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Urinary flavonoids and phenolic acids as biomarkers of intake for polyphenol-rich foods

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Estimation of dietary intake of polyphenols is difficult, due to limited availability of food composition data and bias inherent to dietary assessment methods. The aim of the present study was to evaluate the associations between the intake of polyphenol-rich foods and the urinary excretion of several phenolic compounds and therefore explore whether these phenolic compounds could be used as a biomarker of intake. Fifty-three participants of the SU.VI.MAX study (a randomised primary-prevention trial evaluating the effect of daily antioxidant supplementation on chronic diseases) collected a 24 h urine and a spot urine sample and filled a dietary record during a 2 d period. Thirteen 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, were measured using HPLC–electrospray ionisation–MS–MS. In spot samples apple consumption was positively correlated to phloretin, grapefruit consumption to naringenin, orange to hesperetin, citrus fruit consumption to both naringenin and hesperetin, with *r* coefficients ranging from 0·31 to 0·57 ($P<0·05$). The combination of fruits and/or fruit juices was positively correlated to gallic acid and 4-*O*-methylgallic acid, isorhamnetin, kaempferol, hesperetin, naringenin and phloretin (*r* 0·24–0·44, $P<0·05$). Coffee consumption was positively correlated to caffeic and chlorogenic acids (*r* 0·29 and 0·63, $P<0·05$ respectively). Black tea and wine consumption were positively correlated with gallic and 4-*O*-methylgallic acids (*r* 0·37–0·54, $P<0·001$). The present results suggest that several polyphenols measured in a spot urine sample can be used as biomarkers of polyphenol-rich food intake.

Polyphenols: Flavonoids: Phenolic acids: Biomarker: Urine

Polyphenols are compounds with high antioxidant properties and are probably the most abundant antioxidants in our diet (Scalbert & Williamson, 2000). Both experimental and epidemiological evidence support a role for polyphenols in the prevention of chronic diseases and more particularly CVD and cancer (Scalbert *et al.* 2005). Polyphenol intake has been related to disease in epidemiological studies and especially inverse associations with cardiovascular risk have been observed (Hertog *et al.* 1993*a*; Hirvonen *et al.* 2001; Knekt *et al.* 1996).

Many different types of polyphenol can be found in plant foods, one plant often containing more than one type of polyphenol and progress in epidemiological research on the relation between polyphenol consumption and disease is largely hampered by the lack of complete food composition tables. Although some food composition data are available for polyphenols, especially on the US Department of Agriculture's website, content values for some polyphenol types or foods only consumed in specific countries are still missing. Furthermore, there is no gold standard for collection of dietary intake data, which is always subject to a certain under- or overestimation (Block, 1982; Bingham, 1991). The use of valid biomarkers for intake may be of help where the estimation of dietary intake is particularly difficult (Horner *et al.* 2002); the advantage of biomarkers in dietary assessment being that their random errors are truly random and not dependent on those involved in dietary questionnaires (Bingham, 2002).

So far ideal biomarkers exist for salt and protein intake (sodium/nitrogen measured in a 24 h urine sample) and energy expenditure (the doubly labelled water technique) (Bingham, 2002). This means that with these biomarkers the exact intake of salt and protein and the exact energy expenditure can be calculated. However, collection of 24 h urine is too intensive and the doubly labelled water technique is too expensive for use in large studies. Other biomarkers exist that do not provide information on the exact dietary intake, but which are highly correlated with intake. Examples are the measurement of the fatty acid composition in subcutaneous adipose tissue samples to estimate fatty acid intake, potassium and iodine estimated from urine samples, and measurement of serum carotenoids and vitamin C as biomarkers of fruit and vegetable intake (Walters *et al.* 1973; Plakke *et al.* 1983; Drewnowski *et al.* 1997). We recently showed in a previous study that various polyphenols belonging to the main polyphenol classes can be detected and estimated in urine of free-living subjects. We also showed that their

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daily excretion estimated from 24 h urine samples is correlated to their concentration in spot urine samples. Polyphenol compounds in relatively easily obtained spot urine samples are thus potentially useful biomarkers for polyphenol intake in large epidemiological studies (Noroozi *et al.* 2000).

Few authors have examined the use of urinary or plasmatic concentrations of polyphenols as biomarkers of intake in free-living populations. Isoflavone concentrations in fasting plasma were found to correlate with soy intake calculated from a food-frequency questionnaire (Verkasalo *et al.* 2001; Yamamoto *et al.* 2001; Frankenfeld *et al.* 2003). Correlations between intake of four flavonols and flavanones and their concentration in fasting plasma were also observed in a cohort of fifty-two women following their ordinary diet (Radtke *et al.* 2002). In another study with the same polyphenols, flavanones in fasting plasma appeared to be poor biomarkers of intake (Erlund *et al.* 2002) and this could be explained by the rapid elimination of most polyphenols after intake (Manach *et al.* 2005). We preferred to analyse phenolic compounds in urine samples as their concentrations in urine may better reflect their intake than their concentrations in fasting plasma.

The aim of the present work was therefore to study correlations between the consumption of polyphenol-rich foods and beverages and the concentrations of thirteen polyphenols and metabolites in spot urine samples in a free-living population, and to evaluate whether these polyphenols could be used as a biomarkers of intake for polyphenols or polyphenol-rich foods.

Subjects and methods

Subjects

Subjects were participants of the SU.VI.MAX study, a randomised double-blind placebo-controlled primary-prevention trial evaluating the effect of daily antioxidant supplementation (vitamin C, vitamin E, β-carotene, selenium and zinc) at nutritional doses on the incidence of cancer and ischaemic heart disease. The cohort consisted of women aged 35–60 years (mean 46·4 (SD 6·7) years) and men aged 45–60 years (mean 51·1 (SD 4·7) years) at baseline in 1994 and none of them used vitamin supplements other than those under study. In total 13 077 subjects were included and were followed up for 8 years. Details on recruitment and study design are described elsewhere (Hercberg *et al.* 2004). All SU.VI.MAX subjects living in the Parisian area, who were highly compliant with the total study protocol (completing the dietary record every 2 months, coming to every annual clinical examination) were invited in 2002 to participate in a satellite protocol to evaluate polyphenols in urine as biomarkers of polyphenol intake (*n* 103). Fifty-three of these subjects completed the protocol correctly and were included in the present analyses. Of them, thirty-one were women and twenty-two were men and the mean age was 58 years at the time of the protocol.

