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An LC-MS-Based Metabolomics Approach for Exploring Urinary Metabolome Modifications after Cocoa Consumption

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Cocoa-phytochemicals have been related to the health-benefits of cocoa consumption. Metabolomics has been proposed as a powerful tool to characterize both the intake and the effects on the metabolism of dietary components. Human urine metabolome modifications after single cocoa intake were explored in a randomized, crossed, and controlled trial. After overnight fasting, 10 subjects consumed randomly either a single dose of cocoa powder with milk or water, or milk without cocoa. Urine samples were collected before the ingestion and at 0−6, 6−12, and 12−24-h after test-meals consumption. Samples were analyzed by HPLC-q-ToF, followed by multivariate data analysis. Results revealed an important effect on urinary metabolome during the 24 h after cocoa powder intake. These changes were not influenced by matrix as no global differences were found between cocoa powder consumption with milk or with water. Overall, 27 metabolites related to cocoa-phytochemicals, including alkaloid derivatives, polyphenol metabolites (both host and microbial metabolites) and processing-derived products such as diketopiperazines, were identified as the main contributors to the urinary modifications after cocoa powder intake. These results confirm that metabolomics will contribute to better characterization of the urinary metabolome in order to further explore the metabolism of phytochemicals and its relation with human health.

Keywords: metabolomics • nutrition • cocoa powder • urinary metabolome • food metabolome

Introduction

The consumption of a diet rich in fruit and vegetables is related to a reduction in the development of some kinds of diseases. These positive effects on health have been linked to the phytochemical content, mainly polyphenols[1,2] of plants and/or derived food plants. However, some factors, such as bioavailability and microbiota degradation, have been shown as mandatory keys to these expected healthy effects.[3]

Cocoa and cocoa-derived foods have been considered important sources of phytochemicals such as phenolic compounds, mainly flavan-3-ols,[4] and alkaloids, mainly theobromine.[5] Despite these compounds, cocoa manufacturing processes, such as fermentation or roasting, modify the cocoa powder composition, adding compounds mainly related to the particular cocoa aroma and taste.[6,7] Cocoa-derived products are consumed as chocolate, as beverages, or as other products made with cocoa powder. Regarding consumption, Spain is the country that has the highest consumption of cocoa powder per person (around 1.7 kg/(person/year)) followed by Norway, Sweden, France and Brazil.[8] Spanish cocoa powder consumption shows two particular characteristics: one is that this product is consumed with milk, especially at breakfast, and the other is that this product is present in more than 80% of households with children, representing the main source of flavonoids (around 50% of daily total flavonoid intake) in the young population (children and teenagers younger than 15 years).

An important amount of in vitro and in vivo studies have shown that cocoa and their food-derivates products exert different health-promoting activities mainly related to cardiovascular health.[4,9] In this regard, cocoa consumption has been related to an improvement in antioxidant status,[10] antiplatelet effects, immunoregulatory activity, and vasorelaxation.[9]

Metabolomics aims to assess metabolic changes in a comprehensive and global manner in order to infer biological functions and provide the detailed biochemical responses of cellular systems.[11] Metabonomics and metabolomics approaches have been applied to clinical, pharmaceutical and toxicological applications[12] and recently has also emerged as a field of increasing interest to food and nutrition science.[13,14] Metabolomic and metabonomic strategies produces complex data sets, and therefore, the uses of appropriate multivariate

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statistical and visualization tools are mandatory keys that include efficient and robust methods to model, analyze, and interpret the complex chemical and biological data.[15] Some different chemometric tools, such as principal component analysis (PCA), partial least-squares discriminate analysis (PLS-DA) or their variation with a previous orthogonal signal correction (OSC-PLS-DA), have been proposed as powerful tools for metabolomic studies.

In this context, the aim of the present work was to apply a metabolomic strategy, based on liquid chromatography coupled with time-of-flight mass spectrometry (HPLC-q-TOF) with multivariate analysis, in order to analyze the influences of a single cocoa intake on the 24 h kinetic trajectory of the human urinary metabolic profile identifying the most relevant markers of these modifications.

