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Urinary flavanone concentrations as biomarkers of dietary flavanone intakes in the European Prospective Investigation into Cancer and Nutrition (EPIC) study

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Abstract

In the present study, the aim was to investigate the correlation between the acute and habitual dietary intake of flavanones, their main food sources and the concentrations of aglycones naringenin and hesperetin in 24 h urine in a European population. A 24-h dietary recall (24-HDR) and a 24-h urine sample were collected the same day from a subsample of 475 people from four different countries of the European Prospective Investigation into Cancer and Nutrition study. Acute and habitual dietary data were captured through a standardised 24-HDR and a country/centre-specific validated dietary questionnaire (DQ). The intake of dietary flavanones was estimated using the Phenol-Explorer database. Urinary flavanones (naringenin and hesperetin) were analysed using tandem MS with a previous enzymatic hydrolysis. Weak partial correlation coefficients were found between urinary flavanone concentrations and both acute and habitual dietary flavanone intakes ($R_{partial} = 0{\cdot}14$–$0{\cdot}17$). Partial correlations were stronger between urinary excretions and acute intakes of citrus fruit and juices ($R_{partial} \sim 0{\cdot}6$) than with habitual intakes of citrus fruit and juices ($R_{partial} \sim 0{\cdot}24$). In conclusion, according to our results, urinary excretion of flavanones can be considered a good biomarker of acute citrus intake. However, low associations between habitual flavanone intake and urinary excretion suggest a possible inaccurate estimation of their intake or a too sporadic intake. For assessing habitual exposures, multiple urinary collections may be needed. These results show that none of the approaches tested is ideal, and the use of both DQ and biomarkers can be recommended.

Key words: **Flavanones: Biomarkers: Intake: Urine: European Prospective Investigation into Cancer and Nutrition study**

Abbreviations: 24-HDR, 24-h dietary recall; DQ, dietary questionnaire; EPIC, European Prospective Investigation into Cancer and Nutrition; LOQ, limit of quantification.

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Citrus fruits, raw or as derived products such as juices and jams, are rich sources of flavanones, a subclass of flavonoids[1,2]. There are a plethora of *in vitro* studies showing that flavanones have the ability to modulate several signalling pathways, including enzymatic oxidation, inflammatory response, apoptosis, vascularisation, and cell differentiation and proliferation[3,4]. Indeed, epidemiological studies have also observed that a diet high in flavanones and flavanone-rich foods is associated with a reduction in the risk of cerebrovascular disease, asthma and cancer in several locations[5–8].

The main flavanone aglycones are naringenin (5,7,4′-trihydroxyflavanone), hesperetin (5,7,3′-trihydroxy-4′-methoxyflavanone) and eriodictyol (5,7,3′,4′-tetrahydroxyflavanone), which differ in their hydroxyl and methoxyl substitutions in the flavan A- and B-rings. Naringenin is mainly present in grapefruit, oranges, artichokes and ripe tomatoes; hesperetin is mostly present in lime, lemon, oranges, citric fruit juices and mint and eriodictyol is the main flavanone of lemon and lemon juice, but it also occurs (in lower concentrations) in almonds and orange juice[1,2]. Flavanone intake varies between 20 and 50 mg/d in European adults[9], hesperetin (59–70 %) and naringenin (29–39 %) compounds being the main contributors by far[10].

Flavanones are generally found in nature as glycosides. Their absorption and metabolism depend on their chemical structure and the nature of the sugar moiety. As soon as they reach the small intestine, glycosides are partially hydrolysed by the intestinal β-glucosidase and absorbed. Non-absorbed flavanones reach the colon, where they are hydrolysed by the bacterial enzymes and catabolised into small phenolic acids. In the intestine and in the liver, aglycones and phenolic acids are conjugated into glucuronides and sulphate esters. Finally, these metabolites are largely excreted in the urine and the bile[11,12]. Pharmacokinetic studies show that the recovery in urine is approximately 8·6 and 8·8 % for hesperidin and naringenin, respectively[11].

