins, respectively.

5.3.3 Flavonoids and other compounds included in the biomarker assay

According to the published data on flavonoid intake in Denmark (Dragsted *et al.* 1997, Justesen *et al.* 1997), the intake of flavonoids comes from tea, onions, apples, citrus

fruits and juices, parsley, celery, red pepper, kale, curly broccoli, apples, berries, tea, berries and red wine (see **Table 4**).

The 7 flavonoids included in the flavonoid biomarker assay developed by Nielsen *et al.* 2000 belong to the groups of flavanones (naringenin and hesperetin) found in citrus fruits and tomatoes, flavonols (quercetin, kaempferol, isorhamnetin, tamarixetin) found in, for example, onions, apples, tea, crucifers and wine, and the dihydrochalcones (phloretin) found in apples. These flavonoids were included based on their presence in a variety of commonly consumed fruits and vegetables in Denmark, and on their accessibility as commercial standards. Although the group of catechins and anthocyanidins has been estimated to contribute significantly to the total intake of flavonoids in Denmark (Dragsted *et al.* 1997), these two sub-classes of flavonoids were not included in the flavonoid biomarker assay, as they mainly come from wine and tea, which do not belong to the fruit and vegetable group, and from berries, which, in Denmark, are consumed in small amounts only.

In an attempt to reflect a larger spectrum of different types of fruits and vegetables the biomarker assay was optimized during the course of this Ph.D. project to monitor a few more compounds present in commonly consumed fruits and vegetables. Apigenin was included due to its presence in different types of commonly consumed vegetables (e.g. celery and red pepper). Eriodictyol was included due to its presence in citrus fruits, especially lemons. Consumption of soya in the Asian countries is ≈ 10-35 mg/d, which is equivalent to a mean intake of 25-40 mg isoflavones/d with a maximum intake of 100 mg/d (Messina *et al*. 2006). In Europe and the US, the mean intakes of isoflavones have been estimated to be between 0-1 mg/d (de Kleijn *et al.* 2001, Boker *et al.* 2002, Chun *et al.* 2009). Because of the low suspected intake of isoflavones in Denmark, ^{13}C daidzein and ^{13}C genistein were selected as internal standard in the analytical LC-MS setup. However, it became apparent, when analysing urinary samples from free-living individuals, that many of them had a considerable and quantifiable urinary recovery of genistein and daidzein. We therefore decided to include daidzein and genistein in the biomarker assay. The urinary recovery of isoflavones could indicate a growing incorporation of soya extracts into manufactured food products and we wanted to see if the urinary recovery of daidzein and genistein would correlate with the self-reported intake of selected food groups (processed meat, beans and legumes) in **Paper III-IV**.

The urinary recovery of enterolactone and isolariciresinol have in several studies been explained by the intake of vegetables (Kilkkinen *et al.* 2001, Horner *et al.* 2002, Johansen *et al.* 2004, Milder *et al*. 2005, Touillaud *et al.* 2007). Therefore we decided to include enterolactone in the biomarker assay as well, even though enterolactone is not a flavonoid. This was done in order to investigate if this would improve the capability of the biomarker assay to reflect the self-reported intake of vegetables and thus the total intake of fruits and vegetables as well. In section 8.5, the effect of inclusion of additional flavonoids and enterolactone in the biomarker assay on the observed correlation coefficient between self-reported intake and biomarker is discussed.

Table 4 Most abundant flavonoids and their food sources in the Danish diet (modified after Dragsted *et al.* 1997 and Justesen *et al.* 1997)

Flavonoid subclass	Quantitative important flavonoids	Main dietary sources	Estimated intake (mg/d)
Flavanones	Naringenin Hesperetin	Citrus fruit and juice	6-12
Flavones	Apigenin Luteolin	Parsley, celery, red pepper	1-2
Flavonols	Kaempferol Quercetin Myricetin	Onion, kale, curly broccoli, apple, berries, tea, wine,	15-30
Polymethoxylated flavanols	Tangeretin	Citrus fruit	1-2
Catechins	Epigallocatechin	Black tea	20-50
Anthocyanins	Cyanidin	Red berries, red wine, red cabbage	6-60
Total consumption of flavonoids in Denmark			50-150

5.3.4 Metabolic fate of flavonoids

In general, flavonoids are subjected to extensive metabolism following ingestion. In the upper gastrointestinal tract, dietary flavonoids act as substrates for lactase phloridizin hydrolase (LPH) present in the brush-border of the small intestine epithelial cells. An alternative site of hydrolysis is by cytosolic β-glucosidase (CBG) within the epithelial cells (Spencer *et al.* 2008, Crozier *et al.* 2009). Prior to passage into the blood stream the aglycones undergo metabolism in the enterocytes, forming sulfate, glucuronide and/or methyl conjugated metabolites through the action of the phase II enzymes sulfutransferase (SULT), uridine-5´-diphosphate glucuronosyltransferase (UGT) and catechol-*O*-methyltransferase (COMT), (see **Figure 5**). Once in the bloodstream flavonoid metabolites can be subjected to further phase II metabolism in the liver, where enterohepatic transport in the bile may result in some recycling back to the small intestines (Crozier *et al.* 2009).

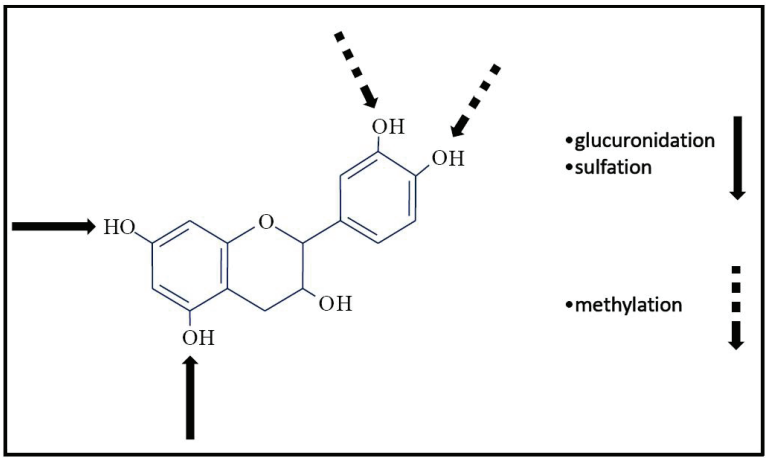

Figure 5 The potential sites of the conjugation process of the polyphenols are schematically illustrated. The *broken arrows* represent the potential methylation sites; the *full arrows* represent the potential glucuronidation and sulfation sites

Flavonoids not absorbed in the small intestines reach the colon, where the sugar moieties are cleaved by enzymes excreted by the microflora (Olthof *et al.* 2003). These enzymes also cleave the ring structure of many polyphenols, including flavonoids, which yields small molecular weight phenolic acids, The colon contains approximately 10^{12} microorganisms/cm^3 and provides an enormous catalytic and hydrolytic potential and produces a wide range of metabolites, which are subsequently absorbed into the systemic circulation from the colon.

Polyphenols, including the flavonoids, markedly differ from one another in their bioavailability and intestinal metabolism. Manach and co-workers have recently summarized all scientific studies (n = 97) that have been conducted so far on the bioavailability of polyphenols (Manach *et al.* 2005), (see **Table 5**). Much of the research covered involved feeding volunteers only one or few compounds within a given subclass of polyphenols and monitoring the levels of flavonoids in plasma and/or urine over a 24h period. As flavonoid metabolites were rarely commercially available, analysis almost invariably involved enzymatic treatment of plasma and urine samples with glucoronidase/sulfatase preparations and subsequent quantifications of the released aglycones. Thus, the information yielded on the metabolites produced is very indirect, and quantitative estimates, although precise, are not necessarily accurate as there are only few data on the efficiency with which the enzymes hydrolyse individual metabolites and release the aglycones (Gu *et al.* 2005, Crozier *et al.* 2009).

As seen in **Table 5**, evidence from bioavailability studies pre-2005 suggest that the bioavailability of flavonoids varies from about 0.3 to 43% (based on urinary recovery) of the dose administered, reaching plasma concentrations of 0.02–4.0 µmol/l at an intake level of 50 mg aglycone equivalents (Manach *et al.* 2005). For most flavonoids included in Table 5, the urinary recovery values are consistent with plasma kinetic data. However, when flavonoids are mainly excreted in bile, as for genistein and epigallocatechin gallate, and

probably also for quercetin, the urinary recovery values are low relative to plasma concentration, as demonstrated for quercetin in **Paper II**.

There is no data concerning the bioavailability of phloretin included in **Table 5**, since the only published study investigating this is from 2009. In this human feeding study with apple cider the sole quantifiable metabolite in plasma was phloretin-2*0*-O-glucuronide, which reached a C_{max} of 73 nmol/L 0.6h after ingestion and had a T½ of 0.7h, indicative of absorption in the proximal part of the small intestine and rapid elimination from the circulatory system (Marks *et al.* 2009). The 0–24h urine contained a total of 2.3 mmol of phloretin metabolites, equivalent to 5.0% of intake (Marks *et al.* 2009). One previously published study has investigated the bioavailability of eriodictyol. Here the urinary excretion of eriodictyol-O-sulfate was reported to be relatively low with recoveries of 0.3 and 0.6%, following consumption of 23 and 5.5 μmol of eriodictyol-C-glucosides contained in the fermented and unfermented beverages, respectively. The majority of eriodictyol-O-sulfate excretion occurred during 5-12 h following ingestion. This is indicative of low level absorption occurring in the large rather than the small intestine (Stalmach *et al.* 2009). Two previously published studies have tried to determine apigenin bioavailability after consumption of parsley. Nielsen *et al.* (1999) detected apigenin in urine samples of 14 participants of a randomized cross-over trail after having administered a parsley supplement providing 3.73–4.49 mg apigenin/MJ and the urinary recovery of apigenin was estimated to be 0.58% of ingested apigenin. This is in good agreement with the findings by Meyer *et al.* (2006) where the average 24h urinary recovery of apigenin was 0.22±0.16% of the ingested dose (Meyer *et al.* 2006). Furthermore, Meyer *et al.* (2006) reported that the average plasma T_{max} was reached after 7.2±1.3h with a high range of variation between subjects.

When polyphenols are present in food as glycosides, the sugar moiety probably has the greatest effect on absorption compared to any other factor (Scholz & Williamson 2007). A study by Nielsen and co-workers demonstrated that the removal of the rhamnose group to yield the corresponding flavonoid glucoside (i.e. hesperetin-7-glucoside) improve the bioavailability of the aglycone hesperetin significantly (Nielsen *et al.* 2006). Likewise, it has previously been shown that quercetin-3-*O*-rhamnoglucoside (rutin) in tea was absorbed more slowly (T_{max}= 6h) than quercetin-4-*O*-glucoside in onions (T_{max}< 0.5h) (Hollman *et al.* 1999).

Absorbed flavonoids are not accumulated in the body, but subjected to excretion through the bile or elimination with the urine often with a relatively short $T_{½}$ ranging from 1-28h, depending on sugar moiety composition and flavonoid sub-class (Manach *et al*. 2005, **Table 5**). Therefore the urinary recovery of flavonoids would represent a short-term biomarker of fruit and vegetable intake. Although not sufficiently studied, it is suggested that variation in flavonoid absorption also occurs due to interactions between the flavonoid and other food components present in milk, bread- and sugar containing meals (Spencer *et al.* 2008). **Table 6** present an overview of the main factors thought to affect the bioavailability of dietary flavonoids in humans.

Table 5 Compilation of pharmacokinetic data from 97 bioavailability studies[1]

	T_{max}		C_{max}		AUC		Urinary recovery		Elimination half-life	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
	H		*µmol/L*		*µmol h/L*		*% of intake*		*h*	
Daidzin	6.3±0.6	4.0–9.0	1.92±0.25	0.36–3.14	21.4±6.5	2.7–38.6	42.3±3.0	21.4–62.0	5.3±0.8	3.4–8.0
Daidzein	4.9±1.0	3.0–6.6	1.57±0.52	0.76–3.00	12.2±2.9	7.5–17.4	27.5		8.5±0.8	7.7–9.3
Genistin	6.5±0.6	4.4–9.3	1.84±0.27	0.46–4.04	23.7±6.7	6.2–45.1	15.6±1.8	6.8–29.7	7.8±0.7	5.7–10.1
Genistein	4.1±0.6	3.0–5.2	2.56±1.00	1.26–4.50	19.8±6.5	10.4–32.2	8.6		7.1±0.3	6.8–7.5
Glycitin	5.0		1.88±0.38	1.50–2.26	7.9		42.9±12.0	19.0–55.3	8.9	
Hesperidin	5.5±0.1	5.4–5.8	0.46±0.21	0.21–0.87	2.7±0.7	1.9–4.1	8.6±4.0	3–24.4	2.2	
Naringin	5.0±0.2	4.6–5.5	0.50±0.33	0.13–1.50	3.7±1.5	0.9–7.0	8.8±3.17	1.1–30.2	2.1±0.4	1.3–2.7
Quercetin glucosides	1.1±0.3	0.5–2.9	1.46±0.45	0.51–3.80	9.8±1.9	5.7–16.0	2.5±1.2	0.31–6.4	17.9±2.2	10.9–28.0
Rutin	6.5±0.7	4.3–9.3	0.20±0.06	0.09–0.52	2.9±0.9	1.6–5.5	0.7±0.3	0.07–1.0	19.9±8.1	11.8–28.1
(Epi)catechin	1.8±0.1	0.5–2.5	0.40±0.09	0.09–1.10	1.1±0.3	0.5–2.0	18.5±5.7	2.1–55.0	2.5±0.4	1.1–4.1
EGC	1.4±0.1	0.5–2.0	1.10±0.40	0.30–2.70	2.0±0.8	1.0–3.6	11.1±3.5	4.2–15.6	2.3±0.2	1.7–2.8
EGCG	2.3±0.2	1.6–3.2	0.12±0.03	0.03–0.38	0.5±0.1	0.2–0.9	0.06±0.03	0.0–0.1	3.5±0.3	2.5–5.1
Gallic acid	1.6±0.2	1.3–1.5	4.00±0.57	2.57–4.70			37.7±1.0	36.4–39.6	1.3±0.1	1.1–1.5
Chlorogenic acid	1.0		0.26				0.3			
Caffeic acid	1.4±0.6	0.7–2.0	0.96±0.26	0.45–1.35			10.7			
Ferulic acid	2.0		0.03				27.6±17.6	3.1–61.7		
Anthocyanins	1.5±0.4	0.7–4.0	0.03±0.02	0.001–0.20			0.4±0.3	0.004–5.1		
Proanthocya-nidin dimers	2.0		0.02±0.01	0.008–0.03						

[1] All data were converted to correspond to a supply of 50 mg aglycone equivalent. T_{max}, time to reach C_{max}; AUC, area under the plasma concentration-time curve; EGC, Epigallocatechin; EGCG, Epigallocatechin gallate.

Table 6 Overview of main factors affecting the bioavailability of dietary flavonoids in humans (adapted from D'Archivio *et al.* 2010)

External factors	Environmental factors (e.g. sun exposure, degree of ripeness).
Food processing related factors	Thermal treatments, homogenization, liophilization, cooking and methods of culinary preparation, storage.
Food related factors	Food matrix, presence of positive or negative effectors of absorption (e.g. fat, fiber).
Interaction with other compounds	Bonds with proteins (e.g. albumin) or with polyphenols with similar mechanism of absorption.
Flavonoid related factors	Chemical structure, concentration in food, amount introduced.
Host related factors	Intestinal factors (e.g. metabolizing enzymes and transporters, gastric emptying, intestinal transit time, colonic microflora). Systemic factors (e.g. gender and age; disorders and/or pathologies, genetics, physiology).

5.3.5 Flavonoids in urine as biomarker of fruit and vegetable intake

The first published study investigating the association between fruit and vegetable intake and the urinary recovery of the 7 flavonoid aglycones (quercetin, naringenin, hesperetin, isorhamnetin, tamarixetin, kaempferol and phloretin) was the strictly controlled dietary intervention study by Nielsen *et al.* (2002). After 6 weeks consumption of a diet either low or high in fruits, vegetables and berries the urinary recovery of quercetin and flavanones, as well as the sum of the 7 flavonoids, were clearly higher after the high fruit and vegetable diet as compared with the low fruit and vegetable diet (Nielsen *et al.* 2002). Furthermore, the study showed that the urinary recovery of flavonoids after intake of habitual diets also correlated significantly with estimates of short-term intake of fruits and vegetables (r_s=0.28-0.38), (Nielsen *et al.* 2002). A few years later, it was found that the urinary recovery of these 7 flavonoids responded in a linear and sensitive manner following 1d consumption of a basic diet supplemented with 300 or 600 g fruits and vegetables at the individual level in 24h urine (r_s=0.86) and in morning spot urine (r_s=0.59) (**Paper I**). In a 14d dose-response study by Brevik *et al.* (2004), a linear relationship between urinary recovery of naringenin and hesperetin in 24h urine and three doses of mixed fruits and vegetables (0, 300 and 750 g/d) were likewise observed.

In **Table 7**, an overview of all previously conducted studies that have been using the urinary recovery of different flavonoids and enterolactone as biomarker of fruit, juice and/or vegetable intake are presented. Included in Table 7 are observations made in different controlled intervention studies and real life observations with different types of dietary assessment methods. Controlled single and multi component bioavailability studies with purified flavonoids and single beverages analysed for their content of flavonoids (e.g. soy products, tea, coffee, wine, orange and grapefruit, black current or apple juice) are covered in **Table 5**.

Table 7 Overview of previously observed correlation coefficient between flavonoids excreted in urine and total intake of fruit, juice and/or vegetables in different types of studies

Flavonoids[1]	Dose ingested	Source	Duration, study design, subjects	Sample	Correlation coefficient	Reference
Q,I,T,K,H,N, P	Low, High	540, 1110g/d FBV diet	6-weeks intervention study (n=77)	Pool of 3X24h urine	Not calculated	Nielsen *et al.* 2002
	3d EFR	Habitual diet	Free living individuals (n=94)		0.35* (FV)	
Q,I,T,K,H,N, P	Trace, 34, 68 mg	Controlled basic diet, basic diet +300 g FV, Basic diet + 600 g FV	1d cross-over intervention study (n=12)	24h urine	0.86* (FV)	**Paper I**
				Morning urine	0.59* (FV)	
Q,I,T,K,H,N, P, E	Low, High	Controlled diet containing 300, 750 g/d FVB	14d intervention study (n=40)	24h urine	Not calculated	Brevik *et al.* 2004
I, K, H, N, P	2d EFR	Habitual diet	Participants of the SU.VI.MAX cohort (n=53)	24h urine	0.38* (FJ)	Mennen *et al.* 2006
				Morning urine	0.47* (FJ)	
Ent, K				24h urine	0.34* (V)	
Ent				Morning urine	-0.09^{ns} (V)	
Q,I,K,H,N,P	FFQ and 7d WFR	Habitual diet	Validation study, participants of the DNBC cohort (n=88)	24h urine	0.30* (FV), (FFQ) 0.39* (FJV) (FFQ) 0.24* (FV), (WFR) 0.39 (FJV), (WFR)	Mikkelsen *et al.* 2007
Q,I,T,K,H,N, P, E, Ent, D, G	FFQ and 4d WFR	Habitual diet	Validation study, participants of the MoBa cohort study (n=99)	24h urine	0.19^{NS} (FFQ), (FV) 0.31* (DH), (FV)	Brantsæter *et al.* 2007
Ent					0.22* (V^2) (FFQ) 0.38* (V^2) (DH)	
N, H, E					0.41* (Citrus FJ) (FFQ) 0.66* (Citrus FJ) (DH)	
D, G					0.17^{ns} (soy products) (FFQ/DH)	
Q,I,T,K,H,N, P, D, G	24h dietary recall	Habitual diet	Validation study, employees at workplaces (n=158)	24h urine	0.31* (F) 0.21* (V) 0.38* (FV)	**Paper III**
Q,I,T,K,H,N, P, E, Ent, A	28d DH and FFQ	Habitual diet	Validation study, participants of the Inter99 cohort (n=191)	24h urine	0.22* (FJV), (FFQ) 0.39* (FJV), (DH)	**Paper IV**
				Morning urine	0.19^{NS} (FJV), (FFQ) 0.29* (FJV), (DH)	
Ent				24h urine	0.22* (legumes) (FFQ) 0.18* (legumes) (DH)	
					0.28* (V) (FFQ) 0.27* (V) (DH)	
				Morning urine	0.24* (V) (FFQ) 0.21* (V) (DH)	

[1]DH, Dietary history; FD, Food diary, WRF, Weighed food record, FFQ, Food frequency questionnaire; F, Fruit; V, Vegetables; B, Berries; J, Juice; *. Significant correlation between fruit and vegetable intake and urinary recovery of flavonoids, Q, Quercetin; K, Kaempferol; I, Isorhamnetin; T, Tamarixetin; N, Naringenin; H, Hesperetin; P, Phloretin; E, Eriodictyol; Ent, Enterolactone; Api, Apigenin, D, Daidzein; G, Genistein, MoBa, Norwegian Mother and Child Cohort Study, SU.VI.MAX, a randomised, double-blind, placebo-controlled primary-prevention trial evaluating the effect of daily antioxidant supplementation; DNBC, Danish National Birth Cohort. [2]Vegetables refer here to all cooked vegetables excl. cruciferous, roots and potatoes.

According to the data presented in **Table 7**, the bivariate correlation coefficient between the urinary recovery of flavonoids and/or enterolactone and estimated dietary intake are in the same range. Mikkelsen *et al.* (2007) calculated the correlation value to be 0.39 between fruit, juice and vegetable intake (estimated by weighed food record) and 24h urinary recovery of 6 flavonoids. In **Paper III,** the correlation coefficient between fruit and vegetable intake (estimated by a single 24h dietary recall) and 24h urinary recovery of 10 flavonoids was 0.38. In **Paper IV**, the correlation coefficient between fruit, juice and vegetable intake (estimated by 28d dietary recall) and 24h urinary recovery of 11 flavonoids and enterolactone was 0.39, and in Brantsæter *et al.* (2007) the correlation coefficient between fruit and vegetables (estimated by a dietary history interview) and urinary recovery of 10 flavonoids and enterolactone was 0.31.

According to Kaaks (1997), the correlation coefficient obtained between biomarker and estimated intake values from FFQ are generally smaller than 0.4 due to interference and the flavonoid biomarker is also within this range. This interference is due to the many different factors described in the previous section (see **Table 6**).

As seen in **Table 7**, almost all of the correlation coefficients between FFQ estimated intake of fruit, vegetable and juice and biomarkers are below the correlation value between biomarker and the other reference methods included (Mikkelsen *et al.* 2007, Brantsæter *et al.* 2007, **Paper IV**).

In **Table 8,** a summary of the studies in which the method of triads was used to calculate VCs between FFQ self-reported intake of fruits and vegetables, different reference methods and the 'true' unknown intake are presented.

In the study by Andersen *et al.* (2005) and Slater *et al.* (2010) one biomarker is included as reference method and one additional dietary assessment method is included as the other reference method. In these two validation studies the VCs for the biomarker were lower than those observed for the FFQ, 24h dietary recall and weighed food record (see **Table 8**). This indicates that the FFQs had good accuracy in measuring the consumption of fruits and vegetables in these population groups. In **Paper IV** and in Brantsæter *et al.* (2007) two biomarkers were included as reference methods, and here the VCs between biomarkers and "true" intake and FFQ and "true" intake were in the same range. When the intension was to measure the intake of citrus fruits and juices the VCs between selected biomarkers (citrus flavanones and β-cryptoxanthin) and 'true' intake were higher as compared to the FFQ. This indicates that citrus flavanones and β-cryptoxanthin are particularly strong in reflecting this food group. The discrepancies between the results in **Table 8** could be consequences of the different reference methods used, different structures of the applied FFQ, differences in sample sizes or differences in the populations being studied.