Experimental Section

Chemicals. The following chemicals were obtained commercially: caffeine, theobromine, tyrosine, vanillic acid, cytosine, leucine, carnitine, betaine, deoxyadenosine, deoxyguanosine, deoxythymidine and formic acid (Fluka, Sigma-Aldrich, St Louis, MO). Water for chromatographic separations was purified with a Milli-Q Gradient A10 system (Millipore, Schwalbach, Germany), and acetonitrile was of HPLC grade (Merck).

Cocoa Powder Composition. The soluble cocoa powder used in the study contained 57% carbohydrates (sucrose, 46%; 104 starch, 1%; complex carbohydrates, 10%), 16% fiber, 5.4% fat, 14.1% protein, 3.97% moisture, 1.3% theobromine, 0.13% caffeine and 2% ash. The phenolic composition (mean ± SD) of the cocoa powder was determined according to the methodology of Andrés-Lacueva et al.:[16] 23.1% monomers with 0.71 ± 0.09 mg/g of (−)-epicatechin and 0.21 ± 0.01 mg/g of (+)-catechin, 13.4% dimers, including 0.64 ± 0.06 mg/g of procyanidin B2, 63.6% 3−8mers,[17,18] and flavonols including 33.87 μg/g isoquercitrin, 5.74 μg/g quercetin, 4.33 μg/g quercetin-3-glucuronide and 36.32 μg/g quercetin-3-arabinoside. The total polyphenolic content of cocoa powder was 11.51 ± 0.95 mg catechin/g.

Subjects and Study Design. Ten healthy volunteers (5 women and 5 men) between 18 and 50 years old with a corporal mass index of 21.6 ± 2.1 were recruited. After overnight fasting, they were provided randomly with three different single doses of (a) 40 g of cocoa powder with 250 mL of water (hereafter termed CW); (b) 40 g of cocoa powder with 250 mL of milk (hereafter termed CM diet); and (c) 250 mL of milk as a control (hereafter termed NM diet). To avoid differences in the rate of stomach emptying (which in turn would influence absorption kinetics), sugar was added to balance energy content, thus, making the three test meals isoenergetic. Test meals were prepared, following a standardized procedure, on each day of the study. The CW and CM macronutrient composition (in 250 mL) was the following: carbohydrates, 30.75 and 58.4 g; fat, 10.91 and 2.16 g; protein, 13.54 and 5.64 g; energy, 1152 kJ (275.35 kcal) and 1158 kJ (276.6 kcal), respectively.

Urine samples were obtained before consumption (0 h) and during the 0−6 (6 h), 6−12 (12 h) and 12−24 h (24 h) periods after test-meal consumption. This protocol was repeated three times on three different days (a week in between) following a crossover experimental design. The volunteers remained in the clinical ward for over 6 h to avoid the possibility of transgressing the proscribed diet in the first study period. For the remaining 18 h, all the volunteers followed a standardized polyphenol-free diet (as they had done the day before the study). None reported any history of heart disease, homeostatic disorders or other medical issues, nor received any medication or vitamin supplements. All gave written informed consent before their inclusion in the trial, and the Institutional Review Board of the Hospital Clinic of Barcelona approved the study protocol. Participants were instructed to abstain from vitamin supplements, drugs, alcoholic beverages and any polyphenol-rich foods for at least 48 h before and during the test day. A list of allowed and forbidden foods and two menus were given to all participants to help them to follow the polyphenol-free diet strictly the day before the study. The urine samples were stored at −80 °C until analysis.

Sample Preparation. The urine samples were thawed before analysis and centrifuged for 5 min at 12 000*g*. A 50 μL aliquot of the supernatant was diluted with 50 μL of Milli-Q water and vortex mixed; the resulting solution was transferred to a 96-well plate for HPLC-q-TOF analysis. To evaluate the quality in this metabolomic study, a procedure with some modifications was used.[19] Three classes of QC samples were used for each batch, injected in randomized order.

QC1: samples were Milli-Q water samples. These samples were injected three times in each batch.

QC2: Standard mixture solution (1 ppm) consisting of cytosine, carnitine, betaine, leucine, deoxyadenosine, deoxyguanosine, deoxythymidine. This sample was injected twice in each batch.

QC3: The reinjection of urine samples. For this purpose six urine samples randomized selected from the whole list of samples were reinjected. Every reinjection was carried out in a randomize order during the same batch of the selected urine sample.