The SU.VI.MAX study has been approved by the ethical committee for studies on human subjects (CCPPRB no. 706) of Paris-Cochin Hospital, and the 'Comité National Informatique et Liberté' (CNIL no. 334641), which advocates that all medical information is confidential and anonymous.

Dietary record and urine collection

Subjects were first visited by a trained dietitian to explain the 2 d protocol. On the morning of the first day the subjects started a 2 d dietary record, for which they wrote down everything they ate or drank during the protocol. The collection of the 24 h urine started in the morning of the second day and lasted until the third day at the same hour (Fig. 1). During the second day, three *p*-aminobenzoic acid tablets were taken, one after the first urine collection, one at 16.00 hours and one at 23.00 hours, in order to check the completeness of the 24 h urine collection (Bingham & Cummings, 1983). The recovery of the *p*-aminobenzoic acid in the 24 h urine was 85 % or more for all subjects included in the present analyses. Urine samples showing a lower *p*-aminobenzoic acid recovery were excluded. The spot sample was taken in the morning of the third day before breakfast and was thus part of the 24 h urine collection. On the first day, the subjects did not eat or drink after 23.00 hours. They brought the two tubes of collected urine (spot and mixed 24 h urine) to the study centre after the completion of the 24 h urine collection. The tubes were stored at −20° until measurement of the polyphenols.

Polyphenol measurements

An HPLC–electrospray ionisation–MS–MS offering a high selectivity of detection for a wide range of phenolic compounds and short run times was developed (Ito *et al.* 2005). In brief, urine samples (250 μl) were supplemented with an internal standard (taxifolin), and incubated with deconjugating enzymes to hydrolyse glucuronides and sulphate esters. The freed aglycones were extracted with ethyl acetate. The organic extracts were redissolved in 25 % aqueous methanol and injected into the HPLC–ESI–MS–MS system using a short Zorbax Eclipse XDB-C18 (2·1 i.d. × 30 mm, 3·5 μm; Agilent Palo Alto, US) and an API-2000 (Applied Biosystems, Ontario, Canada) mass spectrometer. A 4 min gradient of water–formic acid (100:0·1) and acetonitrile–water–formic acid (95:5:0·1) was applied. The whole cycle, including elution and re-equilibration of the column, did not exceed 6 min per sample. Mass detection was carried out with negative ionisation in multiple reaction monitoring mode. Calibration curves were prepared by spiking blank urine with aliquots of standard mixture solutions with duplicated injections at each concentration level.

Statistical analysis

Spearman rank correlation coefficients were calculated to evaluate the relation between the spot and 24 h urine sample and food intake for those variables where a possible association could be

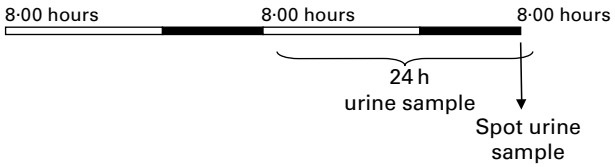

Fig. 1. Time scheme for urine collection during the 2 d food record period. For details of procedures, see this page. □, day ■, night.

expected. Kappa coefficients were calculated to evaluate the agreement across tertiles of polyphenol concentration and food intake. The analyses included the total number of subjects, including non-consumers of the different food products.

Results

Consumption of the main dietary sources of polyphenols was recorded by the fifty-three subjects during the 2 d of the experiment. Almost all subjects consumed fruit, vegetables and coffee (Table 1). Some other foods were eaten by only a few subjects (grapefruit and orange). Thirteen phenolic compounds were estimated in the urine collected by these fifty-three subjects (Table 2). Some of these phenolic compounds are metabolites of dietary polyphenols formed either in the human tissues by methylation (4-*O*-methylgallic acid from gallic acid and isorhamnetin from quercetin) whereas others are formed by the gut microflora (*m*-coumaric acid from caffeic acid, enterolactone and enterodiol from either lignans or lignins). In the 24 h urine samples, enterolactone was found in the highest quantity, followed by caffeic acid and naringenin, whereas isorhamnetin, quercetin and kaempferol were found in the lowest concentration (Table 2). In the spot urine samples enterolactone was also the most abundant, followed by chlorogenic acid and naringenin. Correlations between excretion of these polyphenols and the consumption of polyphenol-rich foods were studied using either 24 h urine samples or spot urine samples. Polyphenols measured in the 24 h urine samples were all well correlated to those measured in the spot sample (r 0·42–0·74, $P<0·0001$ to $<0·02$), with the exception of caffeic acid (r 0·04, $P=0·75$).

Table 1. Mean food intake (g/d) for the total cohort and for consumers of major polyphenol-rich foods*

(Mean values and standard deviations)

	Total (n 53)		Non-consumers excluded		
Food	Mean	SD	Mean	SD	n
Apple	58·7	113·5	239·2	95·0	13
Peach	34·1	55·1	95·1	51·6	19
Red fruits	47·5	128·7	228·6	201·5	11
Grapefruit	18·9	78·6	333·3	57·7	3
Grapefruit juice	17·5	81·1	231·3	219·3	4
Orange	10·8	44·3	190·0	0	3
Orange juice	37·1	87·7	196·5	97·9	10
Citrus fruits	31·5	88·3	238·6	99·9	7
Citrus fruits + juices	92·0	172·6	203·2	209·2	24
Fruits	267·7	228·8	322·5	212·7	44
Fruit juices	64·3	119·7	162·2	143·5	21
Fruits + fruit juices	336·7	265·4	237·1	254·2	48
Vegetables	277·0	180·0	287·8	174·6	51
Onion	6·3	18·4	41·9	28·7	8
Fruits + vegetables	544·7	330·5	544·7	330·5	53
Dark bread	18·8	38·3	82·9	33·6	12
Cereals	33·7	47·6	68·8	47·1	26
Potatoes	56·4	87·8	130·0	90·8	23
Chocolate	11·4	26·9	26·4	36·1	23
Coffee	250·0	262·6	331·3	253·7	40
Black tea	259·9	367·6	510·2	371·1	27
Herbal tea	25·7	78·8	226·7	71·7	6
Wine	104·2	136·2	240·2	98·6	23

* Means are calculated over the 2 d food record period. For details of procedures, see p. 192.