Table 8 Validation studies where validity coefficients (VC) in relation to the 'true' (T) intake of fruits and vegetables were estimated by the triad method

Country	Sample size	Dietary assessment method[1]	Reference method	Validity coefficients (VC_{FFQ-T}, VC_{R1-T}, VC_{R2-T})	Reference
Norway	86-100 military men	180 items FFQ 27 items FFQ	R1 = 14d weighed food record R2 =carotenoids in serum	(0.54, 0.79, 0.47) for all FV (180 items FFQ), R1=14d WFR, R2=α-carotene (0.60, 0.91, 0.43) for all FV (27 items FFQ), R1=14d WFR, R2=α-carotene	Andersen *et al.* 2005
Norway	119 pregnant women	FFQ	R1 = flavonoids in 24h urine, R2 = carotenoids in plasma	(0.65, 0.63, 0.43) for citrus FJ, R1=hesperetin, R2= zeaxanthin (0.37, 0.77, 0.30) for all fruit, R1=phloretin, R2=α-carotene (0.59, 0.34, 0.59) for cooked V, R1=enterolactone, R2=α-carotene	Brantsæter *et al.* 2007
Denmark	191 women and men from the Inter99 cohort in high risk of developing ischemic heart disease	198 items FFQ	R1 = flavonoids in 24h urine, R2 = carotenoids in plasma	(0.56, 0.58, 0.53) for all FJV, R1= 11 flavonoids + enterolactone, R2= total carotenoids (0.52, 0.62, 0.63) for all citrus FJ, R1=citrus flavanones, R2= β-cryptoxanthin	**Paper IV**
Brazil	80 men and women from the Dietary Intake and Physical Activity as Determiners for Changes in Body Mass Index cohort	94 items FFQ	R1 = average of two 24h dietary record R2 = carotenoids in serum	(0.65, 0.38, 0.36) for all FV, R1=24h DR R2=β-carotene	Slater *et al.* 2010

FFQ, Food frequency questionnaire; VC, validity coefficient; F, fruit; V, vegetables; J, juice; R1, reference method 1; R2, reference method 2; T, true, but unknown, intake

The FFQ validation studies described in **Paper IV** and in Brantsæter *et al.* (2007) are the only two studies in which the flavonoids and the carotenoids are used together as biomarkers of fruit and vegetable intake. In **Paper IV**, the VC between 'true' intake of fruits and vegetables and flavonoids are higher (VC=0.58) than VC between 'true' intake and carotenoids (VC=0.53). The VCs between 'true' intake of citrus fruits and juices are reflected equally by the flavonoids (VC=0.62) and carotenoids (VC=0.63). In the study by Brantsæter *et al.* (2007) the flavonoids produces the highest VC with respect to the fruit group (VC=0.63-0.77) while the carotenoids produce the highest VCs with respect to the group of vegetables (VC=0.59), (see **Table 8**). The FFQ validation study described in Mikkelsen *et al.* (2007) is the only other validation study measuring both flavonoids and carotenoids, however without

applying the method of triads. Mikkelsen *et al.* (2007) calculated the bivariate correlation coefficients between estimated fruit, juice and vegetables intake and the flavonoids and β-carotene, and found that β-carotene was more strongly correlated to overall fruit, juice and vegetable intake (r_s=0.46*) than the sum of 6 flavonoids (quercetin, naringenin, hesperetin, phloretin, kaempferol, isorhamnetin), (r_s=0.39*, see **Table 7**).

6 Methods and subjects

Different experimental designs were used in the present Ph.D. thesis. An overview of the studies contained in the papers included in the Ph.D. thesis is shown in **Table 9**.

Table 9 Overview of the studies included in the present Ph.D. thesis

Aim	Development, characterisation and validation of the flavonoid biomarker		Validation of 24h dietary recall	1) Validation of Inter99 FFQ 2) validation of flavonoids in morning spot urine as biomarker of fruit and vegetable intake
Reference	Paper I[1]	Paper II	Paper III	Paper IV
Study design	1d randomised, diet controlled, crossover study	48h, diet-controlled, single-dose, biokinetic study	Intervention study, real life observations	Real life observations in participants of the Inter99 Cohort in high risk of developing IHD
Dietary assessment	Controlled diet spiked with 0/300/600 g of mixed fruit and vegetables. Flavonoids in diet assessed by chemical analysis	Controlled diet spiked with a mixed fruit juice containing 30mg/l quercetin, 28mg/l naringenin, 32 mg/l hesperetin. Flavonoids in diet and fruit juice assessed by chemical analysis	24h dietary recall by personal interviewer	198-items FFQ covering the last month 28d dietary history interview
Subjects	12 healthy M, Age (y), (mean, range): 24.6 (20–28) BMI (kg/m^2), (mean, range): 23.3 (21.2–26.8)	10 healthy M, Age (y), (mean, range): 24.6 (21-28) BMI (kg/m^2),(mean, range): 23.7 (21.2-26.8)	79 M/W (sub-set of free fruit at workplace intervention). Baseline characteristics not significantly different between intervention (n=34) and control group (n=45)	191 M/W in high risk of developing ischemic heart disease (sub-set of Inter99 cohort). Mean age M/W (y): 48.6/48.5 Mean BMI M/W (kg/m^2): 28.0/26.2
Place of conduction	Institute of Human Nutrition, KU-LIFE	Institute of Human Nutrition, KU-LIFE	8 comparable workplaces in the Copenhagen area	Research Centre for Prevention and Health, Glostrup University Hospital
Biological samples collected	24h urine, morning spot urine	Plasma (baseline, 2, 4, 6, 24, 48h post intervention). Each urine void collected separately between 0-24 h. 24-48h urine in one pool	24h urine	24h urine, morning spot urine, fasting plasma
Flavonoids included in the biomarker assay	quercetin, kaempferol, isorhamnetin, tamarixetin, hesperetin, naringenin, phloretin		quercetin, kaempferol, isorhamnetin, tamarixetin, hesperetin, naringenin, phloretin, **daidzein, genistein, eriodictyol**	quercetin, kaempferol, isorhamnetin, tamarixetin, hesperetin, naringenin, phloretin, daidzein, **genistein, eriodictyol, apigenin, enterolactone**

[1]M, Men; W, Woman; BMI, Body mass index; y, year, IHD, ischemic heart disease

6.1 Statistical methodology and calculation of biokinetic indexes

In the current Ph.D. thesis, statistical analyses were performed with SPSS. The calculated p-values were two-sided, and a 5% level of significance was used. Furthermore, the statistical program R was used in **Paper IV** for the bootstrap procedure needed to estimate confidence intervals for the triangular validity coefficients (VCs). Assumptions of normality were checked by visual inspection of histograms and by evaluation of the skewness and kurtosis of the variables.

In general, the data collected on food intake, the urinary recovery of flavonoids and plasma concentrations of carotenoids were not normally distributed and non-parametric tests were therefore used. Wilcoxon matched pairs tests were performed to evaluate changes from baseline to post intervention (**Paper III**) and to compare the selected flavonoids at the different time points or doses (**Paper I -II**). In **Paper II**, the relative bioavailability was calculated by comparing the areas under the plasma concentration-time curves (AUC) up to 48h. AUC was calculated with the linear trapezoidal rule (Rowland & Tozer 1995). The peak plasma concentration (C_{max}) and the time to reach it (T_{max}) were determined based on the plasma concentration curve. Based on two data-points an estimated elimination half live ($T_{½}$) was calculated from the equation $T_{½} = \ln 2/k$. The elimination rate constant (k) was determined in the terminal phase from the slope of the log-transformed plasma concentration curve. The mean excreted amount, rate and relative urinary recovery, expressed as percentage of the dose ingested, were calculated based on urinary recovery data.

The Mann-Whitney *U* test was performed to test differences between two groups (**Paper III** and **IV**). Spearman rank correlation coefficient (r_s) was used to examine associations between the intake of flavonoid-rich foods and the fruit and vegetable biomarkers (**Paper I**, **III** and **IV**). In **Paper IV,** calculation of the percentages of correctly classified and grossly misclassified subjects were calculated based on division of data into quintiles. VCs were calculated for the FFQ and for each of the biomarkers by the equation $VC = \sqrt{r_1 * r_2 / r_3}$, where r_1, r_2 and r_3 are the correlation coefficients between the FFQ and each of the biomarkers and between the two biomarkers (see **Figure1**) (Kaaks, 1997, Ocké & Kaaks 1997). Further details of the statistical analysis are described in each paper included in the Ph.D. thesis.

6.2 Analytical methodology

Liquid chromatography mass spectrometry (LC-MS) was used for determination of flavonoids in the controlled diets, urine and plasma samples in all four papers included in the present Ph.D. thesis. LC-MS is the method of choice for the identification and quantification of natural concentrations of flavonoids in biological matrixes. The main advantages of MS detection are the possibilities for high sensitivity in combination with broad selectivity and short time of analysis.

The concentration of flavonoids in the fruit and vegetable mix applied in **Paper I** was determined after acid hydrolysis by LC-MS as described by Justesen *et al.* 1997, with the exception of the compound phloridzin. Phloridzin was determined by LC-MS without prior hydrolysis, after extraction and solid phase extraction (SPE) cleaning by the method described by Grinder-Pedersen *et al.* 2003. Flavonoid glycosides in the 'juice mix' applied in **Paper II** were identified according to Breinholt *et al.* (2003). In **Paper I**, the urinary concentration of quercetin, kaempferol, phloretin, isorhamnetin, tamarixetin, naringenin and hesperetin were detected by LC-MS after enzymatic hydrolysis and solid phase extraction (SPE) as suggested in Nielsen *et al.* (2000) and Nielsen *et al.* (2002). For each batch of urine and/or plasma samples the performance and reproducibility of the LC-MS analysis were evaluated by external standards. The analysis of every single urine sample was monitored with ^{13}C-labelled internal standards (^{13}C daidzein and ^{13}C genistein) added just prior to the enzymatic hydrolysis and straight before LC-MS analysis.

In **Paper III** we included three additional flavonoid aglycones in the biomarker assay (eriodictyol, daidzein and apigenin), and in **Paper IV,** four additional flavonoid aglycones (eriodictyol, apigenin, daidzein and genistein) and a lignan, enterolactone, were included. In the Inter99 validation study (**Paper IV**) fasting plasma samples (>8h) were collected for determination of 6 carotenoids (lutein, zeaxanthin, b-cryptoxanthin, a-carotene, b-carotene, and lycopene) as an additional biomarker of fruit and vegetable intake by the method described in details in Toft *et al.* (2008). We did also try to set-up a LC-MS method for simultaneous determination of several different flavonoids in plasma. However, it was not possible to obtain enough sensitivity by the method to make reproducible quantifications of the flavonoids in the fasting plasma samples. This was probably due to the very limited amount of flavonoid left in fasting plasma, due to the short plasma $T_{½}$ of flavonoids, which makes urine a more suitable matrix. The effect of including additional flavonoids and enterolactone in the biomarker assay will be discussed in section 8.5.

6.3 Self-reported dietary assessment methods

In **Paper III**, dietary intake was estimated by a 24h dietary recall, which was a modified form of the dietary recall questionnaire from the Danish National Dietary Survey 2000–2002 (Lyhne *et al.* 2005). The software programme, General Intake Estimation System (GIES, version 1.0, Technical University of Denmark, National Food Institute, Division of Nutrition 2008), was used to calculate dietary intake. Details of the FFQ used in the Inter99 cohort is described in **Paper IV** and in Toft *et al.* (2008). In brief, the Inter99 FFQ is a semi-quantitative questionnaire consisting of 198 food items and beverages. Participants were asked to report their average intake of different foods and beverages during the previous month. The food consumption quantity was obtained by multiplying the frequency of consumption of each unit of food by standard portion sizes. Daily food consumption was translated into energy and nutrient intake using the Danish Food Composition Databank,

version 6 (Saxholt *et al.* 2005) and the software program FoodCalc version 1.3 (Lauritsen 1998).

7 Main findings

The Ph.D. thesis is based on the following four scientific papers (**Paper I-IV**);

Paper I. Krogholm KS, Haraldsdóttir J, Knuthsen P, Rasmussen SE. Urinary total flavonoid excretion, but not 4-pyridoxic acid or potassium, can be used as a biomarker for the intake of fruits and vegetables. Journal of Nutrition, 134: 445-451, 2004.

The effect of three different doses of fruits and vegetables intake (0, 300 and 600 g/d) on urinary recovery of 7 flavonoid aglycones (quercetin, phloretin, naringenin, hesperetin, kaempferol, isorhamnetin and tamarixetin) in 12 healthy, normal weighted men (mean BMI=23.7 kg/m^2), with a mean age of 24.6 y, were evaluated in a 1d diet-controlled cross-over intervention study. Fruits and vegetables were combined to match a typical Danish diet and the 300 and 600 g/d of fruits and vegetables corresponded to the actual and recommended daily intake of fruits and vegetables at the time of conduction. We observed that the 24h urinary recovery of the 7 flavonoids increased linearly with increasing fruit and vegetable intakes (r_s=0.86, P=1x10^{-6}). The urinary recovery of the 7 flavonoids in morning spot urine also increased significantly, but the association was somewhat weaker (r_s=0.59, P=0.0001), and the applicability of flavonoids in morning spot urine as a biomarker of fruits and vegetable intake therefore needs further evaluation in free-living individuals. **Paper I** provide evidence that the 24h urinary recovery of the 7 flavonoids is a valid biomarker of fruit and vegetable intake and thus consolidated the previous findings by Nielsen *et al.* (2002). The high inter-individual variation in the urinary recovery of the 7 flavonoids makes it very difficult to separate individuals according to intake by use of the flavonoid biomarker in urine. Because of the low intra-individual variation, the 24h urinary recovery of flavonoids has great potential as a compliance biomarker and as a biomarker of both controlled and uncontrolled changes in the intake level of fruits and vegetables achieved by an intervention.

Paper II. Krogholm KS, Bredsdorff L, Knuthsen P, Haraldsdóttir J, Rasmussen SE. Relative bioavailability of the flavonoids quercetin, hesperetin and naringenin given simultaneously through diet. European Journal of Clinical Nutrition, 64: 432-435, 2010.

This paper reports on the relative bioavailability and urinary recovery of quercetin, naringenin and hesperetin in a 48h diet-controlled intervention study with a single dose (6.3 ml/kg body weight) of juice mix containing equal amounts of the three flavonoids in a dose achievable through normal diet (30mg/l quercetin, 28mg/l naringenin and 32mg/l hesperetin). 10 normal weighted men (mean BMI=23.7 kg/m2) with a mean age of 24.6 y were included in the study. Plasma (baseline, 2, 4, 6, 24 and 48 h post intervention) and urine samples were collected 0-48h post intervention. The study demonstrates that when individuals consume equal amounts of quercetin and hesperetin, the $AUC_{0\text{-}48h}$, and thus the relative bioavailabilities of these flavonoids, are similar. However, the $AUC_{0\text{-}48h}$ of naringenin was significantly higher than

that for quercetin and hesperetin (see **Figure 6**). In addition, plasma C_{max} for quercetin and hesperetin were comparable, whereas the plasma C_{max} for naringenin was significantly higher. The relative 48h urinary recovery of naringenin (22.6% ± 11.5%) and hesperetin (14.2% ± 9.1%) were significantly higher when compared with quercetin (1.5% ± 1%). **Paper II** thus consolidates the apparent paradox that absorption and bioavailability of the three flavonoids seem quite similar, but that their urinary recovery is highly different. This indicates that lower urinary recovery of quercetin is not due to a lower bioavailability of quercetin, but rather reflects different clearance mechanisms.

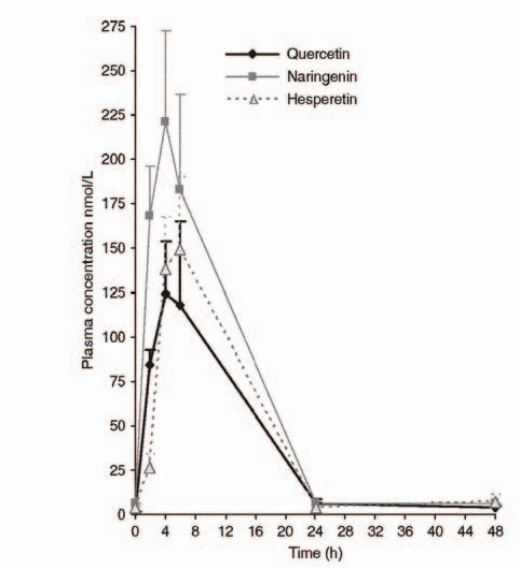

Figure 6 Plasma time versus concentration curves for quercetin (diamond), hesperetin (triangle) and naringenin (square) after ingestion of 'juice mix'. Values are mean ± s.e.m., n=10 (**Paper II**)

Paper III. Krogholm KS, Bredsdorff L, Alinia S, Christensen T, Rasmussen SE, Dragsted LO. Free fruit at workplace increases total fruit intake: a validation study using 24h dietary recall and urinary flavonoid excretion. European Journal of Clinical Nutrition, 64: 1222-1228, 2010.

Employees (n=34) with daily free and easy access to fresh fruit, and control employees (n=45) at workplaces with no free-fruit programme were enrolled in this validation study. The sum of 10 different flavonoids excreted in 24h urine (quercetin, isorhamnetin, tamarixetin, kaempferol, hesperetin, naringenin, eriodictyol, daidzein, genistein, and phloretin) was used to validate the effect of 5 months of free access to fruits at the workplace. Dietary intakes of fruits before and after intervention were assessed by using a 24h dietary recall. According to the 24h dietary recall, the intake of fruits in the intervention group increased significantly from a median fruit intake corresponding to 182 g/d at baseline to 310 g/d after 5 months.

This change in intake of fruits in the intervention group was reflected by a significant increase in the sum of the 10 flavonoids in 24h urine. Furthermore, the sum of the 10 flavonoids in 24h urine correlated significantly to the estimated intake of fruits in both the intervention and control groups (r_s = 0.31, $p<0.01$), and it was therefore concluded that the 24h dietary recall applied in the "free fruit at work intervention" was a valid estimate of the intake of fruits in the population investigated.

Paper IV. Krogholm KS, Bysted A, Brantsæter AL, Jakobsen J, Rasmussen SE, Kristoffersen L, Toft U. 24h and morning spot urine flavonoids and plasma carotenoids in the validation of self-reported intake of fruit and vegetable in the Inter99 cohort study, using the method of triads. British Journal of Nutrition, accepted if revised, 23rd December, 2010.

Using the method of triads, we evaluated the performance of the 198-items Inter99 FFQ covering the last month regarding the intake of fruits, vegetables and beverages rich in flavonoids. The validity of flavonoids excreted in morning spot urine, as a new and more feasible biomarker of fruit and vegetable intake, was in addition evaluated by the method of triads. A total of 191 individuals completed the validation study, including giving one 24h and one morning spot urine collection and blood sampling. The sum of 11 different flavonoids (quercetin, isorhamnetin, tamarixetin, kaempferol, hesperetin, naringenin, eriodictyol, daidzein, genistein, phloretin and apigenin), and enterolactone excreted in 24h and morning spot urine was used as a reference measure of fruit and vegetable intake. The plasma concentration of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene was used as an additional reference method in order to validate the Inter99 FFQ by the method of triads. The strongest bivariate correlations were found between carrots and α-carotene (r_s=0.67), and between β-cryptoxanthin and citrus fruits and juices (r_s=0.50). Regarding the flavonoids, the strongest correlations were found between estimated intake of citrus fruits and juices and citrus flavonoids (r_s=0.47), and for tea intake and kaempferol (r_s=0.43). Using the method of triads resulted in VCs for FFQ that were higher than the bivariate correlation coefficients between total fruit and vegetable intake estimated by FFQ and each of the biomarkers. VCs for the FFQ ranged from 0.43-0.68 using flavonoids in 24h urine and from 0.35-0.64 using flavonoids in morning spot urine, indicating a reasonably stable ranking of individuals in both 24h and morning spot urine, which is of major importance for epidemiologic studies that typically only obtain morning spot urine. The flavonoids in 24h urine are however, in accordance with previous findings (**Paper I**), a stronger biomarker of fruit and vegetable intake as compared to flavonoids in morning spot urine sample. Collection of two or more morning spot urine samples rather than just the one, could probably improve the performance of the flavonoid biomarkers to reflect long term intake of fruits and vegetables assessed by FFQ. Our findings of statistically significant correlations between the Inter99 FFQ and two independent biomarkers indicate that the Inter99 FFQ provides a useful estimate of the overall dietary intake of fruits, juices, tea and vegetables in the Inter99 cohort.

In addition, average % of subjects correctly classified and the % of subjects grossly misclassified by a biomarker and the FFQ or by a biomarker and the dietary recall were comparable and on average below 10%. This further supports that the Inter99 FFQ is a valid method for estimating the fruit and vegetable intake.

8 General discussion, conclusions and perspectives

This section contains a discussion of the applicability and limitations of the flavonoid biomarker on the basis of **Paper I-IV** included in the present Ph.D. thesis and relevant literature. Concluding remarks and perspectives on future work are included at the end of this section.

8.1 Urinary recovery of flavonoids

In **Paper I-II**, the relative urinary recovery (as % of intake) of hesperetin, naringenin and quercetin is investigated and the obtained values (see **Table 10**) are all in the range of previous observations, although there are great variations in the values reported in the literature and in **Paper I-II**. Earlier studies have reported urinary recovery levels in humans to be between 4.1-24.4 % for hesperetin, 1.1–30.2 % for naringenin, and 0.2–1.4 % for quercetin (Erlund *et al.* 2001, Hollman *et al.* 1997, Aziz *et al.* 1998, Ameer *et al.* 1996, Nielsen *et al.* 1997, Manach *et al.* 2003, de Vries *et al.* 1998, Young *et al.* 1999).

Table 10 Relative urinary recovery of quercetin, hesperetin and naringenin (as % of ingested dose) observed in **Paper I-II**

	Paper I		Paper II
Flavonoid source	300g of fruits and vegetables (40g banana, 40g apple, 40g orange, 60g steamed broccoli, 30g cauliflower, 30g sweet pepper, 30g fried sweet pepper, 30g fried onion)	600g of fruits and vegetables (80g banana, 80g apple, 80g orange, 120g steamed broccoli, 60g cauliflower,60g sweet pepper, 60g fried sweet pepper, 60g fried onion)	The juice mix (500 mL) was composed of 95 ml grape fruit juice, 125 ml apple concentrate, 175 ml orange juice and 105 ml water The subjects consumed 6.3 ml juice mix per kg bodyweight (range 380-570 ml)
Flavonoid dose	15.1 mg quercetin 11.6 mg hesperetin 1.7 mg naringenin	30.2 mg quercetin 23.2 mg hesperetin 3.4 mg naringenin	22.8 – 34.2 mg quercetin 23.3 - 36.5 mg hesperetin 21.3 - 32.0 mg naringenin
	Urinary recovery (as % of ingested dose)		
Quercetin	0.2 ± 0.1	0.2 ± 0.1	1.5 ± 1.0
Hesperetin	18.7 ± 24.9	12.9 ± 12.2	14.2 ± 9.1
Naringenin	4.1 ± 4.4	3.1 ± 3.4	22.6 ± 11.5

There may be several explanations of the differences in the urinary recovery of quercetin, hesperetin and naringenin observed between **Paper I** and **II** and previous observations.

First, it should be noted that the inter-individual variability is very high in accordance with other studies (Manach *et al.* 1998, Graefe *et al.* 2001, Moon *et al.* 2000),

indicating that some individuals could be better absorbers than others, possibly due to particular polymorphisms for intestinal enzymes or transporters.

Second, it is clear that, for quercetin, bioavailability differs among food sources, depending on the types of glycosides they contain. For example, onions, which contain glucosides, are better sources of bioavailable quercetin than apples and tea, which contain rutin and other glycosides (Erlund *et al.* 2000, Graefe *et al.* 2001, Olthof *et al.* 2000, Hollman *et al.* 1999). In **Paper I**, major sources of quercetin would be expected to originate from onions and apples according to data in the literature (Crozier *et al.* 2009). However, in **Paper I**, the amount of quercetin in the fruit and vegetable diet was only quantified as the aglycone after acid hydrolysis, and not for each individual fruit and vegetable included in the mixed fruit and vegetable diet. In **Paper II**, apple juice concentrate was the only source of quercetin, and qualitative LC-MS investigation of the 'juice mix' showed that approximately 60 % of the quercetin was present as monoglycosides, 25 % as aglycones and less than 15 % as rutinosides. Based on the supposed food sources of quercetin in **Paper I** (onion and apple) and **Paper II** (apple juice), it seems unlikely that the lower urinary recovery of quercetin in **Paper I,** as compared to **Paper II,** is due to the degree of glycosylation. However, a quantitative determination of the test-diet given in **Paper I** prior to acid hydrolyses would be necessary in order to future investigate this. The controlled, flavonoid free background diet applied in **Paper I** and **II** were similar and are thus not suspected to affect the urinary recovery of the investigated flavonoids.

Third, according to Spencer *et al.* (2008), one of the most important factors thought to determine the subsequent metabolic fate of polyphenols, including flavonoids, in human subjects, is the quantity ingested. Sulphation is generally a higher affinity, lower-capacity pathway relative to glucuronidation, so that as the ingested dose increases, a shift from sulphation toward glucuronidation occurs (Koster *et al.* 1981). The doses ingested in **Paper I** and **II**, are fairly similar for quercetin and hesperetin in **Paper I** and **II**, but for naringenin the dose ingested is much lower in **Paper I**, because the main dietary source of naringenin is grapefruit (see **Table 10**). It might be that this has affected the urinary recovery of naringenin.

Fourth, according to Crozier *et al.* (2009), there can be substantial batch-to-batch variation in the specificity of the glucuronidase/sulfatase preparations used for enzymatic hydrolyses of absorbed flavonoid conjugates. There are no reports of flavonoid bioavailability studies using glucuronidase/sulfatase preparations in which information on the identity, number, and quantity of the individual sulphate and glucuronide conjugates in the samples of interest have been obtained. As a consequence, there are no direct data on the efficiency with which the enzymes hydrolyze the individual metabolites and release the aglycone. This introduces a varying, unmeasured error factor. These limitations of analyses based on enzyme hydrolysis apply to bioavailability studies with all dietary flavonoids and, in this context, it is interesting to note that the use of enzyme hydrolysis has been found to lead to an underestimation of isoflavone metabolites in rat tissues (Gu *et al.* 2005). There is no doubt that the most correct way of quantifying flavonoid conjugates in LC-MS analysis is by

using calibration curves of the identical flavonoid-conjugate, preferably accessible as pure standards. However, one major obstacle in these approaches is the current limited access to commercially available flavonoid-conjugates (Roldán-Marin *et al.* 2010). The future for bioavailability studies must be focused on more than aglycones alone. The quality of these studies is, however, highly dependent on the commercial availability of flavonoid-conjugates.