HPLC-q-TOF Analysis. Chromatography was performed on an Agilent 1200 RRLC system using an RP 18 Luna 5 μm, 50 × 2.0 mm (Phenomenex, Torrance, CA). The mobile phase consisted of (A) 0.1% HCOOH and (B) acetonitrile 0.1% HCOOH. The flow rate was 600 μL/min, and the injection volume was 15 μL for both urine samples and QCs. A linear gradient with the following proportions (v/v) of phase B (*t*, %B) was used: (0, 1), (4, 20), (6, 95), (7.5, 95), (8, 1), (12, 1). The HPLC system was coupled with a hybrid quadrupole time-of-flight QSTAR Elite (Applied Biosystems/MDS Sciex). The MS acquisition was performed in positive ionization and full scan (70−700 Da) modes. Spray parameters were IS +4000, DP 80, FP 380, DP2 10, IRD 6, IRW 5, TEM 400 °C with N_2 as curtain (CUR = 50) and nebulizer (NEB = 60) gases. The TOF was calibrated with reserpine (1 pmol/μL) using the ions at m/z 195.1651 and m/z 609.2812. LC-MS data were acquired in three successive batches of analysis. To avoid possible bias, the sequences of injections were randomized.

Data Processing. LC-MS data were analyzed using MarkerView 1.2 software (Applied Biosystems, MDS Sciex, Toronto, Ontario, Canada) which performs feature extraction by peak finding for each sample and alignment using mass and retention time windows for the peaks. Peak detection was performed using a minimum peak width of 1 ppm, a noise threshold of 5, and a subtraction multiple factor of 1.5. Alignment used 0.04 Da and 0.06 min tolerance windows.

Multivariate Analysis (MVA). Principal component analysis (PCA), partial least-squares discriminant analysis (PLS-DA) and orthogonal signal correction (OSC) PLS and OSC-PLS-DA were used.

Principal component analysis (PCA) is an unsupervised analytical tool involved in the calculation of linear combina-

tions of the original descriptors, the PCs. Each PC is a linear combination of the original variables, whereby each successive PC explains the maximum amount of variance possible in the data set. This method was used for the evaluation of the analytical variability of QCs across the data acquisition.

The PLS-DA is a supervised method that finds directions in a multivariate space for maximum separation of observations (urine samples) belonging to different classes.

To improve data analysis, a preprocessing filter, termed orthogonal signal correction (OSC), was selected.[20] The OSC filter can selectively remove the variation of data X (the LC-MS data set) having no correlation with Y. This filter was carried out by SIMCA-P software using diet and time of urine collection as correction factors.

To better visualize the contribution of ions in the separation of the classes, the S-plot was used. The S-plot combines the contribution (w^*c) and reliability/correlation (p(corr)) from the OSC-PLS model and helps to identify differential markers between classes.[15] With a significance level of 0.05, a p(corr) of 0.5 was adopted as an arbitrary cutoff value to select the variables.

Data were log-transformed, mean-centered and Pareto-scaled (each variable was weighted according to $1/(SD)^{1/2}$) before PCA analysis, and log-transformed and mean-centered before PLS-DA, OSC-PLS and OSC-PLS-DA (SIMCA-P+ 11.5; Umetrics, Umea, Sweden). The quality of the models was evaluated by the goodness-of-fit parameter (R^2X), the proportion of the variance of the response variable that is explained by the model (R^2Y) and the predictive ability parameter (Q^2), which was calculated by a seven-round internal cross-validation of the data using a default option of the SIMCA-P+ 11.5 software. In addition, a permutation test ($n = 20$) was carried out to test possible overfit of the model.[21]

Metabolite Identification. Markers contributing to the discrimination (p(corr) ≥ 0.5) were identified on the basis of their exact mass, which was compared to those registered in the Human Metabolome Database (HMDB; www.hmdb.ca) and the Kyoto encyclopedia of gene and genome (KEGG) (http://www.genome.jp/) (ΔmDa ≤ 5 mDa) using an in-house R script for R environment.[22] The database querying was improved by adding an "*in house*" DB focused on those expected metabolites related to cocoa phytochemicals as described in the literature.[6,7,22−25] In addition, clustering analysis (with Pearson correlation) using the PermutMatrix software 1.9.3[26] facilitated the identification of clusters of ions originating from the same metabolite. All ions in a cluster were characterized by the same retention time including fragments (e.g., loss of water or glucuronide moiety), adducts and ^{13}C isotopes formed in the electrospray source,[27] as well as characteristic neutral losses of important types of phase II conjugates.[28] The LC-MS behavior was also compared with those proposed by the Massbank database (www.massbank.jp) and Human Metabolome Database.