Table 2. Mean amount of polyphenols in 24 h urine (μmol/d) and in the spot sample (μmol/l) collected from fifty-three free-living subjects*

(Mean values and standard deviations)

Polyphenol		Mean	SD
Chlorogenic acid	24 h	4·2	7·8
	Spot	6·6	6·8
Caffeic acid	24 h	11·4	8·5
	Spot	6·2	2·4
m-Coumaric acid	24 h	5·4	13·1
	Spot	2·7	6·2
Gallic acid	24 h	1·6	2·4
	Spot	1·1	1·3
4-*O*-Methylgallic acid	24 h	6·1	10·2
	Spot	4·4	6·8
Quercetin	24 h	0·7	0·5
	Spot	0·4	0·4
Isorhamnetin	24 h	0·5	0·5
	Spot	0·3	0·3
Kaempferol	24 h	0·8	2·0
	Spot	0·4	1·0
Enterolactone	24 h	23·9	26·6
	Spot	10·7	8·8
Enterodiol	24 h	2·0	3·3
	Spot	0·8	1·0
Hesperetin	24 h	3·0	15·2
	Spot	1·8	5·1
Naringenin	24 h	9·9	23·9
	Spot	5·6	13·3
Phloretin	24 h	0·73	1·9
	Spot	0·42	0·5
Total flavonoids†	24 h	22·6	60·8
	Spot	15·4	48·9
Vegetable polyphenols‡	24 h	24·7	27·4
	Spot	11·1	9·0
Fruit polyphenols§	24 h	22·0	60·8
	Spot	15·0	49·0

* For details of procedures, see p. 192.
† Quercetin + isorhamnetin + phloretin + naringenin + hesperetin + kaempferol.
‡ Enterolactone + kaempferol.
§ Hesperetin + narangenin + kaempferol + phloretin.

Urine samples (24 h)

Gallic acid was higher in wine consumers compared to non-consumers (mean 3·2 μg/d *v.* 0·4 μg/d, $P<0·0001$). The same was true for the difference in 4-*O*-methylgallic acid (mean 11·1 μg/d *v.* 2·3 μg/d, $P=0·004$). Also the concentration of naringenin found in urine was higher in consumers of citrus fruits (juices included) than in non-consumers (mean 18·3 μg/d *v.* 3·0 μg/d, $P=0·04$). No other significant differences in polyphenols between consumers and non-consumers were observed.

Correlations between excretion of specific phenolic compounds and intake of particular foods were studied based on their known occurrence in these foods (Table 3). Apple consumption is correlated with *m*-coumaric acid, isorhamnetin, kaempferol and phloretin; orange consumption with hesperetin; citrus fruit and juice intake and total fruit juice intake with hesperetin and naringenin; and fruit and fruit juice intake with naringenin and phloretin. Total fruit intake is associated with kaempferol. Vegetable consumption was positively correlated to enterolactone concentrations and wine consumption to *m*-coumaric acid, gallic and 4-*O*-methylgallic acid (Table 4). For the statistically significant correlations between foods and 24 h urine polyphenol concentrations, kappa coefficients were

Table 3. Correlation between fruit consumption and 24 h urinary polyphenols (μg/d)*
(Spearman rank correlation coefficients and *P* values)

	CGA		CA		mCOU		GA		MeGA		Q		MeQ		K		HESP		NAR		PHLOR		ENL		END	
Fruit	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Apple			0·12	0·39	0·36‡	0·009	0·08	0·55	0·07	0·60	0·25	0·07	0·31‡	0·02	0·45†	0·0007					0·35‡	0·01				
Peach	−0·02	0·87	0·005	0·97	0·02	0·91	−0·15	0·28	−0·14	0·33	−0·09	0·50	−0·09	0·50	0·01	0·92					−0·05	0·74	0·24	0·08	0·009	0·95
Red fruits	0·08	0·55	−0·03	0·81	−0·13	0·35	0·06	0·70	−0·08	0·57	−0·07	0·63	−0·002	0·99	0·19	0·17					0·04	0·78	−0·11	0·44	−0·22	0·12
Grapefruit			−0·06	0·70	0·06	0·68									−0·11	0·43	0·18	0·21	0·20	0·15	0·14	0·33				
Grapefruit juice			0·13	0·36	0·06	0·65									0·04	0·80	−0·06	0·68	0·18	0·21	0·04	0·80				
Orange			−0·15	0·27	−0·11	0·43									−0·11	0·43	0·35‡	0·01	0·08	0·57	−0·03	0·85				
Orange juice			−0·08	0·55	0·04	0·77									−0·12	0·40	0·18	0·21	0·18	0·20	0·09	0·53				
Citrus fruits			−0·11	0·42	−0·09	0·51									−0·17	0·21	0·45‡	0·0007	0·28‡	0·04	0·09	0·54				
Citrus fruits + juices			−0·06	0·69	0·04	0·78									−0·19	0·18	0·46†	0·0006	0·37†	0·007	0·11	0·46				
Fruits	−0·07	0·61	−0·15	0·28	−0·06	0·68	0·06	0·67	0·02	0·90	−0·02	0·87	0·16	0·26	0·27‡	0·05	0·23	0·10	0·19	0·18	0·21	0·14	0·07	0·60	−0·10	0·47
Fruit juices	−0·02	0·87	−0·01	0·93	0·10	0·47	0·18	0·19	0·16	0·24	0·10	0·49	0·14	0·32	−0·12	0·37	0·28	0·04	0·37†	0·007	0·16	0·25	0·11	0·41	0·05	0·72
Fruits + fruit juices	−0·11	0·43	−0·17	0·23	−0·07	0·61	0·10	0·49	0·02	0·87	0·01	0·94	0·19	0·17	0·21	0·13	0·25	0·07	0·35	0·01	0·30	0·03	0·09	0·52	−0·02	0·86