Fifth, it might be that naringenin from grapefruit juice (major source of naringenin in **Paper II**) is more bioavailable that naringenin from orange fruit (source of naringenin in **Paper I**). In a biokinetic study by Erlund *et al.* (2001), the relative urinary recovery varied depending on the source and was 30.2 ± 25.5 % and 1.1 ± 0.8 % for naringenin from grapefruit juice and orange juice, respectively. Erlund and co-workers (2001) suggested that this variation is most likely caused by dose-dependent clearance since the participants ingested a dose of 199 mg naringenin from grapefruit juice and a dose of 23 mg naringenin from orange juice. A biokinetic study providing equal doses of naringenin from different dietary sources would reveal this.

Finally, it might be that there is a significant effect of food matrix on the bioavailability of naringenin. The effects of domestic cooking on the bioavailability of the flavanone naringenin and the phenolic acid chlorogenic acid in fresh or cooked cherry tomatoes was assessed in humans (Bugianesi *et al.* 2004). Both compounds were barely detected in plasma after consumption of fresh tomatoes. After eating cooked tomatoes, both naringenin and intact chlorogenic acid were significantly detected in plasma and it was concluded to be an effect of the food matrix that could be related to cell wall breakage.

8.2 Dose- and time response relationship

In **Paper I**, we investigated the magnitude of the dose-response relationship of the flavonoids included in the biomarker assay and observed a strong, linear relationship between oral doses of 0, 300 and 600 g/d of mixed fruits and vegetables and the 24h urinary recovery of 7 flavonoids (r_s=0.86, P<1x10^{-6}, n=12). The urinary recovery of the 7 flavonoids in morning spot urine also increased, but the association between urinary flavonoids and fruit and vegetable intake was weaker (r_s=0.59, P<0.0001, n=12), due to the biokinetics of the flavonoids and the design of the study. In **Paper I**, the volunteers were consuming citrus fruit, providing them with flavanones, only at breakfast, and the breakfast was consumed more than 12h before the subsequent morning urine collection were initiated. The elimination half lifes of the flavanones are rather short (≈2h, Manach *et al.* 2005) and the urinary recovery of the flavanones is thus limited mainly to the first 12h after intake (Erlund *et al.* 2001, Manach *et al.* 2003, **Paper I**, Kanaze *et al.* 2007, Mullen *et al.* 2008, Bredsdorff *et al.* 2010). As a consequence, these flavonoids were excreted predominantly prior to the morning urine collection, illustrating the great importance of the composition of the diet and the timing of the meals when investigating the urinary recovery of flavonoids from a normal diet. In **Paper I**, the flavonols (quercetin, kaempferol, isorhamnetin and tamarixetin) and phloretin responded in a more linear way to the increasing intakes of fruits and vegetables in morning

spot urine than the flavanones did, further indicating that it was especially due to the flavanones that the flavonoid biomarker in morning spot urine performed worse than in the 24h urine. If the flavanones are to be included in the biomarker assay, an evening spot urine sample would probably respond to the intake in a more linear matter, and this underlines that one should be cautious interpreting results obtained in diet-controlled intervention studies. In **Paper IV** the bivariate correlation coefficients between the urinary recovery in 24h urine and morning spot urine were 0.69 for hesperetin and 0.72 for naringenin. This indicates that the flavanones might work better as biomarkers of intake in morning spot urine in larger groups of free-living individuals than in the study design selected in **Paper I**, (see **Paper IV**, Table 2).

When assessing each individual participating in the dose-response study the dose-response relationship was also linear except in a few occasions. Thus suggesting a strong intra-individual linear relationship between intake of fruits and vegetables and urinary recovery of the 7 flavonoids in the biomarker assay developed by Nielsen *et al.* (2000) (**Figure 7A and 7B**). In a dose-response study by Brevik *et al.* (2004), a linear relationship between 24h urinary recovery of naringenin and hesperetin and three doses of mixed fruits and vegetables (0, 300 and 750 g/d) was likewise observed. Furthermore, in both studies (**Paper I**, Brevik *et al.*2004), the dose of 300 g of mixed fruits and vegetables resulted in a mean 24h urinary recovery of approximately 1000 µg flavonoids. Ideally, a dose-response study should include more than three relevant doses, however when bringing the findings in **Paper I** together with the findings by Brevik *et al.* (2004), the indication of a reproducible and linear dose-response relationship is convincing. The same highly individual, but linear, dose-response relationship was recently seen for one of the other flavonoid sub-classes, the anthocyanins (r_s=0.692, P<0.001, n=40) (Hollands *et al.* 2008) and also previously for the isoflavonoids (Karr *et al.* 1997).

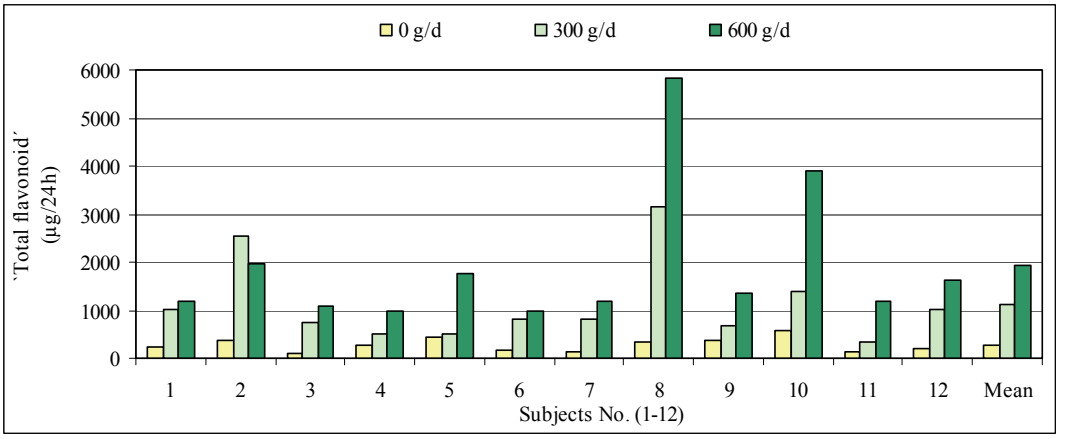

Figure 7A Individual and mean 24h urinary recovery of 7 flavonoids (the sum of tamarixetin, isorhamnetin, quercetin, kaempferol, phloretin, naringenin and hesperetin) after a daily intake of 0, 300 and 600 g of mixed fruits and vegetables (**Paper I**)

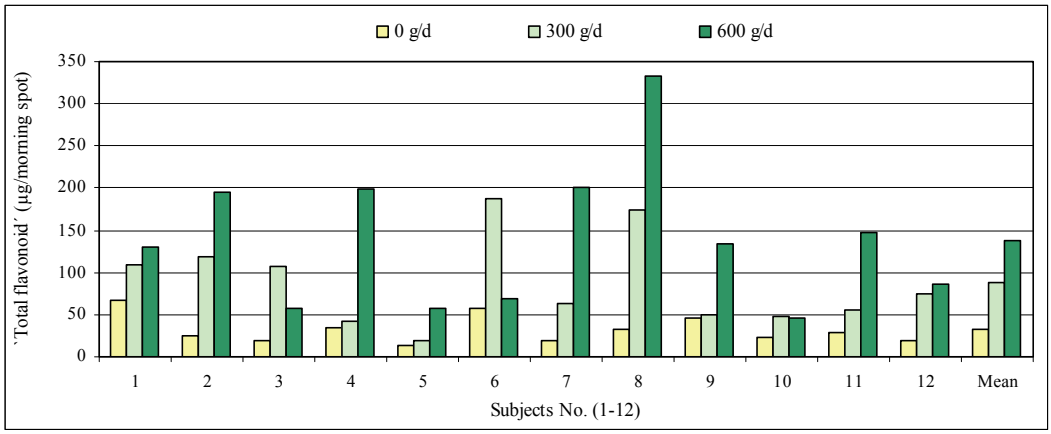

Figure 7B Individual and mean morning spot urinary recovery of 7 flavonoids (the sum of tamarixetin, isorhamnetin, quercetin, kaempferol, phloretin, naringenin and hesperetin) after a daily intake of 0, 300 and 600 g of mixed fruits and vegetables (**Paper I**)

8.3 Inter- and intra-individual metabolic variations

In **Paper I,** we observed a high inter-individual variation in response to the same dose of mixed fruits and vegetables, which makes it very difficult to separate individuals according to intake by use of the flavonoid biomarker in urine. As seen in **Figure 7A and 7B**, two of the 12 subjects are "high excreters", excreting 100-200 % more flavonoid in urine than average. In **Figure 8**, the high inter-individual variation in the urinary recovery of quercetin, naringenin and hesperetin observed in **Paper II** is illustrated, and here we found that two of the 10 subjects recovered more than twice the average urinary recovery of flavonoids. As presented in **Table 6**, in section 5.3.4, the relationship between dietary intake of fruits and vegetables and resulting concentrations of the flavonoid biomarkers in plasma and urine can be affected by many different factors. The study groups in **Paper I** and **II** were, however, relatively homogenous with respect to age, BMI, health, smoking, dieting and alcohol habits, and were all men. Furthermore, they consumed the same standardized diet and test diet. This indicates that the high inter-individual variation in flavonoid absorption and urinary recovery in **Paper I-II** are most likely due to differences in the levels of metabolizing enzymes or transporters and composition of the gut micro-flora rather than external, food processing related, food related, flavonoid related, and systemic related factors. The inter-individual variation in the urinary recovery of quercetin, naringenin and hesperetin is somewhat lower in **Paper II** that in **Paper I (**see **Table 9).** Maybe this could be a result of providing the juice in proportion to body weight in **Paper II** whereas individuals in **Paper I** all consumed the same dose of mixed fruit and vegetables. The high inter-individual variation in the urinary recovery of flavonoids and the presence of a few "high-excreters" agrees with observations in previous studies (Fuhr & Kummert 1995, Nielsen *et al.* 1999, Erlund *et al.* 2001, Nielsen *et al.* 2002, Brevik *et al.* 2004).

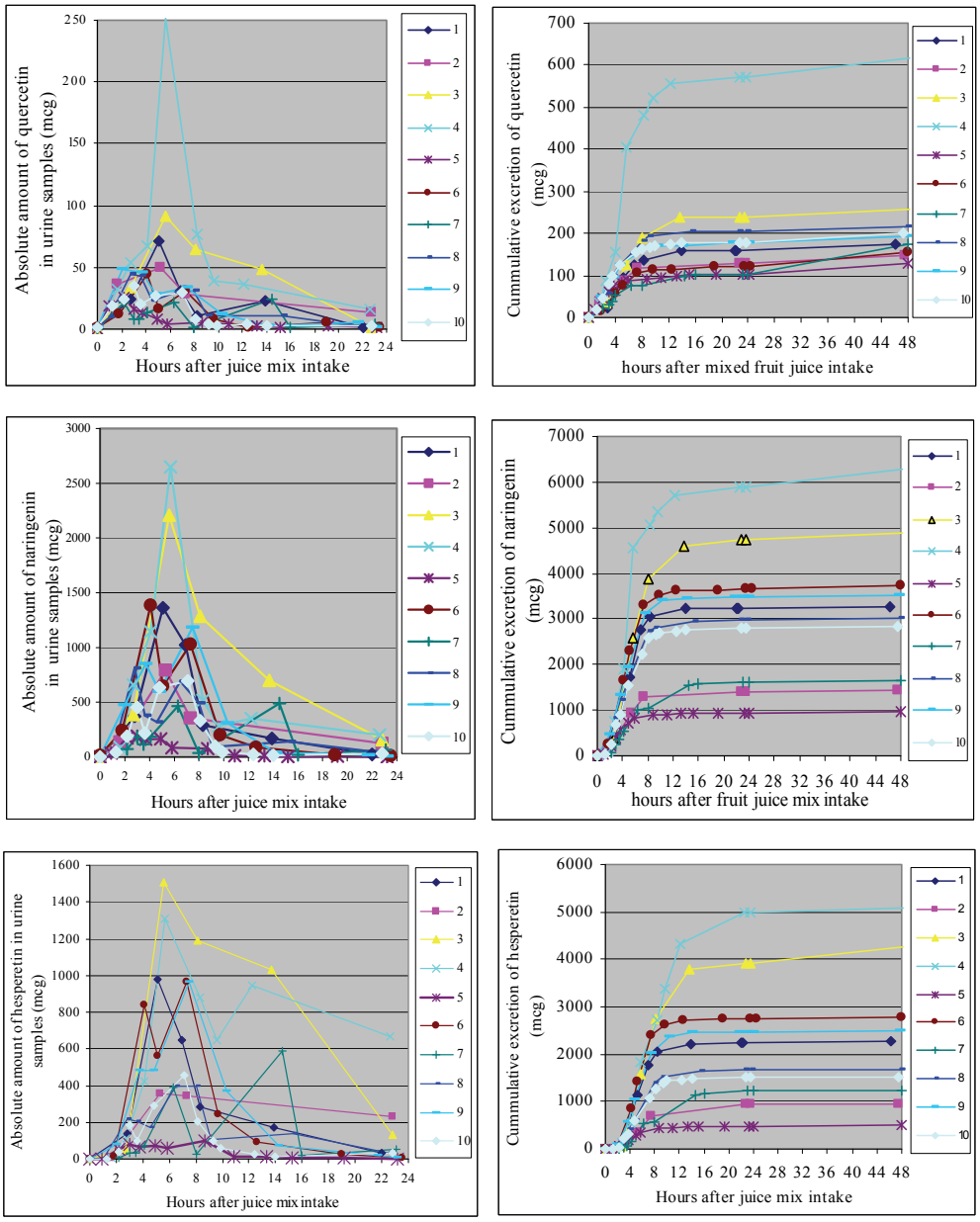

Figure 8 Inter-individual variations in the urinary recovery of quercetin, naringenin and hesperetin in 10 healthy men consuming 6.3 ml of juice mix per kg body weight. The juice mix contained 30 mg/L of quercetin, 28 mg/L of naringenin and 32 mg/L of hesperetin (**Paper II**)

On the contrary, the intra-individual variation was relatively low, as seen previously for quercetin and kaempferol (de Vries *et al.* 1998, Young *et al.* 1999), and the sum of the 7 flavonoids in 24h urine could therefore be useful in studies of changes in urine concentration where the variability due to individual variables is removed, which greatly enhances the opportunity to see small effects of the provided diet. For example, to validate an effect of an intervention programme like the one performed in the present Ph.D. thesis (**Paper III**), where a relatively small increase in fruit intake in the intervention group was followed by a significant ($P<0.01$) increase in the urinary recovery of the 10 flavonoids included in the biomarker assay from median 175 µg in 24h urine to median 635 µg in 24h urine. The urinary recovery of 10 flavonoids also increased significantly in the control group, from a median of 352 µg/24h to 445 µg/24h, but this was not accompanied by an increase in fruit intake according to the 24h dietary recall. This could indicate that the flavonoid biomarker is more sensitive to small changes in dietary intake than is the case for the 24h dietary recall in the employees participating in 'the free-fruit at workplace intervention'.

8.4 Kinetic characterisation

In nutritional research, relative bioavailability is commonly used to describe the bioavailability of a compound from one source compared to another, but in essence bioavailability describes the concentration of a given compound or its metabolites at the target organ (Holst & Williamson 2008). This includes identification, quantification and localization of all conceived metabolites of the ingested compound, which, especially in human subjects, is virtually impossible. In **Paper II,** we use the postprandial test, which is the most frequently used method (Erdman *et al.* 2007), to determine the relative bioavailability of quercetin, hesperetin and naringenin, all of which are included in the flavonoid biomarker assay. Flavonoids are eliminated from the body via urinary and biliary excretion, and **Paper II** demonstrates that probably a much larger fraction of quercetin as compared to hesperetin and naringenin is excreted via the biliary route. The same has previously been seen for the isoflavones daidzein and genistein, where the urinary excretion of daidzein was significantly higher as compared to genistein, because a greater fraction of genistein was eliminated in bile (Setchell *et al.* 2003). Since our flavonoid biomarker is based on the combined sum of several different flavonoids excreted via the urinary elimination route, differences in the route of elimination are not desirable and further rule out the possibility of using the flavonoid biomarker for the quantitative determination of fruit and vegetable consumption. For instance, apples are rich in quercetin while citrus fruits almost exclusively contain hesperetin, naringenin and eriodictyol. Since the citrus flavonoids are excreted in urine to a much higher extend as compared to quercetin, consumption of 100 g of citrus fruits will contribute to a much higher response in the flavonoid biomarker than consumption of 100 g of apples.

When habitual or experimental diets, as the one applied in **Paper I-II**, provides a mixture of flavonoids synergies or antagonism may possible occur between the flavonoids, and this should be taken into consideration when evaluating data from flavonoid

bioavailability studies. For instance, in an experiment by Reboul *et al.* 2007, the individual effect of carotenoids and polyphenols on lutein uptake was investigated, and it was shown that naringenin was the only polyphenol that impaired lutein uptake. The specific effect exerted by naringenin needs to be further elucidated by additional experiments, but it is nevertheless significant that naringenin was the most lipophilic of all the polyphenols tested (log P=2.52 versus 0.86, 0.82 and 0.38 for gallic acid, caffeic acid and (+)-catechin, respectively (Reboul *et al.* 2007). It is possible that naringenin affects lutein uptake through an interaction with scavenger receptor class B type I, which is known to transport lipophilic molecules with low substrate specificity. A second hypothesis might be related to an interaction between naringenin and membrane lipids which may affect the invagination of lipid raft domains containing lutein receptors (Reboul *et al.* 2007).

8.5 The effect of the inclusion of additional flavonoids and other compounds in the biomarker assay

The inclusion of 3 additional flavonoid aglycones (daidzein, genistein, eriodictyol) in the flavonoid biomarker assay applied in **Paper III** only slightly improved the correlation between self-reported intake of fruits and the flavonoid biomarker (from r_s=0.29* to r_s=0.31*), (data not shown in **Paper III**). Daidzein and genistein alone did not significantly correlate to any of the food groups (r_s between 0.03-0.14), and the slight increase in the correlation coefficient therefore must have been due to the inclusion of eriodictyol. Eriodictyol significantly correlated with self-reported intake of the following food groups; fruits, vegetables, apples, and fruit and vegetables (r_s=0.19-0.30).

In **Paper IV,** 4 additional flavonoid aglycones (eriodictyol, apigenin, daidzein and genistein) and one lignan, enterolactone, were included in the biomarker assay. However, only eriodictyol, enterolactone and apigenin, contributed to a stronger association with self-reported fruit and vegetable intake in **Paper IV**, in accordance with the results obtained in **Paper III.** This does, however, not rule out that the urinary recovery of isoflavones could be used as a biomarker of the intake of food rich in isoflavones in some populations. In a cross-sectional study (n=102) the association between urinary isoflavone recovery and intake of soy products was assessed by a dietary questionnaire for soy products consumed during the last year. In this study the soy intake significantly differed among ethnic groups (Caucasian, Native Hawaiian, Chinese, Japanese, and Filipino), and the urinary recovery of isoflavones significantly correlated to the annual dietary soy protein and isoflavone intake (r_s=0.32 and 0.31, respectively) (Maskarinec *et al.* 1998). The dietary assessment methods applied in **Paper III** and **IV** were not designed to estimate intake of food rich in isoflavonoids, and this may have been the reason why the inclusion of these two isoflavones in the biomarker assay did not contribute to a stronger correlation to the overall intake of fruits and vegetables.

Several times enterolactone has been found to correlate with the consumption of vegetables and plant food (Kilkkinen *et al.* 2001, Horner *et al.* 2002, Johansen *et al.* 2004, Milder *et al*. 2005, Touillaud *et al.* 2007). For instance, Mennen and co-workers found that

the 24h urinary recovery of a combination of enterolactone and kaempferol significantly correlated with intake of vegetables estimated by a 2d dietary food record (r_s=0.34), (Mennen *et al.* 2006). Brantsæter *et al.* (2007) likewise found a significant correlation between the 24h urinary recovery of enterolactone and intake of vegetables excl. cruciferous, roots and potatoes (estimated by a food diary) corresponding to 0.38. The inclusion of enterolactone in the biomarker assay applied in **Paper IV** did, in accordance with this, improve the strength of the bivariate correlation coefficient between the biomarker and estimated the intake considerably (from r_s=0.16* to r_s=0.22* when using data from the FFQ, and from r_s=0.28 to r_s=0.39* when using data from the 28d dietary history, data not included in **Paper IV**). Furthermore, the 24h urinary recovery of enterolactone significantly correlated with the recovery of enterolactone in morning spot urine (r_s=0.89), indicating that the urinary recovery of enterolactone in morning spot urine may also be a good biomarker of vegetable intake. The elimination half-life of enterolactone is relatively slow (6-8 h, Manach *et al.* 2005) as compared to the flavanones, and this may be one of the reasons why a morning spot urine sample may be sufficient to reflect the previous days intake of vegetables. Furthermore their urinary recovery are rather high as compared to the flavonols, and this increases the sensitivity of the isoflavones as biomarkers of intake. In conclusion, the inclusion of enterolactone in the biomarker assay applied in **Paper IV** did significantly increase the strength of the correlation to the self-reported intake of fruits and vegetables and enterolactone should thus be included in the biomarker assay in future investigations.

8.6 Validation of the urinary recovery of flavonoids in a morning spot urine sample as biomarker of fruit and vegetable intake in the Inter99 cohort

There is a great need of a larger variety of dietary biomarkers to be developed to reflect wider aspects of diet. In epidemiological studies, where potential health effects of fruits and vegetables are investigated, 24h urinary samples are rarely collected. A biomarker for dietary intake of fruits and vegetables measured in spot urine samples would thus be very valuable.

The time period for which flavonoids and carotenoids reflect dietary exposure differentiates considerably. The carotenoids have an estimated half life of 1-3 months (Burri *et al.* 2001), while flavonoids have a short half-life, often not more than a few hours (Manach *et al.* 2005). As a result the plasma carotenoids reflect the intake of fruits and vegetables over several weeks to months, while 24h urinary recovery of flavonoids would only reflect the intake of fruits and vegetables from the previous day or meal. A pool of multiple urine collections for each person would be needed if the flavonoid biomarker should reflect the habitual dietary intake of fruit, vegetables and/or flavonoid-rich beverages of individuals. Taking the biokinetic characteristics of the flavonoids and enterolactone into consideration, an evening spot urine sample could potentially contain a larger fraction of the flavonoids

consumed during the day than a morning spot urine sample. This would certainly be interesting to investigate further in both controlled and observational studies. In **Paper III-IV** all urine voids were collected in large containers and this further complicated the 24h urine collection for both the participants and the laboratory staff. Application of some kind of urine-collecting aliquot cup, as the one used in Nielsen *et al.* (2002), where 1/21 of the total urine volume was sampled, and the rest was discarded, would ease both the urine collection and the post handling.

According to the results obtained in **Paper I** and **IV**, there is no doubt that a 24h urine sample represents a stronger biomarker of intake than a morning spot urine sample. However, in **Paper IV,** the VCs for the FFQ ranged from 0.46 to 0.64 using 24h urine and from 0.35 to 0.64 using morning spot urine, and this indicates a reasonably stable ranking of individuals in both 24h and morning spot urine. The usefulness of urinary flavonoids and other polyphenolic compounds in morning spot urine as biomarkers of fruit and vegetable intake is supported by the findings of Mennen and co-workers. They measured 13 polyphenols and metabolites (chlorogenic acid, caffeic acid, m-coumaric acid, gallic acid, 4-O-methylgallic acid, quercetin, isorhamnetin, kaempferol, hesperetin, naringenin, phloretin, enterolactone and enterodiol) in morning spot urine samples and found that the apple consumption was significantly correlated to phloretin, grapefruit consumption to naringenin, orange to hesperetin and citrus fruit consumption to both naringenin and hesperetin, with r_s coefficients ranging from 0.31-0.57. The combination of fruits and/or fruit juices was furthermore positively correlated to gallic acid and 4-O-methylgallic acid, isorhamnetin, kaempferol, hesperetin, naringenin and phloretin (r_s= 0.24–0.44), and they concluded that several polyphenols measured in a spot urine sample may be used as biomarkers of polyphenol-rich food intake (Mennen *et al.* 2006).

8.7 Validation of fruit and vegetable intake estimated by 24h dietary recall and FFQ by use of biomarkers

Recently, a working group has been reviewing the validity of dietary assessment methods to be used in epidemiological studies and they developed a scoring system to assess the quality of dietary intake validation studies. The scores ranged from 0 to 7 and highest score was given when;

- the sample population was heterogenic and consisted of more than 100 individuals (50 individuals when the reference method was a biomarker),
- the seasonality and use of supplements were considered,
- the statistical analyses should included comparison between means, calculation of correlation and calculation of % misclassified and if the intake data was be gathered by personal interviews.

Calculation of correlation values are the most frequently applied statistical procedure in epidemiological studies and should, if possible, be adjusted for different variables, particular energy intake, and age and gender, in order to reduce the dependency on inter-individual variation (Serra-Majem *et al.* 2009). Therefore, deattenuation of the correlation and adjustments for energy intake are scored higher than raw correlation values in the scoring system by Serra-Majem *et al.* (2009). If dietary biomarkers are available, as is the case for fruits, juice, tea and vegetables, the problem of correlation errors between test and reference method however diminishes (Kaaks *et al*. 2002). The integration of biomarkers in validation studies is thus highly desirable whenever possible. This can provide a measure of relative validity of the dietary assessment method and, if using the method of triads, furthermore provide an estimate of the correlation of each method with true, but unknown, intake.