Traditional methods for estimating flavanone intake (i.e. self-reported questionnaires + food composition tables) have several limitations that could be overcome using biomarkers[13–16]. However, to date, very few studies have investigated biomarkers of intake of flavanones and flavanone-rich fruits (i.e. citrus fruit)[17]. For example, in a small subsample of the SU.VI.MAX (SUpplementation en VItamines et Minéraux AntioXydants) cohort, acute grapefruit consumption was positively correlated with urinary naringenin concentration (r 0·20), orange intake with hesperetin (r 0·35) and citrus fruit consumption with both naringenin and hesperetin (r 0·46 and 0·37, respectively) ($P < 0·05$) using a 2-d dietary record[18]. However, further studies are warranted to discover and validate useful biomarkers of flavanones and flavanone-rich fruits, especially long-term biomarkers. In order to tackle this issue, our objective was to investigate the correlation between the acute and habitual dietary intake of flavanones, their main food sources and the concentrations of the naringenin and hesperetin aglycones in 24 h urine in a multicentre European study.

Materials and methods

Study population

The European Prospective Investigation into Cancer and Nutrition (EPIC) is a prospective cohort study including more than 521 000 study participants enrolled mostly from the general population, between 1992 and 2000, from twenty-three centres in ten Western European countries[19,20]. The study was approved by the ethics committees of the International Agency for Research on Cancer and all participating institutions. All participants also signed an informed consent.

In the present study, a subsample of 475 men and women was used, aged between 33 and 77 years from four different countries: France, Italy (Florence, Varese, Ragusa, Turin and Naples), Greece and Germany (Heidelberg and Potsdam). In France and Naples, only women were recruited. In all participants, a single 24-h dietary recall (24-HDR) and a 24-h urine sample were taken on the same day. The 24-HDR were collected over all four seasons and over different days of the week, in order to cover day-to-day and season variations[21].

Dietary and lifestyle data

In the present study, short- and long-term diet was assessed. Acute dietary data were evaluated using a single 24-HDR, which was administered by a face-to-face interview using a standardised software EPIC-Soft (renamed GloboDiet)[21]. Habitual dietary data were collected at baseline using a centre-specific quantitative dietary questionnaire (DQ) with an estimation of individual average portions structured by meals, except in Greece where the food items were not structured by meals. In Naples, a semi-quantitative DQ with the same standard portion assigned to all subjects was used[19,20]. DQ were developed and validated in each country or centre[22].

The average time interval between the DQ and the 24-HDR varied by country, from 1 to 3 years later[23]. The intakes of naringenin and hesperetin and their glycosides (as they are found in nature) were assessed with the Phenol-Explorer database, a comprehensive food composition database on polyphenols[19]. Total flavanone was calculated as the sum of naringenin and hesperetin and their glycosides. Flavanone intake was also expressed as aglycones, summing both flavanone free form and glycosides after their conversion of glycosides to aglycone equivalents on the basis of their molecular weight. Total energy intake was estimated using the standardised EPIC nutrient database[24].

Data on lifestyles, such as physical activity, smoking history and education, were collected at recruitment by standardised questionnaires[23,25]. Data on age, body weight and height were self-reported during the 24-HDR interview.

Samples and analytical method

A total of 24-h urine samples were collected and stored at −20°C using boric acid as a preservative. The integrity of the 24-h urine samples was monitored using *p*-aminobenzoic acid (PABA). Samples with recovery of PABA <70 and >110 % were excluded from the present study.

