Fasting plasma concentrations of selected flavonoids as markers of their ordinary dietary intake

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Summary *Background* Dietary flavonoids, especially flavonols, are discussed as potentially preventive agents in the etiology of diseases such as coronary heart disease, stroke, and cancer. However, their consideration in epidemiologic studies is hampered by difficulties in exposure assessment. *Aim of the study* By comparison with dietary intake estimates, fasting plasma flavonoid concentrations should be evaluated as possible biomarkers of the ordinary dietary intake. *Methods* 7-d dietary records were completed by 48 healthy female students. Flavonoid intake was estimated by means of available literature data on the flavonoid content of foods. Fasting plasma samples were taken at the end of the record period for flavonoid determination (HPLC). *Results* The mean intake estimates (7-d period) of quercetin, kaempferol, naringenin, and hesperetin amounted to 17.9, 4.7, 12.1, and 17.4 mg/d, respectively; the corresponding mean plasma concentrations were 22.9, 10.7, 8.2, and 22.2 nmol/l. For all four flavonoids significant correlations between 7d-intake results and fasting plasma concentrations ($r = 0.30–0.46$, $p < 0.05$) existed. As expected from the known short elimination half-life of some plasma flavonoids, distinctly higher correlation coefficients were found for the relationship between intake estimates for the last day before blood sampling and the fasting plasma concentrations ($r = 0.42–0.64$; $p < 0.01$). The intraindividual variation in fasting plasma flavonoid concentrations during ad libitum intake was found to be rather high (mean coefficient of variation between 82 and 91%; $n = 4$). *Conclusions* The flavonoid content in fasting plasma samples seems to be a suitable biomarker of short-term intake and a possible biomarker of the medium-term intake. Due to the high intraindividual variation the combined use of plasma flavonoid concentrations and dietary intake estimates may be the best choice in epidemiologic studies.

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Introduction

Flavonoids, which are widespread in plant and plant-derived foodstuff such as vegetables, fruit, tea, and wine represent one of the most attracting phytochemical subgroups [1]. For a series of single flavonoid compounds several biological effects were demonstrated in experimental studies with the most prominent being their antioxidative and anticancerogenic activities [2–7]. However, so far the restricted amount of epidemiologic evidence from studies on their role in human disease prevention gave conflicting results. In studies on cardiovascular disease and stroke risk or on the risk of cancer of different sites for flavonoids (mainly flavonols) either protective effects [8–14] or no effects [15–18], were reported.

Difficulties in assessing the exact exposure level are among the most important reasons for this inconsistency. Due to the increasing information on the flavonoid content in food the estimation of their dietary intake was improved in the last years although not opti-

mised [1, 19]. Considering also differences in food choice habits the published estimates for flavonoid intake in different populations vary considerably, e. g. for flavonols between 3 mg/d and 68 mg/d [13, 16, 19–22]. On the other hand, there is still a distinct lack of information over the degree of bioavailability for most polyphenolic compounds [1, 23, 24]. Both problems are in favour of the search for reliable biomarkers of exposure. The most common biomarker approaches refer to measurements of the original compound or its metabolites in blood and urine [25]. In fact, this has been tested successfully in studies supplementing the human diet with high-flavonoid food [26–29] or with isolated compounds [30]. However, for the search on biomarkers applicable in epidemiologic studies, markers of the ordinary dietary intake of subjects over a longer period of time are deeply needed.

The present study deals with two of the quantitatively most important representatives of each of the two subgroups, flavonols and flavanones, in the human diet, i. e. quercetin and kaempferol, and naringenin and hesperetin, respectively. Average dietary intake of all four compounds is estimated by means of 7-d dietary records in combination with a data base on the flavonoid content of food. The results are being compared to the corresponding flavonoid concentrations in fasting plasma samples taken at the end of the dietary assessment period. Supplementary, an indication of the within-person variability of flavonoid concentrations in fasting plasma samples will be given.

Subjects and methods

Subjects and dietary assessment

52 female University students were recruited for the study. All of them stated to be healthy, not suffering from metabolic disorders, not to be pregnant or breast-feeding, and not following a special diet. Total dietary intake was assessed by means of estimated dietary records over a period of 7 consecutive days (7-d DR) [31]. The students were given oral instructions at the start of the protocol period by an experienced nutritionist; during the protocol phase, all of them were recontacted at least once by telephone. The completed 7-d DR were handed out personally enabling a brief check of the record booklets. The nutrition software PRODI 3+ (Wissenschaftliche Verlagsgesellschaft, Stuttgart/Germany) was used for coding of the 7-d DR because it easily allows the definition of additional nutrients, such as flavonoids, to the nutrient data base (Souci/Fachmann/Kraut 1986). Data on the flavonoid content of food was taken from the literature as previously described [19]. For the present evaluation, the food content of quercetin, kaempferol, naringenin, and hesperetin was considered; all included data refer to the aglycon form of the compounds. Individual flavonoid intake data were calculated as mean over the 7 day period (7-d DR) as well as for the last day before blood sampling only (1-d DR).