Results and Discussion

Evaluation of Data: Quality Parameters. Small variations between LC-MS runs are to be expected in any LC-MS-based method; therefore, the inclusion of different tools to evaluate the quality of acquisition data steps is mandatory in a large metabolomic study. Several artifacts could occur during the acquisition steps in large LC-MS metabolomic studies, including possible trends caused by the order of acquisition, carryover, sensitivity changes or ion suppression.[29] Alterations

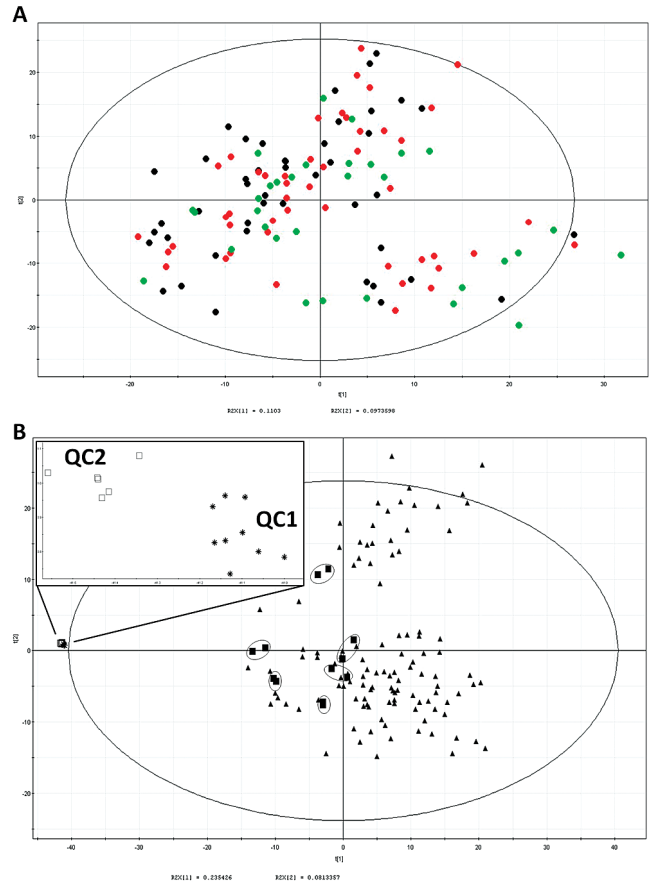

Figure 1. (A) PCA scores plot (PC1 vs PC2) obtained from urine samples. Black dots, first batch; red dots, second batch; and green dots, third batch. (B) PCA score plot (PC1 vs PC2) obtained from urine and QC samples. Black boxes indicate reinjected samples and circles have been used to link corresponding reinjected samples. Zoomed area corresponds to QC1 (stars) and QC2 (squares).

occurred during data acquisition could cause drastic artifacts in data profiles which, using an unsupervised multivariate analysis such as PCA, could be shown by the identification of either particular trends or sample clusters. An evaluation of possible trends dependent on the acquisition order was carried out using a PCA analysis with Pareto scaling. Figure 1A shows the PCA results with sample labels indicating batches. The PC1/PC2 score plots revealed that urine samples were not clustered according to batches. Another possible artifact that could occur during experiment is the carryover that could be enhanced by the randomization of sample injections as this procedure could place low-level samples following intense ones, and therefore, possible traces could still remain in the autosampler. This phenomenon could affect the next sample or, more probably, several samples in a batch. Moreover, carryover could also be arbitrary, whereby late-eluting residues on chromatographic columns could affect chromatograms several samples later.[30] To study the possible occurrence of carryover, blank samples (QC1) were injected randomly into each batch. As depicted in Figure 1B, QC1 samples were tightly clustered, suggesting that carryover did not occur.