Naringenin and hesperetin were analysed in 24-h urine samples using ultra-HPLC coupled to tandem MS[26]. Briefly, the urine samples were treated with a mixture of β-glucuronidase/sulphatase and extracted twice with ethyl acetate. All phenolic groups in naringenin and hesperetin were quantitatively dansylated using a differential isotopic labelling method and quantified

Table 1. Urinary flavanone excretion (μmol/24 h) according to socio-demographic and lifestyle factors in the European Prospective Investigation into Cancer and Nutrition (EPIC) study (*n* 475)
(Medians and 10th and 90th percentile (P10 and P90) values)

		Naringenin			Hesperetin			Flavanones		
Characteristics	*n*	Median	P10	P90	Median	P10	P90	Median	P10	P90
Centre										
Paris (FRA)	67	0·91	0·32	10·67	0·45	0·14	1·43	1·54	0·54	10·44
Florence (ITA)	45	2·23	0·57	18·28	2·67	0·15	11·91	4·21	0·74	31·15
Varese (ITA)	51	2·60	0·83	10·64	2·89	0·20	12·83	5·50	1·29	23·89
Ragusa (ITA)	17	9·93	1·93	17·29	7·79	1·08	17·41	19·74	3·31	38·81
Turin (ITA)	42	1·42	0·28	7·68	0·55	0·11	8·28	1·94	0·48	14·50
Naples (ITA)	20	1·77	0·55	4·15	0·67	0·17	5·38	2·26	0·69	9·26
Greece	56	1·54	0·44	8·49	0·66	0·15	6·86	2·35	0·60	15·14
Heidelberg (GER)	59	1·55	0·57	7·45	0·91	0·17	4·47	2·75	0·76	19·96
Potsdam (GER)	118	1·88	0·50	7·08	1·33	0·16	6·94	3·06	0·76	12·86
Sex										
Men	198	2·23	0·61	12·35	1·60	0·18	12·22	3·87	0·88	25·16
Women	277	1·43	0·37	7·86	0·81	0·15	6·04	2·47	0·61	13·69
Age (years)										
<50	151	1·67	0·46	10·63	0·68	0·13	7·79	2·56	0·64	21·51
50–60	201	1·50	0·41	8·61	1·01	0·15	8·12	2·95	0·70	15·59
>60	123	1·76	0·49	9·36	1·52	0·19	8·36	3·90	0·66	19·87
BMI (kg/m^2)										
<25	207	1·37	0·34	7·53	0·73	0·13	6·61	2·33	0·57	13·23
25 to <30	186	1·75	0·54	10·18	1·30	0·17	10·51	3·09	0·79	20·18
>30	82	2·10	0·53	12·19	1·18	0·17	8·92	3·95	0·77	23·40
Smoking status										
Never smoker	241	1·62	0·45	8·66	0·99	0·14	7·08	2·94	0·66	15·82
Former smoker	129	1·98	0·59	14·62	1·09	0·17	11·30	3·05	0·84	26·36
Current smoker	92	1·41	0·34	8·05	0·91	0·16	9·48	2·59	0·58	20·82
Unknown	13	1·73	0·54	18·67	1·03	0·18	14·18	2·80	0·70	33·92
Energy intake (kJ/d)										
4184–8368	170	1·35	0·39	6·48	0·71	0·13	5·90	2·16	0·63	13·88
>8368–12552	238	2·00	0·44	10·16	1·40	0·17	8·81	3·87	0·70	20·27
>12552–20920	67	1·75	0·55	11·13	0·97	0·15	9·10	2·66	0·78	22·45

FRA, France; ITA, Italy; GER, Germany.

using a tandem MS. The limit of quantification (LOQ) for naringenin and hesperetin was 0·21 and 0·19 μM, respectively. The upper LOQ for both compounds was 18·2 μM. Intra-batch CV were 5·2 % for naringenin and 4·9 % for hesperetin. Inter-batch CV were 10·2 and 8·2 % for naringenin and hesperetin, respectively.

The 475 urine samples were analysed in nineteen batches, with a ten-point calibration curve and three quality controls injected in duplicate within each batch. A small number of urinary excretion measurements of naringenin (*n* 5) and hesperetin (*n* 6) did not fulfill the established analytical quality control criteria (i.e. the presence of an interfering compound and/or a concentration above the upper LOQ) and, therefore, were excluded from the statistical analysis.

