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Metabolomics Provide New Insight on the Metabolism of Dietary Phytochemicals in Rats[1,2]

Anthony Fardet,[3] Rafael Llorach,[3,4] Alexina Orsoni,[3] Jean-François Martin,[3] Estelle Pujos-Guillot,[3] Catherine Lapierre,[5] and Augustin Scalbert[3]*

[3]Unité de Nutrition Humaine, Institut National de la Recherche Agronomique, Centre de Recherche de Clermont-Ferrand/Theix, F-63122 St-Genès-Champanelle, France; [4]Nutrition and Food Science Department, XarTA, Pharmacy School, University of Barcelona, 08028 Barcelona, Spain; and [5]Unité Mixte de Recherche de Chimie Biologique, Institut National de la Recherche Agronomique-Institut National Agronomique Paris-Grignon, 78850 Thiverval-Grignon, France

Abstract

Foods of plant origin contain a large number of phytochemicals that may positively affect health. Phytochemicals are largely excreted in urine as metabolites that are formed in host tissues or by the microbiota and constitute a great proportion of the urinary metabolome. The latter can be characterized by a metabolomics approach. In this work, we compared the metabolism of lignins to that of the structurally related ferulic acid (FA) and sinapic acid (SA). Five groups of rats ($n = 5$) were fed for 2 d a purified diet alone [control (C)] or supplemented with lignin-enriched wheat bran (3% of the diet, wt:wt), poplar wood lignins (0.42%), FA (0.42%), or SA (0.42%). The metabolomes of urine samples collected after 1 and 2 d of supplementation were analyzed by high-resolution MS (liquid chromatography/quadrupole time-of-flight). Comparing metabolic fingerprints by gathering semiquantitative information on several hundreds of metabolites and using multivariate statistical analysis (partial least squares for discriminant analysis) showed the similarity between both lignin-supplemented and C groups and confirmed that lignins are largely inert and not absorbed in the body. One the other hand, metabolic fingerprints of the 2 phenolic acid-supplemented groups were clearly distinct from the C group. Differences between the groups were mainly from nonmetabolized FA and SA and metabolites excreted in urine. Thirteen of them were identified as sulfate esters and glucuronide and glycine conjugates of the same phenolic acids, and of dihydrosinapic, vanillic, and benzoic acids. This study shows that metabolomics allows the identification of new metabolites of phytochemicals and can be used to distinguish individuals fed different phytochemical-containing foods. J. Nutr. 138: 1282–1287, 2008.

Introduction

A large variety of phytochemicals are found in foods and beverages commonly consumed in the human diet. Some of these phytochemicals, such as polyphenols, carotenoids, glucosinolates, or sulfur compounds have raised considerable interest for their favorable effects on health and the prevention of various diseases. They may prevent cardiovascular diseases, cancers, diabetes, osteoporosis, and neurodegenerative diseases (1–3). However, these effects are still a matter of intense debate and few health claims on such phytochemicals have so far been approved by regulatory authorities. More evidence on the associations between phytochemical intake and positive health effects is needed as well as a better understanding on the mechanisms of action involved. Metabolic profiles can be characterized today in a more comprehensive way using high-throughput analytical tools such as NMR spectroscopy or MS. Recent developments in MS allow characterizing in a semi-quantitative way a large fraction of the plasma or urine metabolome (4). Subtle metabolic differences can be identified between individuals or for the same individual between different environmental conditions using metabolomics. In this approach, metabolic profiles or fingerprints are compared using proper multivariate statistical methods (5). Metabolomics opens new perspectives in the field of nutrition to explore the complex metabolic effects of diets or nutrients (6). A few metabolomics studies have shown that it allows the unraveling of new metabolic effects of phytochemicals as previously described for isoflavones or catechins (7–9).

A major fraction of the metabolome in human urine is made up of the products of food digestion. We propose to call this fraction "food metabolome," which is part of the xenometabolome derived from the incomplete catabolism of xenobiotics (drugs, environmental pollutants, or dietary components) (10).

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[2] Author disclosures: A. Fardet, R. Llorach, A. Orsoni, J.-F. Martin, E. Pujos-Guillot, C. Lapierre, and A. Scalbert, no conflicts of interest.
* To whom correspondence should be addressed. E-mail: scalbert@clermont.inra.fr.

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Phytochemical metabolites are prominent features of this food metabolome (11). Metabolomics studies of the food metabolome may lead to the discovery of new phytochemical metabolites and new markers of phytochemical intake. Such an approach is therefore potentially useful to study the metabolism of phytochemicals and to search for further associations between phytochemical intake and health or disease risk.