Other artifacts could occur relating to the retention time and mass precision shifts. In this context, QC2 samples, corresponding with the mixture of standards, were randomly injected. Retention time shift ranged from 0.009 min up to 0.08

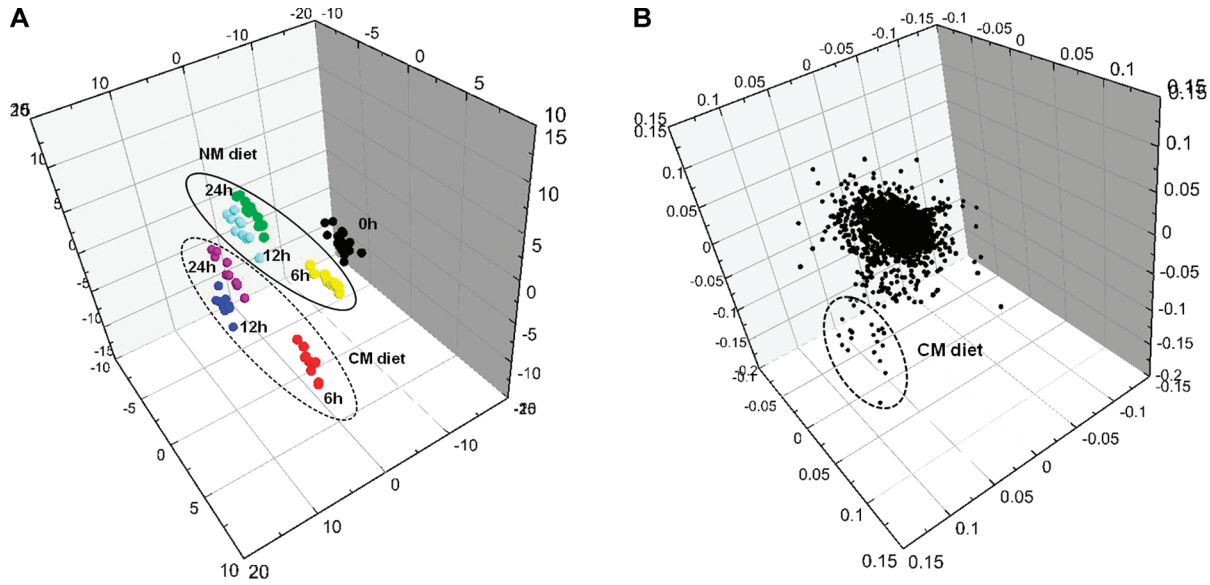

Figure 2. (A) 3D OSC-PLS scores plot LC-MS deriving from the urine samples collected before test-meal consumption (CM and NM) (0 h) and at 6 h (CM: red spheres; NM: yellow spheres), 12 h (CM: blue spheres; NM: teal spheres) and 24 h (CM: violet spheres; NM: green spheres). (B) 3D loadings plot. Dotted line highlights markers related to CM diet.

min, and mass accuracy deviation ranged from 1.6 mDa up to 3.7 mDa. These data are in accordance with data proposed elsewhere.[19,31,32] Concerning the peak area, the coefficient of variation ranged from 11.5% up to 23.3% with a mean value of 15.21%, agreeing with those recently proposed for metabolomic studies.[32]

Reinjection of urine samples was also used as QC. The hypothesis is based on the ability of PCA analysis to cluster samples in an unsupervised way, taking into consideration that if some problem took place, the reinjection would be placed in a different position in the score plot. As depicted in Figure 1B, replicates are very closely related in the PCA scores plot. Other authors[19] have proposed a particular QC by mixing equal volumes from each of the samples and reinjecting this pooled sample several times during the whole experiment. According to the obtained data, the quality of data was accepted as well as the data following further steps of the metabolomic workflow.

Urinary Metabolome Modifications. The LC-MS data obtained in positive mode were submitted to MVA analysis. Preliminary analysis showed that positive ionization produced more information (number of ions) than negative ionization. Therefore, the positive mode was used in the data acquisition procedure. A PLS-DA analysis was conducted to investigate the possible differences between times before (0 h) test-meal consumptions of CM, CW and NM. The autofit function of SIMCA-P produces a model with one latent variable and with R^2X, R^2Y and Q^2 values of 0.07, 0.29 and −0.1, respectively. These low values indicated that differences between the times before test-meal consumption were not detected. This behavior is probably explained by the effect of previous standardization of diet, agreeing with the results obtained by Walsh et al.[33] For further multivariate analysis, times before test-meal consumption (0 h) were grouped in a single class.