Because of underreporting (ratio of energy intake to basal metabolic rate < 1.1 [32]) or special dieting, four persons were excluded from the study. The remaining study sample comprised of 48 female students with a mean age of 24.3 years (range: 22–36 y) and a mean body mass index (BMI) of 21.7 kg/m^2 (range: 17.2–29.9).

To describe the intraindividual variation in flavonoid plasma concentrations during ad libitum intake, fasting blood samples were drawn from four persons (two male and two female) with a mean age of 39.5 years and a mean BMI of 23.6 kg/m^2. From each person four plasma samples were taken with the sampling being separated at least by two weeks time.

All participants were informed about the aims of the study and gave their written consent.

Blood sampling and HPLC analysis

Fasting blood samples were obtained from each participant at the end of the protocol period. The venous blood samples were drawn into EDTA containing tubes and centrifuged (3500 rpm, 10 min). Plasma was stored at –80 °C until analysis.

The plasma concentrations of quercetin, kaempferol, naringenin, and hesperetin were analysed by means of HPLC. A described methodology [33] was modified for the hydrolysis and extraction procedure of the concerning flavonoid compounds. Plasma (2 ml) was spiked with fisetin as internal standard, acidified with 200 µl acetic acid (0.58 M) and vortexed. After addition of 30 mg of a mixture of β-glucuronidase and sulfatase (β-glucuronidase, 300 U/mg; sulfatase, 15–40 U/mg; Sigma, Deisenhofen, Germany) the samples were incubated for 30 min at 37 °C for enzymatic hydrolysis. The flavonoid aglycons were extracted twice with acetone (5.5 ml and 3 ml, respectively). The pooled supernatants were evaporated to dryness and redissolved in 200 µl methanol. For HPLC analysis 30 µl of the final solution were injected (autosampler Gina 50, Gynkotek, Germering, Germany) on a C–18 Hypersil ODS column (150 x 4.6 mm, particle size 5 µm, Knauer, Germany), protected by an integrated precolumn (5 x 4 mm; 5 µm). As mobile phase two solvents, solvent A (demineralised water adjusted to pH 2.1 with phosphoric acid) and solvent B (methanol) were used. The gradient elution program started with 90 % A and 10 % B and continuously changed to 35 % A and 65 % B after 85 min. A constant flow rate of 0.8 ml/min was applied (pump: P 580, Gynkotek, Germering, Germany). Detection of flavonols (quercetin, kaempferol) and flavanones (naringenin,

hesperetin) was performed by means of a diode array detector at 370 nm and 287 nm, respectively (UVD 340 S, Gynkotek, Germering, Germany). Peaks of the chromatograms were evaluated with the chromatography software Chromeleon (version 4.32, Gynkotek) and the substances were identified by retention time and spectra.

Mean recovery of flavonoid standards added to plasma (n = 4) amounted to 98 % (quercetin), 105 % (kaempferol), 103 % (naringenin), and 106 % (hesperetin). The method showed a good reproducibility with coefficients of variation of 6 % (quercetin), 4.7 % (kaempferol), 3.7 % (naringenin), and 4.1% (hesperetin).

Statistical analysis

The results are presented as means, standard deviation (SD), minimum (Min) and maximum (Max) values, and percentiles (10 %, 90 %). Deviation of the flavonoid intake estimates and plasma concentrations from normal distribution was tested (Kolmogorov-Smirnov-test) and Spearman correlation coefficients for the relationship between intake and plasma concentrations were calculated. Regression analysis was applied for normally distributed data (sum of flavonols). The intraindividual variation of the flavonoid plasma concentration was expressed as coefficient of variation (CV) from the mean. All analyses were performed with the statistics software SPSS version 10.0 (SPSS Inc., Chicago/USA). Statistical significance was accepted with $p < 0.05$.

Results

The subjects' diet provided on average an energy intake of 2178 ± 346 kcal/d and a fat intake of 82 ± 19 g/d.