We apply here this metabolomics approach to study the metabolism of some dietary polyphenols in rats. Lignins are cell wall phenylpropanoid polymers widespread in all vascular plants and notably present among foods in cereal brans, beans, and some vegetables (12). We have shown that lignins, which were generally considered as inert in the body, are actually metabolized into enterolactone phytoestrogen (13). We further explored its fate in rats using metabolomics and compared its metabolism to that of 2 structurally related phenolic acids, ferulic (FA)[6] and sinapic (SA) acids, both known as precursors in the biosynthesis of lignins (14). This proof of concept study illustrates the capacity of this new metabolomics approach to identify new metabolites of phytochemicals and to recognize individuals fed different phytochemical-containing foods.

Materials and Methods

Animals and diets. Male Wistar rats weighing ~390–430 g were fed for 16 d a standard purified diet containing 75.5% wheat starch, 15% casein, 5% peanut oil, 1% vitamins [AIN-93VX mix (15) supplemented with 0.25% choline bitartrate, UPAE, INRA], and 3.5% minerals [AIN-93 G-MX mix (15), MP Biomedicals]. After this period of adaptation, rats were divided into 5 groups ($n = 5$) and fed for 2 d a control diet (C) or that diet supplemented with 3% lignin-enriched wheat bran (LEWB), 0.42% poplar lignins (PL), 0.42% FA, or 0.42% SA. These 2 lignin materials were chosen because of their importance in the diet (LEWB) or the relative facility to isolate pure lignin fractions in good yield (PL). We determined the level of supplementation to provide the same amount of lignins or FA as a 60% whole-grain cereal-based diet. LEWB was prepared from extractive-free wheat bran treated with enzymes to remove most of the cell wall polysaccharides, as described previously (13). PL was obtained by dioxane-water extraction of ultraground poplar wood (16). LEWB and PL preparations contained 17 and 82% lignins, respectively [Klason determination (17)]. Nonlignin components in these fractions were mainly cell wall polysaccharides. The monomeric composition of these lignins was determined by thioacidolysis according to Lapierre et al. (18): PL was composed of 60% syringyl and 40% guaiacyl units and the lignin fraction of LEWB was composed of 49% syringyl, 42% guaiacyl, and 8% *p*-hydroxyphenylpropane units. In addition, LEWB contained 3.2 mg/g of ester-linked FA as determined according to Lapierre et al. (19). Animals consumed food and water ad libitum between 1600 and 0800. Rats were maintained in a temperature-controlled room (22°C) with a dark period from 2000 to 0800. They were handled according to the recommendations of the Institutional Ethics Committee (Clermont-Ferrand University, France). Urine was collected in metabolic cages during the dark period (from 1600 to 0900) at d 0 (before switching to polyphenol-supplemented diets) and at d 1 and d 2 (1 and 2 d after the beginning of the polyphenol-supplemented diets) and was stored at −20°C for metabolomic analyses.

Metabolite standards. FA, isoFA, SA, hippuric acid, and enterolactone were obtained from Sigma-Aldrich. Standards were dissolved in a Milli-Q water/acetonitrile solution (50:50 v:v) + 0.1% formic acid to obtain solutions of 1 mg/L.

Urine sample analysis by liquid chromatography-quadrupole time-of-flight MS. Liquid chromatography (LC) was performed on a HPLC Alliance 2695 (Waters) using a Waters SymmetryShield RP18 column (2.1 × 150 mm; 5 μm). The HPLC system was coupled to a Micromass quadrupole time-of-flight (QTof)-micro equipped with an electrospray source operating in positive ion mode. Urine samples were centrifuged at 13,000 × *g*; 5 min; the supernatant was diluted with deionized water (1:3) before injection. The column, equilibrated with solvent A (water + 1% formic acid), was eluted with increasing concentrations of solvent B (acetonitrile + 1% formic acid): a linear gradient of 0 to 20% B from 0 to 4 min, 20 to 95% B from 4 to 8 min, and 95% B from 8 to 9 min. Flow rate was 300 μL/min. The source temperature in the mass spectrometer was set at 120°C with a cone gas flow of 50 L/h, a desolvatation gas temperature of 300°C, and a nebulization gas flow of 400 L/h. The capillary voltage was set at 3.0 kV and the cone voltage at 30 V. A scan time of 0.4 s with an interscan delay of 0.1 s was applied. A lock-mass of leucine enkephalin at a concentration of 0.25 mg/L in 50:50 methanol:water + 0.2% formic acid was employed with an infusion flow of 10 μL/min via a lock spray interface. The mass spectrometry data were collected in continuum and full scan mode [mass to charge ratio (*m/z*) 100–1000].