The sample size in the validation of the 24h dietary recall was 79 individuals in **Paper III**, and thus reasonable for a validation study. Our initial aim was to include a minimum of 100 employees. However recruitment was much slower than anticipated and the drop-out was high mainly due to unexpected ending of employment and illness. In the 'free fruit at workplace intervention' the study sample was heterogeneous and randomly selected, as all workplaces in the Copenhagen area (n>1000) and all employees at these workplaces were invited to participate in the study. Further details are described in Alinia *et al.* (2010) and in **Paper III**. Correlation values were calculated between the 24h urinary recovery of 10 flavonoids and fruit intake was estimated by 24h dietary recall, performed by a trained interviewer, at baseline and after five months of intervention. Calculation of % misclassified was not included in **Paper III** since the aim with the "free fruit at workplace intervention" was to validate the effect of the intervention. Furthermore, it is possible that the interpretation of the effect of the "free-fruit at workplace intervention" was confounded by seasonality and use of supplements, since the length of the intervention was approximately five months, beginning between June and September. As a result of these limitations, a total score of about 3.5 out of 7 was obtained, corresponding to the lower limit of the category; Good, $3.5 \leq$ score ≤ 5. 41.9% of 124 validation studies evaluated by Serra-Majem *et al.* (2009) were classified into this category.

In the Inter99 FFQ validation study the final sample size was 191 individuals (**Paper IV**) and this must be considered rather large, as compared to the suggested minimum of 50 individuals when the reference method is a biomarker (Serra-Majem *et al.* 2009). All participants in the validation study were selected from an age and gender-stratified random sample of 13 016 individuals who were invited for a health screen at the hospital centre. A total of 53% turned up for the investigation and individuals at high risk of developing ischemic heart disease were re-invited after one and three years, and completed the Inter99 FFQ at all three occasions. Individuals attending the hospital centre between 10 June 2002 and 16 February 2004 were personally and sequentially invited to participate in the validation study. The participants in the Inter99 FFQ validation study also gave information concerning their use of dietary supplements and the time for completion of the Inter99 FFQ was recorded,

as stated to be important in a validation study (Serra-Majem *et al.* 2009). However, the quality and level of detail of this information varied considerably between individuals, and the data was therefore not included in the data analysis. As a result the total score, according to the scoring system, was 5.0 out of 7.0, corresponding to the lower limit of the category; Very good/excellent, score ≥ 5.0, in which only 5.6% of the previously 124 conducted validation studies have been categorised (Serra-Majem *et al.* 2009).

The inclusion of supplements in the scoring system developed by Serra-Majem *et al.* (2009) is clearly very important in validation studies of micronutrients, since intake of a multivitamin-mineral supplement is quite widespread. For instance, about 45% of the young and adult Danish population and 64% of the Danish children reported regular or occasional use of dietary supplements (Andersen *et al.* 2002, Gille *et al.* 2010). However, the most common supplements used are multivitamin-mineral supplements followed by single vitamins or minerals, and supplements containing proteins, amino acids, carnitine, creatine, caffein, and bicarbonate. Consumption of other types of supplements, possibly containing flavonoids, is still rather uncommon (Andersen *et al.* 2002, Radimer *et al.* 2004, Alves & Lima 2009, Gille *et al.* 2010). For instance, the use of flavonoid- and carotenoid-containing dietary supplements have been found to have only little impact on the correlations found between these biomarkers and the fruit and vegetable intake (Brantsæter *et al.* 2007). This was due to the majority of the women in the Norwegian Mother and Child Cohort not taking such supplements. Therefore it could be argued, if the total score given to validation studies of self-reported dietary intake of fruits and vegetables by objective biomarkers such as flavonoids and carotenoids should be subtracted a score of 1.5 or if it should be less?

The method of triads has rarely been used for validating FFQ estimated intake of fruits and vegetables (Andersen *et al.* 2005, Brantsæter *et al.* 2007, **Paper IV**, Slater *et al.* 2010), (see **Table 8**), and the VCs for the biomarkers applied were either lower (Andersen *et al.* 2005, Slater *et al.* 2010), or in the same range (Brantsæter *et al.* 2007, **Paper IV**), as those observed for the FFQ, 24h dietary recall and weighed food record when considering the whole group of fruits and vegetables. Most other published studies that apply the method of triads investigate the validity of different estimated micro- or macronutrient intakes. In Yokota *et al.* (2010), a fine overview of all previous studies using the method of triads for validation is presented. Like others, who have used the method of triads, the 95% confidence intervals obtained in **Paper IV** were wide. However, the range between the lower confidence limit and the calculated VCs, e.g. 0.40–0.65 for citrus fruits and juice, indicates satisfying validity of the Inter99 FFQ. Furthermore, the upper limit of the intervals was not greater than 1 in **Paper IV**, reflecting the rather large sample size and the application of two independent reference methods for validation. At present, the Danish Inter99 cohort is among the largest epidemiological databases in Denmark and the total population is followed through central registers, lifestyle questionnaires (including the Inter99 FFQ) and measurements of cholesterol, blood pressure and body mass index. The findings, that the Inter99 FFQ provide a valid estimate of the fruit, juice, vegetable and tea intake, is the foundation for further

investigation of relations between intake of fruits and vegetables, lifestyle and risk of diseases in the Inter99 cohort.

8.8 Conclusions

The research presented in this Ph.D. thesis has shown that the sum of 7 flavonoids in 24h urine respond in a linear and sensitive manner with moderate increases in the intake of fruits and vegetables, and thus consolidates previous observations by Dr. Nielsen, and suggests that the flavonoids are a valid short-term biomarker of fruit and vegetable intake. The correlation between the urinary recovery of the 7 flavonoids in morning spot urine and controlled intake of fruits and vegetables was weaker than for 24h urine in the dose-response study, mainly due to the short elimination half-life of the flavanones included in the biomarker assay.

The biokinetic intervention study consolidated the apparent paradox that absorption and bioavailability of the three dietary important flavonoids, quercetin, hesperetin and naringenin, seem quite similar when they are given in equal amounts achievable through normal diet, but that their urinary recovery are highly different. In addition, a high degree of inter-individual variation in response to test-meals was observed in both the biokinetic study and in the dose-response study, whereas the intra-individual variation was low. Differences in urinary recovery between flavonoids included in the biomarker assay, due to flavonoid related and host related factors, are undesirable, and make it impossible to use the flavonoid biomarker for the quantitative determination of fruit and vegetable intake.

The present work has demonstrated the value of the flavonoid biomarker in the validation of self-reported dietary intake of fruits and vegetables. In the `free fruit at workplace´ intervention, the flavonoid biomarker in 24h urine significantly correlated with the fruit intake estimated with a 24h dietary recall questionnaire. This shows that the 24h dietary recall was a valid and useful tool for estimating of the intervention effect.

In a sub-sample of the Inter99 cohort, the FFQ estimated intake of fruits and vegetables was confirmed by two independent biomarkers (urinary flavonoids and plasma carotenoids). In addition, our results demonstrated the potential applicability of the flavonoids in morning spot urine instead of 24h urine as a biomarker of fruits and vegetables intake in the Inter99 cohort. This opens up for new and promising perspectives in the flavonoid biomarker research, since collection of one or several spot urine samples would be more feasible than collection of 24h urine in larger epidemiological studies.

The inclusion of apigenin, eriodictyol and especially enterolactone, in the biomarker assay significantly increased the strength of the correlations between the self-reported intake of fruit and/or vegetables and the flavonoid biomarker. These compounds should therefore be included in the biomarker assay in future investigations.

So far, it seems that the flavonoid biomarker is not a stronger method for measuring fruit and vegetable intake than plasma carotenoid or self-reported dietary assessment methods. However, by now it is recommended to use several of the different

assessment methods, since the different methods give complementary information and provide better and different estimates of the dietary intake than each of the individual methods alone.

8.9 Perspectives and future research

The flavonoid biomarker reflect short term intake of fruits and vegetables, which can be a limiting factor because quite often validation studies are intended to relate food consumption over time with the development of chronic diseases. In such studies, it is important to include repeated samples of the biomarker to account for the intra-individual variation, which can create the false appearance of correlated errors between diet assessment methods. Furthermore, the timing of collection of biomarkers is important; if they are collected close in time to a short-term method, such as a 1-week record, this can exaggerate their correlation (Willett *et al*. 2009).

The work presented in this Ph.D. thesis indicates that the intake of fruits, vegetables, juices and tea can be reflected by flavonoids in both 24h and morning spot urine. This opens up for new and promising perspectives in the flavonoid biomarker research, since collection of several morning spot urine samples would be more feasible than collection of 24h urine. A pool of several morning spot urine samples, for instance four collections evenly distributed over a whole year, furthermore could turn the flavonoid biomarker into a long-term dietary biomarker also taking seasonality into consideration. This would increase the applicability and value of the flavonoid biomarker in future diet-disease investigations.

New perspectives for the carotenoid biomarker have also recently been published. Mayne and co-workers (2010) have developed a new, valid and non-invasive method for assessing dermal carotenoids as a biomarker for studies of nutrition and health, and this now makes the use of carotenoids as biomarkers of fruit and vegetable intake more feasible and allows for the collection of several measurements over a long period of time.

Assessing bioavailability by measuring flavonoid aglycones after enzymatic hydrolysis with glucuronidases and sulphatases has been criticized for risk of quantitative underestimation and lack of information on metabolites (Gu *et al.* 2005, Crozier *et al*. 2009). A few studies have investigated bioavailability of flavonoids and other dietary polyphenols by identifying and quantifying all metabolites instead of only converted aglycones of the ingested compound (González-Barrio *et al*. 2010, Bredsdorff *et al.* 2010, Stalmach *et al* 2010, Stalmach *et al*. 2010b). However, quantification and characterization of all metabolic forms are difficult due to the current lack of commercially available flavonoid-conjugate standards and the most common approach is still to quantify the aglycones following the hydrolysis of flavonoid conjugates with the enzymes β-glucoronosidase and aryl-sulphatase, as described in **Paper I-IV**. At the beginning of the current Ph.D. thesis, the LC-MS method developed by Nielsen *et al.* (2000) was the only thoroughly validated method available for determination of flavonoids in urine. Since then, the research group of Dr. Scalbert in France has proposed an alternative method for identification of flavonoid aglycones and polyphenols in human urine

samples by LC-MS (Ito *et al.* 2005). However, it took until 2008 before this method was validated in free-living subjects (Mennen *et al.* 2008). It is clear, that the future for bioavailability studies must be to focus on more than aglycones alone.

In order to increase the power of the flavonoid biomarker more effort should be put into the elucidation of which factors are affecting the absorption and metabolism of flavonoids. To the extend that factors causing these variations can be measured, adjustments can be made for them and thus improve the observed correlations. For instance, it would be useful to determine why 10-20% of a population excretes more than double the average amount of flavonoids in urine. Interactions between flavonoids and colonic microflora are probably of great importance since the colonic microflora is unique to each individual and has great impact on which metabolites that are produced from the ingested compounds (Licht *et al.* 2010).

The sum of several different key flavonoids and enterolactone excreted in urine has repeatedly shown to be a more robust biomarker of the total consumption of fruits, juices and vegetables as compared to individual flavonoid measurements (Brantsæter *et al.* 2007, **Paper III** and **IV**). However, common for both the flavonoids and carotenoids is unfortunately, that a few foods are responsible for the major part of the carotenoid and flavonoid intake. Tea and red wine are, as mentioned in section 5.3.3, some of the major dietary flavonoid sources, but they do not belong to the fruit and vegetable food group. Therefore it is important not to include flavonoids (e.g. anthocyanidins and catechins) in the flavonoid biomarker assay that mainly comes from food groups other than the group of fruits and vegetables. Instead, more effort should be put into the inclusion of flavonoids with a high urinary recovery value that are present in quantitative important types of fruits (e.g. banana, apples, grapes, pears, citrus fruits) and vegetables (carrots, onions, peppers, cruciferous vegetables, tomatoes, cucumbers and lettuce).

The research strategy applied in the present development of the flavonoid biomarker can be referred to as a metabolic targeting strategy. In biomarker discovery research the metabolic targeting strategy is not always obvious, but instead a holistic metabolic profiling strategy could be the choice of strategy. Metabolomics is more suited to exploit unexpected metabolic variations of complex organisms like humans. Metabolomics is a new technique that aims to profile all small molecules that are present in a biological sample, e.g. human urine, and the analytical techniques applied in metabolomics are usually based on Nuclear Magnetic Resonance (NMR) or MS (Kussmann *et al.* 2006, Kussmann *et al.* 2008). Although still in its infancy, a number of studies applying this technology have been performed. For instance, NMR spectroscopy has been used to investigate the role of dietary phytochemicals on shaping human urinary metabolomic profiles (Walsh *et al.* 2007). Multivariate statistical analysis indicated a clear distinction between the individuals consuming a diet low in phytochemicals and the individuals consuming either a normal diet or a diet containing high amounts of phytochemicals (Walsh *et al.* 2007). Another example is the study by Xu *et al.* (2010), investigating the variability in the metabolic urinary profiles of healthy populations from four groups (lactovegetarian males, lactovegetarian females,

omnivorous males and omnivorous females) by NMR spectroscopy. The study revealed that the most influential metabolites responsible for the differences between the diet groups were N-acetyl glycoprotein, succinate, citrate, trimethylamine-N-oxide, taurine, glycine, hippurate, phenylalanine and methylhistidine. Some of these metabolites might be new potential fruit and vegetable biomarkers and could be further explored by including some of these metabolites in a targeted LC-MS analysis on urine samples from subjects supplemented with different fruit and vegetable diets and also in free-living individuals.

So far, it seems that the use of fruit and vegetable biomarkers are not a stronger tool for measuring fruit and vegetables intake than self-reported dietary assessment methods. The two validation studies applying both the carotenoids and flavonoids as reference biomarkers of fruit and vegetable intake indicates that the flavonoid biomarker produces the highest VCs with respect to the fruit group while the carotenoids produce the highest VCs with respect to the group of vegetables (see Table 8) (**Paper IV** and Brantsæter *et al.* 2007). However, this may vary considerably with the different structures of the applied FFQ, differences in sample size or differences in the populations being studied. Furthermore, two biomarkers may give complementary information and provide better and different estimates of dietary intake than anyone of the individual methods alone. The use of several different assessment methods, whenever possible, should be the new future strategy. Future dietary assessment might benefit from the growing prominence of internet and telecommunication technologies to further enhance the available data on food consumption for each study participant. However, the self-reported nature of the data itself will always lead to bias, and therefore the need for biomarkers will continue in the future.

9 List of abbreviations

AUC: Area under the curve

Biomarker: A biological parameter measurable in body fluid or tissue that relates to substances in the diet

CBG: Cytosolic β-glucosidase

C_{max}: Maximum concentration in plasma

COMT: Catechol-*O*-methyltransferase

FFQ: Food frequency questionnaire

24h: 24 hours

HPLC: High Performance Liquid Chromatography

Inter99: Population based cohort in Denmark

LPH: Lactase phloridizin hydrolase

Morning spot urine sample: All urine voids from midnight including the first morning void

MS: Mass spectrometry

NMR: Nuclear magnetic resonance

Reference method: Method against which the test method is being compared and validated

RR: Relative Risk

R_s: Spearman Correlation Coefficient

SULT: Sulfotransferase

Test method: Dietary assessment method being validated

$T_{1/2}$: Elimination half life

T_{max}: Time to reach maximum concentration in plasma

UGT: Uridine-5'-diphosphate glucuronosyltransferase

Validity: The ability of an instrument to measure what it is intended to measure

VC: Validity Coefficient

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Paper I

Urinary Total Flavonoid Excretion but Not 4-Pyridoxic Acid or Potassium Can Be Used as a Biomarker for the Intake of Fruits and Vegetables[1]

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ABSTRACT To gain better insight into the potential health effects of fruits and vegetables, reliable biomarkers of intake are needed. The main purpose of this study was to investigate the ability of flavonoid excretion in both 24-h and morning urine samples to reflect a low intake and moderate changes in fruit and vegetable consumption. Furthermore, the urinary excretions of 4-pyridoxic acid (4-PA) and potassium were investigated as other potential biomarkers of fruit and vegetable intake. The study was designed as a 5-d randomized, controlled crossover study. On d 1–3, the men ($n = 12$) consumed a self-restricted flavonoid-free diet. On d 4, they were provided a strictly controlled diet containing no fruits or vegetables (basic diet). On d 5, they consumed the basic diet supplemented with 300 or 600 g of fruits and vegetables. The total excretion of flavonoids in 24-h urine samples increased linearly with increasing fruit and vegetable intakes ($r_s = 0.86$, $P < 1 \times 10^{-6}$). The total excretion of flavonoids in morning urine also increased, but the association was weaker ($r_s = 0.59$, $P < 0.0001$). Urinary 4-PA in 24-h and morning urine samples increased significantly only with the 600-g increase in fruit and vegetable intake, whereas the excretion of potassium in urine did not reflect the changes in fruit and vegetable intake. We conclude that the total excretion of flavonoids in 24-h urine may be used as a new biomarker for fruit and vegetable intake. J. Nutr. 134: 445–451, 2004.

KEY WORDS: • *biomarkers* • *flavonoids* • *fruits and vegetables* • *diet-controlled* • *human intervention*

A high intake of fruits and vegetables has been associated with a reduced risk of coronary heart disease (1–4) and certain types of cancer (5–7). In epidemiologic studies on the association between diet and disease, dietary intake data are often based on self-reported data, which carries a risk for subjective bias (8–11). Furthermore, consumption of healthy foods such as fruits and vegetables may be overreported (12). Dietary biomarkers represent an objective alternative to the traditional food registration methods. Flavonoids are widely distributed in fruits and vegetables regularly consumed by humans and are thus a potential biomarker for the dietary intake of fruits and vegetables. We recently reported that 7 dietary flavonoids could be determined simultaneously in urine by a sensitive and analytically validated method (13), and that the 24-h urinary excretion of these flavonoids may have potential as a new biomarker for fruit and vegetable intakes (14,15). These studies demonstrated that high intakes of fruits and vegetables are easily detected by the flavonoid biomarker, both in subjects consuming controlled diets and in those consuming their habitual diet (14,15). An essential property of a biomarker in studies of diet-disease associations is that the biomarker changes in a linear manner with varying exposures. It is therefore important to further validate the flavonoid biomarker and to evaluate its responsiveness in intervention studies with more moderate changes in fruit and vegetable intake. In the present study, we therefore investigated the 24-h urinary excretion of 7 dietary flavonoids, including the flavonols quercetin, kaempferol, isorhamnetin, tamarixetin, the flavanones hesperetin and naringenin, and the dihydrochalcone, phloretin (**Fig. 1**), after 0, 300, and 600 g/d of fruit and vegetable intake. Because collection of complete 24-h urine samples is both difficult and time-consuming, we investigated further whether a morning urine sample could substitute for the collection of 24-h urine samples.

Because the analytical methods by which the flavonoids are determined are quite complicated and time consuming, a simpler fruit and vegetable biomarker would be preferable. On the basis of earlier studies on potassium and vitamin B-6, we decided to investigate the use of the urinary excretion of potassium and the major vitamin B-6 metabolite, 4-pyridoxic acid (4-PA),[3] as biomarkers for the intake of fruits and vegetables (16–22). Even though vitamin B-6 and potassium are less specific for fruits and vegetables than are the flavonoids, these nutrients are widely distributed in many types of frequently consumed fruits and vegetables (23,24). The intake of vitamin B-6 and potassium from fruits and vegetables is sub-

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[3] Abbreviations used: DMSO, dimethyl sulfoxide; FVDiet, fruit and vegetable diet; LC-MS, liquid chromatography-mass spectrometry; 4-PA, 4-pyridoxic acid; r_s, Spearman's correlation coefficient; SPE, solid phase extraction.

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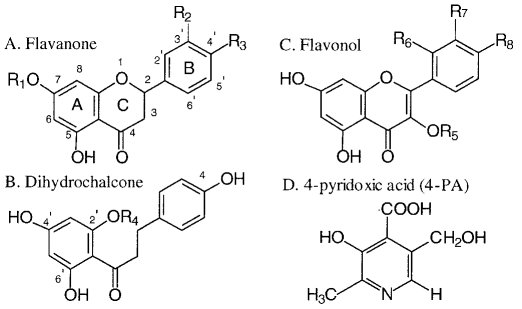

FIGURE 1 Chemical structures of all 7 dietary flavonoids and 4-PA.

stantial and was shown to increase with increased fruit and vegetable consumption (16–18,21,22). Furthermore, they are easily detected in urine by simple analytically validated methods (25,26).

MATERIALS AND METHODS

Subjects. Male volunteers were recruited among students and employees from the universities in Copenhagen. A total of 13 healthy men were enrolled in the study. One volunteer dropped out at the beginning of the study for reasons not related to the study. Complete urinary data were obtained from 12 men. Their mean age was 24.6 y (range 20–28 y) and their mean BMI was 23.3 kg/m^2 (range 21.2–26.8 kg/m^2). Exclusion criteria included regular intake of medicine, smoking, intake of dietary supplementation < 14 d before study start, > 10 h/wk of physical activity, > 160 mL of pure alcohol/wk, blood donation or participation in other experiments during the study period and major weight changes during the last 3 mo.

Study design and diet. Each volunteer participated in two 5-d periods; d 1–3 of each period were "run-in-days" in which subjects followed a self-restricted diet without any fruits, vegetables, potatoes, red wine, tea, or coffee. On d 4 of each period, the men were provided a strictly controlled diet containing no fruits or vegetables (basic diet). On d 5 of each period, they consumed the basic diet supplemented with either 300 g of fruit and vegetable mixture (low FVDiet) or 600 g fruit and vegetable mixture (High FVDiet) in random order. On d 4 and 5, breakfast and lunch were provided at the Department of Human Nutrition between 0715 and 0735 h and 1215 and 1245 h, respectively. The dinner meal provided was consumed at home between 1800 and 2000 h. The basic diet contained no fruits and vegetables and was based on bread, meat, pasta, rapeseed oil, butter, and low-fat milk. The fruit and vegetable diet included items commonly eaten in Denmark (17) (**Table 1**). The menus were prepared in advance and frozen at −18°C until use. With a few exceptions (white bread and low-fat milk), all of the ingredients were from the same batch. All of the men received the same energy level. The differences in energy intake among the 3 types of diets were equalized with a drink rich in carbohydrates. The energy intake and the concentration of macronutrients in the diet were calculated with Dankost2000 software based on the Danish Veterinary and Food administration food composition database; the concentrations of flavonoids, potassium, and vitamin B-6 in the diet were based on chemical analysis (see **Table 2**). The study was approved by the Ethics Committee of Copenhagen and Frederiksberg municipality (J. No. KF01–161/01). Informed written consent was obtained from all participants before the study.

Collection of urine samples. All urine samples were collected in 2500- or 500-mL plastic bottles containing 10 or 2 mL of a 100 g/L freshly prepared ascorbic acid solution and 50 or 10 mL of 1 mol/L HCl, respectively, as stabilizing agents. The 24-h and morning urine samples were collected on d 4 and 5 of each period. The excretions of 7 different dietary flavonoids (see Fig. 1), 4-PA, and potassium were determined in all urine samples. On d 4, the first morning urine void was discarded and all subsequent voids until 2400 h on the same day were collected. The morning urine, defined as all urine voids from 2400 h including the first morning void, was collected separately to determine whether it was useful to collect this sample compared with the 24-h urine sample. All subsequent voids on d 5 were collected in the same manner as on d 4. The morning urine was again collected separately. A mixture of the morning urine sample and the urine collected during the daytime until 2400 h served as the 24-h urine sample. The plastic bottles were kept in black bags at room temperature during the collection period. The volunteers received careful instruction for the collection of the urine, and the importance of complete samples was stressed. Completeness of the urine collection

TABLE 1

Content of fruits and vegetables in low and high FVDiets

Fruits and vegetables	Time of consumption[1]	Low FVDiet	High FVDiet
		g/d	
Banana	B	40	80
Apple	B	40	80
Orange	B	40	80
Lemon juice[2]	B	10	10
Broccoli (steamed)	L/D	30/30	60/60
Cauliflower	L	30	60
Sweet red pepper	L	30	60
Sweet red pepper (fried)	D	30	60
Onion (fried)	D	30	60
Total fruits		120	240
Total vegetables		180	360
Total fruits and vegetables		300	600

[1] B, breakfast; L, lunch; D, dinner.
[2] Lemon juice was added the fruit salad to prevent browning.