The mean intake (7-d DR) of the flavonols quercetin and kaempferol amounted to 17.9 ± 9.5 and 4.7 ± 4.8 mg/d, respectively; the average intake of the flavanones naringenin and hesperetin was 12.1 ± 15.6 and 17.4 ± 14.3 mg/d, respectively. A huge range for the estimated individual mean flavonoid intakes was found (Table 1). However, for all of the compounds the 10th percentile was above zero. As the main sources for flavonol intake green and black tea (40 % of total intake), onions (25 %), and apples (11 %) were identified, whereas the flavanones were exclusively provided by citrus fruits.

The intake of flavonols and flavanones on the day before blood sampling (1-d DR) was calculated in order to enable comparison of short-term intake data with the corresponding fasting plasma concentrations. As compared to the 7-d DR data, mean dietary intake results for the day before blood sampling were higher for quercetin (+ 6.1mg/d), lower for naringenin (–2.7 mg/d) and hesperetin (–3.0 mg/d), and nearly equal for kaempferol (Table 1).

In the fasting plasma samples, which were drawn at the end of the protocol period, the concentrations of the four flavonoids quercetin, kaempferol, naringenin, and hesperetin were determined by means of HPLC. The mean values of quercetin and kaempferol were 22.9 ± 16.6 and 10.7 ± 7.9 nmol/l, respectively, with slightly lower values for the medians (Table 2). For naringenin and hesperetin mean concentrations of 8.2 ± 15.4 nmol/l and 22.2 ± 44.9 nmol/l were obtained; the much lower median values indicate a shaped distribution of the values. Hesperetin and especially naringenin could not be detected in several samples (below detection limit); even the median value for naringenin was zero. On the other hand the maximum values demonstrate that also comparably high plasma concentrations could be found for all of the four flavonoids.

For all four plasma flavonoids, a statistically significant relationship between the dietary intake estimates and the corresponding fasting plasma concentrations could be established (Table 3). Referring to the results of the 7-d DRs, correlation coefficients ranged between 0.30 (quercetin) and 0.46 (kaempferol) ($r < 0.05$). However, distinctly higher coefficients (0.42–0.64; $p < 0.01$) were obtained when comparing the fasting plasma con-

Table 1 Dietary intake (mg/d) of selected flavonols and flavanones in 48 female students as derived from 7-d dietary records and 1-d dietary records.

Flavonoids	Mean ± SD	Median (mg/d)	Min. (mg/d)	Percentiles 10 %	Percentiles 90 %	Max.
7-d dietary records						
Quercetin	17.89±9.5	17.25	4.86	6.66	30.56	49.62
Kaempferol4.72±4.8	3.16	0.30	0.61	10.19	24.81	
Naringenin	12.11±15.6	5.45	0.00	0.76	34.86	63.49
Hesperetin	17.37±14.3	13.06	0.00	1.89	38.54	62.68
1-d dietary records						
Quercetin	24.00±16.5	16.53	0.00	4.19	44.34	120.30
Kaempferol5.27±7.2	2.21	0.00	0.15	13.83	35.39	
Naringenin	9.38±23.6	0.43	0.00	0.00	20.68	152.32
Hesperetin	14.39±21.9	2.43	0.00	0.00	34.49	92.86

Table 2 Concentrations (nmol/l) of quercetin, kaempferol, naringenin, and hesperetin in fasting plasma samples of 48 female students.

Flavonoids	Mean ± SD	Median (nmol/l)	Min. (nmol/l)	Percentiles 10%	Percentiles 90%	Max.
Quercetin	22.87±16.61	20.26	0.00	3.84	49.91	81.10
Kaempferol	10.65±7.89	8.72	0.00	0.00	20.29	40.65
Naringenin[a]	8.15±15.43	0.00	0.00	0.00	26.92	77.17
Hesperetin[b]	22.16±44.85	5.75	0.00	0.00	59.37	203.26

[a] n = 45; [b] n = 47

Table 3 Relationship[a] between the flavonoid concentration in fasting plasma samples and the mean dietary flavonoid intake (mg/d) over the previous 7 days (7-d DR) or on the last day before blood sampling (1-d DR), obtained in 48 female students.

Flavonoid intake	Fasting plasma concentrations: Quercetin	Kaempferol	Σ Flavonols	Naringenin	Hesperetin	Σ Flavanones
7-d DR						
Quercetin	**0.30***	0.14	0.26	–0.05	0.02	0.01
Kaempferol	**0.40****	**0.46****	**0.50****	–0.09	0.04	0.00
Σ Flavonols	**0.36***	0.27	**0.37****	–0.07	0.04	0.01
Naringenin	0.08	–0.10	0.02	**0.35***	**0.50****	**0.45****
Hesperetin	–0.16	–0.08	–0.15	**0.30***	**0.32***	**0.30***
Σ Flavanones	0.02	–0.12	–0.02	**0.36***	**0.46****	**0.42****
1-d DR						
Quercetin	**0.42****	0.28	**0.45****	–0.02	0.08	0.03
Kaempferol	0.21	**0.50****	**0.43****	–0.07	0.07	0.00
Σ Flavonols	**0.42****	**0.37****	**0.49****	–0.06	0.08	0.01
Naringenin	0.07	–0.07	0.02	**0.47****	**0.65****	**0.60****
Hesperetin	–0.02	–0.03	–0.01	**0.43****	**0.64****	**0.59****
Σ Flavanones	0.03	–0.07	–0.01	**0.44****	**0.63****	**0.57****