Data analysis. The LC-MS data were analyzed using Micromass MarkerLynx Application Manager (version 4.0 SP4 integrated to the MassLynx software, Waters Corporation). The data obtained consisted of a large set of variables corresponding to the detected ions, each variable being defined by its retention time and exact mass. Raw data were filtered: a variable was selected if present in at least 2 urine samples in a given rat group (15 groups defined by diet and time). The 5682 variables selected were then centered scale, log-transformed, and analyzed by partial least squares for discriminant analysis (PLS-DA) using SIMCA-P (version 11, Umetrics). The quality of the PLS-DA models was evaluated by the goodness-of-fit parameter (R^2) and the predictive ability parameter (Q^2), which is calculated by a 7-round internal cross-validation of the data. R^2 and Q^2 values at levels ≥0.5 indicated good quality of PLS-DA models. Variables were also classified according to their dependence on diet and sampling day by using repeated-measures ANOVA (proc mixed SAS, v8.01, SAS Institute). The ANOVA gives all the variables having a "diet" effect ($P < 0.05$) and/or an interaction between diet and sampling day ($P < 0.05$) and, among them, those discriminating the 5 groups of rats at each day of urine sampling following diet consumption ($P < 0.05$ at both d 1 and 2). Variables were considered as characteristic of a given group of rats only if present in more than one-half of the urine samples of that group. Among them, the variables present in 1, 2, or 3 groups and absent from the other groups were counted. Pearson correlation coefficients between each variable in urine samples were calculated using Excel software (Microsoft Office).

Metabolite identification. Characteristic markers of the different groups of rats were identified according to their exact mass compared with those registered in KEGG (20) and Human Metabolome (21) databases and to those of expected metabolites previously described in the literature (mass difference <5 mDa). Identification was confirmed by comparison with standards when available. Variables corresponding to fragments or adducts formed in the electrospray source were recognized by a retention time identical to that of the parent metabolite and an expected mass loss or gain.

Results

Urinary metabolic profiles following polyphenol-supplemented diets. We first conducted a PLS-DA analysis of the samples collected on d 0, 1, and 2 for rats fed the C, FA, SA, LEWB, and PL diets, all d 0 samples being clustered with d 1 and 2 samples of C group (total of 9 classes). The model showed a poor predictability to explain the differences between all 9 classes ($R^2X = 0.104$, $R^2Y = 0.377$, $Q^2 = 0.120$). More particularly, LEWB and PL groups could not be distinguished from the C group (data not shown). On the other hand, FA and SA groups could clearly be differentiated from C, LEWB, and PL groups. A

[6] Abbreviations used: C, control; FA, ferulic acid; LC-QTof, liquid chromatography-quadrupole time-of-flight; LEWB, lignin-enriched wheat bran; PL, poplar lignin; PLS-DA, partial least squares for discriminant analysis; SA, sinapic acid.

new model was therefore constructed using 3 classes defined as follows: FA group at d 1 and 2; SA group at d 1 and 2; C, LEWB, and PL groups at d 0, 1, and 2, and FA and SA groups at d 0 (**Fig. 1**). The model showed a good predictability to explain the differences between these 3 classes ($R^2X = 0.286$, $R^2Y = 0.986$, $Q^2 = 0.914$).

Variables characterizing the different groups of rats fed phenolic acids and lignins. There were 1039 and 1036 variables discriminating the 5 groups of rats at d 1 and 2, respectively (1-way ANOVA, $P < 0.05$). At d 1, 404 variables were present in only the urine of rats fed FA and SA diets and absent in other groups (**Fig. 2**). Few of the variables were shared by both FA and SA groups. The LEWB group was characterized by 24 variables and the PL group by 26 variables not shared with the LEWB group. The LEWB and PL groups had only a very few variables in common with the FA and SA groups (from 1 to 5). Of all variables affected by the different diets, 118 (d 1) and 110 (d 2) variables were also common to the C group and therefore not derived from the metabolism of lignins or phenolic acids. Similar results were obtained at d 2 (not shown).