Statistical analyses

Urinary concentrations of naringenin and hesperetin <LOQ were set at values corresponding to half of the LOQ. Both urinary concentrations and dietary intakes of flavanones of all participants, including consumers and non-consumers, were presented using descriptive statistics (medians, 10th and 90th percentiles). Kruskal–Wallis tests were used to compare the levels of urinary naringenin, hesperetin and total flavanones between subject groups with different demographic and lifestyle characteristics. Spearman's correlations were used to calculate the relationships between urinary flavanone levels and their intakes and main food sources extracted from the DQ and the 24-HDR. Partial Spearman's correlations ($R_{partial}$) were calculated to evaluate the previous associations adjusted for several potential confounders, such as centre, sex, BMI, age of recruitment, smoking status (never, former and current smoker) and total energy intake (obtained from the DQ or in 24-HDR as appropriate). All statistical analyses were conducted using R 3.2.1 software (R Foundation for Statistical Computing).

Results

The number of participants with urinary concentrations <LOQ was eleven and 122 for naringenin and hesperetin, respectively. In our study, the highest median of urinary flavanone level was in Ragusa, Italy (19·7 μmol/24 h), while the lowest was in France (1·5 μmol/24 h) (Table 1). Flavanone excretion was likely to be greater in men, in older individuals, in those with BMI ≥ 30 kg/m^2, in former smokers and in those with a total energy intake between 8368 and 12 552 kJ/d.

Median intake of flavanones was higher in the DQ (21·0 mg/d) than in the 24-HDR (1·8 mg/d), as well as their major food source citrus fruit + juices (61·7 *v.* 7·5 g/d) (Table 2). In the DQ, the median intake of hesperetin compounds (15·8 mg/d) was more abundant than naringenin compounds (4·7 mg/d). Hesperidin (15·6 mg/d) and narirutin (2·7 mg/d) were the most abundant

Table 2. Dietary intakes of flavanones, glycosides and aglycones and their main food sources and urinary excretions of flavonols in the European Prospective Investigation into Cancer and Nutrition (EPIC) study (*n* 475)
(Medians and 10th and 90th percentile (P10 and P90) values)

Variables	LOQ or non-consumers	Median	P10	P90
Urine 24 h (μmol/24 h)				
Naringenin	11	1·63	0·43	9·27
Hesperetin	122	0·99	0·15	8·08
Total flavanones	–	2·82	0·65	19·68
Acute flavanone intake (mg/d)				
Naringenin	294	0·00	0·00	0·48
Naringenin 7-O-glucoside	192	0·04	0·00	0·34
Naringin	191	0·00	0·00	3·00
Narirutin	255	0·00	0·00	12·04
Naringenin compounds*	191	0·63	0·00	12·50
Naringenin aglycones†	191	0·35	0·00	5·89
Hesperetin	264	0·00	0·00	0·57
Hesperidin	241	0·00	0·00	78·42
Neohesperidin	312	0·00	0·00	0·02
Hesperetin compounds*	241	0·49	0·00	78·63
Hesperetin aglycones†	241	0·46	0·00	38·93
Total flavanones*	191	1·82	0·00	102·61
Total flavanone aglycones†	191	1·07	0·00	50·54
Acute food intake (g/d)				
Fruit	77	254·1	0·0	485·7
Citrus fruit	290	0·0	0·0	123·5
Citrus juices	344	0·0	0·0	121·8
Citrus fruit + citrus juices	191	7·5	0·0	203·3
Habitual flavanone intake (mg/d)				
Naringenin	5	0·03	0·00	0·19
Naringenin 7-O-glucoside	0	0·05	0·02	0·18
Naringin	1	1·68	0·04	11·35
Narirutin	0	2·66	0·40	12·29
Naringenin compounds*	0	4·70	1·09	25·10
Naringenin aglycones†	0	2·23	0·53	11·86
Hesperetin	264	0·03	0·00	0·15
Hesperidin	241	15·56	2·45	63·54
Neohesperidin	312	0·04	0·01	0·29
Hesperetin compounds*	0	15·77	2·50	63·96
Hesperetin aglycones†	0	7·83	1·25	31·68
Total flavanones*	0	20·97	4·06	86·39
Total flavanone aglycones†	0	10·27	2·06	42·49
Habitual food intake (g/d)				
Fruit	0	233·5	75·9	485·7
Citrus fruit	0	36·0	3·7	123·5
Citrus juices	16	4·5	0·0	121·8
Citrus fruit + citrus juices	12	61·7	10·7	203·3