To evaluate possible differences between CW and CM diets consumption in the urinary metabolome modifications, an OSC-PLS-DA was constructed. Applying OSC filter to the data set removed seven components representing 29% of the variation in the original data set. The PLS-DA analysis of the resultant data set resulted in a two latent variable model characterized by R^2X, R^2Y and Q^2 values of 0.175, 0.373, and 0.319, respectively. These low levels showed that this model was not able to discriminate samples according to the diets. This result suggests a weak global influence of either the milk or water matrix on the urinary metabolome modifications after cocoa intake. A major controversy has grown around the effect of milk on the bioavailability of some dietary compounds, mainly polyphenols, from different dietary sources such as tea or cocoa.[34,35] In fact, studies carried out with cocoa consumed either with milk or water does not show significant matrix influence on the bioavailability of polyphenols.[34,36] In this context, a recent study proposed that the possible influence of milk on cocoa-flavonoid absorption is more relevant for drink with lower flavan-3-ol content, which is typical of many commercial cocoas, than for drink with higher content.[37]

It should be noted that food matrix is an important factor to take into account in dietary intervention studies as it could modulate absorption and further biological activity. In this respect, Visioli et al.[38] provided evidence that n-3 fatty acids from fish are more effectively incorporated into plasma lipids than when administered as capsules.

Detection and Identification of Markers Related to Cocoa Powder Intake. Pursuing the hypothesis that cocoa intake could alter urinary metabolome, an OCS-PLS was constructed using samples from the consumption of the CM diet and urine samples from the NM diet used as a control diet (milk intake). Applying this filter to the data set removed eight components representing 32.6% of the variation in the original data set. The PLS analysis of the resultant data set resulted in a four-component model characterized by R^2X, R^2Y and Q^2 values of 0.29, 0.91, and 0.825, respectively, indicating the robustness of the model. In addition, a permutation test ($n = 20$) was carried out to evaluate the overfit of the model. This test showed a R^2 intercept of 0.247 and a Q^2 intercept of −0.319, validating the model. The scores plot (Figure 2A) revealed a kinetic evolution during the 24 h after test-meal intake in the urine fingerprint of both diets, suggesting that the circadian rhythm of urine

Table 1. Summary of Parameters for Assessing OSC-PLS-DA Modeling Quality

models	no.[a]	R^2X_{cum}[b]	R^2Y_{cum}[b]	Q^2_{cum}[b]	R intercept[c]	Q intercept[c]
6 h	1	0.18	0.97	0.89	0.61	−0.26
12 h	1	0.16	0.98	0.85	0.67	−0.23
24 h	1	0.13	0.97	0.80	0.63	−0.23

[a] No., number of components. [b] R^2X_{cum} and R^2Y_{cum} are the cumulative modeled variation in **X** and **Y** matrix, respectively, and Q^2Y_{cum} is the cumulative predicted variation in **Y** matrix. [c] Obtained after permutation test ($n = 20$).

metabolome probably plays an important role in urinary modifications. Important effects of the circadian rhythm on the urinary metabolome have been demonstrated in several metabolomic studies on mice,[39] rats[40] and humans.[41,42] In addition, Figure 2A depicts a clear separation between both diets. In fact, loadings plot investigation enables the detection of several markers responsible for this difference (Figure 2B). To identify the markers responsible for cocoa intake modifications, samples from the consumption of the CM diet and NM diet as control were used. Three OSC-PLS-DA models were carried out comparing urine samples at 6, 12, and 24 h. These analyses allow the possible influence of noncocoa intake related markers (i.e., circadian rhythm markers) to be avoided. The quality parameters obtained for the different models are summarized in Table 1. All models showed high-quality parameters and were validated by a permutation test.

The "S-plots"[43] associated with the scores plots were generated to reveal the markers responsible for the cocoa consumption (Figure 3). The markers with higher w^*c and p(corr) values were the more relevant ions for explaining the separation between diets. The higher-right quadrant of each S-plots in Figure 3 displays the most important ions in urine samples related to cocoa powder consumption (CM diet), whereas those placed in the lower-left quadrant correspond to those related to the NM diet. The ions in the middle of the figure did not show any relevance in the model.