CA, caffeic acid; CGA, chlorogenic acid; END, enterodiol; ENL, enterolactone; GA, gallic acid; HESP, hesperetin; K, kaempferol; mCOU, *m*-coumaric acid; MeGA, 4-*O*-methylgallic acid; MeQ, isorhamnetin; NAR, naringenin; PHLOR, phloretin; Q, quercetin.
* For details of procedures, see p. 192.
† Kappa coefficient $P < 0{\cdot}05$.
‡ Kappa coefficient was not calculated because of unequal numbers in rows and columns.

Table 4. Correlation between consumption of vegetables, cereals, potatoes, chocolate and beverages and 24 h urinary polyphenols (μg/d)*
(Spearman rank correlation coefficients and *P* values)

	CGA		CA		mCOU		GA		MeGA		Q		MeQ		K		ENL		END	
Food	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Vegetables	−0·14	0·32	−0·05	0·71	−0·10	0·47	−0·26	0·06	−0·23	0·10	0·02	0·89	−0·03	0·85	0·17	0·22	0·31†	0·02	−0·04	0·79
Onion			−0·13	0·36	0·05	0·74	−0·10	0·47	−0·21	0·13	0·05	0·72	−0·05	0·73	−0·06	0·70				
Dark bread			−0·20	0·16											−0·009	0·95	0·18	0·19	0·24	0·08
Cereals			−0·08	0·56											−0·07	0·63	−0·002	0·99	0·06	0·69
Potatoes	0·10	0·46	0·02	0·90											0·03	0·84	0·06	0·67	−0·06	0·65
Chocolate			−0·04	0·79	0·17	0·22	−0·09	0·50	−0·07	0·64					0·05	0·73				
Coffee	0·24	0·09	0·24	0·08											0·02	0·90				
Black tea	−0·13	0·37	−0·22	0·11	−0·14	0·32	0·06	0·69	−0·006	0·97	−0·10	0·48	−0·006	0·97	−0·03	0·85				
Herbal tea	−0·13	0·36	0·02	0·89	0·21	0·14	−0·02	0·89	−0·27	0·05	0·10	0·49	0·04	0·79	0·15	0·27				
Wine	0·25	0·07	0·38†	0·005	0·18	0·19	0·70†	<0·0001	0·52†	<0·0001					0·11	0·42				

CA, caffeic acid; CGA, chlorogenic acid; END, enterodiol; ENL, enterolactone; GA, gallic acid; HESP, hesperetin; K, kaempferol; mCOU, *m*-coumaric acid; MeGA, 4-*O*-methylgallic acid; MeQ, isorhamnetin; NAR, naringenin; PHLOR, phloretin; Q, quercetin.
* For details of procedures, see p. 192.
† Kappa coefficient $P < 0{\cdot}05$.

mostly statistically significant, ranging from 0·23 to 0·62, indicating an acceptable agreement between consumption and urine measurement.

Correlations with food intake and excretion of a combination of polyphenols were also studied. Urinary excretion of enterolactone + kaempferol, both known to be present or formed after vegetable consumption, were positively related to vegetable intake (r 0·34, $P=0·01$) with a statistically significant kappa coefficient (κ 0·23; 95 % CI 0·02, 0·45). Urinary excretion of isorhamnetin + hesperetin + naringenin + kaempferol + phloretin, all present in fruits or formed after fruit consumption, were positively related to intake of fruits (r 0·27, $P=0·06$), fruit juices (r 0·28, $P=0·04$) and the total of fruits and fruit juices (r 0·38, $P=0·006$). Kappa coefficient was only statistically significant for fruit juices (κ 0·24; 95 % CI 0·04, 0·45) and the total of fruits and fruit juices (κ 0·21; 95 % CI 0·001, 0·41).

Spot urine samples

When evaluating the spot urine samples only phloretin remained positively correlated to apple consumption (Table 5). Red fruit consumption was positively associated with kaempferol, but inversely with *m*-coumaric acid. The positive correlations between hesperetin and naringenin and the different citrus fruits were stronger for the spot samples than for the 24 h samples. The consumption of orange, and thereby of total citrus fruits, was also correlated to caffeic acid. The combination of fruits and/or fruit juices was positively correlated to gallic and 4-*O*-methylgallic acid, isorhamnetin, kaempferol, hesperetin, naringenin and phloretin. Coffee consumption was positively correlated to caffeic and chlorogenic acid, the latter being inversely correlated to black tea consumption (Table 6). Black tea consumption was furthermore inversely associated to *m*-coumaric acid and positively with gallic and 4-*O*-methylgallic acid. Wine was also positively associated to these two latter polyphenols. For the statistically significant correlations between foods and urine polyphenol concentrations kappa coefficients were mostly statistically significant, ranging from 0·26 to 0·49, for those correlations where these statistics could be calculated.

Correlations between the sum of selected phenolic compounds and food consumption was also examined. The sum of isorhamnetin + hesperetin + naringenin + kaempferol + phloretin was positively associated to intake of fruits (r 0·34, $P=0·01$), fruit juices (r 0·44, $P=0·001$) and the total of fruits and fruit juices (r 0·47, $P=0·0004$). The kappa coefficient was only statistically significant for fruit juices (κ 0·36; 95 % CI 0·17, 0·55) and the total of fruits and fruit juices (κ 0·26; 95 % CI 0·06, 0·46), but these statistics were difficult to interpret due to the low number of observations in each cell.

