Polyphenol levels in human urine after intake of six different polyphenol-rich beverages

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Dietary polyphenols are suggested to participate in the prevention of CVD and cancer. It is essential for epidemiological studies to be able to compare intake of the main dietary polyphenols in populations. The present paper describes a fast method suitable for the analysis of polyphenols in urine, selected as potential biomarkers of intake. This method is applied to the estimation of polyphenol recovery after ingestion of six different polyphenol-rich beverages. Fifteen polyphenols including mammalian lignans (enterodiol and enterolactone), several phenolic acids (chlorogenic, caffeic, *m*-coumaric, gallic, and 4-*O*-methylgallic acids), phloretin and various flavonoids (catechin, epicatechin, quercetin, isorhamnetin, kaempferol, hesperetin, and naringenin) were simultaneously quantified in human urine by HPLC coupled with electrospray ionisation mass-MS (HPLC-electrospray-tandem mass spectrometry) with a run time of 6 min per sample. The method has been validated with regard to linearity, precision, and accuracy in intra- and inter-day assays. It was applied to urine samples collected from nine volunteers in the 24 h following consumption of either green tea, a grape-skin extract, cocoa beverage, coffee, grapefruit juice or orange juice. Levels of urinary excretion suggest that chlorogenic acid, gallic acid, epicatechin, naringenin or hesperetin could be used as specific biomarkers to evaluate the consumption of coffee, wine, tea or cocoa, and citrus juices respectively.

Polyphenols: Flavonoids: Phenolic acids: High-throughput analysis: Urine: Beverages

The relationship between the diet and prevention of diseases in man has been the topic of numerous investigations during recent years. A reduction of disease risk has been attributed to antioxidants present in foods, due to their ability to scavenge free radicals and thus reduce oxidative damage (Gutteridge & Halliwell, 1994). Polyphenols are the most abundant antioxidants in our diets (Scalbert & Williamson, 2000) and their consumption may contribute to prevent cancers, stroke, CHD, neurodegenerative diseases or diabetes (Scalbert *et al.* 2005). Polyphenols are found in many foods of plant origin and are particularly abundant in fruits and beverages such as tea, wine or coffee. They form a highly diverse class of compounds. They are distributed in several categories according to their structures with phenolic acids, flavonoids and lignans being the most widespread in food (Shahidi & Naczk, 2003). Their biological properties and bioavailability depends on their chemical structure (Manach *et al.* 2004*a*,*b*) and it is important to study the effects on health of the different types of polyphenols. A number of epidemiological studies have been carried out on some flavonoids (flavonols, flavones, flavanols and isoflavones) and lignans (Arts & Hollman, 2005). These studies largely rely on the accurate estimation of polyphenol intake or exposure. This estimation was most often based on the use of food composition tables. Variations in isoflavone or

lignan intake have also been evaluated in case–control studies through the measurement of their concentrations in urine or plasma (Adlercreutz *et al.* 1982; Akaza *et al.* 2002; Hulten *et al.* 2002). The use of these biomarkers of exposure was validated in free-living populations by a good correlation between urinary excretion or serum concentration of polyphenols and the consumption of polyphenol-rich foods such as soya (isoflavones), whole grains (lignans) or fruit and vegetables (various flavonoids, lignans) (Maskarinec *et al.* 1998; Lampe *et al.* 1999; Kilkkinen *et al.* 2001; Nielsen *et al.* 2002).

The present study reports variations in the urinary excretion of major dietary polyphenols after consumption of six polyphenol-rich beverages. The recent developments of MS have made possible the current estimation of a wide range of polyphenols with good sensitivity of analysis (Nielsen *et al.* 2000; Franke *et al.* 2002; Gonthier *et al.* 2003*b*). A high-throughput method for the simultaneous quantification in human urine of fifteen polyphenols and related compounds using the HPLC-electrospray-tandem mass spectrometry (HPLC-ESI-MS-MS) method was developed and applied to the measurement of polyphenol recovery in urine. The short run time makes the method particularly adapted to the analysis of large batches of samples in epidemiological studies.

Abbreviation: HPLC-ESI-MS-MS, HPLC-electrospray-tandem mass spectrometry.

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Materials and methods

Materials

Chlorogenic acid, caffeic acid, *m*-coumaric acid, gallic acid, cate-chin, epicatechin, quercetin, kaempferol, hesperetin, naringenin, phloretin, taxifolin, and type H-2 β-glucuronidase (G-0876) from *Helix pomatia* were purchased from Sigma (St Louis, MO, USA). Enterodiol and enterolactone were obtained from Fluka (St Quentin Fallavier, France). 4-*O*-Methylgallic acid was pre-pared by refluxing overnight a mixture of gallic acid, dimethyl sulfate and potassium carbonate in dry acetone. The resulting methyl ester was purified by preparative TLC and hydrolysed with 5 % methanolic potassium hydroxide for 3 h to afford 4-*O*-methylgallic acid, which was purified by column chromatography over MCI-GEL CHP-20P (Mitsubishi, Tokyo, Japan).