LOQ, limit of quantification.
* Free forms + glycosides expressed as such.
† Free forms + glycosides expressed as aglycones.

glycosides of hesperetin and naringenin, respectively. In the 24-HDR, naringenin (0·63 mg/d) and hesperetin (0·59 mg/d) compounds were similarly consumed.

Weak Spearman's correlation coefficients between urinary flavanone excretion and their acute and habitual intake ($R = 0{\cdot}15$–$0{\cdot}20$) were observed (Table 3). Urinary naringenin and hesperetin were highly correlated with citrus fruit + juice intake in the 24-HDR ($R \sim 0{\cdot}6$) but weakly correlated in the DQ ($R \sim 0{\cdot}25$). Conversely, correlations between dietary flavanones and citrus fruit + juice consumption were higher in habitual intakes ($R = 0{\cdot}65$–$0{\cdot}69$) than in acute intakes ($R = 0{\cdot}22$–$0{\cdot}32$).

The results of the Spearman's partial correlations adjusted for sex, BMI, age, smoking status and total energy intake (Table 4) were similar to the simple Spearman's correlations (Table 3). Weak partial correlation coefficients between urinary flavanone concentrations and both acute and habitual dietary flavanone intakes ($R_{partial} = 0{\cdot}14$–$0{\cdot}17$) were observed. Partial correlations were stronger between urinary flavanone excretions and acute intakes ($R_{partial} \sim 0{\cdot}6$) than with habitual intakes ($R_{partial} \sim 0{\cdot}24$).

Discussion

In the present study, 24-h urine concentrations of flavanones were weakly correlated with acute and habitual dietary flavanones. Partial and simple correlations were high between urinary flavanone levels and acute intake of citrus fruits and juices and relatively low with habitual intakes of citrus fruits and citrus juices. These differences between correlations with flavanones or flavanone-rich foods (citrus fruits and juices) could be due to inaccuracies in the estimation of flavanone intake, seasonality of citrus consumption and differences in bioavailability of flavanones varying between food sources.

In a validation study, the flavanone biomarker should be compared with the true value, but these data are not available, and for this reason, nutritional biomarkers are often compared with self-reported questionnaires[14]. Thus, if both measurements are significantly correlated, it is assumed that they can be used equivalently and therefore, urinary levels of flavanones can be valid biomarkers of flavanone intakes. In contrast, nothing can be learned from a lack of correlation, which can be explained either by limitations in the value of the biomarkers or by errors in self-reported intake measurement. Despite efforts to estimate the flavanone intake accurately using the available DQ and food composition tables, results presented in the present study may be influenced by random and systematic errors in the dietary assessment using questionnaires, which may weaken our correlations. Nevertheless, the 24-HDR was collected using a standardised software[21], and the DQ were validated and centre/country-specific[22]. A possible error in the estimation of intake could be linked to the variability of the composition of flavanones in foods. However to mitigate this limitation, food composition data in Phenol-Explorer are the means of various food composition data, for example, 4·5–73·0 mg/100 g concentration values for hesperidin in blond orange juice[2]. Flavanone biomarkers also have their limitations. Enzymatic hydrolysis could negatively affect the recovery of urinary concentrations of polyphenol conjugates[27]; however, the conditions of enzymatic hydrolysis were optimised, testing different enzyme preparations, incubation times and enzyme quantities, and the method for polyphenol estimation was validated based on the criteria of the Food and Drug Administration guidelines[26]. Finally, the use of one single 24-h urinary sample does not consider within-person variability and is as such potentially less reliable for assessing habitual exposures, unless the food sources are regularly consumed or unless the biomarker has a relatively long half-life. Indeed, several studies have been able to find associations between habitual polyphenol intake and 24-h urinary samples, such as resveratrol[28] and isoflavones[29].