Marker identification. Several of the variables discriminating FA and SA groups were identified by comparison of exact mass with those given in public databases and by comparison of experimental retention time with that of authentic standards (**Table 1**). For a given metabolite characterized by its retention time, several ions were often observed corresponding to fragments and/or adducts of the parent compound formed in the electrospray ionization source. For example, the ion at *m/z* 177 (RT = 9.67 min) originated from the loss of water from FA and was highly correlated ($r = 0.99$; $P < 0.05$) to the $[M + H]^+$ ion (*m/z* 195). Similarly, the marker with *m/z* 195 (RT = 8.22 min) originated from the loss of the glucuronide moiety from FA glucuronide. Similar fragment ions were observed for SA and SA glucuronides. Other markers showed *m/z* values characteristic of FA sulfate ester (*m/z* 275) and feruloylglycine sulfate ester (*m/z* 332). Other metabolites such as hippuric acid (*m/z* 180) were present in the urine of all groups but at a significantly higher level (from 2- to 11-fold increase) in the FA group. Of all the 15 metabolites tentatively identified (Table 1), the identity of 4 of them was confirmed by comparison with authentic standards (FA, isoFA, SA, and hippuric acid). Finally, no variables

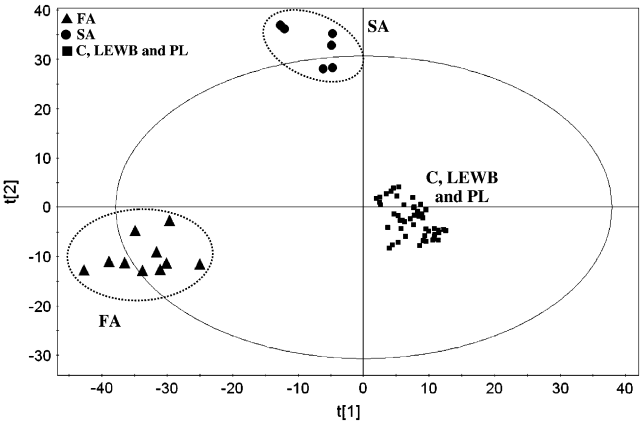

FIGURE 1 PLS-DA score plot (axes 1 and 2) of the urine LC-QTof metabolic profiles of rats fed C, LEWB, PL, SA, or FA diets. Three classes were predefined: FA group at d 1 and 2; SA group at d 1 and 2; C, LEWB and PL groups at d 0, 1, and 2, and FA and SA groups at d 0.

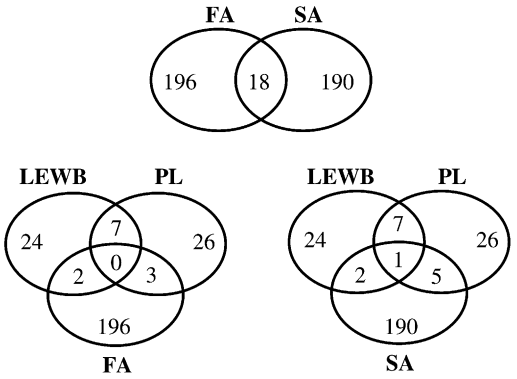

FIGURE 2 Numbers of variables (ions) characterizing the urine samples collected from rats fed FA, SA, LEWB, and PL diets at d 1, $P < 0.05$ (repeated-measures ANOVA). Only variables present in more than one-half of the urine samples of a given group of rats were considered as characteristic of that group.

characteristic of lignin metabolism or metabolic effects of lignin could be identified.

Discussion

The main objective of this work was to evaluate the metabolic fate of lignins and to compare it to that of 2 structurally related phenolic acids using a metabolomic approach. In addition, metabolomics allowed us to identify in urine new metabolites of phytochemicals that may be used as biomarkers of intake. We anticipate that the present data together with data relative to other phytochemicals to be collected in future studies will form the basis of a new database on the food metabolome. This database should allow researchers to discriminate individuals according to their diet, based on urine analysis.