Subjects

The study was approved by the ethical committee for human experimentation of the University Hospital of Clermont-Ferrand, France (ethics reference CCPPRB-AU362). Exclusion criteria were as follows: signs of diseases related to the gastrointestinal tract, any form of liver disease or gall bladder problems, use of drugs that influenced gastrointestinal transit, present illness, preg-nancy or lactation. Nine subjects (five women and four men), with a mean age of 25 (SD 1) years (range 20–32 years), and a mean BMI of 22·1 (SD 0·6) kg/m^2 (range 18·9–24·8 kg/m^2) were admitted to participate and signed an informed consent form. The subjects were healthy, based on a medical questionnaire, had normal blood values for Hb, packed cell volume and leuco-cyte counts, and absence of glucose and protein in urine. They had stable food habits and were not vegetarians.

Polyphenol-rich beverages

The composition of polyphenol extracts used for beverage prep-aration is detailed in Table 1. Decaffeinated coffee powder was from the Nestlé Product Technology Centre (Vevey, Switzerland). Green tea extract was provided by Unilever Research (Colworth, Beds, UK) and contained caffeine (14·1 mg/g). Cocoa powder was from Mars, Inc. (Hackettstown, NJ, USA) and contained caffeine (2·1 mg/g), theobromine (21·1 mg/g) and fat (106·3 mg/g). Grape skin extract was from CHR Hansen (St-Julien-de-Peyrolas, France). Grapefruit and orange juices (100 % pure juice) were bought in a local supermarket. Phenolic composition was deter-mined by HPLC by the suppliers of the materials. Flavanones were determined by HPLC as previously described (Manach *et al.* 2003).

Study design

Subjects followed a diet low in polyphenols for 2 d before the test meal. To ensure adherence to the dietary guidelines, volunteers were given a list of forbidden foods and beverages; fruits and fruit-containing foods, vegetables, whole cereals, chocolate and beverages rich in polyphenols (coffee, tea, fruit juice, cocoa drinks, wine, cider, and beer) were excluded from the diet. Poly-phenol-rich beverages were made of 4 g instant coffee (equivalent to two cups of coffee), 0·3 g green tea extract (equivalent to one cup of tea), 10 g cocoa powder (equivalent to one cup of hot cho-colate) or 18 g grape-skin extract, dissolved in 200 ml hot water.

Table 1. Phenolic composition of the extracts (mg/g DM) and juices (mg/l) consumed by the human subjects

Green tea extract*		Grape-skin extract*		Cocoa powder*		Instant coffee*		Grapefruit juice		Orange juice	
Polyphenol	Content	Polyphenol	Content	Polyphenol	Content	Polyphenol	Content	Polyphenol	Content	Polyphenol	Content
EGCG	452·2	Malvidin 3-*O*-Glc	14·7	Epicatechin	6·6	Chlorogenic acid	20·6	Narirutin	104·8	Narirutin	68·6
ECG	162·1	Gallic acid	0·7	Catechin	2·8	4-CQA	17·3	Naringin	384·7	Hesperidin	423·5
EGC	10·4	Caffeic acid	0·04	PC dimers	4·2	3-CQA	13·6	Hesperidin	14·3		
GCG	132·3	*p*-Coumaric acid	0·02	PC trimers	3·6	3,4-diCQA	2·8				
Epicatechin	70·2	Quercetin	0·08	PC tetramers	3·3	3,5-diCQA	1·6				
Catechin	48·1	Catechin	0·04	PC pentamers to decamers†	8·4	4,5-diCQA	2·9				
						FQA	5·9				

EGCG, epigallocatechin gallate; Glc, glucoside; ECG, epicatechin gallate; CQA, caffeoylquinic acid; EGC, epigallocatechin; PC, procyanidin; GCG, gallocatechin gallate; FQA, feruloylquinic acid.
*Content values were determined by the suppliers of the extracts.
†Pentamers, 2·6 mg/g; hexamers, 2·5 mg/g; heptamers, 1·0 mg/g; octamers, 1·0 mg/g; nanomers, 0·9 mg/g; decamers, 0·4 mg/g.

Quantities were determined in order to provide the equivalent of 250–290 mg polyphenols. On day 3, after an overnight fast, all subjects consumed one of the polyphenol-rich beverages or hot water as a control (200 ml) between 08.00 and 08.30 hours at the laboratory, together with a breakfast that was provided. Breakfast consisted of bread, butter and milk. After breakfast, subjects stayed at the laboratory all day and were allowed to drink water from 12.00 hours. They received a dinner without polyphenols at 18.00 hours and were asked to continue the polyphenol-free diet on day 4. All test meals were consumed once by each subject in a randomised order at intervals of 14 d. Volunteers collected 24 h urine samples from breakfast time until the next day at the same hour. Urine samples were stored at 4°C and immediately treated with sodium azide (1 g/l) and acidified to pH 4 with HCl (40 mmol/l) on reception in the laboratory. Samples were stored at −20°C until analysis. Citrus juices were tested in a separate protocol. Nine male volunteers ingested after an overnight fast 430 ml grapefruit juice and 1 week later 550 ml orange juice. Urine was collected in different fractions (0–6, 6–12 and 12–24 h periods) from breakfast time until the following day in plastic bottles containing 1 g ascorbic acid. Sodium azide (1 g/l) was added at the end of each collection period. Samples of urine were stored at −80°C. Only the 6–12 h samples, corresponding to the peak of flavanone excretion (Manach *et al.* 2003), were analysed.