In our study, 24-h urinary concentrations of naringenin, hesperetin and total flavanones were poorly correlated with acute

Table 3. Spearman's correlations between urinary flavanones concentrations and intakes of glycosides and their main food sources estimated with 24-h dietary recall (acute intake) and dietary questionnaire (habitual intake) in the European Prospective Investigation into Cancer and Nutrition (EPIC) study

	Urinary naringenin	Urinary hesperetin	Urinary flavanones	Total naringenin glucosides	Naringenin aglycone	Total hesperetin glucosides	Hesperetin aglycone	Total flavanone glucosides	Total flavanone aglycone	Total fruit	Citrus fruit	Citrus juices	Citrus fruits + citrus juices
Acute intake													
Urinary naringenin	1	0·79***	0·96***	0·16**	0·16**	0·14**	0·14**	0·16***	0·16***	0·33***	0·56***	0·15**	0·58***
Urinary hesperetin		1	0·90***	0·13**	0·12**	0·18***	0·18***	0·16**	0·16**	0·28***	0·60***	0·15**	0·62***
Urinary flavanones			1	0·17***	0·17***	0·17***	0·17***	0·18***	0·18***	0·31***	0·60***	0·17***	0·63***
Naringenin compounds†				1	1·00***	0·72***	0·73***	0·90***	0·90***	0	0	0·49***	0·22***
Naringenin aglycones‡					1	0·71***	0·72***	0·89***	0·89***	0	0	0·48***	0·22***
Hesperetin compounds†						1	1·00***	0·93***	0·93***	0	0	0·68***	0·32***
Hesperetin aglycones‡							1	0·93***	0·94***	0	0	0·67***	0·32***
Total flavanones†								1	1·00***	0	0	0·64***	0·29***
Total flavanone aglycones‡									1	0	0	0·63***	0·29***
Total fruit										1	0·50***	0	0·39***
Citrus fruit											1	0	0·80***
Citrus juices												1	0·49***
Citrus fruit + citrus juices													1
Habitual intake													
Urinary naringenin	1	0·79***	0·96***	0·20***	0·20***	0·19***	0·19***	0·20***	0·20***	0·14**	0·21***	0·13**	0·26***
Urinary hesperetin		1	0·90***	0·15**	0·15**	0·17***	0·17***	0·17***	0·17***	0·13**	0·17***	0·15**	0·22***
Urinary flavanones			1	0·19***	0·20***	0·20***	0·20***	0·20***	0·20***	0·14**	0·20***	0·15**	0·26***
Naringenin compounds†				1	1·00***	0·91***	0·91***	0·95***	0·95***	0·21***	0·28***	0·52***	0·65***
Naringenin aglycones‡					1	0·90***	0·90***	0·95***	0·95***	0·21***	0·28***	0·51***	0·65***
Hesperetin compounds†						1	1·00***	0·99***	0·99***	0·17***	0·25***	0·55***	0·66***
Hesperetin aglycones‡							1	0·99***	0·99***	0·17***	0·25***	0·55***	0·66***
Total flavanones†								1	1·00***	0·22***	0·28***	0·57***	0·69***
Total flavanone aglycones‡									1	0·22***	0·28***	0·57***	0·69***
Total fruit										1	0·74***	–0·19***	0·47***
Citrus fruit											1	–0·15**	0·70***
Citrus juices												1	0·45***
Citrus fruit + citrus juices													1

** $P < 0·01$, *** $P < 0·001$.
† Free forms + glycosides expressed as such.
‡ Free forms + glycosides expressed as aglycones.