Two lignins isolated from either PL or LEWB were compared with FA and SA. Of the thousand variables affected by the 4 polyphenol-supplemented diets, very few were specific of the LEWB and PL groups or common to lignin- and phenolic acid-supplemented diets. None of these variables could be identified on the basis of database querying. They could be either lignin metabolites or endogenous metabolites affected by lignin metabolism. The statistical weight of these variables was not sufficient to distinguish metabolic fingerprints of LEWB and PL groups from that of C group in PLS-DA analyses. In a previous study on rats fed deuterated wheat bran lignins, we showed that lignins were metabolized within 1 or 2 d into enterolactone (a mammalian lignan) (13). However, enterolactone was not detected in the present study due to a lower sensitivity of the LC-QTof method used here for this compound. The limit of detection (5 μmol/L) was higher than the concentration previously determined in urine of the lignin-supplemented rats ($\sim$1.4 μmol/L) (13). The present results therefore suggest that lignin metabolism is very limited in rats. Enterolactone may well be one of the main metabolites formed from the lignins and absorbed through the gut barrier.

The absence of common variables in lignin-supplemented and phenolic acid-supplemented rats suggests that the lignin monomer units (mainly guaiacyl and syringylpropane units) are not degraded in rats into metabolites common to FA and SA.

SA and FA groups presented urinary metabolic fingerprints clearly distinct from the C, LEWB, and PL groups. Both FA and SA were rapidly metabolized in rats and their metabolites

TABLE 1 Urinary markers in rats fed FA or SA diets

RT	Detected mass	*P*-value[1]	Putative identification	Ions seen	Theoretical mass	Mass difference
min	*m/z*				*m/z*	*Δ mDa*
9.67	195.0667	0.0200	FA[2]	$[M+H]^+$	195.0657	1.0
	177.0553	<0.0001		$[M+H\text{-}H_2O]^+$	177.0551	0.2
9.64	275.0237	<0.0001	FA sulfate	$[M+H]^+$	275.0225	1.2
8.22	371.0964	0.0110	FA glucuronide	$[M+H]^+$	371.0978	−1.4
	195.0676	0.0003		[M+H-glucuronide]$^+$	195.0657	1.9
9.44	195.0659	<0.0001	IsoFA[2]	$[M+H]^+$	195.0657	0.2
	178.0614	0.0003		(^{13}C $[M+H]^+$-H_2O)	178.0629	−1.5
	177.0553	<0.0001		$[M+H\text{-}H_2O]^+$	177.0551	0.2
7.98	371.0986	<0.0001	IsoFA glucuronide	$[M+H]^+$	371.0978	0.8
	177.0560	<0.0001		[M+H-glucuronide-H_2O]$^+$	177.0551	0.9
8.41	252.0887	<0.0001	Feruloylglycine	$[M+H]^+$	252.0872	1.5
	177.0557	<0.0001		[M+H-glycine]$^+$	177.0551	0.6
7.55	254.1026	<0.0001	Dihydroferuloylglycine	$[M+H]^+$	254.1022	0.4
	255.1068	0.0001		(^{13}C $[M+H]^+$)	255.1101	−3.3
	179.0715	<0.0001		[M+H-glycine]$^+$	179.0708	0.7
	137.0600	<0.0001		[M+H-glycine-C_2H_2O]$^+$	137.0602	−0.2
8.86	332.0441	<0.0001	Feruloylglycine sulfate	$[M+H]^+$	332.0440	0.1
	257.0114	<0.0001		[M+H-glycine]$^+$	257.0119	−0.5
	252.0891	<0.0001		[M+H-sulfate]$^+$	252.0872	1.9
8.23	334.0596	<0.0001	Dihydroferulloylglycine sulfate	$[M+H]^+$	334.0591	0.5
	351.0918	0.0007		$[M+NH_4]^+$	351.0862	5.6
6.93	226.0729	<0.0001	Vanilloylglycine	$[M+H]^+$	226.0715	1.4
	151.0386	<0.0001		[M+H-glycine]$^+$	151.0389	−0.3
9.07	225.0768	<0.0001	SA[2]	$[M+H]^+$	225.0763	0.5
	247.0631	<0.0001		$[M+Na]^+$	247.0577	5.4
	226.0830	<0.0001		(^{13}C $[M+H]^+$)	226.0835	−0.5
	208.0716	<0.0001		(^{13}C $[M+H\text{-}H_2O]^+$)	208.0730	−1.4
	207.0614	<0.0001		$[M+H\text{-}H_2O]^+$	207.0651	−3.7
	175.0412	<0.0001		[M+H-CH_3O-H_2O]$^+$	175.0389	2.3
9.19	305.0360	<0.0001	SA sulfate	$[M+H]^+$	305.0331	2.9
	225.0773	<0.0001		[M+H-sulfate]$^+$	225.0763	1.0
	207.0644	<0.0001		[M+H-sulfate-H_2O]$^+$	207.0651	−0.7
8.01	439.0661	<0.0001	SA glucuronide	$[M+K]^+$	439.0637	2.4
	423.0888	<0.0001		$[M+Na]^+$	423.0897	−0.9
	418.1351	<0.0001		$[M+NH_4]^+$	418.1343	0.8
	383.0990	0.0014		$[M+H\text{-}H_2O]^+$	383.0972	1.8
	242.1047	0.0416		[(M+H-glucuronide)+NH_4]$^+$	242.1028	1.9
	226.0786	<0.0001		(^{13}C [M+H-glucuronide]$^+$)	226.0835	−4.9
	225.0759	<0.0001		[M+H-glucuronide]$^+$	225.0763	−0.4
	175.0411	<0.0001		[M+H-glucuronide-CH_3OH-H_2O]$^+$	175.0389	2.2
7.77	227.0911	<0.0001	Dihydrosinapic acid	$[M+H]^+$	227.0919	−0.8
7.24	180.0657	0.0118	Hippuric acid[2]	$[M+H]^+$	180.0661	−0.4
	181.0713	0.0497		(^{13}C $[M+H]^+$)	181.0733	−0.2
	105.0334	0.0055		[M+H-glycine]$^+$	105.0334	0