TABLE 2

Daily intakes of energy, flavonoids, vitamin B-6 and potassium during the dietary intervention

Daily intake	Basic diet	Low FVDiet	High FVDiet
Total energy,[1] *MJ*	14.4	14.4	14.4
Protein,[1] *en%*	14	14	15
Total fat,[1] *en%*	28	28	29
Total carbohydrates,[1] *en%*	58	57	56
Fiber,[1] *g*	31.0	37.0	44.0
Quercetin,[2] *mg*	Trace[3]	15.1	30.2
Kaempferol,[2] *mg*	Trace	3.9	7.8
Isorhamnetin,[2] *mg*	ND[4]	1.2	2.4
Tamarixetin,[2] *mg*	ND	ND	ND
Hesperitin,[2] *mg*	Trace	11.6	23.2
Naringenin,[2] *mg*	ND	1.7	3.4
Phloretin,[2] *mg*	Trace	0.4	0.8
Total flavonoid, *mg*	Trace	34.0	68.0
Vitamin B-6,[2] *mg*	1.8	2.6	3.4
Potassium,[2] *mg*	2730	3470	4200

[1] Values are calculated with Dankost2000 software based on the Danish Veterinary and Food administration food composition database (48).
[2] Values are based on chemical analysis. Flavonoids and vitamin B-6 were determined as single determinations, potassium as duplicates.
[3] Traces of flavonoids in the diet were below 0.05 mg/100 g fresh weight.
[4] ND, not detectable. Detection limit = 0.02 mg/100 g fresh weight.

was verified by oral questionnaire and by study diaries. The volunteers brought the urine collections daily to the Department of Human Nutrition, where they were weighed, their density measured, and samples were adjusted to pH 3–4 with 1 mol/L HCL. Aliquots of 10 mL of both morning and 24-h urine samples were stored at −80°C (for flavonoids and 4-PA analysis) or −20°C (for potassium analysis) until analysis. The laboratory personnel had no knowledge of treatments and all samples were analyzed in random order.

Determination of urinary flavonoids. The 7 dietary flavonoids (Fig. 1) were determined in urine by liquid chromatography-mass spectrometry (LC-MS) using the method of Nielsen et al. (13) with small alterations with respect to the internal standards used. In short, to 2-mL aliquots of each urine sample were added 500 ng of 3X ^{13}C isotopic labeled genistein and 500 ng of genistein as an internal standard [20 ng/μL dimethyl sulfoxide (DMSO)] and hydrolyzed enzymatically as previously described (13). The hydrolyzed samples were evaporated completely under vacuum and redissolved in 10% aqueous methanol and 1% formic acid. Then a DMSO stock solution containing 500 ng of 3X ^{13}C-daidzein and 500 ng of daidzein was added as an additional internal standard (20 ng/μL DMSO). The samples were then centrifuged at 11000 × *g* for 10 min at 20°C and the entire amount of the supernatant (~300 μL) was injected into the LC-MS system. The flavonoid concentrations in the urine samples were determined as single determinations based on calibration curves generated by spiked blank urine samples as described previously (13). In addition, supplementary calibration samples were included at the beginning, during, and at the end of each series of samples, and a final adjustment was performed based on the level of the internal standards in each sample. Before and after each series of samples, the performance of the entire LC-MS assay was controlled by injections of aliquots containing all of the employed flavonoid standards, including the internal standards. The enzymes used for the enzymatic hydrolysis of the flavonoid glycosides in the urine samples were arylsulphatase (*Aerobacter aerogenes*, 16.8 standard kU/L) from Sigma Chemical and β-glucoronidase (*Escherichia coli*, >200 standard kU/L) obtained from Boehringer Mannheim. Methanol and acetonitrile were of HPLC grade and obtained from Rathburne. The flavonoid standards, quercetin and naringenin, were obtained from Aldrich. Kaempferol, isorhamnetin, tamarixetin, genistein, and daidzein were obtained from Apin Chemicals. Phloretin and hesperetin were purchased from Sigma Chemical. All standards were of HPLC grade. A stock solution of 100 ng/μL of a mixture of all of the flavonoid aglycone standards was prepared in DMSO. Stock solutions of 20 ng/μL of the internal standards 3X ^{13}C genistein, genistein, 3X ^{13}C-daidzein and daidzein were prepared in DMSO. The isotopically labeled internal standards, 3X ^{13}C genistein, and 3X ^{13}C-daidzein were obtained from the School of Chemistry, University of St. Andrews, United Kingdom. All stock solutions were stored at −20°C and were stable for at least 3 mo.

Determination of urinary 4-PA. The urinary concentration of 4-PA was determined by HPLC (26). The interassay CV for 4-PA was 2.2% (n = 16).

Determination of urinary potassium. Potassium was determined by flame photometry as described by Boling (25). Analysis was done with the Ciba-corning flame photometer 410. Calibration of the instrument was performed before analysis with 1 mL diluent concentrate (Sherwood, Cat/Best. No. 011 56 681) in 1 L deionized water. For every 20 samples, an internal standard [Multical TM 200Na/100^{K}/45^{CO2} (Bayer, commissions no. 478524), Bio-Rad] and an external standard [Lyphocheck 174Na/110^{K}, Quantitative Urine Control (abnormal), Bio-Rad 377, 2 (lot. no. 60152)] were analyzed. The interassay CV for potassium was 1.7% (n = 22).

Chemical analysis of the diet. The concentration of flavonoids in the fruit and vegetable supplements was determined after acid hydrolysis by HPLC and MS as described by Justesen et al. (27), with the exception of the compound phloridzin. Phloridzin was determined by HPLC and MS without prior hydrolysis, after extraction and solid phase extraction (SPE) cleaning by the method described by Grinder-Pedersen et al. (28), which filters the extract through a 5-μm filter before SPE. The SPE eluate was filtered through a 0.45-μm filter before HPLC, which was performed on a Jupiter Phenomenex RP C18 column (250 × 4.6 mm, 5 μm). The mobile phase consisted of 1% formic acid:99% water (A), 23% acetonitrile: 23% methanol:1% formic acid:47% water (B) and 100% methanol (C). The gradient was 100% A to 100% B in 21 min,100% B to 100% C from 21 to 26 min, and 100% C from 26 to 38 min. Phloridzin was quantified at 289 nm. The concentration of flavonoids in the basic diet was determined by the method of Grinder-Petersen et al. (28). The concentration of potassium was determined by inductively coupled plasma-atomic emission spectroscopy after high-pressure wet-ashing of the samples with nitric acid in polytetrafluoroethylene-lined steel bombs (internal method FM.061.1). Potassium quantification was done by external calibration and in all analytical series, a sample of certified reference material and a blank were included to ensure analytical quality. The potassium concentration was determined as duplicates. The concentration of vitamin B-6 in the diet was determined by HPLC using the method of Kall et al. (29).

Statistical analysis. The statistical analyses were performed using the SPSS package program version 10.0. P-values < 0.05 (two-tailed) were considered to be significant. Variables were examined for normality and skewness and were tested for carry-over and treatment-period interactions. The concentrations of flavonoids, 4-PA, and potassium in 24-h and morning urine samples were not normally distributed, and because logarithmic transformation did not normalize them, nonparametric tests were used in the statistical analysis of the data (30). Wilcoxon matched pairs tests were performed to compare the urinary excretion of the selected biomarkers at the different intake levels of fruits and vegetables. Spearman's correlation (r_s) was used to examine associations between the intake level of fruits and vegetables and the excretion of biomarkers in urine.

RESULTS

The 24-h and morning urine excretions of flavonoids, 4-PA, and potassium did not differ between the two groups of men at baseline, with the exception of potassium excreted in morning urine. No carry-over effects or treatment-period interactions were observed; the groups did not differ during the 2 d on which they consumed only the basic diet (d 4) and there were no differences in the response to the fruit and vegetable supplements, regardless of the order of treatment. Thus, the data from the two study periods were pooled and treated as independent observations.

Flavonoids. The mean 24-h urinary excretion of all the individual flavonoids and the sum of all 7 flavonoids determined in urine (total flavonoids) increased significantly with increased fruit and vegetable intake (see **Table 3**). Furthermore, there was a strong linear correlation between the mean excretion of total flavonoids and the fruit and vegetable dose in 24-h urine ($r_s = 0.86$, $P < 1 \times 10^{-6}$ (**Fig. 2**A). Most of the individual flavonoids tested had a similar positive and significant correlation with the intake of fruits and vegetables in 24-h urine (r_s between 0.73 and 0.83, $P < 1 \times 10^{-5}$) except for phloretin ($r_s = 0.28$, $P = 0.1$). Among the 12 men, two were high-excreters with a flavonoid excretions > 2 times the mean excretion of the other 10 men (Fig. 2A).

The excretion of quercetin, kaempferol, isorhamnetin, tamarixetin, hesperetin, naringenin, and total flavonoids in morning urine also increased significantly with increased intake of fruits and vegetables, but to a lesser extent than in the 24-h samples (Table 3 and Fig. 2*B*). In morning urine samples, the correlation of the individual flavonoids also was weaker (r_s between 0.43 and 0.79, $P < 0.01$) again with the exception of phloretin showing no correlation with the increase in the intake of the fruit and vegetable mixture. Because naringenin, hesperetin, and phloretin were consumed together only with breakfast, the association with the intake of fruits and vegetables was improved by excluding these flavonoids (data not shown).

To investigate the linear responsiveness of the individual

TABLE 3

Excretion of flavonoids, 4-PA, and potassium in 24-h and morning urine samples from 12 men supplemented with either 300 or 600 g of fruit and vegetable mixture[1]

	Basic diet[2]	Low FVDiet	High FVDiet	LOW FVDiet[3]	High FVDiet[4]
				% flavonoid excreted	
24-h urine					
Flavonoids, *μg*					
Quercetin	17 ± 9[a]	47 ± 22[b]	74 ± 30[c]	0.2 ± 0.1	0.2 ± 0.1
Isorhamnetin	12 ± 6[a]	23 ± 16[b]	33 ± 25[c]	1.0 ± 1.1	0.9 ± 0.9
Tamarixetin	10 ± 3[a]	16 ± 7[a]	25 ± 16[a]	—[5]	—[5]
Kaempferol	11 ± 5[a]	79 ± 62[b]	148 ± 120[c]	1.8 ± 1.5	1.8 ± 1.5
Phloretin	62 ± 36[a]	81 ± 65[a,b]	116 ± 91[b]	4.5 ± 17.0	6.5 ± 11.2
Hesperitin	17 ± 13[a]	486 ± 510[b]	735 ± 794[c]	12.9 ± 12.2	18.7 ± 24.9
Naringenin	73 ± 99[a]	288 ± 212[b]	697 ± 925[c]	4.1 ± 4.4	3.1 ± 3.4
Total flavonoid	201 ± 103[a]	1020 ± 804[b]	1829 ± 1408[c]	2.5 ± 2.3	2.4 ± 2.0
4-PA, *μg*	1319 ± 586[a]	1429 ± 678[a,b]	1648 ± 733[b]		
Potassium,[6] *mg*	2162 ± 344[a]	2216 ± 434[a]	2357 ± 864[a]		
Morning urine					
Flavonoids, *μg*					
Quercetin	4 ± 2[a]	16 ± 12[b]	26 ± 14[c]	0.08 ± 0.08	0.07 ± 0.04
Isorhamnetin	3 ± 2[a]	7 ± 5[a,b]	12 ± 8[b]	0.36 ± 0.35	0.36 ± 0.31
Tamarixetin	3 ± 1[a]	6 ± 4[b]	11 ± 9[c]	—[5]	—[5]
Kaempferol	3 ± 1[a]	39 ± 30[b]	60 ± 48[c]	0.90 ± 0.77	0.70 ± 0.62
Phloretin	20 ± 15[a]	19 ± 24[a]	30 ± 30[a]	0.15 ± 3.17	1.27 ± 3.60
Hesperitin	3 ± 1[a]	55 ± 88[a,b]	29 ± 39[b]	0.44 ± 0.76	0.11 ± 0.17
Naringenin	41 ± 89[a]	47 ± 49[a]	49 ± 49[b]	0.34 ± 6.20	0.24 ± 3.00
Total flavonoid	76 ± 88[a]	189 ± 168[a,b]	216 ± 151[b]	0.34 ± 0.57	0.21 ± 0.28
4-PA, *μg*	476 ± 256[a]	495 ± 201[a,b]	549 ± 256[b]		
Potassium,[6] *mg*	324 ± 141[a]	422 ± 235[a]	340 ± 125[a]		
	461 ± 164[a]				

[1] Values are means ± SD, $n = 12$. Means in a row without a common letter differ, $P < 0.05$.
[2] The data for basic diet represents a mean of d 4 of each intervention period. 24-h urine = 0700–0700 h. Morning urine = 2400 h-first morning void.
[3] The values are calculated as the difference in flavonoid excretion after low FVDiet in relation to the basic diet.
[4] The values are calculated as the difference in flavonoid excretion after high FVDiet in relation to the basic diet.
[5] The relative excretion of tamarixetin was not calculated because tamarixetin was not detected in the diet.
[6] The excretion of potassium in morning urine at d 4 of each period of the study differed (Wilcoxon, $P = 0.003$).

flavonoids to the dietary treatments, the excreted fraction of the two different flavonoid doses was calculated (see Table 3). Ideally, the fraction excreted in urine should be independent of the given flavonoid dose, and this was in general the case in the 24-h urine sample. The citrus flavonoids, hesperetin and naringenin, however, had a different excretion profile, and the fraction of hesperetin excreted was ~10 times higher than the investigated flavonols (Table 3); in addition, the excretion of naringenin and phloretin exceeded the excretion of the flavonols. In general, a much lower fraction of the flavonoid dose was found in the morning urine.

4-PA and potassium. The urinary excretion of the vitamin B-6 metabolite 4-PA was used as a measure of the intake of vitamin B-6. We found that the excretion of 4-PA in 24-h and morning urine samples increased significantly only with the 600 g increase in the fruit and vegetable intake, whereas an increase of 300 g did not affect 4-PA excretion (see Table 3).

Because the excretion of potassium in morning urine on d 4 of the first crossover period of the study was lower than that on d 4 of the second crossover period ($P = 0.003$, see Table 3), the potassium data could not be combined. Instead, Wilcoxon matched-pairs tests were performed to investigate the response to the fruit and vegetable supplementation in relation to the treatment order. However, the changes in the fruit and vegetable intake employed in the present study did not affect the excretion of potassium in urine (Table 3).

DISCUSSION

We found a significant, dose-dependent association between the intake of fruits and vegetables and the sum of 7 selected flavonoids excreted in 24-h urine samples and with the individual flavonoids quercetin, kaempferol, and isorhamnetin. Thus, our results suggest that the sum of these 7 flavonoids in 24-h urine samples can serve as a biomarker of fruit and vegetable intake. This consolidates our previous observations and suggests that the flavonoids are a valid biomarker for fruit and vegetable intakes (14,15). The present study demonstrates furthermore that the flavonoid biomarker reflects low intakes and relatively moderate changes in the intake of fruits and vegetables. Earlier diet-controlled, intervention studies with flavonoids used higher fruit and vegetable doses than those in the present study (14,15). However, in population studies, the collection of complete 24-h urine samples is difficult, and a morning or spot urine sample would therefore be more applicable and reliable. In the present study, we also investigated the concentration of the 7 flavonoids in morning urine samples and found that it rose significantly, but not linearly with increased fruit and vegetable intake. This study therefore demonstrates that the excretion of flavonoids in a single morning urine sample probably does not have the potential to be a general biomarker for fruit and vegetable intake. The flavonoid biomarker thus has limited applicability in large-scale research projects due to the difficulties related to

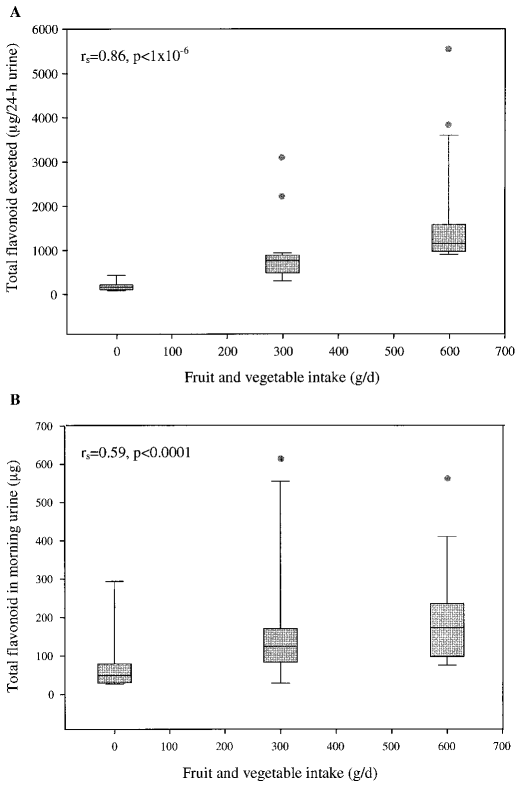

FIGURE 2 Box plot presenting the association between the excretion of total flavonoids and the intake of fruits and vegetables for all 12 men in 24-h urine (*A*) and in morning urine samples (*B*). The boundaries of the box indicate the 25th and the 75th percentiles and the line within the box marks the median. Whiskers above and below the box indicate the 90th and 10th percentiles. Subjects outside the 90th percentiles are illustrated as outliers.

collection of 24-h urine samples. The lack of a dose-dependent association between the excretion of flavonoids in morning urine and the fruit and vegetable intake can be explained by the biokinetics of the flavonoids. The short half-life of the citrus flavonoids in particular limits the excretion of these flavonoids to the first 12 h after intake (31–33). In the present study, the volunteers were provided only an orange and apple at breakfast, containing hesperetin, naringenin, and phloretin. Breakfast was consumed > 12 h before the preceding morning urine collection; consequently, these flavonoids were excreted predominantly before the morning urine collection. Thus, the composition of the diet and the timing of the fruit and vegetable meals are of great importance when using morning urine samples or other spot urine samples for flavonoid determination. In large cohort studies, spot urine samples are very often collected randomly throughout the day, e.g., in connection with a visit to the local physician. Flavonoids determined in this kind of spot urine samples will very likely result in an even poorer association with the fruit and vegetable intake than that observed in the present study with specific timeframes for each sample collection. The composition of a test meal is in general of great importance for the outcome of a diet-controlled intervention; thus, one should always have the diet composition in mind when using such data.

In the present study, the 24-h urinary excretion as a percentage of intake of total flavonoids and the majority of the individual flavonoids was independent of the flavonoid dose, demonstrating that the urinary excretion of these flavonoids reflects the dietary intake in a dose-dependent manner, within the dosage range tested. It was shown previously that the flavonoids excreted in 24 h reflect the preceding flavonoid dosages (14); however, more information on the dose dependency is required. The excretion levels observed in the present study are in the range of previous observations, although there are great variations reported in the literature. Earlier studies reported excretion levels in humans between 4.1 and 24.4% for hesperetin, 1.1–30.2% for naringenin 0.2–1.4% for quercetin, and 0.9–2.5% for kaempferol (31–38). More studies are thus warranted to explain these large variations in the fractions excreted in urine.

Because the flavanones and dihydrochalcones are excreted to a greater extent than the flavonols, they may also be absorbed to a greater extent than the flavonols and thus have an increased potential of exerting biological activity, thereby preventing diseases. On the other hand, the relatively longer plasma half-life of the flavonol quercetin (15.1–28.0 h) (32,39) in relation to the flavanones (1.3–2.9 h) (31,40) may diminish the biological importance of these flavonoids. Further studies are warranted, however, before final conclusions on the biological importance of these observations can be made.

All 7 flavonoids employed in the analytical set-up were measurable in the urine. A recent study suggests, however, that some of the phloretin present in urine could be a result of endogenous metabolism of naringenin to phloretin (41). To our knowledge, few reports exist on the excretion of phloretin in human urine (14,15,42). In the present study, ~5.5% of the phloretin dose was excreted in the 24-h urine. In a study performed by DuPont and co-workers (42) with alcoholic apple cider, as much as 21% of the phloretin dose was excreted in 24-h urine. In the present study, the correlation between the urinary excretion of phloretin and the fruit and vegetable intake was not significant. The lack of a correlation may be due to the rather low amount of phloretin consumed in the present study (0.4 or 0.8 mg/d) and to the high variation in the urinary phloretin data. The amount of apple consumed in the present study (40 or 80 g/d) was low compared with the average weight of an apple (~150 g); thus, urinary phloretin may be sensitive enough to reflect higher and more realistic apple intakes.

The presence of tamarixetin in urine is probably a result of endogenous metabolism of quercetin catalyzed by catechol-*O*-methyltransferase, which results in the formation of isorhamnetin and tamarixetin by monomethylation of the B-ring (43,44). Tamarixetin was positively affected by the fruit and vegetable intervention; thus, the effect of this endogenous metabolism apparently increases with increasing quercetin intake.

Even though the study group in the present study was relatively homogenous and consumed a standardized diet, there was a high interindividual variation in the amount of flavonoids excreted in urine (Table 3). This was caused mainly by 2 high-excreters, with flavonoid excretion 2–3 times the mean of the other 10 men (see Fig. 2*A*). Exclusion of the 2 high-excreters from the data analysis did not considerably affect the correlation coefficients between the fruit and vegetable intake and the excretion of total flavonoid in 24-h or

morning urine ($r_s = 0.89$, $P < 1 \times 10^{-6}$ and $r_s = 0.56$, $P < 0.001$, respectively). Because of the high interindividual variation in the excretion of the flavonoids, the flavonoid biomarker is not a particularly precise biomarker for intake levels of fruits and vegetables at the individual level. The high interindividual variation in the flavonoid excretion and the presence of a few "high-excreters" agrees with observations in previous studies (14,31,40,45). In contrast, the intraindividual variation was low, which also agrees with previous studies (37,38). Because of the low intraindividual variation, the excretion of flavonoids in 24-h urine has great potential as a compliance biomarker and as a biomarker for both controlled and uncontrolled changes in the intake level of fruits and vegetables achieved by an intervention.

In the present study, we also determined the concentration of 4-PA and potassium in the urine samples collected to investigate whether these compounds reflected changes in the fruit and vegetable intake. The present study showed, however, that the responsiveness of 4-PA was insufficient to serve as a biomarker of fruit and vegetable consumption levels because the increase in the fruit and vegetable intake did not result in a linear response in the excretion of 4-PA in 24-h or morning urine. Potassium also did not reflect the changes in the intake level of fruits and vegetables. According to Jacobsen et al. (46) and Bingham et al. (47), the amount of potassium excreted in the urine is dependent on the amount of fiber consumed. Thus, increased fiber consumption results in increased excretion of vitamin B-6 in the feces. Because fruits and vegetables are a rich source of fiber, it is possible that the lack of an association between fruit and vegetable intake and potassium excretion in the present study could be explained in part by the higher fiber intake. Further studies with feces collection or fiber-adjusted diets are required before such a conclusion can be drawn.

In conclusion, the present study demonstrated that the excretion of flavonoids in 24-h urine could be used as a new biomarker for fruit and vegetable intake, and that it is useful also for lower intake levels. Flavonoids in morning urine samples responded to the different fruit and vegetable doses in a nonlinear manner and are therefore not useful for detecting modest changes in dietary intakes of fruits and vegetables. The other urinary markers investigated, 4-PA and potassium, are not applicable as quantitative biomarkers of fruit and vegetable intake.

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Paper II

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SHORT COMMUNICATION

Relative bioavailability of the flavonoids quercetin, hesperetin and naringenin given simultaneously through diet

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The bioavailability and urinary excretion of three dietary flavonoids, quercetin, hesperetin and naringenin, were investigated. Ten healthy men were asked to consume a 'juice mix' containing equal amounts of the three flavonoids, and their urine and plasma samples were collected. The resulting mean plasma area under the curve $(AUC)_{0-48h}$ and C_{max} values for quercetin and hesperetin were similar, whereas the AUC_{0-48h} of naringenin and, thus, the relative bioavailability were higher after consumption of the same dose. The study consolidates a significantly lower urinary excretion of quercetin (1.5 ± 1%) compared with hesperetin (14.2 ± 9.1%) and naringenin (22.6 ± 11.5%) and shows that this is not due to a lower bioavailability of quercetin, but rather reflects different clearance mechanisms.
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Keywords: bioavailability; biokinetics; humans; quercetin; hesperetin; naringenin

To compare the impact of dietary important flavonoids, it is necessary to study them in the same food matrix and at similar realistic doses. The present study investigates the bioavailability and urinary excretion of the flavonoids, quercetin, hesperetin and naringenin, in a 48-h intervention study with a single dose (6.3 ml/kg b.w.) of 'juice mix' containing the three flavonoids.

Complete urine and plasma samples were obtained from 10 healthy men, aged 21–28 years. The study was approved by the ethics committee of Copenhagen and Frederiksberg municipality (J.No.(KF)01-161/01). The 'juice mix' was provided to fasting individuals in the morning, along with a standardized flavonoid-free diet (0–24 h), after which the individuals maintained a flavonoid-free diet (24–48 h). Blood and urine samples were collected as described previously (Nielsen *et al*., 2006). Flavonoid aglycones were quantified in the 'juice mix' (30 mg/l quercetin, 28 mg/l naringenin and 32 mg/l hesperetin) and flavonoid glycosides in the 'juice mix' were identified according to Breinholt *et al*. (2003).

Flavonoids in plasma were completely hydrolysed as described in Nielsen *et al*. (2006). Plasma samples were added 25 µl aqueous ascorbic acid (20 mg/ml) and 0.5% formic acid to pH = 4 and applied to Evolute ABN columns (25 mg, Mikrolab, Aarhus, Denmark). The eluted flavonoid aglycones were evaporated to dryness and re-dissolved in 200 µl 0.5% formic acid and 10% methanol, and 250 ng ^{13}C-daidzein was added as external standard.

Determination of flavonoids in urine is essentially described elsewhere (Nielsen *et al*., 2006), except for the inclusion of solid-phase extraction (Isolute SPE 100, Mikrolab) before injection into the liquid chromatography–mass spectrometry system.