Discussion

The present study shows various correlations between the consumption of polyphenol-rich foods or beverages and urinary excretion of polyphenols. Such a link has been well established in controlled intervention studies with specific foods, but few studies have examined these correlations in free-living populations. The present results suggest that phenolic compounds in spot urine samples may be useful biomarkers of polyphenol intake in an epidemiological setting. Phenolic compounds considered here were selected for their widespread occurrence in the human diet, and for being representative of the main types of polyphenol class (Ito *et al.* 2005). Isoflavones were not included due to the low consumption in France of soy-containing products, the main dietary origin of isoflavones. Furthermore, catechins were not included due to a too limited sensitivity of the analytical method.

Most observed correlations between urinary excretion of polyphenols and food intake were expected from their content in food and established recovery in urine in intervention studies with specific foods (Manach *et al.* 2004). The main dietary source of phloretin is apple (Spanos & Wrolstad, 1992) and this explains the correlation between phloretin excretion and apple consumption. Naringenin is the main flavonoid of grapefruit (Rousseff *et al.* 1987). It is also present in orange and orange juice, but in lower amounts than hesperetin, the main flavanone in orange. Both have been found in urine after grapefruit and orange juice administration (Ameer *et al.* 1996; Erlund *et al.* 2001; Ito *et al.* 2005). As expected, naringenin excretion correlates with grapefruit and grapefruit juice intake, and hesperetin with orange and orange juice intake. Both also correlate with intake of citrus fruits and citrus juices.

Gallic acid is a common constituent of wine and tea (Landrault *et al.* 2001; Hodgson *et al.* 2004). Correlations between urinary excretion of gallic acid and its metabolite 4-*O*-methylgallic acid and intake of these beverages were clearly observed in the present work. Positive correlations with tea consumption have been previously observed in two studies evaluating long-term and acute black tea consumption (Hodgson *et al.* 2000, 2004). The same metabolites were also recovered in urine after wine ingestion (Caccetta *et al.* 2000; Cartron *et al.* 2003). Quercetin is present in the skin of apple and in the Netherlands apple has been identified as a main dietary source of quercetin together with tea (Hertog *et al.* 1993*b*). We could only observe a weak correlation between isorhamnetin, the O-methylated metabolite of quercetin, in 24 h urine and apple intake. Phloretin therefore appears to be a better biomarker than quercetin or isorhamnetin for apple intake.

4-*O*-Methylgallic acid and isorhamnetin are, respectively, the two major metabolites of gallic acid and quercetin, formed by methylation in various human tissues and more particularly in the liver (Manach *et al.* 2004). Strong correlations were observed between each polyphenol and its methylated metabolite (r 0·75, $P=0·0001$ and r 0·83, $P=0·0001$ for gallic acid and quercetin, respectively).

Chlorogenic acid is a major constituent of coffee (Clifford, 1999). Chlorogenic acid has been detected in urine after consumption of coffee (Ito *et al.* 2005). A strong correlation is observed here between urinary excretion of chlorogenic acid in the spot urine sample and coffee consumption. A correlation is also observed with caffeic acid. However, it is weaker than for chlorogenic acid, due to some variations in the yield of hydrolysis between subjects and samples (Ito *et al.* 2005). Enterolactone was positively associated with vegetable intake, as has been previously reported (Lampe *et al.* 1999).

Inverse associations can mainly be explained by simultaneous non-consumption of other foods; coffee drinkers for example will not drink tea (Mennen *et al.* 2003), and this explains the inverse correlation coefficients between tea consumption and chlorogenic and *m*-coumaric acid. Similarly, the positive association between caffeic acid and wine in the

Table 5. Correlation between fruit consumption and polyphenol concentrations (μM) measured in spot samples*
(Spearman rank correlation coefficients and *P* values)

	CGA		CA		mCOU		GA		MeGA		Q		MeQ		K		HESP		NAR		PHLOR		ENL		END	
Fruit	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Apple			−0·06	0·66	0·19	0·18	0·003	0·98	−0·04	0·79	−0·03	0·86	−0·02	0·89	0·14	0·33					0·60‡	<0·0001				
Peach	−0·09	0·51	0·09	0·50	−0·20	0·15	−0·06	0·66	−0·05	0·70	0·06	0·65	0·04	0·78	0·12	0·39					−0·007	0·96	0·24	0·08	−0·04	0·77
Red fruits	−0·10	0·47	0·05	0·73	−0·34	0·01	−0·002	0·98	0·02	0·90	−0·15	0·29	0·03	0·85	0·28	0·04					−0·01	0·92	−0·03	0·86	−0·19	0·17
Grapefruit			0·12	0·40	−0·07	0·63									0·05	0·73	0·11	0·45	0·31‡	0·02	0·06	0·68				
Grapefruit juice			0·13	0·34	−0·08	0·59									0·16	0·25	−0·11	0·41	0·36‡	0·008	0·14	0·33				
Orange			0·37‡	0·007	0·20	0·15									−0·12	0·38	0·40‡	0·003	0·26	0·06	−0·05	0·72				
Orange juice			−0·18	0·19	−0·06	0·66									−0·04	0·79	0·46‡	0·0006	0·21	0·13	−0·06	0·66				
Citrus fruits			0·27‡	0·05	0·08	0·59									−0·07	0·61	0·42‡	0·002	0·48‡	0·0003	0·01	0·95				
Citrus fruits + juices			0·04	0·75	0·02	0·90									−0·07	0·64	0·52†	<0·0001	0·56†	<0·0001	−0·02	0·92				
Fruits	−0·10	0·48	0·04	0·79	−0·26	0·06	−0·007	0·96	0·05	0·72	−0·21	0·14	0·10	0·47	0·30‡	0·03	0·22	0·11	0·28	0·04	0·28	0·04	0·13	0·34	−0·07	0·61
Fruit juices	−0·25	0·07	−0·14	0·31	−0·13	0·34	0·33†	0·02	0·37†	0·006	0·07	0·62	0·30†	0·03	0·007	0·96	0·39†	0·004	0·44†	0·001	0·09	0·51	0·09	0·53	0·04	0·77
Fruits + fruit juices	−0·16	0·26	−0·006	0·97	−0·32†	0·02	0·16	0·26	0·22	0·12	−0·13	0·35	0·21	0·12	0·29‡	0·04	0·32	0·02	0·44†	0·001	0·31†	0·03	0·13	0·37	−0·007	0·96

CA, caffeic acid; CGA, chlorogenic acid; END, enterodiol; ENL, enterolactone; GA, gallic acid; HESP, hesperetin; K, kaempferol; mCOU, *m*-coumaric acid; MeGA, 4-*O*-methylgallic acid; MeQ, isorhamnetin; NAR, naringenin; PHLOR, phloretin; Q, quercetin. *For details of procedures, see p. 192. †Kappa coefficient $P < 0{\cdot}05$. ‡Kappa coefficient was not calculated because of unequal numbers in rows and columns.