[a] Spearman correlation coefficient; * $p < 0.05$; ** $p < 0.01$

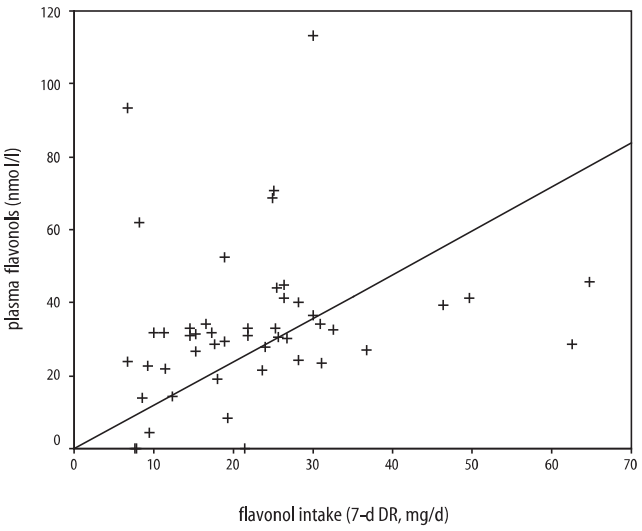

Fig. 1 Relationship between the dietary intake (7-d dietary records, mg/d) and the fasting plasma concentrations (nmol/l) for the sum of flavonols (quercetin, kaempferol) in 48 female students ($r^2 = 0.622$, $p < 0.001$).

centrations with the intake results of the day before blood sampling. Only for kaempferol, the relationship didn't change markedly. It has to be reminded that there was nearly no difference in the mean kaempferol intake between the calculation over 7 days or over 1 day (see above). Regarding the sums of flavonols and flavanones, respectively, the correlation coefficients were within the range given for the single compounds (except for the sum of flavanones, 7-d DR estimate). For the sum of flavonols, the relationship between the dietary intake and the plasma concentration is shown in Fig. 1, including results of the regression analysis (data fit to normal distribution).

The intraindividual (i. e. within-person) variation of the flavonoid concentrations in fasting plasma samples was analysed in four persons. Mean coefficients of variation ranged between 82% and 91% (Table 4). Naringenin could not be detected in a sufficient number of these plasma samples. Considering the individual CVs, values as low as 10% are opposed to values up to

Table 4 Intraindividual variation (CV*, %) of quercetin, kaempferol, and hesperetin concentrations in fasting plasma samples of four healthy subjects (P1, P2, P3, P4). Four samples each were taken with an interval of at least two weeks.

Flavonoid	Mean ± SD	CV* (%) P1	P2	P3	P4
Quercetin	82±77	116	118	74	21
Kaempferol	89±46	38	85	34	
Hesperetin**	91±72	10	147	–	116

* CV, coefficient of variation; ** n = 3

200 % without clear differences between quercetin, kaempferol, and hesperetin.

Discussion

With respect to the average daily energy intake, the students' diet was fairly comparable to the population-based average sex- and age-matched data in Germany (German National Food Consumption Survey) [34]. However, in the present study the mean flavonol intake was much higher (22.6 mg/d for quercetin and kaempferol) than reported previously in a Bavarian subgroup of the German National Food Consumption Survey (12.5 mg/d) [19]. This hints towards a higher consumption of flavonoid-rich foodstuff than found on average in a Bavarian subpopulation. On the other hand, the flavonol intake data fit well with those published for Dutch populations which were in a range of 20–26 mg/d; moreover, the main food sources of flavonols (tea, onions, and apples) were readily comparable [8, 9, 22]. Regarding the dietary intake of naringenin and hesperetin (12.1 and 17.4 mg/d), also higher values were noted than found for the above mentioned subgroup of the German National Food Consumption Survey (7.2 and 13.9 mg/d [19]); from Finland [35] somewhat higher intake values were reported for hesperetin intake (28.3 mg/d). Because flavanones occur nearly exclusively in citrus fruits, mainly oranges and grapefruits [36, 37], the intake strongly depends on the consumption of these fruits (including juices) implicating also a likewise high variation in intake. Data on the flavonol and flavanone content of food was taken from the literature as already described [19]. As for most other flavonol intake calculations, the comprehensive food data published by Hertog and coworkers [38–40] are the basis of the here presented flavonol intake calculations. Thus, it can be assumed that the calculated values are fairly good estimates of the true intake and comparable to other recent studies. Considering the few flavanone-providing kinds of food for which data are available, a valid estimate can be expected as well.