Analysis of polyphenols in urine

Urine samples (250 μl) were acidified with 0·58 M-acetic acid (20 μl) containing taxifolin (50 μM) as internal standard and incubated with 1300 units of β-glucuronidase and thirty-eight units of sulfatase (from *H. pomatia*, Sigma G0876; Sigma, St Louis, MO, USA) for 45 min at 37°C. The duration of hydrolysis of 45 min was selected as it is intermediate between optimal times for different polyphenols present in the analysed samples (Morand *et al.* 2001). After addition of 1 M-HCl (10 μl), samples were extracted twice with ethyl acetate (400 and 300 μl), and centrifuged for 10 min at 5000 *g*. The combined organic layers were evaporated under N_2, re-dissolved by vortexing in 25 % aqueous

methanol (200 μl), filtered (PTFE membrane, 0·45 μm; Millipore, Bedford, MA, USA) and injected (20 μl) into the HPLC-ESI-MS-MS system.

HPLC-ESI-MS-MS analysis was performed on a Hewlett-Packard 1100 HPLC system (Waldbronn, Germany) coupled to a triple quadrupole mass spectrometer, API-2000 (Applied Biosystems, Creemore, ON, Canada). The chromatographic column was a Zorbax Eclipse XDB-C18 (internal diameter 2·1 × 30 mm, 3·5 μm, Agilent) maintained at 20°C, and the mobile phase consisted of water–formic acid (100:0·1, v/v) (solvent A) and aceto-nitrile–water–formic acid (95:5:0·1, by vol.) (solvent B). A gradient was applied as follows: the proportion of solvent B in the eluent increased from 0 % to 10 % (t = 1 min), 100 % (t = 4 min) and decreased back to 0 % (t = 4·1 min) until the next injection (t = 6 min). The flow rate was 0·8 ml/min with 0·2 ml/min split directed to the mass spectrometer. Mass detection was carried out in multiple reaction monitoring mode using an electrospray interface operating in negative-ion mode at 450°C with nebuliser pressure of 90 pounds per square inch, a drying N_2 gas flow of 11 litres/min, a fragmentor voltage of 20 V and capillary voltage of 4000 V. Ionisation and fragmentation was optimised for each polyphenol by direct infusion of a standard solution (five parts per million in 40 % aqueous CH_3CN containing 0·1 % formic acid solution). Parent and product ions are given in Table 2. Two acquisition periods were defined for each analysis with dwell times of 170 ms for the first period and 85 ms for the second period. Peak identity was established by both the characteristic parent and product ion pair and retention time.

Calibration curves and limits of detection and quantification

Calibration curves were prepared by spiking blank urine with samples of standard mixture solutions at different concentrations with duplicate injections at each level. Different blank urine samples were compared by checking for the absence of any significant ion suppression effect. Spiked urine samples were treated with enzymes and extracted with ethyl acetate as earlier. The following concentration ranges were validated: 0·5–5 μM for chlorogenic, caffeic and *m*-coumaric acids, quercetin, kaempferol,

Table 2. Parent and product ions, retention time, and limits of detection and quantification of polyphenols, their metabolites and internal standard

Compound	Parent ion (*m/z*)	Product ion (*m/z*)	Retention time (min)	LOD (μM)	LOQ (μM)
Chlorogenic acid	353	191	2·53	0·2	1
Caffeic acid	179	135	2·56	0·01	0·05
m-Coumaric acid	163	119	2·95	0·01	0·05
Gallic acid	169	125	0·43	0·5	1
4-*O*-Methylgallic acid	183	124	2·22	0·5	1
Catechin	289	109	2·51	1	5
Epicatechin	289	109	2·71	1	5
Quercetin	301	151	3·33	0·1	0·5
Isorhamnetin	315	300	3·52	0·05	0·2
Kaempferol	285	93	3·49	1	5
Hesperetin	301	164	3·50	0·2	1
Naringenin	271	151	3·46	0·01	0·05
Phloretin	273	167	3·44	0·05	0·2
Enterodiol	301	253	3·24	0·1	0·5
Enterolactone	297	107	3·50	0·01	0·02
Taxifolin (internal standard)	303	125	2·98	0·1	0·2

LOD, limits of detection; LOQ, limits of quantification.

isorhamnetin, enterodiol and phloretin; $5-50\,\mu\text{M}$ for gallic and 4-O-methylgallic acids, naringenin, and enterolactone; $0.5-50\,\mu\text{M}$ for hesperetin. Peak areas were plotted against the corresponding standard concentrations. A good linearity ($r^2\ 0.986-0.999$) was observed in the range of concentrations observed for all urine samples, except for hesperetin where a weighted 3rd polynominal expression was used for calibration.

Standard samples for limits of detection and limits of quantification were prepared by spiking blank urine samples over a concentration range of $0.01-10\,\mu\text{M}$ with ten different concentration levels, and duplicate extractions and injections at each level.

Accuracy and intra- and inter-day assay precision

Accuracy and intra- and inter-day assay precision of the method were determined by assaying six replicates of blank urine samples spiked with solutions of the fifteen phenolic compounds at six separate concentrations (1, 2, 5, 10, 20 and $50\,\mu\text{M}$) over 6 d for inter-day CV (reproducibility) and in six independent assays on the same day for intra-day CV (repeatability). Accuracy was measured as the percentage deviation from the added concentrations.

Statistical analyses

Values are given as means with their standard errors. Paired comparisons between control and polyphenol-rich beverage intake were performed for urinary concentrations of all polyphenols and their metabolites by a non-parametric Mann–Whitney U test

(Excel statistics version 5.0; Esumi Co. Ltd, Tokyo, Japan). All of the statistical tests were two-tailed.