[1] *P*-values (diet effect) were obtained from repeated-measures ANOVA including all 5 groups at both d 1 and 2.
[2] Identity was confirmed with authentic standards.

recovered in urine. FA glucuronide, FA sulfate ester, feruloylglycine, and vanilloylglycine were identified as major metabolites in rat urine. All have been described in previous bioavailability studies (22–25). Few studies have been carried out on SA. Dihydrosinapic acid is the only metabolite described thus far in rat urine following SA supplementation in the diet (26). We also detected the glucuronide and sulfate ester of SA in the SA group. Despite the presence of ester-linked FA in LEWB, no FA metabolites were found in urine of rats fed LEWB. This is due to the low concentration of FA in LEWB (3.2 mg/g) and in the LEWB diet (0.01%) compared with that in the FA diet (0.42%) and also to its esterified nature. Indeed, ester-linked FA is poorly available in rats compared with free FA (27).

Other metabolites can be formed from dietary phenolic acids without being exclusively derived from these precursors. Metabolomics analysis suggests that FA was also metabolized into hippuric acid. Hippuric acid has repeatedly been described as a polyphenol metabolite. A similar metabolomics approach showed formation of hippuric acid from tea catechins (28). To our knowledge, no study has linked increased hippuric acid excretion with FA consumption. However, FA, after demethylation and dehydroxylation, is degraded within the colon by

resident flora into dihydroxyphenylpropionic acid before yielding phenylpropionic, benzoic, and, in the end, hippuric acids (29,30).

Apart from the variables identified here as phenolic acid metabolites, other variables affected by one or the other diets may originate from the influence of the polyphenol supplementation on rat metabolism or from the digestion of the constituents of the basal diet. Over 100 variables common to the C group and 1 or more polyphenol-supplemented groups belong to this category. None of these variables could be identified when comparing their exact mass with those registered in publicly available MS-based metabolomics databases.

In conclusion, metabolomics allowed us to distinguish groups of rats that consumed FA or SA from those that consumed LEWB or PL based on their different urine metabolomes. Such an approach allowed us to confirm the limited degradation of lignins in rats compared with the rapidly metabolized phenolic acids, FA and SA. A large number of phenolic acid metabolites were also detected. There is a considerable diversity of phytochemicals present in the various plants regularly consumed with the human diet. These phytochemicals and their metabolites formed in the body and excreted in urine constitute a major part of the food metabolome (31). Metabolomics will contribute to better characterize this fraction of the food metabolome to further explore the metabolism of phytochemicals. The present study shows a considerable potential to identify a large variety of metabolites formed from these phytochemicals. Not all the metabolites detected here in urine could be identified. A major limit of MS-based metabolomics is the difficulty in identifying the markers revealed by statistical analyses. Metabolite databases such as KEGG or Human Metabolome Database contain only a very limited number of phytochemicals or phytochemical metabolites. Databases including all phytochemical metabolites still need to be constructed in collaboration with plant scientists who have undertaken the task of building up databases for plant secondary metabolites (32,33). Exploitation of these databases will also require standardization of the analytical methods used and of the minimum reporting requirements for marker identification (34). Such databases will also facilitate the discovery of new markers of phytochemical exposure useful for epidemiological studies (35).

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