Table 4. *R* partial coefficients between the intake of dietary flavanones (mg/d) or foods (g/d) and urinary flavanone concentrations (μmol/24 h) in the European Prospective Investigation into Cancer and Nutrition (EPIC) study†

	Urinary naringenin	Urinary hesperetin	Urinary flavanones
Acute intake			
Naringenin compounds‡	0·14**		
Naringenin aglycones§	0·14**		
Hesperetin compounds‡		0·16**	
Hesperetin aglycones§		0·16**	
Total flavanones‡			0·15**
Total flavanone aglycones§			0·16**
Total fruit	0·24***	0·23***	0·26***
Citrus fruit	0·55***	0·60***	0·59***
Citrus juices	0·16**	0·16**	0·17***
Citrus fruit + citrus juices	0·58***	0·62***	0·63***
Habitual intake			
Naringenin compounds‡	0·17***		
Naringenin aglycones§	0·17***		
Hesperetin compounds‡		0·16**	
Hesperetin aglycones§		0·16**	
Total flavanones‡			0·17***
Total flavanone aglycones§			0·17***
Total fruit	0·11*	0·12**	0·12**
Citrus fruit	0·22***	0·22***	0·20***
Citrus juices	0·14**	0·13**	0·16**
Citrus fruit + citrus juices	0·25***	0·23***	0·24***

* $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
† Spearman's correlation model adjusted for centre, sex, age, BMI, smoking status and total energy intake.
‡ Free form + glycosides expressed as such.
§ Free form + glycosides expressed as aglycones.

naringenin, hesperetin and total flavanones intake, respectively (all $R_{partial} \sim 0·16$). Results were identical when flavanones were measured as they are found in nature (mainly as glycosides) or expressed as aglycones. A systematic review has examined all studies assessing urinary polyphenols as biomarkers of polyphenol intake, including flavanones[17]. Naringenin was investigated in six intervention studies using pure compounds (e.g. naringin and naringenin) or flavanone-rich foods, such as grapefruit juice, orange juice, decoction honey-treated *Pericarpium citrigrandis*. The average correlation coefficient was modest (*r* 0·32) but higher than that in our observational study. For hesperetin, seven intervention studies were reported and the mean correlation was even higher (*r* 0·52), although the authors of the review concluded that urinary concentrations of flavanones were weak biomarkers of the intake[17]. To our knowledge, the present study is the first one investigating the correlation between habitual flavanone intake and urinary flavanone concentrations, showing similar low correlations as for acute intake. It is important to highlight that the correlation between acute and habitual flavanone intake was also low ($R = 0·20$) as well as for citrus intake ($R = 0·30$). This could be explained by the seasonal and, in some cases, sporadic consumption of flavanones and flavanone-rich foods. Thus, the number of non-consumers of flavanones was higher in the 24-HDR (*n* 191) than in the DQ (*n* 0), similar to citrus fruit + juices (*n* 191 *v.* 12). Other issues could be the different main food sources of flavanones between the DQ (blond orange juices and oranges) and the 24-HDR (fruit and juices of blond oranges, grapefruit, blood orange, lemon and tangerine) and the variability of the content of flavanones in foods[2].

Flavanones are typically found in citrus fruits and their derived products such as juices and jams. Citrus contribute to more than 90 % of total flavanones in the EPIC-Europe study[10]. Thus, their intake strongly depends on the consumption of these fruits (including juices). In the EPIC-Europe cohort, Spain had the highest flavanone consumption because of high citrus fruit intakes[10]. In South European region, including Italy and Greece, flavanone intake was moderate (ranging between 25·1 and 37·1 mg/d) and citrus fruit was also the main food source, contributing to 70 % of total flavanones. However, Central European region (such as Germany and France) had a high flavanone intake (from 33·1 to 46·5 mg/d), but it was due to the citrus-based fruit juices (60 %)[10]. It is important to bear in mind that concentrations of flavanones are lower in orange fruit compared with orange juice[1]. Moreover, bioavailability of flavanones from juices is twice higher than from fruit, probably because they are entrapped in the fibre-rich matrix of the fruit[30].