Statistical analyses were performed using Wilcoxon matched pair tests (SPSS version 14.0). The relative

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Contributors: KSK contributed to the study design and is responsible for conducting the experiment, for LC-MS analysis of urine, plasma and juice, for data interpretation, and for writing the paper; LB contributed to the LC-MS analysis of plasma and juice, to data interpretation and to the editing of the paper; PK is responsible for the LC-MS analysis of the diet, and contributed to the LC-MS analysis of the 'juice mix' and to the editing of the paper; JH contributed to the study design, to conducting the experiment and to the editing of the paper; SER is responsible for the study design, and contributed to the LC-MS analysis of urine, plasma and juice, to the data interpretation, and to the editing of the paper.
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bioavailability, area under the curve $(AUC)_{0-48h}$, was calculated using the linear trapezoidal rule.

Results and discussion

This study demonstrates that when individuals consume equal amounts of quercetin and hesperetin, the AUC_{0-48h}, and thus the bioavailabilities of these flavonoids, is similar. However, the AUC_{048h} of naringenin was significantly higher than that for quercetin and hesperetin (Figure 1). In addition, plasma C_{max} levels achieved for quercetin and hesperetin were comparable, whereas the level achieved for naringenin was significantly higher (Table 1). In the study design we assumed that the low and natural amounts of flavonoids in the 'juice mix' excluded any interference with respect to pharmacokinetics of the flavonoids investigated. Previous studies dealing with flavanone and flavonol bioavailability report AUC values in the same range (Hollman *et al*., 1997; Erlund *et al*., 2001; Manach *et al*., 2003, 2005) as observed in the current study. However, the lack of blood sampling between 8–24 h in the present study may have caused an overestimation of the AUCs, especially for the flavanones.

In the present study, we observed a 9–15-fold higher urinary excretion of naringenin and hesperetin compared with quercetin (Table 1), consolidating the apparent paradox that absorption and bioavailability of the three flavonoids seem quite similar, but that urinary excretion is highly different. The low urinary recovery (1.5%) of the ingested quercetin leaves a large amount of the ingested dose unaccounted for compared with hesperetin and naringenin. The most likely fate of the absorbed quercetin is excretion via bile (Matsukawa *et al*., 2009) and further degradation to low-molecular-weight phenolic acids that were not analysed in the current and most previous flavonol bioavailability studies (Mullen *et al*., 2006, 2008). Only small amounts of the 3′-methylated form of quercetin, isorhamnetin, were present in the 'juice mix' (1.8 mg/500 ml 'juice mix'), and of this only 4.1 ± 3.3% of isorhamnetin was excreted in urine after 48 h, excluding extensive methylation of quercetin. Only trace amounts of tamarexetin (4′-OMe-quercetin) were found in juice, plasma and urine.

Hydrolysis of the flavonoid glycoside moiety is a necessary step for absorption of flavonoids from the gut. In the present study, apple juice was the source of quercetin and qualitative liquid chromatography–mass spectrometry investigation of the 'juice mix' showed that approximately 60% of the quercetin was present as monoglycosides, 25% as aglycone and less than 15% as rutinosides. It has been shown that only quercetin-3-glucoside and quercetin-4′-glucoside are absorbed in substantial quantities, whereas only small quantities of quercetin-3-galactoside, quercetin-3-rhamnoside and quercetin-3-arabinoside are absorbed in the small

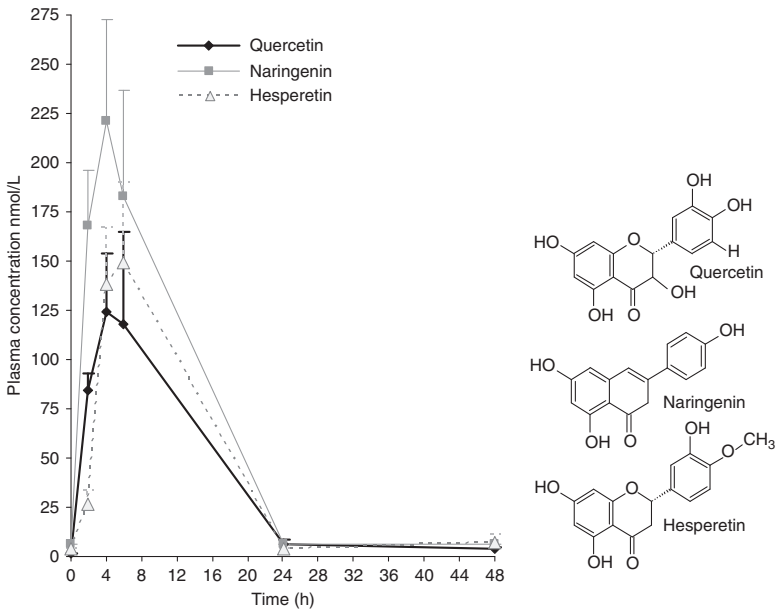

Figure 1 Plasma time versus concentration curves for quercetin (diamond), hesperetin (triangle) and naringenin (square) after ingestion of 'juice mix'. Values are mean + s.e.m., $n = 10$.

Table 1 Plasma and urinary biokinetic parameters for quercetin, naringenin and hesperetin after administration of 'juice mix' (mean ± s.d., $n = 10$)

	Mean excreted amount (μg)	*Accumulated relative urinary excretion (% of the dose)*	T_{max} *plasma (h)*	C_{max} *plasma (μmol/l)*	C_{max}/AUC_{0-48h}	AUC_{0-48h} *(μmol h/l)*	AUC_{0-48h} *(μmol h/l)*[a]
Quercetin (30 mg/l 'juice mix')			3.6 ± 1.6	0.15 ± 0.13	0.09 ± 0.02	1.77 ± 1.63	1.77 ± 1.63
0–3 h	31 ± 13	0.3 ± 0.2					
3–6 h	73 ± 29	0.9 ± 0.7					
6–12 h	74 ± 11	1.2 ± 0.9					
12–24 h	21 ± 17	1.3 ± 0.9					
24–48 h	28 ± 18	1.5 ± 1.0					
0–48 h	227 ± 142[b,c]	1.5 ± 1.0[b,c]					
Naringenin (28 mg/l 'juice mix')			3.6 ± 1.6	0.25 ± 0.13[d]	0.11 ± 0.05	2.64 ± 1.95	2.82 ± 2.09[d]
0–3 h	456 ± 231	3.3 ± 1.6					
3–6 h	1390 ± 1060	13.2 ± 8.2					
6–12 h	952 ± 482	20.0 ± 10.0					
12–24 h	278 ± 282	22.0 ± 10.9					
24–48 h	83 ± 114	22.6 ± 11.5					
0–48 h	3160 ± 1612	22.6 ± 11.5					
Hesperetin (32 mg/l 'juice mix')			4.9 ± 1.4	0.18 ± 0.13[b]	0.09 ± 0.03	2.13 ± 1.59	1.99 ± 1.49[b]
0–3 h	103 ± 78	0.6 ± 0.5					
3–6 h	802 ± 595	5.7 ± 3.6					
6–12 h	880 ± 462	11.2 ± 6.3					
12–24 h	436 ± 542	13.9 ± 8.7					
24–48 h	58 ± 103	14.2 ± 9.1					
0–48 h	2278 ± 1457[b]	14.2 ± 9.1[b]					

[a]Adjusted for ingested dose. Ingested dose is based on chemical analysis. The contents of flavonoids were calculated on the basis of internal and external standards.
[b]Significantly different from naringenin (Wilcoxon, $P < 0.01$).
[c]Significantly different from hesperetin (Wilcoxon, $P < 0.01$).
[d]Significantly different from quercetin (Wilcoxon, $P < 0.01$).

intestine, but are hydrolysed and absorbed in the distal part of the colon together with diglycosides such as naringin and hesperidin (Arts *et al*., 2004). More than 99% of the hesperetin and naringenin in the 'juice mix' were diglycosides, presumably naringin and hesperidin. The nature of the glycoside moieties bound to the three flavonoids was, thus, mainly in a form that favours absorption from the colon. The similar T_{max} values for quercetin, hesperetin and naringenin in the current study support this assumption. T_{max} for quercetin in the literature is between 1–9 h, depending on sugar moiety composition (Manach *et al*., 2005), and the intermediate T_{max} (3.6 ± 1.6 h) in the present study correlates with the mixed content of quercetin glycosides. The average T_{max} in the literature for naringenin is between 2.0–4.6 h (Manach *et al*., 2003) and that for hesperetin between 5.4–5.8 h (Manach *et al*., 2003), and are thus also in the same range as observed here.

In conclusion, the current study confirms a lower urinary excretion of quercetin compared with naringenin and hesperetin. The study furthermore shows that the significantly lower urinary excretion of quercetin is not due to a low bioavailability of quercetin, but rather reflects the different clearance mechanisms of this flavonoid.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Paper III

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ORIGINAL ARTICLE

Free fruit at workplace intervention increases total fruit intake: a validation study using 24 h dietary recall and urinary flavonoid excretion

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Background/Objectives: To validate 24 h dietary recall of fruit intake by measuring the total 24 h excretion of 10 different flavonoids in 24 h urine during an intervention with free fruit at workplaces.
Subjects/Methods: Employees at workplaces offering a free-fruit program, consisting of daily free and easy access to fresh fruit, and controls employees at workplaces with no free-fruit program were enrolled in this validation study ($n=103$). Dietary intake was assessed by using a 24 h dietary recall questionnaire at baseline and approximately 5 months later. Ten flavonoids, quercetin, isorhamnetin, tamarixetin, kaempferol, hesperetin, naringenin, eriodictyol, daidzein, genistein, and phloretin, were measured using HPLC–electrospray ionization–MS.
Results: The 24 h urinary excretion of total flavonoids and the estimated intake of fruits were significantly correlated ($r_s=0.31$, $P<0.01$). The dietary intake of citrus fruits and citrus juices was significantly correlated with total excretion of citrus specific flavonoids ($r_s=0.28$, $P<0.01$), and orange was positively correlated with naringenin ($r_s=0.24$, $P<0.01$) and hesperetin ($r_s=0.24$, $P<0.01$). Phloretin in urine was correlated with apple intake ($r_s=0.22$, $P<0.01$) and also with overall estimated intake of fruit ($r_s=0.22$, $P<0.01$).
Conclusions: This study shows that a 24 h dietary recall can be used as a valid estimate of the intake of fruits in agreement with an objective biomarker of fruit intake in free fruit at workplace interventions.
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Keywords: validation; fruit; vegetables; biomarker; flavonoids; 24 h dietary recall

Introduction

Meta-analyses of observational studies have repeatedly shown that a high fruit and vegetable intake is associated with a reduced risk of cardiovascular disease, even though causality and mechanism remain to be shown (Riboli and Norat, 2003; Dauchet *et al*., 2006; He *et al*., 2007; Duijnhoven *et al*., 2009). On the basis of these results, authorized food-based dietary guidelines recommend to eat more fruits and vegetables, for example the '5–10 a day' program in Canada, the 'go for 2&5' program in Australia or the '6 a day' program in Denmark. Fruit and vegetable intake is, however, still inadequate among the majority of the population in many countries (Ashfield-Watt *et al*., 2004; Lassen *et al*., 2004; Guenther *et al*., 2006; Ovesen *et al*., 2007; Kimmons *et al*., 2009). The average intake of fruits and vegetables in Denmark is currently 434 g/day with only 16% of the population consuming an amount, corresponding to the Danish recommendations of 600 g fruits and vegetables per day for adults and children above 10 years old (Astrup *et al*., 2005; Fagt *et al*., 2008).

It has been shown that simple provision of information is not enough to achieve significant changes in the dietary behavior of a population (Eertmans *et al*., 2001; Stockley,

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Contributors: KSK contributed to the study design, carried out the study, was responsible for statistical calculations, interpreted the data, and wrote the manuscript; LB was responsible for determination of flavonoids in urine by LC–MS, contributed to manuscript editing, and carried out the study; SA carried out the study and contributed to manuscript editing; TC calculated the dietary intake and contributed to manuscript editing; SER contributed to study design and manuscript editing; LOD was responsible for study design and contributed to manuscript editing.
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2001). Free-fruit campaigns on worksites are, therefore, well suited as an environmental strategy with the potential for increasing the daily fruit intake in a given population. This study will investigate whether a 24 h dietary recall is a valid tool to measure if a free-fruit intervention at workplace will increase fruit intake among employees.

Estimation of fruit and vegetable intake is complicated by a tendency to over-report intake of healthy foods (Gibson, 1990). Furthermore, food registration methods are often based on self-reported data, which implies the risk for subjective bias (Kipnis *et al*., 2001). Biological markers of exposure as an alternative to the more traditional dietary assessment tools have been used for many years to provide semi-quantitative indexes of the exposure to individual food constituents or food groups such as fruits or vegetables (Spencer *et al*., 2008). A combination of several different flavonoids quantified in 24 h urine samples has recently been shown to correlate more strongly to fruit intake than plasma carotenoids. Therefore, at the present, a combination of urinary flavonoids seems to be the best performing objective biomarker of fruit intake (Nielsen *et al*., 2002; Brantsæter *et al*., 2007; Mikkelsen *et al*., 2007). This biomarker has, therefore, been used in a workplace-based intervention study providing free fruit or no free fruit to employees as validation of a 24 h dietary recall.

Materials and methods

Workplaces and subjects
Eight comparable workplaces in the Copenhagen area signed up for the study. Five were enrolled as intervention workplaces and the remaining three workplaces, without any actual consideration of free fruit at the workplace, were enrolled as control workplaces. At each workplace, collaboration with a contact person was established. Recruitment of the employees, interested in participating in the study, occurred through this contact. Altogether, 146 employees were enrolled at baseline in the free fruit at work intervention study. Of the 146 employees, a total of 103 accepted participation in the validation study. Pregnant and lactating women, and individuals, not expecting to be at the particular workplace 5 months later were excluded. The study protocol was accepted by the Ethics Committee of the Copenhagen and Frederiksberg municipalities (J. No. KA-20060047).

The intervention
Workplaces entered the study at distinct points in time, starting between June and September 2006. Assessments were made both at baseline and at termination approximately 5 months later. The intervention was a fruit program, consisting of a fruit basket placed where employees had free and easy access, that is in the reception or in a kitchenette. There was at least one piece of fruit per employee each day. The fruit intervention was not accompanied with informational, counselling or other health promotion activities to make a minimal difference to the controls.

Dietary assessment
Dietary intake was assessed by a 24 h dietary recall questionnaire, which was a modified form of the dietary recall questionnaire from the Danish National Dietary Survey 2000–2002 (Lyhne *et al*., 2005). The recall was performed by trained interviewers both at baseline and after 5 months. The software program, General Intake Estimion System (version 1.0, Technical University of Denmark, National Food Institute, Division of Nutrition, 2008), was used to calculate dietary intake.

Urine samples
Urine samples were collected by the employees through 24 h at baseline and 5 months later to determine the concentration of flavonoids as an objective biomarker for fruit intake. The collection of the 24 h urine samples started in the morning the day before completion of the 24 h recall questionnaires and lasted until the next day at the same hour. Hence, the 24 h urine sample and the 24 h dietary recall questionnaire covered the same period of time. All urine samples were collected as previously described by Krogholm *et al*. (2004). The employees were instructed on how to collect the urine and in the importance of providing complete samples. Completeness of the urine collection was checked by oral questionnaires and study diaries. Anthropometric measurements (height, bodyweight, and waist circumference) were also performed, and will be published elsewhere.

Determination of urinary flavonoids
The flavonoids were determined by LC–MS after enzymatic hydrolysis of conjugates, as described by Nielsen *et al*. (2000) with slight modifications. In short, 2 ml aliquots of each urine sample were initially added 500 ng of ^{13}C *O*-desmethylangolensin internal standard (20 ng/μl dimethylsulfoxid) and enzymatically hydrolyzed. The hydrolyzed samples were evaporated to dryness under vacuum. The urine samples were then dissolved with 50 μl 100% methanol and 2.0 ml 0.5% formic acid and added on an Isolute SPE 100 column preconditioned with 2.0 ml 100% methanol and 2.0 ml 0.5% formic acid. The column was washed and the flavonoids eluted and evaporated as described in Nielsen *et al*. (2000). After evaporation, the samples were redissolved in 25 μl 100% methanol and 200 μl 0.5% formic acid and added 500 ng of ^{13}C-daidzein as an additional standard (20 ng/μl dimethylsulfoxid). The entire amount of supernatant was injected into the LC–MS system.

Reagents and standards
Enzymes used for the hydrolysis of the flavonoid glycosides in urine samples were arylsulfatase (*Aerobacter aerogenes*, 16.8

standard units/ml) from Sigma Chemicals Co. (St Louis, MO, USA) and β-glucoronidase (*Escherichia coli*, >200 standard units/ml) obtained from Boehringer Mannheim (Mannheim, Germany). Methanol and acetonitrile were of HPLC grade and obtained from Rathburne Ltd. (Walkerburn, UK). The flavonoid standards quercetin, kaempferol, isorhamnetin, tamarixetin, and naringenin were obtained from Aldrich (Steinheim, Germany). Hesperetin, eriodictyol, and phloretin were purchased from Sigma Chemicals Co. Genistein and daidzein were obtained from LC Laboratories (Woburn, MA, USA). The isotopically labeled internal standards, $3 \times {}^{13}$C *O*-desmethylangolensin, and $3 \times {}^{13}$C-daidzein were obtained from School of Chemistry, University of St Andrews, UK. Dimethylsulfoxid (Urasol) was purchased from Merck & Co., Inc. (Whitehouse Station, NJ, USA). The Isolute SPE 100 was purchased from Mikrolab, Aarhus, Denmark. All standards were HPLC grade. The concentration of flavonoids in urine samples were determined as single determinations based on calibration curves generated by spiked blank urine samples as described by Nielsen *et al*. (2000).

Statistical analysis

The concentration of flavonoids in urine and intake of fruit and flavonoid-rich foods did not have a normal distribution even after logarithmic transformation, so nonparametric tests were used in the statistical analysis of the data. Wilcoxon's two-related samples test were performed in the intervention ($n = 34$) and control ($n = 45$) group separately to evaluate changes from baseline to after 5 months in the urinary excretion of flavonoids, in intakes of fruit, and individual flavonoid-rich foods. The Mann–Whitney *U*-tests were performed to compare the two groups (control and intervention) on one variable at the time. Spearman's correlation (r_s) was used to examine associations between the intake of fruits, flavonoid-rich foods, and the excretion of the flavonoid biomarkers in urine ($n = 158$). The analyses were made using the SPSS package program version 14.0 (SPSS, Inc., Chicago, IL, USA). *P*-values <0.05 (two tailed) were regarded as statistically significant.

Results

Participants and food intake

Altogether, 103 employees were enrolled at baseline in the validation study. Of these, 99 employees did complete the 24 h dietary recall and 24 h urine collection at baseline. At the second measurement, after 5 months, the 24 h dietary recall and the collection of 24 h urine sample was completed by 79 out of the 99 employees. Main causes of drop out or exclusion were unexpected termination of employment (16 individuals) and incomplete 24 h urine collection. Data from the 79 employees were analyzed using Wilcoxon's two-related samples test and the Mann–Whitney *U*-tests.

At baseline, there were no significant differences between the control and intervention groups in the estimated dietary intakes of fruits, citrus fruits and citrus juices, vegetables, fruits and vegetables, apples, and oranges based on 24 h

Table 1 Median (P5, P95) dietary intake estimated by 24 h dietary recall (g/day) and 24 h urinary excretion of flavonoids (μg) in control and intervention group at baseline and after 5 months of a free fruit at workplace campaign

	Control group (n = 45)			*Intervention group (n = 34)*		
	Baseline	*After 5 months*	*Change*	*Baseline*	*After 5 months*	*Change*
Dietary intake estimated by 24 h dietary recall (g/day)						
Fruit	193 (2, 541)	267 (0, 732)	2 (−308, 490)	182 (0, 584)	310 (15, 1312)[a,b]	72 (310, 792)[c]
Citrus fruit and juice	0 (0, 119)	0 (0, 200)[d]	0 (−104, 200)	0 (0, 245)	50 (0, 610)[a]	20 (−139, 610)[e]
Vegetable	191 (44, 594)	194 (9, 521)	−19 (−334, 318)	153 (29, 417)	113 (4, 539)[f]	−22 (−341, 340)
Fruit and vegetables	411 (120, 990)	474 (15, 1049)	22 (−441, 465)	322 (55, 850)	470 (132, 1600)[a]	114 (−369, 1279)
Apple	61 (0, 231)	61 (0, 317)	0 (−125, 293)	0 (0, 281)	56 (0, 600)[d]	0 (−125, 511)
24 h urinary excretion of flavonoids (μg/24 h urine)						
Total flavonoids	352 (66, 1506)	445 (61, 4570)[d]	105 (−1371, 3585)	175 (65, 2569)	635 (78, 8262)[a]	258 (−752, 7285)
Citrus flavonoids	169 (8, 1166)	317 (8, 4244)[a]	143 (−1012, 4009)	91 (8, 1852)	493 (9, 7899)[d]	156 (−816, 7267)
Naringenin	72 (3, 576)	210 (3, 1802)[d]	66 (−517, 1701)	70 (3, 738)	226 (3, 2720)[d]	93 (−347, 2603)
Hesperetin	5 (2, 521)	7 (1, 2105)[d]	3 (−465, 1724)	5 (2, 1198)	177 (2, 4516)[a,f]	73 (−1021, 4172)
Eriodictyol	3 (1, 135)	17 (1, 154)	0 (−133, 150)	3 (1, 197)	24 (1, 1074)	2 (−89, 1071)

[a]Significant different from baseline in same group ($P < 0.01$, Wilcoxon).
[b]Significant different from 'after 5 months' in control group ($P < 0.01$, Mann–Whitney *U*-test).
[c]Significant different from change in control group ($P < 0.01$, Mann–Whitney *U*-test).
[d]Significant different from baseline in same group ($P < 0.05$, Wilcoxon).
[e]Significant different from change in control group ($P < 0.05$, Mann–Whitney *U*-test).
[f]Significant different from 'after 5 months' in control group ($P < 0.05$, Mann–Whitney *U*-test).
Citrus flavonoids refers to the sum of hesperetin, naringenin, and eriodictyol. Total flavonoids refer to the sum of quercetin, isorhamnetin, tamarixetin, kaempferol, hesperetin, naringenin, eriodictyol, daidzein, genistein, and phloretin.

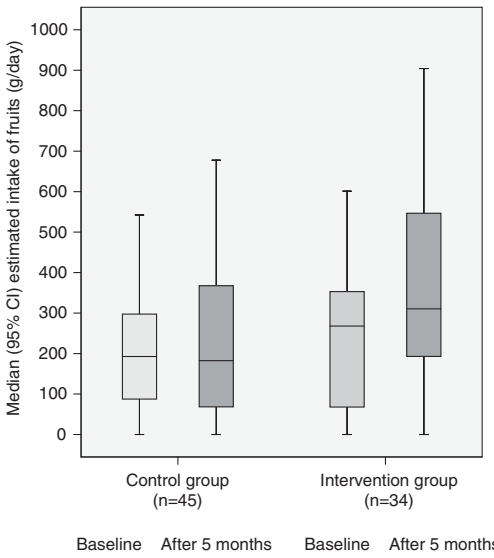

Figure 1 Error bars reflecting the change over time (from baseline to after 5 months) in the median (95% CI) estimated intake of fruits (g/day) between control and intervention group.

dietary recall questionnaires (see Table 1). After 5 months of intervention, the total intake of fruits was higher in the group with free access to fruit at work every day, compared with the control group receiving no additional fruit (median 310 (15, 1312) g/day vs median 265 (0, 732) g/day, $P<0.01$) (see Figure 1). In addition, the overall change in fruit intake from baseline to after 5 months was significantly higher in the intervention group (median 72 (310, 792) g/day, $P<0.01$) compared with the control group (median 2 (−308, 490) g/day). As seen in Table 1, the median intake of citrus fruits and juices increased from 0 g/day at baseline to 50 g/day after 5 months, and this change in intake in the intervention group was significant higher than in the control group ($P<0.05$). Moreover, the intake of fruit in the control group tended to increase over the 5 months study period (from median 193 (2, 541) g/day to median 265 (0, 732) g/day), though the change was not statistically significant (see Figure 1).

Biomarkers of fruit intake

The median (P5, P95) 24 h urinary excretion of total flavonoids (that is the sum of quercetin, isorhamnetin, tamarixetin, kaempferol, hesperetin, naringenin, eriodictyol, daidzein, genistein, and phloretin) was similar in the control and intervention groups at baseline (median 352 (66, 1506) μg vs median 175 (65, 2569) μg) (see Table 1). After 5 months of free access to fruit at workplace every day, the 24 h urinary excretion of total flavonoids, in the intervention group, had increased significantly from median 175 (65, 2569) μg to median 635 (78, 8262) μg. Moreover, the amount of total citrus flavonoids (that is the sum of hesperetin, naringenin, and eriodictyol) increased significantly after 5 months in the intervention group ($P<0.05$). In the control group, the urinary excretion of total flavonoids and total citrus flavonoids was also increased significantly after 5 months ($P<0.05$) (see Table 1).