Table 6. Correlation between consumption of vegetables, cereals, potatoes, chocolate and beverages and polyphenol concentrations (μM) measured in spot samples*
(Spearman rank correlation coefficients and *P* values)

	CGA		CA		mCOU		GA		MeGA		Q		MeQ		K		ENL		END	
Food	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Vegetables	0·11	0·45	−0·19	0·17	−0·12	0·38	−0·27	0·05	−0·23	0·10	0·04	0·77	−0·12	0·41	0·12	0·38	−0·09	0·53	−0·17	0·22
Onion			−0·14	0·32	−0·06	0·65	0·02	0·86	0·01	0·92	0·15	0·30	−0·03	0·81	0·13	0·34				
Dark bread			−0·09	0·51											0·03	0·81	0·05	0·74	0·18	0·20
Cereals			0·15	0·28											0·05	0·74	−0·004	0·98	0·10	0·48
Potatoes	0·10	0·49	0·01	0·92											−0·02	0·89	0·23	0·10	0·01	0·94
Chocolate			0·21	0·13	−0·03	0·85	0·02	0·84	0·08	0·55					0·10	0·49			−0·11	0·45
Coffee	0·63†	<0·0001	0·29†	0·03											−0·21	0·13				
Black tea	−0·31†	0·03	−0·20	0·16	−0·44†	0·001	0·45†	0·0008	0·54†	<0·0001	0·18	0·20	0·18	0·21	0·27	0·05				
Herbal tea	−0·11	0·44	0·01	0·93	0·14	0·33	0·09	0·54	0·14	0·33	0·12	0·38	0·25	0·07	−0·005	0·97				
Wine	−0·01	0·93	−0·06	0·69	0·16	0·26	0·45†	0·0007	0·37†	0·006					0·12	0·39				

CA, caffeic acid; CGA, chlorogenic acid; END, enterodiol; ENL, enterolactone; GA, gallic acid; HESP, hesperetin; K, kaempferol; mCOU, *m*-coumaric acid; MeGA, 4-*O*-methylgallic acid; MeQ, isorhamnetin; NAR, naringenin; PHLOR, phloretin; Q, quercetin.
*For details of procedures, see p. 192.
†Kappa coefficient $P < 0{\cdot}05$.

24 h urine samples can be explained by the fact that wine consumers are also coffee consumers. This last correlation could also be explained by the presence in wine of caftaric acid, a caffeoyl tartrate ester (Baderschneider & Winterhalter, 2001).

A few other positive correlations (most often weak) were observed: kaempferol with apple, red fruit and fruits; gallic acid and 4-*O*-methylgallic acid with fruit juices; caffeic acid with orange and citrus fruits; or *m*-coumaric acid with apple. These compounds or their metabolic precursors are known to be present in these food sources although not necessarily in high amounts.

Several authors have proposed some urinary or plasmatic biomarkers to compare fruit and vegetable intake in a population. Nielsen *et al.* (2002) observed a good correlation between the sum of quercetin, kaempferol, isorhamnetin, tamarixetin, naringenin, hesperetin and phloretin measured in 24 h urine samples, and fruit and vegetable consumption by human subjects following their free-living diet (r 0·35, $P<0·005$, n 94) and concluded that it was a good biomarker for fruit and vegetable consumption. A good correlation was also observed between the sum of the same flavonoids in urine and the consumption of fruit and vegetables in a controlled study with two levels of intake (Krogholm *et al.* 2004). We did not observe a correlation between the sum of these flavonoids excreted in urine and the total fruit and vegetable consumption, but this sum was correlated to fruit and fruit juice intake. This combination of flavonoids could therefore be used as a biomarker of fruit and fruit juice intake. It compares well with other biomarkers previously proposed to assess fruit and vegetable consumption. Drewnowski *et al.* (1997) observed correlation coefficients of 0·29 and 0·36, respectively, between serum β-carotene, vitamin C and total fruit and vegetable intake. In the study by Block *et al.* (2001), these correlation coefficients were 0·35 and 0·59, respectively. Correlations were also reported between serum total carotenoids or serum lutein and carotenoid intake estimated from three 24 h recalls with respective correlation coefficients of 0·35 and 0·41 (Resnicow *et al.* 2000). A validation study of the dietary methods used in the UK arm of the EPIC study showed a correlation of 0·48 for vitamin C and 0·21 for β-carotene when a 7 d diary record was compared with the plasma concentrations of these nutrients (Bingham *et al.* 1997), whereas another study in the UK showed correlations of 0·64, 0·47 and 0·45 for lutein, lycopene and β-carotene, respectively, with intakes estimated from a 4 d weighed food record (Scott *et al.* 1996). The study by Jansen *et al.* (2004) showed a correlation of 0·21 between total plasma carotenoids and total fruit and vegetable intake estimated from a food frequency questionnaire, with the highest correlation for β-cryptoxanthin (r 0·41). The strongest correlation between total plasma carotenoids and total fruit and vegetable intake has been observed in a study by Campbell *et al.* (1994; r 0·59). At these levels of correlation, carotenoids are accepted as sufficiently valid biomarkers for fruit and vegetable intake. The correlations we observed with phenolic compounds are all in the same line as these studies and support the use of urinary polyphenols as biomarkers for intake of polyphenol-rich fruits. The polyphenol combination considered in the present study might be particularly useful as a biomarker of fruit intake rather than fruit and vegetable intake, in accordance with the high polyphenol content in fruits (Manach *et al.* 2004). Adding more polyphenols from fruits might still improve the accuracy of such a biomarker to take into account the diversity of the fruit sources.