Results

Validation of a high-throughput method for the analysis of polyphenols in urine

Fifteen polyphenols (Fig. 1) were analysed by HPLC-ESI-MS-MS analysis using a short reverse-phase column and multiple reaction monitoring detection. They were selected for their abundance in the human diet and in the beverages considered in the present study, and for being representative of the main types of polyphenol classes. A short solvent gradient was applied, which allowed the analysis of all polyphenols within 6 min (Fig. 2). Typical chromatograms of human urine samples collected after intake of the polyphenol-rich beverages are shown in Fig. 3. Most extracted traces showed a single peak for a given ion pair. Chromatograms of blank urine samples showed only two main peaks in addition to the internal standard (Fig. 3 (A)). One is enterolactone, not significantly affected by the 2 d wash-out period.

The limits of detection ($S/N > 3$) and limits of quantification ($S/N > 10$) were determined for all standards (Table 2). These data indicated wide differences in sensitivity between polyphenols and sufficient sensitivity to estimate them in urine samples. Repeatability, reproducibility and accuracy were evaluated at six different concentrations (Table 3). Accuracy varied from -4.1 to 3.2%, and intra- and inter-day variability were lower than 10.1 and 10.3 % respectively. Thus, validation of
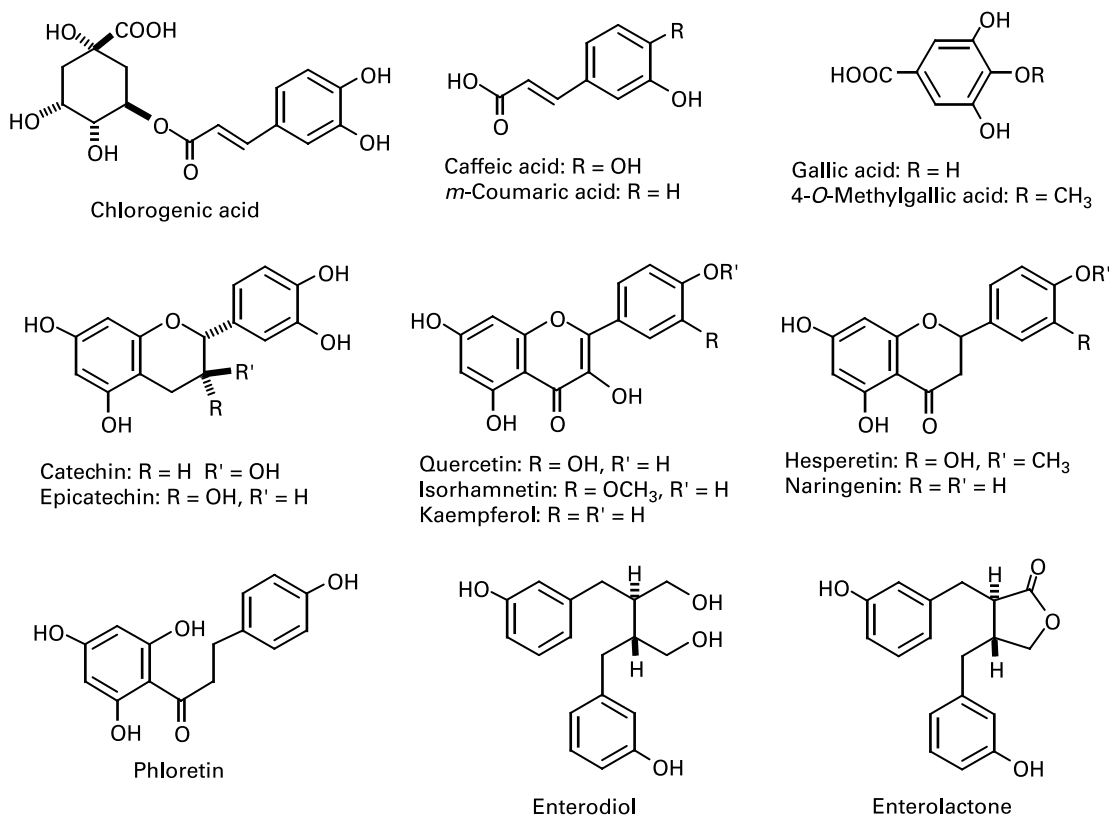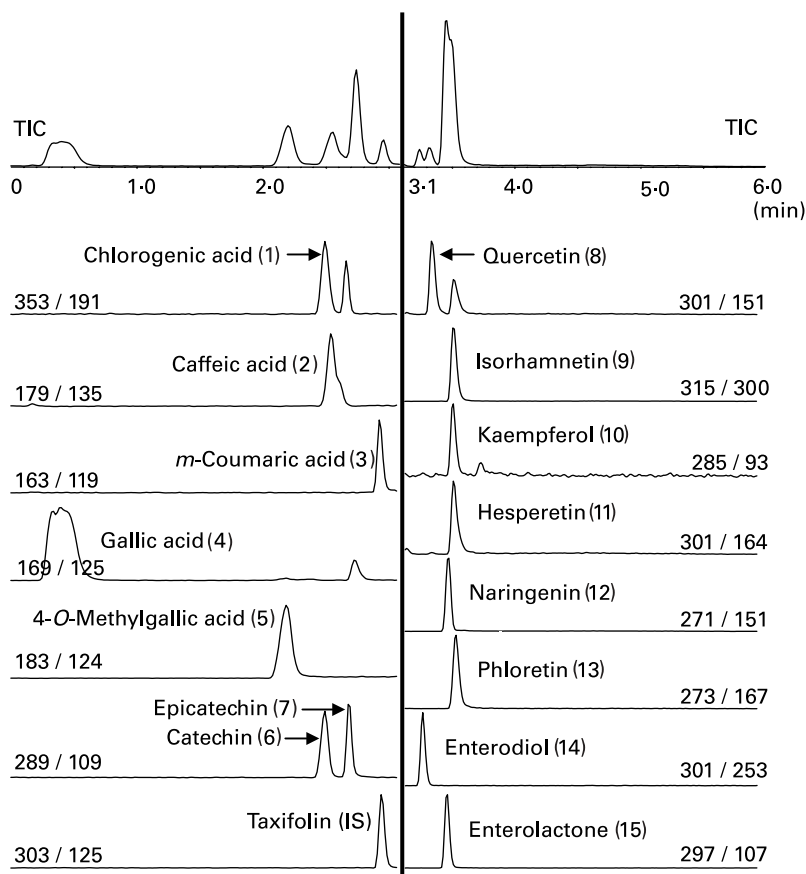