The quantitatively largest amounts of flavonoids excreted in urine were the citrus flavonoids hesperetin, naringenin, and eriodictyol (see Table 1). Kaempferol, phloretin, quercetin, genistein, daidzein, isorhamnetin, and tamarixetin were excreted in lower amounts and did not change significantly in any of the two groups over time (data not shown).

Correlations

In this study, we use the Spearman's correlations coefficients for validation of the self-reported fruit intake. Spearman's correlations were calculated for the employees with complete 24 h dietary recall and 24 h urine collection at both baseline and after 5 months, corresponding to a total of 158 employees. Spearman's correlations between dietary intake estimated by 24 h dietary recall and 24 h urinary excretion of different combinations of flavonoids showed that total flavonoids in urine was highly and significantly correlated ($r_s=0.31$, $P<0.01$, $n=158$) with the total intake of fruits (see Table 2). Adding the intake of vegetables to the fruit intake did make the strength of the Spearman's correlation even stronger to total flavonoids in urine ($r_s=0.37$, $P<0.01$). Urinary excretion of the combination of hesperetin, naringenin, phloretin, and eriodictyol (referred to as the 'fruit marker'), all present in fruits or formed after fruit consumption, were also positively correlated to intake of fruits ($r_s=0.29$, $P<0.01$), but the correlation was stronger when including the isoflavonids, genistein, and daidzein, and the flavonols quercetin, kaempferol, isorhamnetin, and tamarixetin, which all are present in both fruits and vegetables ($r_s=0.31$, $P<0.01$) (see Table 2). Correlations between excretion of specific flavonoids and intake of particular foods were studied based on their known occurrence in these foods. The dietary intake of citrus fruits and citrus juices was significantly correlated with total citrus flavonoids ($r_s=0.24$, $P<0.01$), and oranges were positively correlated with naringenin ($r_s=0.24$, $P<0.01$) and hesperetin ($r_s=0.24$, $P<0.01$). Furthermore, the total citrus flavonoids were significantly correlated to the intake of fruits in general ($r_s=0.28$, $P<0.01$). Phloretin in urine was correlated with apple intake ($r_s=0.22$, $P<0.01$) and also with overall estimated intake of fruit ($r_s=0.22$, $P<0.01$). Finally, some highly significant correlations between individual flavonoids were observed: urinary excretion of hesperetin was correlated with excretion of naringenin ($r_s=0.60$, $P<0.0001$) and eriodictyol ($r_s=0.58$, $P<0.0001$), naringenin with eriodictyol ($r_s=0.52$, $P<0.0001$) and phloretin ($r_s=0.23$, $P<0.001$),

Table 2 Spearman's correlations (lower 95% confidence interval–upper 95% confidence interval) between intake of fruit and flavonoid-rich foods determined by 24 h dietary recall and 24 h urinary excretion of flavonoids ($n=158$)

	Fruits	*Citrus fruits and citrus juices*	*Vegetables*	*Fruits and Vegetables*	*Orange*	*Apple*
Total flavonoids	0.31 (0.16–0.45)[a]	0.23 (0.08–0.37)[a]	0.21 (0.06–0.36)[a]	0.37 (0.23–0.50)[a]	0.23 (0.08–0.37)[a]	0.24 (0.09–0.38)[a]
Citrus flavonoids	0.28 (0.13–0.43)[a]	0.24 (0.09–0.38)[a]	0.23 (0.08–0.37)[a]	0.36 (0.22–0.49)[a]	0.26 (0.11–0.40)[a]	0.20 (0.05–0.35)[b]
Hesperetin	0.29 (0.14–0.43)[a]	0.27 (0.12–0.41)[a]	0.13 (0.03–0.28)	0.31 (0.16–0.45)[a]	0.24 (0.09–0.38)[a]	0.19 (0.04–0.34)[b]
Naringenin	0.23 (0.08–0.37)[a]	0.19 (0.04–0.34)[b]	0.25 (0.10–0.39)[a]	0.33 (0.18–0.46)[a]	0.24 (0.09–00.038)[a]	0.19 (0.04–0.34)[b]
Eriodictyol	0.25 (0.10–0.39)[a]	0.08 (−0.08–0.23)	0.19 (0.04–0.34)[b]	0.30 (0.15–0.44)[a]	0.11 (−0.05–0.26)	0.16 (0.00–0.31)[b]
Quercetin	0.18 (0.03–0.33)[b]	0.07 (−0.09–0.24)	0.15 (0.00–0.30)	0.20 (0.05–0.35)[b]	−0.06 (−0.21–0.10)	0.17 (0.01–0.32)[b]
Phloretin	0.22 (0.07–0.36)[a]	0.09 (−0.07–0.24)	0.12 (−0.04–0.27)	0.23 (0.08–0.37)[a]	−0.04 (−0.20–0.12)	0.22 (0.07–0.36)[a]
Daidzein	0.11 (−0.05–0.26)	0.12 (−0.04–0.27)	0.07 (−0.09–0.22)	0.10 (−0.06–0.25)	0.08 (−0.08–0.23)	0.03 (−0.13–0.19)
Genistein	0.12 (−0.04–0.27)	0.03 (−0.13–0.19)	0.12 (−0.04–0.27)	0.14 (−0.02–0.29)	−0.08 (−0.23–0.08)	0.12 (−0.04–0.27)
Kaempferol	0.18 (0.03–0.33)[b]	0.08 (−0.08–0.23)	0.15 (0.00–0.30)	0.20 (0.05–0.35)[b]	−0.06 (−0.21–0.10)	0.16 (0.00–0.31)[b]
Fruit marker	0.29 (0.14–0.43)[a]	0.24 (0.09–0.38)[a]	0.22 (0.07–0.36)[b]	0.37 (0.23–0.50)[a]	0.25 (0.10–00.39)[a]	0.22 (0.07–0.36)[a]

[a]Correlation is significant at the 0.01 level two tailed.
[b]Correlation is significant at the 0.05 level two tailed.
Citrus flavonoids refers to the sum of hesperetin, naringenin, and eriodictyol. Total flavonoids refer to the sum of quercetin, isorhamnetin, tamarixetin, kaempferol, hesperetin, naringenin, eriodictyol, daidzein, genistein, and phloretin. Fruit marker refers to the sum of citrus flavonoids and phloretin.

quercetin with phloretin ($r_s=0.96$, $P<0.0001$), isorhamnetin ($r_s=1$, $P<0.0001$) and tamarixetin ($r_s=1$, $P<0.0001$), and daidzein was correlated with genistein ($r_s=0.52$, $P<0.0001$).

Discussion

In this study, we used the combination of several different flavonoids excreted in 24 h urine as a reference biomarker to the self-reported intake of fruits and flavonoid-rich foods. The link between intake of fruits and vegetables and the urinary excretion of flavonoids has been well established in controlled intervention studies with specific foods (Nielsen *et al*., 2002; Brevik *et al*., 2004; Krogholm *et al*., 2004) and in free-living populations (Nielsen *et al*., 2002; Brantsæter *et al*., 2007; Mikkelsen *et al*., 2007). Therefore, the significant correlation between the urinary total flavonoids and fruit intake in this study shows that the estimation of intake of fruits by the 24 h dietary recall is a valid estimate of the true effect of the free-fruit intervention program. The correlation values and excretion levels in this validation study are in the same range as previously reported (Nielsen *et al*., 2002; Brevik *et al*., 2004; Krogholm *et al*., 2004; Brantsæter *et al*., 2007; Mikkelsen *et al*., 2007).

In this study, the quantitatively largest amounts of flavonoids excreted in urine were the citrus flavonoids (hesperetin, naringenin, and eriodictyol). The main reasons for this is that the urinary excretion relative to the intake of the citrus flavonoids, hesperetin, and naringenin is roughly 10 times higher than for the flavonols (Manach *et al*., 2005; Krogholm *et al*., 2010). Owing to the high relative urinary excretion of citrus flavonoids, citrus fruit and citrus juice intake is more easily reflected by our flavonoid biomarker than the fruits containing only flavonols. This was for instance seen in this study as highly significant correlations between intake of citrus fruits and citrus juices and total citrus flavonoids. Moreover, the previously reported high correlation between phloretin in urine and intake of apples (Mennen *et al*. 2006; Brantsæter *et al*., 2007) was confirmed in this study ($r_s=0.22$, $P<0.01$). Urinary phloretin was also correlated with overall estimated intake of fruits ($r_s=0.22$, $P<0.01$), indicating that individuals consuming apples are 'high fruit consumers' in general. We could only observe weak correlations between the 24 h urinary excretion of quercetin ($r_s=0.17$, $P<0.05$) and intake of apple, including apple juice. Phloretin, therefore, appears to be a better biomarker for apple intake than quercetin, even though apples are also a dietary important source of quercetin. Ito *et al*. (2005) has suggested that phloretin is a metabolite of naringenin formed by reductive opening of the heterocyclic C-ring, and Mennen *et al*. (2006) has found high correlations between phloretin and naringenin ($r_s=0.51$, $P<0.0001$). In this study, the correlation between phloretin and naringenin was also significant ($r_s=0.23$, $P<0.01$), although the association was weaker than in the study by Mennen *et al*. (2006).

Although the citrus flavonoids are dominating among the flavonoids measured in our biomarker, this study consolidates the importance of including several different flavonoids and/or metabolites when using flavonoids as an overall biomarker for intake of fruits, as the combination of all the flavonoids measured in this study result in the strongest correlation to estimated intake of fruits ($r_s=0.31$, $P<0.01$), with a 95% confidence interval from 0.16 to 0.45, which is acceptable when validation is the overall purpose. Adding more flavonoids and/or flavonoid metabolites present in fruits might further improve the accuracy of the flavonoid biomarker. Further research investigating this is needed. The magnitude of a given correlation between a biomarker and self-reported food intakes cannot be expected to be very high even if both methods are quite accurate, and there is at present no agreement in literature as to what constitutes a satisfactory level of correlation. The magnitude

of the presented correlations between total flavonoid in urine and the estimated dietary intake of fruits, fruits and vegetables, and citrus fruits and citrus juices, given the relatively small sample size and the rather narrow range in dietary intake in this study, is rather high.

Being in a distinct protocol can result in changes in lifestyle and exercise, also referred to as the placebo effect. In this study, this was reflected by an increased fruit intake in both the control and intervention group because volunteers in both groups were informed about the purpose of the study. Over the 5 months study period, the intake of fruits in the control group changed from median 193 (2, 541) g/day to median 265 (9, 732) g/day. According to the 24 h dietary recall, this increase was not significant, whereas the urinary total flavonoid responded significantly to this change in intake. This could indicate that the 24 h dietary recall is less sensitive as a marker of fruit intake than our flavonoid biomarker. With a larger sample size, the power would increase and the 24 h dietary recall would then be a cheap and apparently accurate marker of fruit intake. In conclusion, this study shows highly significant correlations between intake of fruit, estimated by 24 h dietary recall, and the reference biomarker for fruit intake, the 24 h urinary excretion of total flavonoids.

Conflict of interest

The authors declare no conflict of interest.

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Paper IV

Title

24h and morning spot urine flavonoids and plasma carotenoids in the validation of self-reported intake of fruits, vegetables and beverages in the Inter99 study, using the method of triads.

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Short running head

Biomarker validation of FFQ using the method of triads

Abstract

The aim of the present study was to evaluate the usefulness of flavonoids in 24h and in morning spot urine samples to validate the intake of flavonoid-rich foods like fruits and vegetables measured by the food frequency questionnaire (FFQ) used in the population-based Inter99 cohort in Denmark. Individuals invited for follow up within the Inter99 study (n=264) were recruited to a validation study. Participants were asked to provide one 24h and one morning spot urine collection, to donate blood and to participate in a 28-day's diet history interview. Urinary flavonoids and enterolactone were determined and the intake of fruits, vegetables, and beverages by the FFQ was evaluated against the two flavonoid measures and plasma carotenoids using Spearman correlations and triangular validity coefficients (VCs). A

total of 191 participants were included in the study. The FFQ intake of citrus fruits/juices significantly correlated with the citrus flavonoids in both 24h and morning spot urine (r_s=0.38 and r_s=0.23). The same was found for vegetables and enterolactone (r_s=0.28 and r=0.24) and for tea and kaempferol (r_s=0.37 and r_s=0.16) (all $p<0.05$). Validity coefficients for the FFQ ranged from 0.43-0.68 using 24h and from 0.35-0.64 using morning spot urine, indicating that the intake of fruits, vegetables and tea were reflected by flavonoids both in morning spot and 24h urine. In conclusion: The Inter99 FFQ provides valid estimates of the fruit, juice and vegetable intake in the inter99 cohort and this were reflected by flavonoids both in morning spot urine and in 24h urine.

Keywords: Validation; Food Frequency Questionnaire; Diet history; Urinary flavonoids; Polyphenols, 24h urine, morning spot urine, plasma carotenoids, triangular validity coefficients

Introduction

Most validation studies show that the FFQ method is a rather low-precision instrument, and there is an ongoing debate regarding the applicability of the food frequency methods with regard to diet – disease relationships (Byers 2001; Kristal *et al.* 2005). Estimates of diet-disease relative risks suffer from inaccuracies introduced by dietary measurements errors, and diet-disease associations in epidemiological studies are likely to be attenuated. However, no other methods have so far been able to replace the food frequency method, which has been the dominant instrument in nutritional epidemiology for more than 30 years due to its low cost, easy administration (Prentice, 2003) and because it in theory represent intake over an extended period, which is the usual temporal frame of interest for chronic diseases (Willett *et al.* 1985). Therefore, thorough evaluation of FFQ estimates relative to independent reference methods including biomarkers is particularly important (Cade *et al.* 2004).

The FFQ has typically been validated by daily methods, but the FFQ and daily methods has the same sources of errors and therefore they could be correlated or might not be independent. The use of biomarkers as the reference method represents therefore a valuable and independent method for validation of self-reported intake data.

Previously, we have shown that the 24h urinary excretion of flavonoids may be used effectively as a biomarker for fruit and vegetable intake in general and for individual food groups like apple, orange, tea and fruit (Nielsen *et al.* 2002; Krogholm *et al.* 2004; Brevik *et al.* 2004; Brantsaeter *et al.* 2007; Mikkelsen *et al.* 2007; Krogholm *et al.* 2010).

Collection of complete 24h urine samples is however difficult in large populations studies, whereas a morning spot urine sample would be more applicable. Some studies indicates that the excretion of flavonoids, lignans and phenolic acids in morning spot urine can be used as biomarkers of intake for polyphenol-rich foods (Krogholm *et al.* 2004; Mennen *et al.* 2006; Mennen *et al.* 2008). In the

present study we wanted to investigate this further by comparing the excretion of flavonoids in 24h and morning spot urine samples with respect to their Spearman correlations, triangular validity coefficients and percentage of subjects correctly/grossly classified/misclassified. In order to strengthen our previously developed flavonoid biomarker (Nielsen *et al.* 2002), we have incorporated 4 more flavonoids and also the lignan, enterolactone, in the assay so that the biomarker now is the sum of twelve compounds, rather than seven; quercetin, kaempferol, apigenin, hesperetin, naringenin, eriodictyol, phloretin, enterolactone, daidzein, genistein, isorhamnetin and tamarixetin were measured using liquid chromatography-mass spectrometry.
The aim of the present study was to evaluate the usefulness of urinary flavonoids in 24h urine and in morning spot urine samples to validate the intake of flavonoid-rich foods like fruits and vegetables measured by the FFQ used in the population-based Inter99 cohort in Denmark.
The FFQ that was used in the Danish Inter99 study has previously been validated using a 28-day's diet history interview and plasma carotenoid concentrations as reference methods (Toft *et al.* 2008). In the present study the urinary flavonoid biomarker was used as an addition reference method, and triangular validity coefficients (VSs) were calculated using FFQ intakes of fruits and vegetables items and the two independent biomarkers. The method of triads is an approach to calculate the correlation between the FFQ measure and the "true" but not known intake.

Materials and methods

Subjects and study design

The aim of the Inter99 study was to decrease the incidence of ischemic heart diseases. An age- and sex-stratified random sample of 13016 individuals born from 1939-1970 and living in the south-western part of Copenhagen was drawn from the study population that comprised 61301

individuals. Of these, 82 individuals were non-eligible, as they had died or could not be traced. The remaining 12934 subjects were invited for a health-screening programme at the Research Centre for Prevention and Health. A total of 6906 (53.4%) turned up for the investigation. Out of these 122 were excluded because of alcoholism, drug abuse or linguistic barriers. Individuals at high-risk of developing ischemic heart disease were re-invited after one and three years, and completed a 198-item FFQ at each time point.

The 198-item food frequency questionnaire

The FFQ applied on the Inter99 cohort is described elsewhere (Toft *et al.* 2008). In short, the FFQ is a semi-quantitative questionnaire consisting of 198 food items and beverages. Participants were asked to report their average intake of different foods and beverages the last month. The questionnaire consisted of 16 sections: number of meals; breakfast; bread and fat spread on bread; cheese, meat and fish etc. laid on bread; hot meals; accompaniments to hot meals; sauces; fats for cooking; fast food; vegetables; salad dressing; fruits; snacks; candy/ice cream/chocolate; cookies; beverages. The food consumption quantity was obtained by multiplying the frequency of consumption of each unit of food by standard portion sizes. Daily food consumption were translated into energy and nutrient intakes using the Danish Food Composition Databank, version 6 (Saxholt *et al.* 2005) and the software program FoodCalc version 1.3 (Lauritsen, 1998).

The validation study

Individuals attending the 3-years follow up visit were invited to participate in the validation study that in addition to answering the FFQ included: a 28-day's diet history (DH) interview, collection of a 24h and a morning spot urine sample and donating a blood sample. Participants included in the

validation study did not differ from other participants at 3-years follow-up with respect to age, sex, physical activity, dietary habits, smoking, employment, diabetes, BMI and cholesterol (data not shown). The DH interview was performed by a trained clinical dietician. The participants were questioned about their habitual diet using a structured interview guide as described previously (Toft *et al.* 2008).

Collection and preparation of urine samples

Participants collected the 24h and morning spot urine sample within 2 weeks after completing the FFQ. At the day for urine collection, the first morning urine void was discarded and all subsequent voids until 2400 h on the same day were collected. The morning urine, defined as all urine voids from 2400 h including the first morning void, was collected separately. All urine samples were kept cool and dark during collection. The subjects brought the urine collections to the study site immediately after ending the collection, where put in fridge until weighed and adjusted to pH 3-4 with 1 M hydrochloric acid and stored at -20^{o}C for maximum 1 month and afterwards at -80^{o}C until analysis. To verify the completeness of the 24h urine collections the PABA method was used, and any urine collection with a PABA recovery level below 78% was classified as incomplete and excluded (Jakobsen *et al.* 1997).

Determination of flavonoids in urine

The concentration of flavonoids and enterolactone were determined by liquid chromatography-mass spectrometry (LC-MS). Urine samples (2 ml) were subjected to enzymatic hydrolysis and solid-phase extraction prior to LC-MS analysis (Nielsen *et al.* 2000; Nielsen *et al.* 2003).

Determination of carotenoids in plasma

Fasting blood samples were drawn at the day of completing the FFQ. Plasma was treated with ethanol to precipitate proteins, and the carotenoids were extracted and determined as described in detail previously (Toft *et al.* 2008).

Statistical analysis

The concentration of biomarkers and intake of fruit, vegetables and beverages did not have a normal distribution, so nonparametric tests were used in the statistical analysis of the data. We used linear regression to examine whether sex influenced the associations between the biomarkers and intake estimates, with the cross product of sex and the biomarker as an interaction term. In order to fulfil model assumptions, square root transformation was applied to the variables. The Mann-Whitney U-test were used to test differences between groups and calculated Spearman correlation coefficients (r_s) was used for calculation of the correlations between the intake of various food groups and the references measures.

We ranked carotenoid biomarkers, 24h and morning spot flavonoid biomarkers and FFQ intakes of fruits and vegetables into quintiles and calculated the percentage of subjects classified into same or adjacent quintiles (correctly classified) by the FFQ and a biomarker, or classified into opposing quintiles (grossly misclassified) for fruits and vegetables.

Validity coefficients (VCs) were calculated for the FFQ and each of the biomarkers by the equation $VC=\sqrt{r_1 * r_2 / r_3}$, where r_1, r_2 and r_3 are the correlation coefficients between the FFQ and each of the biomarkers and between the two biomarkers (Ocke and Kaaks, 1997). We used maximum likelihood estimation and bootstrap sampling with the statistical program R (http://www.r-project.org/) to estimate VCs and 95% confidence intervals for these. Use of maximum likelihood

estimation eliminates the problems related to possible negative correlations or calculating validity coefficients larger than one, as explained elsewhere (Brantsæter *et al.* 2007).

All other analyses were performed with SPSS, version 14.0 (SPSS, Inc., Chicago, IL). P values < 0.05 (two-tailed) were regarded as statistically significant.

Results

Subjects

A total of 264 individuals participated in the validation study. The urine collections were incomplete for 37 participants according to the PABA methods. Furthermore 33 participants were excluded due to laboratory failure of urine analyses and 4 participants were excluded because of incomplete dietary data leaving 191 participants for the present study.

There were no differences in age, BMI, or any flavonoids or enterolactone excreted in 24h urine between the male and female participants, while enterolactone, isoflavonoids and kaempferol in morning spot urine were higher for women than for men (**Table 1**). Furthermore, we found no significant interactions with sex when examining the correlations between intakes and biomarkers and, consequently we performed all further analyses without stratifying by sex.

Biomarkers

The median (5^{th} and 95^{th} percentile) excretion of the sum of flavonoids and enterolactone in 24h urine was 1884 µg/g createnine (299, 15606). The quantitative largest amounts of flavonoids in 24h were the citrus flavonoids naringenin and hesperetin, and daidzein (**Table 2**). The median excretion of enterolactone was 233 µg/mg createnine (13, 1306). In morning spot urine the excretion of the sum of flavonoids and enterolactone was 738 µg/g createnine (234, 5640) and also here the citrus

flavonoids, but not daidzein, were the qualitative largest group of flavonoids excreted. For the quantitatively major flavonoids, the excretion was higher in 24h urine than in morning spot urine. The flavonoid and enterolactone excretion in morning spot urine strongly correlated with flavonoid and enterolactone excretion in the 24h urine samples, and were >0.5 for all but the isoflavonoids, phloretin and apigenin (Table 2).

The median concentration of the sum of eight carotenoids in plasma was 0.9 μmol/l (0.4, 2.2).The largest amounts of carotenoids were: lycopene 0.3 μmol/l (0.1, 0.54), β-carotene 0.14 μmol/l (0.03, 0.54), phytoene 0.11 μmol/l (0.04, 0.33) and lutein 0.11 μmol/l (0.05, 0.25).

Correlations between biomarkers and intake of fruits and vegetables

The correlations were predominately strongest between the biomarkers and DH interview than between biomarkers and FFQ (**Table 3**). However the average percentage of subjects correctly classified into the (same or adjacent quintiles), and the percentage of subjects grossly misclassified (opposing quintiles) by a biomarker and the FFQ or by a biomarker and the DH were comparable (**Table 4**). On average, the degree of misclassification by biomarker quintiles was less than 10% for both the FFQ and the DH. Furthermore, the 24h urinary biomarker and plasma carotenoids significantly distinguished between high and low intakes of most fruits and vegetables items by the FFQ as well as by the DH (Table 4).

Both the fruit and fruit juice intake and the vegetable intake were more strongly correlated with the carotenoid concentrations than with the urinary flavonoids and enterolactone in the present study. On the contrary the intake of tea and wine were more strongly correlated to the excretion of flavonoids in urine (Table 3). The strongest correlations were found between carrots and α-carotene (r_s=0.67), and between β-cryptoxeanthin and citrus fruits and juices (r_s=0.50). The strongest

correlation between the flavonoids in urine and food intake were seen for citrus fruits and juices and citrus flavonoids (r_s=0.47), and for tea intake and kaempferol (r_s=0.43). No significant correlations were seen between biomarkers and coffee consumption. Potato intake showed significant negative correlation with lutein and non-significant negative correlation with the sum of all the eleven flavonoids and enterolactone.

Statistically significant correlations between urine and plasma biomarkers were found between plasma β-cryptoxanthin and naringenin in 24h urine (r_s=0.39) and naringenin in morning spot urine (r_s=0.26). Likewise, β-cryptoxanthin also correlated with nearly all flavonoids and enterolactone, e.g. hesperetin in 24h urine (r_s=0.34) and in morning spot urine (r_s =0.23), with the sum of all flavonoids and enterolactone (ex. daidzein and genistein) in 24h urine (r_s=0.33) and in morning spot urine (r_s =0.28). Furthermore α-carotene correlated with enterolactone in both 24h urine (r_s=0.30) and morning spot urine (r_s=0.28), the sum of lutein, α- and β-carotene with enterolactone in 24h urine (r_s =0.32) and morning spot urine (r_s =0.32), and all carotenes with the sum of all flavonoids and enterolactone (ex. daidzein and genistein) in 24h urine (r_s=0.31) and in morning spot urine (r_s =0.31).

The method of triads

Triangular validity coefficients were first calculated for FFQ intakes, 24h urinary flavonoid and enterolactone and carotenoids, and then for FFQ intakes, morning spot urine flavonoids and enterolactone and carotenoids (**Table 5**). For all food items the VCs for the FFQ (VC_{FFQ-T}) were higher for the 24h urinary flavonoids and enterolactone (range 0.43 to 0.68) than for the morning spot urine biomarkers (range 0.35 to 0.64). The same was seen for the flavonoid biomarker VCs (VC_{BM1-T}) (Table 5).