The present work provides a validation of phenolic biomarkers in spot urine using a 2 d dietary record. It is therefore not known whether they reflect the true habitual intake of polyphenol-rich foods. Finally, when a complete food composition table on polyphenols becomes available, it will also be possible to correlate the total polyphenol intake calculated using these data to the concentrations of the phenolic compounds in the spot samples and to validate their use as biomarkers of polyphenol intake.

In conclusion, the present results indicate that several phenolic compounds in spot urine samples collected from free-living subjects can be used as biomarkers of specific polyphenol-rich foods: chlorogenic acid for coffee consumption, phloretin for apple consumption, naringenin for grapefruit consumption and hesperetin for orange consumption. The combination of several polyphenols (isorhamnetin + hesperetin + naringenin + kaempferol + phloretin) may be a good indicator for total fruit consumption. Confirmation of the quality of these biomarkers when evaluating long-term dietary intake remains necessary.

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References

Ameer B, Weintraub RA, Johnson JV, Yost RA & Rouseff RL (1996) Flavanone absorption after naringin, hesperidin, and citrus administration. *Clin Pharmacol Ther* **60**, 34–40.

Baderschneider B & Winterhalter P (2001) Isolation and characterization of novel benzoates, cinnamates, flavonoids, and lignans from Riesling wine and screening for antioxidant activity. *J Agric Food Chem* **49**, 2788–2798.

Bingham S (1991) Limitations of the various methods for collecting dietary intake data. *Ann Nutr Metab* **35**, 117–127.

Bingham SA (2002) Biomarkers in nutritional epidemiology. *Public Health Nutr* **5**, 821–827.

Bingham S & Cummings JH (1983) The use of 4 amino benzoic acid as a marker to validate the completeness of 24 h urine collections in man. *Clin Sci* **64**, 629–635.

Bingham SA, Gill C, Welch A, *et al.* (1997) Validation of dietary assessment methods in the UK arm of EPIC using weighed records, and 24-hour urinary nitrogen and potassium and serum vitamin C and carotenoids as biomarkers. *Int J Epidemiol* **26**, Suppl. 1, S137–S151.

Block G (1982) A review of validations of dietary assessment methods. *Am J Epidemiol* **115**, 492–505.

Block G, Norkus E, Hudes M, Mandel S & Helzlsouer K (2001) Which plasma antioxidants are most related to fruit and vegetable consumption? *Am J Epidemiol* **154**, 1113–1118.

Caccetta RA, Croft KD, Beilin LJ & Puddey IB (2000) Ingestion of red wine significantly increases plasma phenolic acid concentrations but does not acutely affect ex vivo lipoprotein oxidizability. *Am J Clin Nutr* **71**, 67–74.

Campbell DR, Gross MD, Martini MC, Grandits GA, Slavin JL & Potter JD (1994) Plasma carotenoids as biomarkers of vegetable and fruit intake. *Cancer Epidemiol Biomarkers Prev* **3**, 493–500.

Cartron E, Fouret G, Carbonneau MA, Lauret C, Michel F, Monnier L, Descomps B & Leger CL (2003) Red-wine beneficial long-term effect on lipids but not on antioxidant characteristics in plasma in a study comparing three types of wine—description of two O-methylated derivatives of gallic acid in humans. *Free Radic Res* **37**, 1021–1035.

Clifford MN (1999) Chlorogenic acids and other cinnamates—nature, occurrence and dietary burden. *J Sci Food Agric* **79**, 362–372.

Drewnowski A, Rock CL, Henderson SA, Shore AB, Fischler C, Galan P, Preziosi P & Hercberg S (1997) Serum beta-carotene and vitamin C as biomarkers of vegetable and fruit intakes in a community-based sample of French adults. *Am J Clin Nutr* **65**, 1796–1802.

Erlund I, Meririnne E, Alfthan G & Aro A (2001) Plasma kinetics and urinary excretion of the flavanones naringenin and hesperetin in humans after ingestion of orange juice and grapefruit juice. *J Nutr* **131**, 235–241.

Erlund I, Silaste ML, Alfthan G, Rantala M, Kesaniemi YA & Aro A (2002) Plasma concentrations of the flavonoids hesperetin, naringenin and quercetin in human subjects following their habitual diets, and diets high or low in fruit and vegetables. *Eur J Clin Nutr* **56**, 891–898.

Frankenfeld CL, Patterson RE, Horner NK, Neuhouser ML, Skor HE, Kalhorn TF, Howald WN & Lampe JW (2003) Validation of a soy food-frequency questionnaire and evaluation of correlates of plasma isoflavone concentrations in postmenopausal women. *Am J Clin Nutr* **77**, 674–680.

Hercberg S, Galan P, Preziosi P, Bertrais S, Mennen L, Malvy D, Roussel AM, Favier A & Briancon S (2004) The SU.VI.MAX study: a randomised, placebo-controlled trial of the health effects of antioxidant vitamins and minerals. *Arch Int Med* **164**, 2335–2342.

Hertog MGL, Feskens EJM, Hollman PCH, Katan MB & Kromhout D (1993*a*) Dietary antioxidant flavonoids and risk for coronary heart disease: the Zutphen Elderly Study. *Lancet* **342**, 1007–1011.

Hertog MGL, Hollman PCH, Katan MB & Kromhout D (1993*b*) Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutr Cancer* **20**, 21–29.

Hirvonen T, Pietinen P, Virtanen M, Ovaskainen ML, Hakkinens S, Albanes D & Virtamo J (2001) Intake of flavonols and flavones and risk of coronary heart disease in male smokers. *Epidemiology* **12**, 62–67.