Fig. 1. Chemical structures of polyphenols and their metabolites analysed by the HPLC-electrospray-tandem mass spectrometry method.

Fig. 2. Total ion chromatogram (TIC) and extracted single ion chromatograms of a mixture of fifteen dietary polyphenols (10 μM) obtained by an HPLC-electrospray-tandem mass spectrometry method in multiple reaction monitoring mode with negative ionisation. IS, internal standard.

this method for estimation of sixteen polyphenols and their metabolites in human urine was achieved.

*Urinary excretion of polyphenols after consumption of polyphenol-rich beverages*

These fifteen polyphenols were estimated in urine samples collected in the 24 h following the consumption of different polyphenol-rich beverages. The composition of the beverages is given in Table 1. Coffee, green tea, cocoa and grape-skin beverages were ingested hot by nine volunteers at intervals of 2 weeks in a random order. Consumption of instant coffee provided 259 mg phenolic acids, green tea, 263 mg catechins, cocoa beverage, 289 mg catechins and proanthocyanidin, and the grape-skin-extract beverage, 280 mg of different polyphenols including 267 mg malvidin 3-glucoside. Two citrus juices were also tested, grapefruit and orange juice, providing respectively 252 mg (largely naringin) and 271 mg (largely hesperidin) flavanone glycosides.

Large differences in the urinary excretion of the fifteen polyphenols were observed between beverages (Table 4). Enterolactone and low amounts of caffeic acid, *m*-coumaric acid, naringenin and enterodiol were observed in control urine samples. Ingestion of the grape-skin extract and of coffee induced a significant increase of the urinary excretion of caffeic acid. An increase in the urinary excretion of *m*-coumaric acid, a microbial metabolite of caffeic acid and chlorogenic acid, was also observed

after coffee intake. Consumption of grape-skin extract and green tea induced an increase of the urinary excretion of gallic acid and its metabolite, 4-*O*-methylgallic acid. Epicatechin was observed in green tea and cocoa urine samples. A high level of naringenin and hesperetin urinary excretion was observed after consuming grapefruit and orange juices respectively. Isorhamnetin and phloretin were also specifically detected after consumption of citrus juices. Catechin and kaempferol could not be detected in any of the urine samples. No significant difference in urinary recovery could be observed between men and women for any of the polyphenols analysed.