Discussion

Correlations between biomarkers and estimated intake of fruits and vegetables

Our findings of statistically significant correlations between the Inter99 FFQ and two independent biomarkers indicates that the Inter99 FFQ provides a useful estimate of the overall dietary intake of fruits, juices and vegetables in the Inter99 cohort. This information can be used in future studies investigating the association between fruit and vegetable intake (estimated by FFQ) and risk for a disease in the Inter99. The Spearman correlations were predominately strongest between the biomarkers and DH than between biomarkers and FFQ. This is not surprisingly since the DH is regarded as a more precise and accurate dietary assessment method that the FFQ. Overall, the percentage of subjects correctly classified (same or adjacent quintile) and the percentage of subjects grossly misclassified (opposing quintiles) by a biomarker and the FFQ or by a biomarker and the DH were comparable.

For the total intake of fruits, juices and vegetables the FFQ and the DH showed better agreement with the carotenoid biomarkers than with the flavonoid biomarkers, and this is in contrast to the few previous studies including both flavonoids and carotenoids as biomarkers for fruit and vegetable intake (Nielsen *et al.* 2002; Brantsæter *et al.* 2007; Mikkelsen *et al.* 2007). The Inter99 FFQ and the DH are both retrospective methods that cover the same period of time. Urinary flavonoids and enterolactone is a short term dietary biomarker and thus the intakes of flavonoid-rich foods estimated in the Inter99 cohort would be expected to have a stronger correlation with the carotenoid biomarker, since the carotenoid biomarker is covering a much longer time frame (weeks to months) than the flavonoids in 24h and morning spot urine.

Choice of timeframe for the flavonoid biomarker

In the present validation study the VCs for the FFQ ranged from 0.46 to 0.64 using 24h and from 0.37 to 0.56 using morning spot urine, indicating that the intake of fruits, vegetables, juices and tea were reflected by flavonoids both in morning spot urine and in 24h urine. This indicate reasonably stable ranking of individuals in both 24h and morning spot urine, which is of major importance for large epidemiologic studies that typically only obtain morning spot urine samples. If the spot urine sample available is not a morning spot urine sample, but is collected randomly throughout the day, it is likely that the association between the concentration of flavonoids and fruit and vegetable intake is weaker. In order to strength the flavonoid biomarkers performance, collection of two or more (morning) spot samples rather that just one, could be a choice of strategy. The major reason why a 24h urine collection is considered difficult is, that the participants has to bring the collection bottles along through the whole day, e.g. to work or school. It would properly be much easier to give several (morning) spot urine samples than one whole 24h collection, and if the (mornings) spot urine collections furthermore were pooled before the chemical LC-MS analysis, the cost would be held on the approximately same level as if only one morning spot urine sample were used. A pool of several (morning) spot samples furthermore offers the advantages that it then would reflect a much longer time span than hours-days as a single 24h urine sample does.

Comparison between VCs for the applied biomarkers

Using the method of triads, we have evaluated the performance of the Inter99 FFQ regarding the intake of fruits, vegetables and beverages rich in flavonoids. The main strength of the present study is the inclusion of two independent biomarkers of fruit and vegetable intake, enabling correct use the method of triad. Most other validation studies using the triangular method have used two dietary methods and one biomarker, and as the dietary methods are likely to have correlated errors, the results will be an overstatement (Kabagambe *et al.* 2001; McNaughton *et al.* 2005; Brevik *et al.*

2005; McNaughton *et al.* 2007). The results indicated that the relative validity of the FFQ regarding fruit, juice and vegetable intake was acceptable whether using 24h urine or morning spot urine. Furthermore, evaluating the VCs for the biomarkers, this study showed that a morning spot urine sample may correctly reflect the intakes of fruits, juices and vegetables, even though the VCs for 24h urine were stronger than for morning spot urine. The VCs obtained here may be used for future adjustments of diet-disease relative risks estimates in this Inter99 cohort. The VCs for vegetable intake estimated for the Inter99 FFQ were relatively high in both 24h urine (0.61) and in morning spot urine (0.56), but the confidence intervals were wide, as seen in other studies using this method (Andersen *et al.* 2005, McNaughton *et al.* 2005; Brantsaeter *et al.* 2007). There was however, no upper limit of the intervals greater than one, reflecting the rather large sample size, and the range between the lower confidence limit and calculated VC, i.e. 0.47 - 0.64 in 24h urine for citrus fruits and juices and 0.35 - 0.55 in morning spot urine, indicates that the relative validity of the questionnaire is satisfactory.

Conclusions

The main strength of the present study is the inclusion of two independent biomarkers of fruit and vegetable intake, enabling correct use the method of triad, for validation of fruit and vegetable intake estimated by a FFQ. Our study indicates that the Inter99 FFQ provides valid estimates of the fruit, juice and vegetable intake in the inter99 Cohort and this were reflected by flavonoids in both morning spot and 24h urine. Collection of 24h urine is difficult and time consuming, and therefore morning spot urine may be a more convenient tool than 24h urine for evaluating fruit and vegetable consumption in large population studies.

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Table 1 Age, BMI, urinary excretion of flavonoids and enterolactone according to sex in the validation study (n=191) within the Inter99 study

		Male n=84 Median (P5, P95)[§]	Female n=107 Median (P5, P95) [§]	p -value
Age, years		48.6 (38.3, 63.1)	48.5 (38.3, 63.2)	0.055
BMI, kg/m^2		28.0 (22.3, 37.8)	26.2 (20.4, 39.3)	0.800
Hesperetin, µg/g createnine	24h urine	47.4 (0, 2647)	36.5 (0, 2011)	0.468
	Morning spot urine	42.1 (0, 3044)	32.1 (0, 1431)	0.457
Naringenin, µg/g createnine	24h urine	166 (30.1, 1607)	205 (36.7, 1283)	0.107
	Morning spot urine	104 (19.9, 2099)	143 (28.9, 1768)	0.233
Eriodictyol, µg/g createnine	24h urine	17.6 (8.3, 91.9)	22.2 (3.4, 281)	0.559
	Morning spot urine	0.87 (0, 123)	0 (0, 129)	0.210
Phloretin, µg/g createnine	24h urine	29.7 (0, 120)	32.6 (0, 154)	0.187
	Morning spot urine	0 (0, 177)	0 (0, 66.1)	0.287
Quercetin, µg/g createnine	24h urine	17.6 (3.4, 97.2)	20.6 (5.0, 67.8)	0.240
	Morning spot urine	21.6 (0, 93.7)	28.9 (0, 74.8)	0.080
Kaempferol, µg/g createnine	24h urine	7.55 (0, 78.8)	10.8 (0, 71.7)	0.863
	Morning spot urine	30.2 (0, 80.9)	37.1 (0, 106.8)	0.034
Isorhamnetin, µg/g createnine	24h urine	3.28 (0, 28.4)	4.18 (0, 33.2)	0.128
	Morning spot urine	19.4 (0, 64.0)	22.5 (0, 65.5)	0.578
Tamarixetin, µg/g createnine	24h urine	4.30 (0, 24.3)	6.19 (0, 24.0)	0.426
	Morning spot urine	22.7 (0, 62.2)	24.7 (0, 71.3)	0.691
Apigenin, µg/g createnine	24h urine	10.2 (0, 276)	12.6 (0, 287)	0.601
	Morning spot urine	17.4 (0, 48.0)	17.3 (0, 66.5)	0.650
Enterolactone, µg/g createnine	24h urine	191 (6.76, 880)	261 (37.8, 1514)	0.060
	Morning spot urine	173 (0, 879)	241 (40.8, 2176)	0.007
Genistein, µg/g createnine	24h urine	14.8 (0, 1089)	21.8 (0, 1002)	0.703
	Morning spot urine	16.2 (0, 45.5)	20.3 (0, 55.1)	0.010
Daidzein, µg/g createnine	24h urine	91.1 (5.6, 12686)	120 (8.0, 10046)	0.380
	Morning spot urine	15.9 (0, 264)	24.5 (0, 307)	0.150
Sum all flavonoids and enterolactone, µg/g createnine	24h urine	1627 (308, 17833)	2052 (299, 13329)	0.635
	Morning spot urine	682 (211, 5974)	838 (248, 5502)	0.099

[§] P5: 5th percentile, P95: 95th percentile
*Mann-Whitney U-test

Table 2 Flavonoid and enterolactone excretion and correlations between 24h urine and morning spot urine samples in the validation study (n=191) within the Inter99 study

Flavonoid µg/g createnine	24hour urine Median (P5, P95) [§]	Spot urine Median (P5, P95) [§]	Spearman r (95% CI)
Hesperetin	40.0 (0.0, 2443)	33.8 (0, 1542)	0.69 (0.61, 0.76)
Naringenin	192 (33.6, 1563)	118 (23.6, 1914)	0.72 (0.67, 0.80)
Eriodictyol	20.3 (4.94, 198)	0.0 (0, 143)	0.52 (0.40, 0.61)
Phloretin	31.2 (0, 153)	0.0 (0, 99.9)	0.28 (0.14, 0.40)
Quercetin	19.5 (4.19, 76.1)	24.1 (0, 92.1)	0.64 (0.55, 0.72)
Kaempferol	9.47 (0, 80.3)	34.2 (0, 104)	0.62 (0.53, 0.70)
Isorhamnetin	3.78 (0, 33.5)	20.8 (0, 65.5)	0.59 (0.49, 0.67)
Tamarixetin	4.99 (0, 25.5)	24.0 (0, 72.3)	0.58 (0.48, 0.67)
Apigenin	12.2 (0, 289)	17.3 (0, 58.5)	0.08 (-0.07, 0.22)
Enterolactone	233 (12.7, 1306)	218 (13.3, 1601)	0.89 (0.87, 0.92)
Sum all above	864 (258, 4659)	672 (184, 5433)	0.76 (0.69, 0.81)
Genistein	17.8 (0, 1100)	18.2 (0, 54.9)	0.26 (0.13, 0.39)
Daidzein	116 (6.0, 12244)	22.1 (0, 304)	0.43 (0.30, 0.54)
Sum isoflavonoids	139 (10.6, 12529)	41.4 (0, 340)	0.46 (0.34, 0.57)
Sum all flavonoids and enterolactone	1884 (299, 15606)	738 (234, 5640)	0.50 (0.39, 0.60)

[§] P5: 5th percentile, P95: 95th percentile

Table 3 Bivariate correlations between intake of fruits and vegetables determined by dietary methods (FFQ and DH) and biomarkers (BM), n=191

Fruits, vegetables and beverages	Flavonoids and enterolactone, 24h urine	rFFQ-BM	rDH-BM	Flavonoids and enterolactone, , morning spot urine	rFFQ-BM	rDH-BM	Carotenoids, plasma	rFFQ-BM	rDH-BM
Apples	Phloretin	0.19**	0.27**	Phloretin	0.12^{NS}	0.14	α -carotenee	0.43**	0.31**
Tomato	Flav+ent	0.17*	0.22**	Flav+ent	0.13^{NS}	0.17*	Phytoen	0.24**	0.27**
							Lycopene	0.12^{NS}	0.20**
Citrus fruits and juices	Naringenin	0.37**	0.46**	Naringenin	0.18*	0.28**	β-cryptoxanthin	0.43**	0.50**
	Hesperetin	0.33**	0.40**	Hesperetin	0.29**	0.31**	Zeaxanthin	0.28**	0.22**
	Citrus flavonoids	0.38**	0.47**	Citrus flavonoids	0.23**	0.31**			
All fruit juices	Naringenin	0.30**	0.27**	Naringenin	0.21**	0.18*	β-cryptoxanthin	0.33**	0.26**
	Hesperetin	0.27**	0.24**	Hesperetin	0.24**	0.21**	Zeaxanthin	0.30**	0.18*
	Citrus flavonoids	0.32**	0.29**	Citrus flavonoids	0.24**	0.21**			
All fruits, berries, juice and jam	Phloretin	0.03^{NS}	0.24**	Phloretin	0.18*	0.09^{NS}	Lutein & α- & β-carotene	0.27**	0.40**
	Citrus flavonoids	0.14^{**}	0.35**	Citrus flavonoids	0.10^{NS}	0.20**		0.17*	0.45**
	Flav+ent (ex. isoflav)	0.19**	0.32**	Flav+ent (ex. isoflav)	0.13^{NS}	0.20**	β-cryptoxanthin	0.20*	0.31**
							all carotenoids		
Legumes	Quercetin	0.12^{NS}	0.21**	Quercetin	0.06^{NS}	0.15*	Lutein	0.37**	0.17*
	Ent	0.22**	0.18**	Ent	0.19**	0.15*	Lutein& α- & β-carotene	0.39**	0.10^{NS}
	Flav+ent	0.14^{NS}	0.23**	Flav+ent	0.17*	0.19*			
Potatoes	Flav+ent	-0.00^{NS}	-0.07^{NS}	Flav+ent	0.02^{NS}	-0.05^{NS}	All Carotenoids	-0.14*	-0.08^{NS}
	Isoflav	-0.06^{NS}	-0.09^{NS}	Isoflav	-0.17*	-0.21*	Lutein	-0.19**	-0.08^{NS}
Carrots	Flav+ent (ex. isoflav)	0.19**	0.20**	Flav+ent (ex. isoflav)	0.15*	0.23**	α-carotene	0.41**	0.67**
	Ent	0.21**	0.21**	Ent	0.19**	0.19**	β-carotene	0.36**	0.49**
Crucifiers vegetables	Ent	0.16*	0.03^{NS}	Ent	0.13^{NS}	0.04^{NS}	α-carotene	0.38**	0.28**
	Flavonols	0.08^{NS}	-0.08^{NS}	Flavonols	0.16*	0.03^{NS}	Lutein	0.36**	0.18*

All vegetables including potatoes	Ent	0.26**	0.22**	Enterolactone	0.21**	0.21**	α-carotene	0.43**	0.42**
	Flav+ent (ex. isoflav)	0.33**	0.24**	Flav+ent (ex. isoflav)	0.18*	0.22**	all carotenoids	0.27**	0.36**
All vegetables except potatoes	Ent	0.28**	0.27**	Ent	0.24**	0.21**	α-carotene	0.43**	0.50**
	Flav+ent (ex. isoflav)	0.25**	0.33**	Flav+ent (ex. isoflav)	0.15*	0.28**	all carotenoids	0.34**	0.44**
All fruits, juices and vegetables including potatoes	Flav+ent (ex. isoflav)	0.33**	0.36**	Flav+ent (ex. isoflav)	0.19**	0.28**	α-carotene	0.44**	0.47**
	Ent	0.32**	0.27**	Enterolactone	0.26**	0.26**	all carotenoids	0.30**	0.41**
	Naringenin	0.27**	0.28**	Naringenin	0.10^{NS}	0.16*	Lutein&α- & β-carotene	0.42**	0.48**
	Hesperetin	0.20**	0.19**	Hesperetin	0.19**	0.22**			
	Citrus flavonoids	0.27**	0.29**	Citrus flavonoids	0.14^{NS}	0.24**			
All fruits, juices and vegetables except potatoes	Flav+ent (ex. isoflav)	0.22**	0.39**	Flav+ent (ex. isoflav)	0.14^{NS}	0.29**	α-carotene	0.36**	0.52**
	Ent	0.23**	0.27**	Ent	0.20**	0.26**	all carotenoids	0.26**	0.45**
	Naringenin	0.16*	0.32**	Naringenin	0.09^{NS}	0.18*	Lutein&α- & β-carotene	0.34**	0.54**
Tea	Kaempferol	0.37**	0.43**	Kaempferol	0.16*	0.17**	Lutein&α- & β-carotene	0.17*	0.19**
Coffee	Kaempferol	-0.17*	-0.15*	Kaempferol	-0.05^{NS}	-0.01^{NS}			
Red wine	Quercetin	0.17*	0.13^{NS}	Quercetin	0.04^{NS}	-0.00^{NS}	Phytoen	0.16*	0.15*
White wine	Flav+ent (ex. isoflav)	0.13^{NS}	0.11^{NS}	Flav+ent (ex. isoflav)	0.17*	0.17*			

P5 – 5th percentile;

P95 - 95th percentile;

Flav+ent - all eleven flavonoids and enterolactone;

Ent – enterolactone;

Isoflav – the two isoflavonoids daidzein and genistein;

Citrus flavonoids – sum of hesperetin, naringenin and eriodictyol;

Flav+ent (ex. isoflav) – all flavonoids and enterolactone except the isoflavonoids daidzein and genistein;

Flavonols – sum of quercetin, kaempferol, isorhamnetin and tamarixetin;

* $P < 0.05$;

** $P < 0.01$

Table 4 Cross-classification of subjects (n=191) by quintiles based on food-frequency questionnaire (FFQ), Diet history (DH) and biomarker (BM). Correctly classified if classified into the same or adjacent quintiles, grossly misclassified if classified into opposing quintiles

Food (g/day)	Biomarker	FFQ-BM % Correctly classified	FFQ-BM % Grossly misclassified	DH-BM % Correctly classified	DH-BM % Grossly misclassified	p-value FFQ*	p-value DH*
Citrus fruit and juices	Citrus flavonoids[§]	69.1	7.9	73.8	10.5	<0.001	<0.001
	β-cryptoxanthin	68.6	6.8	73.3	8.1	<0.001	<0.001
All fruits and juice	Flav+ent (ex.isoflav)[§]	56.0	6.6	63.9	9.2	0.002	<0.001
	Sum carotenoids	59.2	9.2	64.4	10.5	0.025	0.005
Carrots	Enterolactone[§]	59.7	9.2	56.0	6.6	0.004	0.033
	α-carotene	62.3	3.9	77.5	0.0	<0.001	<0.001
Legumes	Enterolactone[§]	60.2	7.9	57.6	15.8	0.004	0.237
	Lutein	66.0	10.8	56.5	17.6	<0.001	0.241
All vegetables including potatoes	Enterolactone[§]	61.8	10.5	60.7	10.5	0.034	0.013
	Sum carotenoids	64.4	13.2	68.1	9.2	0.005	<0.001
All vegetables except potatoes	Enterolactone[§]	60.2	10.5	57.6	6.6	0.002	0.001
	Sum carotenoids	68.6	14.5	67.5	5.3	0.002	<0.001
All fruits, juices and vegetables	Flav+ent (ex.isoflav)[§]	64.4	7.9	64.9	9.2	<0.001	<0.001
including potatoes	Sum carotenoids	63.9	11.8	67.0	6.6	0.004	<0.001
All fruits, juices and vegetables except potatoes	Flav+ent (ex.isoflav)[§]	58.1	11.8	68.1	5.3	0.039	<0.001
	Sum carotenoids	61.3	13.2	68.6	6.6	0.013	<0.001
Average		62.7	9.7	65.3	8.6		

Flav+ent - all eleven flavonoids and enterolactone;

Flav+ent (ex. isoflav) – all flavonoids and enterolactone except the isoflavonoids daidzein and genistein;

Citrus flavonoids – sum of hesperetin, naringenin and eriodictyol;

[§] Flavonoids excreted in 24h urine; *p for difference in the biomarker excretion/concentration between intake quartiles 1 and 5 (Mann-Whitney U-test)

Table 5 Validity coefficients (VCs) and 95% confidence intervals for fruit and vegetable and tea intake by the FFQ and two independent biomarkers (BM), n =191

	24h urine	(95% CI)		Spot urine	(95% CI)
FFQ: citrus fruit and juices, BM1: citrus flavonoids: BM2: β-cryptoxanthin					
VC_{FFQ-T}	0.64	(0.49, 0.80)	VC_{FFQ-T}	0.59	(0.38, 0.83)
VC_{BM1-T}	0.59	(0.44, 0.74)	VC_{BM1-T}	0.39	(0.22, 0.56)
VC_{BM2--T}	0.66	(0.51, 0.82)	VC_{BM2--T}	0.72	(0.50, 1.00)
FFQ: All fruit juice, BM1: citrus flavonoids, BM2: β-cryptoxanthin					
VC_{FFQ-T}	0.52	(0.28, 0.67)	VC_{FFQ-T}	0.53	(0.28, 0.75)
VC_{BM1-T}	0.62	(0.28, 0.66)	VC_{BM1-T}	0.46	(0.25, 0.63)
VC_{BM2--T}	0.63	(0.48, 1.00)	VC_{BM2--T}	0.62	(0.40, 1.00)
FFQ: Legumes, BM1: enterolactone, BM2: lutein					
VC_{FFQ-T}	0.51	(0.31, 0.73)	VC_{FFQ-T}	0.48	(0.30, 0.71)
VC_{BM1-T}	0.43	(0.27, 0.60)	VC_{BM1-T}	0.40	(0.24, 0.57)
VC_{BM2--T}	0.73	(0.52, 1.00)	VC_{BM2--T}	0.78	(0.56, 1.00)
FFQ: All vegetables and potatoes, BM1: ent, BM2: all carotenes					
VC_{FFQ-T}	0.49	(0.27, 0.72)	VC_{FFQ-T}	0.42	(0.21, 0.64)
VC_{BM1-T}	0.53	(0.33, 0.82)	VC_{BM1-T}	0.49	(0.28, 0.70)
VC_{BM2--T}	0.55	(0.35, 0.82)	VC_{BM2--T}	0.64	(0.40, 1.00)
FFQ: All vegetables and potatoes, BM1: ent, BM2: α-carotene					
VC_{FFQ-T}	0.61	(0.42, 0.84)	VC_{FFQ-T}	0.56	(0.36, 0.80)
VC_{BM1-T}	0.43	(0.26, 0.58)	VC_{BM1-T}	0.37	(0.19, 0.54)
VC_{BM2--T}	0.70	(0.50, 0.87)	VC_{BM2--T}	0.76	(0.52, 1.00)
FFQ: All vegetables, not potatoes, BM1: ent, BM2: all carotenes					
VC_{FFQ-T}	0.57	(0.37, 0.80)	VC_{FFQ-T}	0.52	(0.31, 0.76)
VC_{BM1-T}	0.49	(0.32, 0.66)	VC_{BM1-T}	0.47	(0.30, 0.64)
VC_{BM2--T}	0.61	(0.40, 0.86)	VC_{BM2--T}	0.66	(0.43, 0.94)
FFQ: All vegetables, not potatoes, BM1: ent, BM2: α-carotene					
VC_{FFQ-T}	0.63	(0.41, 0.91)	VC_{FFQ-T}	0.62	(0.40, 0.90)
VC_{BM1-T}	0.43	(0.28, 0.58)	VC_{BM1-T}	0.39	(0.23, 0.54)
VC_{BM2--T}	0.69	(0.47, 0.97)	VC_{BM2--T}	0.71	(0.46, 1.00)
FFQ: All fruit, juice, vegetables and potatoes, BM1: Flav+ent (ex isoflav), BM2: all carotenoids					
VC_{FFQ-T}	0.56	(0.38, 0.76)	VC_{FFQ-T}	0.43	(0.22, 0.64)
VC_{BM1-T}	0.58	(0.39, 0.82)	VC_{BM1-T}	0.45	(0.26, 0.66)
VC_{BM2--T}	0.53	(0.34, 0.72)	VC_{BM2--T}	0.69	(0.43, 1.00)
FFQ: All fruit, juice, vegetables and potatoes, BM1: ent, BM2: α-carotene					
VC_{FFQ-T}	0.68	(0.50, 0.92)	VC_{FFQ-T}	0.64	(0.42, 0.89)
VC_{BM1-T}	0.46	(0.29, 0.62)	VC_{BM1-T}	0.40	(0.23, 0.55)

VC_{BM2--T}	0.64	(0.46, 0.83)	VC_{BM2--T}	0.69	(0.46, 0.99)
FFQ: All fruit, juice and vegetables (not potatoes), BM1: Flav+ent (ex isoflav), BM2: all carotenoids					
VC_{FFQ-T}	0.43	(0.24, 0.62)	VC_{FFQ-T}	0.35	(0.17, 0.55)
VC_{BM1-T}	0.50	(0.28, 0.77)	VC_{BM1-T}	0.41	(0.22, 0.65)
VC_{BM2--T}	0.61	(0.37, 0.98)	VC_{BM2--T}	0.76	(0.42, 1.00)
FFQ: All fruit, juice and vegetables (not potatoes), BM1: ent, BM2: α-carotene					
VC_{FFQ-T}	0.53	(0.32, 0.76)	VC_{FFQ-T}	0.50	(0.28, 0.76)
VC_{BM1-T}	0.44	(0.27, 0.62)	VC_{BM1-T}	0.39	(0.22, 0.55)
VC_{BM2--T}	0.68	(0.45, 1.00)	VC_{BM2--T}	0.71	(0.42, 1.00)
FFQ: Tea, BM1: Kaempferol, BM2: Lutein + α-carotene + β-carotene					
VC_{FFQ-T}	0.57	(0.32, 0.95)	VC_{FFQ-T}	0.37	(0.14, 0.67)
VC_{BM1-T}	0.66	(0.37, 1.00)	VC_{BM1-T}	0.44	(0.16, 1.00)
VC_{BM2--T}	0.29	(0.14, 0.47)	VC_{BM2--T}	0.45	(0.18, 1.00)

Ent – enterolactone;

Flav+ent - all eleven flavonoids and enterolactone;

Flav+ent (ex. isoflav) – all flavonoids and enterolactone except the isoflavonoids daidzein and genistein;

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