Hodgson JM, Chan SY, Puddey IB, *et al.* (2004) Phenolic acid metabolites as biomarkers for tea- and coffee-derived polyphenol exposure in human subjects. *Br J Nutr* **91**, 301–306.

Hodgson JM, Morton LW, Puddey IB, Beilin LJ & Croft KD (2000) Gallic acid metabolites are markers of black tea intake in humans. *J Agric Food Chem* **48**, 2276–2280.

Horner NK, Patterson RE, Neuhouser ML, Lampe JW, Beresford SA & Prentice RL (2002) Participant characteristics associated with errors in self-reported energy intake from the Women's Health Initiative food-frequency questionnaire. *Am J Clin Nutr* **76**, 766–773.

Ito H, Gonthier MP, Manach C, Morand C, Mennen L, Remesy C & Scalbert A (2005) Polyphenol levels in human urine after intake of 6 different polyphenol-rich beverages. *Br J Nutr* **94**, 500–509.

Jansen MC, Van Kappel AL, Ocke MC, Van't Veer P, Boshuizen HC, Riboli E & Bueno-de-Mesquita HB (2004) Plasma carotenoid levels in Dutch men and women, and the relation with vegetable and fruit consumption. *Eur J Clin Nutr* **58**, 1386–1395.

Knekt P, Jarvinen R, Reunanen A & Maatela J (1996) Flavonoid intake and coronary mortality in Finland: a cohort study. *BMJ* **312**, 478–481.

Krogholm KS, Haraldsdottir J, Knuthsen P & Rasmussen SE (2004) Urinary total flavonoid excretion but not 4-pyridoxic acid or potassium can be used as a biomarker for the intake of fruits and vegetables. *J Nutr* **134**, 445–451.

Lampe JW, Gustafson DR, Hutchins AM, Martini MC, Li S, Wahala K, Grandits GA, Potter JD & Slarin JL (1999) Urinary isoflavonoid and lignan excretion on a Western diet: relation to soy, vegetable, and fruit intake. *Cancer Epidemiol Biomarkers Prev* **8**, 699–707.

Landrault N, Poucheret P, Ravel P, Gasc F, Cros G & Teissedre PL (2001) Antioxidant capacities and phenolics levels of French wines from different varieties and vintages. *J Agric Food Chem* **49**, 3341–3348.

Manach C, Scalbert A, Morand C, Rémésy C & Jimenez L (2004) Polyphenols—food sources and bioavailability. *Am J Clin Nutr* **79**, 727–747.

Manach C, Williamson G, Morand C, Scalbert A & Rémésy C (2005) Bioavailability and bioefficacy of polyphenols in humans. A review of 97 bioavailability studies. *Am J Clin Nutr* **81**, 230S–242S.

Mennen L, Malvy D, Galan P, Preziosi P, Bertrais S, Bruckert E, Maurel M, Franchisseur C & Hercberg S (2003) Tea consumption and cardiovascular risk in the SU.VI.MAX study: are life-style factors important? *Nutr Res* **23**, 879–890.

Nielsen SE, Freese R, Kleemola P & Mutanen M (2002) Flavonoids in human urine as biomarkers for intake of fruits and vegetables. *Cancer Epidemiol Biomarkers Prev* **11**, 459–466.

Noroozi M, Burns J, Crozier A, Kelly IE & Lean MEJ (2000) Prediction of dietary flavonol consumption from fasting plasma concentration or urinary excretion. *Eur J Clin Nutr* **54**, 143–149.

Plakke T, Berkel J, Beynen AC, Hermus RJ & Katan MB (1983) Relationship between the fatty acid composition of the diet and that of the subcutaneous adipose tissue in individual human subjects. *Hum Nutr Appl Nutr* **37**, 365–372.

Radtke J, Linseisen J & Wolfram G (2002) Fasting plasma concentrations of selected flavonoids as markers of their ordinary dietary intake. *Eur J Nutr* **41**, 203–209.

Resnicow K, Odom E, Wang T, Dudely WN, Mitchell D, Vaughan R, Jackson A & Baranowski T (2000) Validation of three food frequency questionnaires and 24-hour recalls with serum carotenoid levels in a sample of African-American adults. *Am J Epidemiol* **152**, 1072–1080.

Rousseff RL, Martin SF & Youtsey CO (1987) Quantitative survey of narirutin, naringin, heperidin, and neohesperidin in Citrus. *J Agric Food Chem* **35**, 1027–1030.

Scalbert A, Manach C, Morand C, Remesy C & Jimenez L (2005) Dietary polyphenols and the prevention of diseases. *Crit Rev Food Sci Nutr* **45**, 287–306.

Scalbert A & Williamson G (2000) Dietary intake and bioavailability of polyphenols. *J Nutr* **130**, 2073S–2085S.

Scott KJ, Thurnham DI, Hart DJ, Bingham SA & Day K (1996) The correlation between the intake of lutein, lycopene and beta-carotene from vegetables and fruits, and blood plasma concentrations in a group of women aged 50–65 years in the UK. *Br J Nutr* **75**, 409–418.

Spanos GA & Wrolstad RE (1992) Phenolics of apple, pear, and white grape juice and their changes with processing and storage—a review. *J Agric Food Chem* **40**, 1478–1487.

Verkasalo PK, Appleby PN, Allen NE, Davey G, Adlercreutz H & Key TJ (2001) Soya intake and plasma concentrations of daidzein and genistein: validity of dietary assessment among eighty British women (Oxford Arm of the European Prospective Investigation into Cancer and Nutrition). *Br J Nutr* **86**, 415–421.

Walters GO, Miller FM & Worwood M (1973) Serum ferritin concentration and iron stores in normal subjects. *J Clin Pathol* **26**, 770–772.

Yamamoto S, Sobue T, Sasaki S, *et al.* (2001) Validity and reproducibility of a self-administered food-frequency questionnaire to assess isoflavone intake in a Japanese population in comparison with dietary records and blood and urine isoflavones. *J Nutr* **131**, 2741–2747.