Discussion

Most often, analytical methods are developed to estimate polyphenols of specific types in urine or plasma samples. It is important, particularly for epidemiological studies in which the subjects ingest a large variety of polyphenols with their regular diet, to be able to simultaneously estimate the main polyphenols provided by the different foods and beverages. In an earlier study, an HPLC-APCI-MS method was developed to quantify twelve flavonoids in human urine (Nielsen *et al.* 2000). However, the single ion monitoring mode used to detect the flavonoids required a proper separation of the different compounds and a run time of more than 90 min. In the present study, a rapid and selective method was developed for the simultaneous quantification of fifteen polyphenols including flavonoids, mammalian lignans, phenolic acids and
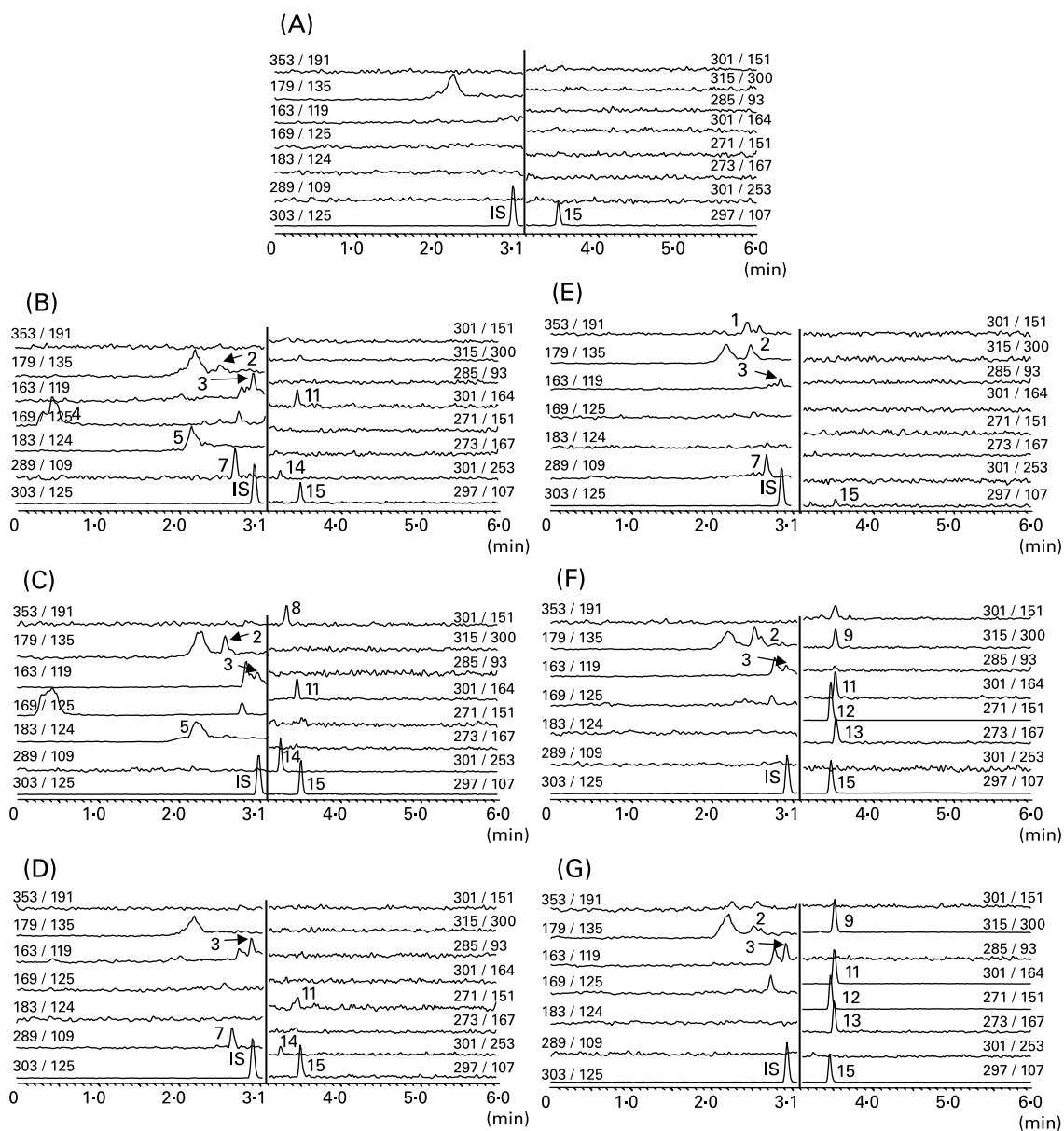
Fig. 3. Typical extracted single ion chromatograms of human urine samples collected after consumption of various polyphenol-rich beverages obtained by an HPLC-electrospray-tandem mass spectrometry method in multiple reaction monitoring mode with negative ionisation. (A), Blank urine sample; (B), green tea intake urine; (C), grape-skin extract; (D), cocoa; (E), coffee; (F), grapefruit juice; (G), orange juice. For identity of the peaks, see Fig. 2.

phloretin. Detection with multiple reaction monitoring allowed us to considerably shorten the run time to 6 min. Accuracy and precision were well within the limits recommended by the Food and Drug Administration's guidelines for bioanalytical method validation (US Department of Agriculture, 2001). Such a short run time makes this method particularly adapted to the analysis of large batches of samples as commonly found in epidemiological studies.

Using this method, urinary excretion of the main polyphenols consumed with six different polyphenol-rich beverages could be measured. Beverages were selected for their high consumption or for their content in specific flavonoids or phenolic acids representative of the main types of dietary polyphenols (Table 1). Most polyphenols are quickly excreted in the 24 h following their ingestion (Manach *et al.* 2004*a*,*b*). Their excretion in 24 h urine

samples should therefore be directly related to the amounts ingested during the same and previous day.

Coffee is known to be a major dietary source of chlorogenic acid (Clifford, 2000). Chlorogenic acid and caffeic acid were the main phenolic compounds recovered in urine after coffee intake (Table 4). No chlorogenic acid could be detected with any of the other beverages. Some previous authors failed to detect chlorogenic acid in either urine or plasma after ingestion of chlorogenic acid or chlorogenic acid-containing food (Booth *et al.* 1957; Bourne & Rice-Evans, 1998; Choudhury *et al.* 1999; Azuma *et al.* 2000; Nardini *et al.* 2002; Rechner *et al.* 2002). However, chlorogenic acid has been found in urine after ingestion of pure chlorogenic acid or prunes (Cremin *et al.* 2001; Olthof *et al.* 2001, 2003; Gonthier *et al.* 2003*c*). After ingestion of chlorogenic acid by healthy volunteers, a 1·7 %

Table 3. Evaluation of precision and accuracy in the analysis of polyphenols and their metabolites in human urine by HPLC-electrospray-tandem mass spectrometry

(Means of six measurements for all standards, and ranges)