The reported data on flavonol concentrations in individual fasting plasma samples show a broad range of variation. For quercetin, fasting plasma concentrations between 0 and 142 nmol/l were analysed [33, 41] as compared to the here reported values of 0–81.1 nmol/l. Most likely as a consequence of the lower dietary kaempferol intake, the mean fasting plasma concentration of kaempferol (10.7 nmol/l) was below the value of quercetin. No fasting kaempferol concentrations in plasma could be found in the literature.

It was already described in supplementation studies, that differences in the intake of quercetin and kaempferol could be distinguished by their concentrations in plasma and urine [27, 28]. Noroozi et al. [26] quantified the relationship between dietary intake and the plasma and urine concentrations in a study supplementing the usual diet with meals containing high amounts of flavonols. They found statistically significant correlations for the dietary quercetin intake and the quercetin concentrations in plasma ($r = 0.74$) and urine ($r = 0.81$). In the present study focussing on the ordinary dietary intake, statistically significant correlations between the mean intake (7-d DR) and the fasting plasma concentrations of quercetin ($r = 0.30$) and kaempferol ($r = 0.46$) were observed as well. Moreover, considering the dietary intake at the day before blood sampling, the fasting plasma concentrations were better markers of short-term intake. Although the higher correlation coefficients for the 1-d DR results seem not surprising with respect to the observed fast turnover of polyphenolic compounds [29, 42–44], it is important to note that the difference in the correlation coefficients using the 1-d DR or the 7-d DR results is moderate. A link between medium-term flavonol intake through consumption of ordinary foodstuff, i. e. without supplementation of flavonol-rich food, and fasting plasma concentrations has not been established so far.

It is worth mentioning that all flavonoid aglycons regardless of their real binding situation in plasma (free, or as glucuronide or sulfatide) are estimated by means of the applied methodology (enzymatic hydrolysis). With respect to biological activity this may be a crucial step [45]. The investigation of the metabolic handling of flavonols and flavanones is ongoing [1], with the determinants of inter-personal variation being largely unclear. The situation for the diversity of flavonoids is similar at the absorption site although the information increased distinctly in the last years [46–48]. However, the present knowledge in bioavailability and metabolism of flavonoid compounds as a whole seems insufficient to be used for detailed explanation of their fasting plasma concentrations.

Initially, fasting naringenin and hesperetin plasma concentrations were expected near or below the detection limit because of the described short elimination half-life [29, 44]. In contrast, some students with a higher flavanone intake showed considerably high fasting plasma concentrations, and contributed to the high standard deviations found (8.2 ± 15.4 and 22.2 ± 44.9 nmol/l). The high variability is also reflected by the here reported data on the intra-individual variation in flavanone plasma concentrations. Besides the differences in dietary intake, a rather high inter-individual variation in the bioavailability of flavanones has been described [29] which may also contribute to the variation in plasma flavanone results. Therefore, Erlund and co-workers [29] suggested that plasma values of both naringenin and hesperetin may not be appropriate biomarkers for their dietary intake. However, the restriction was made that when juices were given together

with a meal, plasma flavanones could be detected over a longer time period. In fact, in the present study naringenin and hesperetin could be detected in quite a lot of fasting plasma samples and this is dependent upon the ingested amount. The correlation coefficients were distinctly higher when comparing 1-d DR results with the corresponding fasting plasma values, but these were also significantly predictive of the average individual 7-d intake estimates of naringenin and hesperetin.

In conclusion, fasting plasma flavonol and flavanone concentrations seem to be promising biomarkers of their dietary intake. Single fasting plasma values do not only reflect dietary intake on the day before blood sampling but are a measure for the usual dietary intake over one week, too. The strength of the correlation seems comparable to other nutritional biomarkers, e. g. fatty acids or carotenoids [25]. The impact of other parameters (besides diet) affecting plasma concentrations, i. e. the bioavailability and the metabolic handling of the compounds, have to be defined more precisely. Clearly, the intra- and inter-individual variation both in intake and plasma concentrations of flavonols and flavanones is comparably high. Thus, the best approach for epidemiologic research on health-relevant effects of flavonols and flavanones might be the application of their plasma concentrations in combination with their dietary intake estimates.

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