In the present study, we found high partial correlations between 24-h urinary concentrations of naringenin, hesperetin and especially total flavanones and the acute consumption of total citrus (fruits and juices) ($R_{partial} \sim 0·6$), estimated using a standardised 24-HDR. Similar correlations have been previously reported by Mennen *et al.*[18], who examined the associations between acute dietary intakes and levels of urinary polyphenols of fifty-three participants of the SU.VI.MAX study using a 2-d dietary record. Correlation coefficients between concentrations of naringenin and hesperetin in 24-h urine and intake of citrus fruit and juices ranged between 0·37 and 0·46. Similarly, in a Norwegian study including 119 pregnant women, a correlation between 24-h urinary concentration of hesperetin and citrus fruits and juice consumption measured with a 4-d weighed food diary was observed[31]. However, in a small intervention study, Erlund *et al.* showed that after the orange or grapefruit juice, urinary concentrations of flavanones varied a lot between subjects (the C_{max} for naringenin from grapefruit juice varies between 0·7 and 14·8 μmol/l), and therefore they concluded that flavanone concentrations in the urine are poor biomarkers of dietary intake[32]. In contrast, in a small French study, correlations between two consecutive days were moderate: 0·50 for hesperetin and 0·27 for naringenin[33]. Similarly, a pharmacokinetic study reported moderate intra- and inter-individual variation for hesperetin (CV = 1·21 and 2·28, respectively)[34]. Urinary concentrations of flavanones have been revealed as useful biomarkers of acute intake of flavanone-rich foods with correlation coefficients moderate to high, and intra- and inter-person variability was relatively weak.

It is clear that urinary flavanone excretions increase with the intake of fruits and vegetables, particularly citrus fruits[35]. In our study, weak correlations between 24-h urinary levels of naringenin, hesperetin and total flavanones and habitual citrus fruit and juice intakes were observed ($R_{partial}$ approximately 0·24). Slightly higher results were reported by Krogholm *et al.* in 191 participants of the Inter99 cohort in Denmark. Correlation coefficients between 24-h urine samples and habitual intake of citrus fruits and juices, assessed using a FFQ, were 0·30 for naringenin, 0·27 for hesperetin and 0·32 for citrus flavonoids[36]. As expected, correlations with habitual citrus intake were lower than its acute

intake due to the seasonality and sporadicity of the consumption of citrus, especially citrus fruits.

Another potential biomarker of citrus consumption is proline betaine, which has received a lot of attention recently. In an acute intervention study, Heinzmann *et al.* found proline betaine as a biomarker of citrus intake (sensibility 86·3 % and specificity 90·6 %)[37]. Lloyd *et al.* also identified proline betaine as a citrus biomarker of an acute breakfast[38]. Unfortunately, we did not analyse proline betaine in our study, so we cannot compare both potential biomarkers.

In conclusion, urinary excretion of flavanones was strongly correlated with the acute consumption of citrus fruits and juices and weakly correlated with their habitual intake. Therefore, flavanones can be considered good short-term biomarkers of citrus intake. Correlations between naringenin, hesperetin and flavanone intakes and urinary flavanone concentrations were low. It probably means that the estimation of flavanone intake may be inaccurate, since the correlations with citrus fruit and juices are much higher than with flavanones, especially for acute intakes. For assessing habitual exposures, multiple urinary collections may be needed. Thus, studies on associations between flavanone exposure and diseases should be best evaluated using a combination of dietary and biomarker data in order to confirm preliminary results, which were obtained using solely DQ.

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A. S. and R. Z.-R. designed the research; D. A. carried out the laboratory analyses; I. T. and V. C. performed the statistical analyses; I. T., Y. G.-A. and R. Z.-R. drafted the manuscript; A. M. and A. S. largely contributed to the interpretation of the results; F. R. M., Y. M.-S., M.-C. B.-R., T. K., V. K., H. B., A. T., A. K., E. V., D. P., S. S., M. S. d. M., R. T., A. M., I. H. and A. A. provided data and biological samples. All authors reviewed, edited and approved the final manuscript.

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