Concentration (μM)								
	Found		Accuracy (%)		Intra-day precision (CV %)		Inter-day precision (CV %)	
Added	Mean	Range	Mean	Range	Mean	Range	Mean	Range
1	1·0	0·9 to 1·1	2·2	−6·4 to 8·2	10·1	3·2 to 21·8	10·3	4·0 to 19·6
2	2·0	1·7 to 2·2	−2·1	−13·6 to 10·1	6·8	2·5 to 12·1	6·7	4·7 to 9·8
5	4·8	4·2 to 5·1	−4·1	−16·8 to 1·7	3·9	0·2 to 12·8	4·0	0·5 to 12·2
10	10·1	9·7 to 10·3	1·0	−2·7 to 3·0	3·5	2·1 to 5·9	4·3	3·6 to 5·3
20	20·6	20·0 to 21·0	3·2	−0·1 to 5·2	3·5	1·1 to 6·5	4·8	1·0 to 6·5
50	50·2	49·6 to 52·1	0·4	−0·7 to 4·2	2·5	0·2 to 10·6	2·5	0·7 to 8·3

recovery of chlorogenic acid in urine was reported (Olthof *et al.* 2003). In the present study, consumption of coffee resulted in a 2·3 % recovery of chlorogenic acid in urine. Actual excretion may still be higher. Indeed, when the same analytical method was applied to a blank urine sample spiked with pure chlorogenic acid, not only chlorogenic acid but also caffeic acid were detected, showing that part of chlorogenic acid was hydrolysed during sample processing (data not shown), most likely by some esterase activity present in the *H. pomatia* enzyme mixture.

The higher concentration of caffeic acid in urine observed after coffee consumption as compared with other beverage groups (Table 4) can be explained by the hydrolysis of chlorogenic acid by the microflora in the colon (Couteau *et al.* 2001; Gonthier *et al.* 2003c), as well as by the deconjugating enzymes during processing of urine samples. A significant increase of caffeic acid urinary excretion was also observed after ingestion of the grape-skin extract. Most probably caffeic acid originates from caftaric acid, a caffeoyl tartrate ester present in grape (Gonthier *et al.* 2003a). *m*-Coumaric acid was detected in all urine samples.

It is a known metabolite of caffeic acid (Gonthier *et al.* 2003c), and in all samples it is present together with caffeic acid (Table 4). Expectedly, the highest concentrations were observed in urine samples collected after coffee consumption.

Gallic acid was found together with its major 4-*O*-methylated metabolite in urine samples collected after intake of green tea or grape-skin extract (Table 4) (Shahrzad & Bitsch, 1998). 4-*O*-Methylgallic acid was previously described as a major metabolite of gallic acid identified in plasma after wine consumption (Caccetta *et al.* 2000). Both account together for 29 % of the gallic acid ingested with the grape-skin extract. A similarly high value (36–40 %) was obtained after ingestion of pure gallic acid (Shahrzad *et al.* 2001). Lower amounts of gallic acid and its 4-*O*-methylated metabolite were also excreted in urine after green tea consumption (Table 4). Most probably they originate from the hydrolysis of gallated catechins, as the content of free gallic acid is very low in green tea as compared with the content of gallated catechins (Del Rio *et al.* 2004). In the present study, both gallic acid and its *O*-methylated metabolite accounted for

Table 4. Urinary excretion of polyphenols and their metabolites after polyphenol-rich beverage intake by nine healthy volunteers

(Mean values with their standard errors)

	Urinary excretion (μmol/24 h)											Urinary excretion (μmol/6 h)			
	Control		Green tea		Grape skin extract		Cocoa		Coffee		Grapefruit†		Orange†	
Compound	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Chlorogenic acid	n.d.		n.d.		n.d.		n.d.		5·3**	2·0	n.d.		n.d.	
Caffeic acid	0·1	0·2	0·3	0·5	0·7**	0·3	0·4	0·4	2·1**	0·5	0·4	0·2	0·2	0·1
m-Coumaric acid	0·5	0·9	1·2	1·3	0·8	1·0	1·4	1·4	2·6*	2·4	0·3	0·2	0·2	0·1
Gallic acid	n.d.		0·3	0·8	5·9**	1·4	n.d.		n.d.		n.d.		n.d.	
4-*O*-Methylgallic acid	n.d.		0·9	1·6	15·7**	2·8	0·6	1·1	n.d.		n.d.		n.d.	
Catechin	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
Epicatechin	n.d.		2·1	3·1	n.d.		4·3*	4·2	n.d.		n.d.		n.d.	
Quercetin	n.d.		0.1	0·2	n.d.		n.d.		n.d.		n.d.		n.d.	
Isorhamnetin	n.d.		n.d.		n.d.		n.d.		n.d.		0·1	0·1	0·3**	0·1
Kaempferol	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
Hesperetin	n.d.		0·5	1·4	n.d.		n.d.		n.d.		n.d.		16·6**	7·3
Naringenin	0·1	0·2	0·5*	0·5	0·3*	0·2	0·3	0·4	0·0	0·1	24·7**	12·6	8·4**	3·1
Phloretin	n.d.		n.d.		n.d.		n.d.		n.d.		1·5	1·6	0·7	0·4
Enterodiol	0·5	1·1	0·2	0·3	1·0**	0·6	0·2	0·3	0·1	0·3	0·0	0·1	0·1	0·1
Enterolactone	3·8	2·8	3·0	3·3	6·7	4·8	3·6	2·9	2·8	2·0	1·4	1·4	1·8	1·1

n.d., Not detected.

Mean value was significantly different from control: * $P<0.05$, ** $P<0.01$.

†Urine collected between 6 and 12 h after juice intake.

0·2 % of the gallic acid ingested as esters with catechins. This suggests a low hydrolysis of tea galloyl esters in the body. A low bioavailability of esters of gallic acid or cinnamic acids as compared with the free acids has been reported previously (Hodgson *et al.* 2000; Adam *et al.* 2002).

Epicatechin was excreted in urine after green tea and cocoa intake. The amounts recovered in urine accounted for 2·9 and 1·9 % of the epicatechin ingested with the green tea and cocoa beverage respectively, values much lower than previously reported after cocoa or chocolate consumption (25–30 %) (Baba *et al.* 2000) but similar to that reported after ingestion of pure catechin (1–3 %) (Goldberg *et al.* 2003). Catechin present in lower amounts in the green tea and cocoa beverage was not detected in urine due to its lower intake and low ionisation in our experimental conditions (Table 2).

Quercetin was detected in low concentration in urine after green tea consumption. Quercetin was not analysed in the green tea extract used in the present study but green tea is known to contain several quercetin glycosides (Del Rio *et al.* 2004). No quercetin could be detected in urine samples collected after intake of the other beverages, in agreement with the lower concentration or absence of quercetin in these beverages (Hertog *et al.* 1993).

Naringenin was excreted in high concentration after ingestion of grapefruit and orange juice, the main dietary source of flavanones. Urinary recovery of naringenin in the 6–12 h urine samples accounted for 6·8 % (mol/mol) of the naringenin glycosides ingested with grapefruit juice. Recovery of naringenin and hesperetin accounted respectively for 12·9 and 4·3 % of the corresponding glycosides ingested with orange juice. Excretion of flavanones in this time period accounts for about two-thirds of the total excretion over 24 h (C Manach and C Morand, unpublished results). The present recovery figures are close to average values (8·8 and 8·6 % for naringenin and hesperetin respectively) calculated from previously published data (Manach *et al.* 2004*b*).

A small amount of naringenin was also found in urine samples after ingestion of the other beverages, suggesting that a 2 d washout period was not sufficient to excrete all naringenin ingested before the intervention period. The very high concentrations of naringenin reached after citrus fruit ingestion as compared with those of all other polyphenols ingested in similar quantities (see Table 4) and the widespread consumption of orange juice most probably explains the specific residual occurrence of naringenin in these samples. Isorhamnetin, known as an *O*-methylated metabolite of quercetin, but not quercetin itself, was also found in urine after grapefruit and orange consumption. Its presence could derive from the presence of low amounts of quercetin in these juices but it would not explain its absence in other urine samples collected after consumption of other beverages which may also contain low amounts of quercetin (Hertog *et al.* 1993).

Phloretin was detected in the urine samples collected after consumption of both grapefruit and orange juice. This is the first time that its presence is reported after consumption of citrus juices. It can be assumed that phloretin was produced from naringenin by reductive opening of the C-ring, since phloretin corresponds to the dihydrochalcone form of naringenin. Naringenin has been shown to isomerise to naringenin chalcone in simulated gut conditions (Gil-Izquierdo *et al.* 2003), which could be itself dihydrogenated to phloretin by the gut microflora. The only foods containing phloretin are

apple and apple-derived products, and its use as a biomarker of apple consumption has been suggested (DuPont *et al.* 2002). The present results raise some doubts about its possible use as a biomarker of apple intake.

Enterolactone was detected in all urine samples including those of the control group. Enterodiol, a metabolite of enterolactone, was also present in most urine samples, although in lower concentrations. These lignans, also called mammalian lignans or enterolignans, are produced by the intestinal microflora. They have a dietary origin but their exact precursors and food sources are still a matter of debate (Heinonen *et al.* 2001; Begum *et al.* 2004). Intake of dietary fibre and fibre from grains has been associated with enterolignan excretion (Adlercreutz *et al.* 1981, 1987; Lampe *et al.* 1999). Lignan excretion levels measured in the present study were well within the range of previously published values (Horn-Ross *et al.* 1997). They are not significantly influenced by the consumption of beverages, suggesting that the beverages considered in the present paper do not contain major precursors of mammalian lignans. Mammalian lignans identified in the control group are not easily washed out either, because of a relatively slow elimination as compared with other polyphenols, or because of the existence of unknown precursors still present in the polyphenol-free diet consumed during the 2 d wash-out period. The lower values for lignans in the citrus juice groups are explained by the shorter period over which the urine samples were collected.

Conclusion

We developed a rapid analytical method for the simultaneous quantification of fifteen phenolic compounds in human urine using HPLC-ESI-MS-MS. With a run time of 6 min, the assay is faster than any other published method developed for the simultaneous determination of various polyphenols in urine samples. It is particularly adapted to the analysis of a large series of urine samples, as required for epidemiological studies. The results obtained in the present trial suggest that compounds such as chlorogenic acid, gallic acid, epicatechin, naringenin or hesperetin could be used as biomarkers to evaluate the consumption of respectively coffee, wine, tea or cocoa, and citrus juices. Validation is underway based on the analysis of urine samples collected from 171 subjects following their free-living diet (L Mennen, D Sapinho, H Ito, P Galan, S Hercberg and A Scalbert, unpublished results). This should help estimate more precisely polyphenol intake or exposure, and to determine, in epidemiological studies, optimal levels of polyphenol intake for better health.

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