A list of markers detected in the three S-Plots was submitted for metabolite identification procedure. The results were summarized in Table 2 which provides compound information including retention time, detected calculated mass and theoretical mass, statistical parameters and postulated identifications.

Ten identified metabolites were present as a group of ions, the metabolites being termed "metabolite cluster" even if only one ion was detected (Table 2). Although ESI is considered as a soft ionization technique, it was expected that the metabolite clusters would be composed of different kinds of ions such as molecular ions, possible in-source fragments related to a loss of water and/or fragment coming from the loss of glucuronide moiety (−176 Da), or even loss of glycine moiety (−75 Da). An important characteristic is that daughter ions show similar retention time to their respective quasi-molar ion due to the fragmentation occurring after chromatography. These nonquasi-molar ions could reach the same statistical relevancy or even more than the quasi-molar ions (Table 2). Therefore, the correct assignation of these fragments or isotopes is important to avoid metabolite identification mistakes as well as helping to postulate tentative metabolite identifications.[44] To illustrate this procedure, some examples of metabolite cluster from Table 2 have been selected. Metabolite cluster 7 shows an in-source loss of 43 Da for the ion at m/z 167.0575, giving rise to the

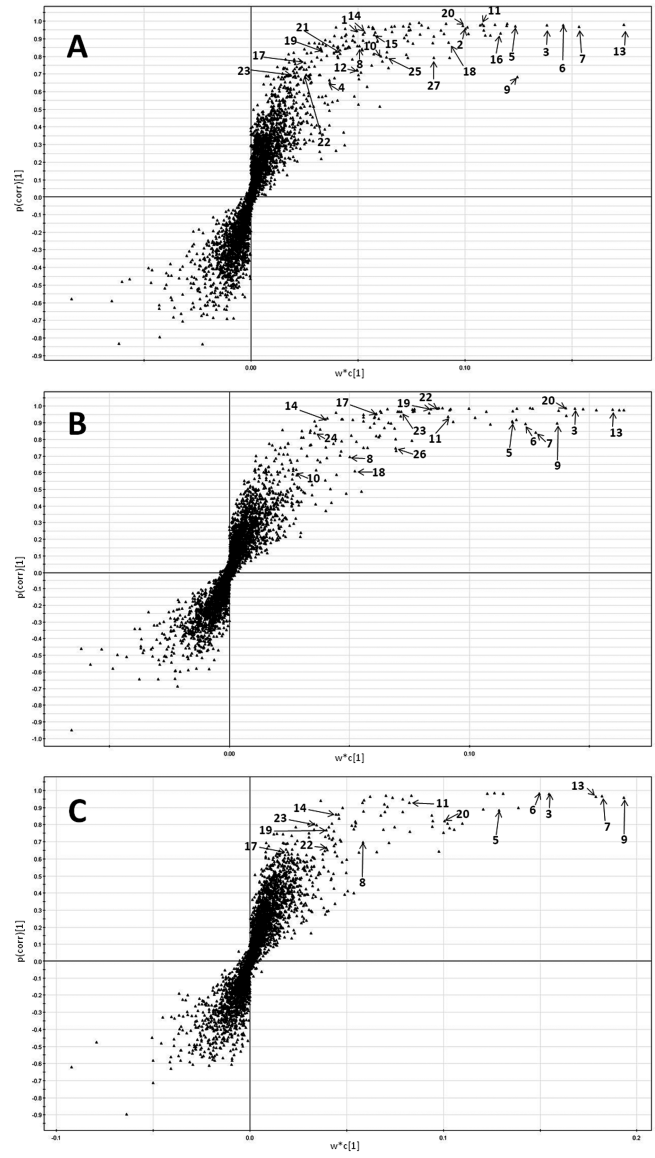

Figure 3. S-plots associated with the OSC-PLS-DA score plots obtained for data derived from LC-MS of urine obtained at 6 h (A), 12 h (B) and 24 h (C) after both test-meal consumptions (CM and MN). The numbers for the metabolites used (quasi-molar ions) are as given in Table 2.

product ion at m/z 124.0491. This pattern is similar to that proposed by HMDB for the compound 7-methylxanthine (metabocard HMDB01991). Because of its exact mass and its LC-MS behavior, the ion at 167.0575 was tentatively identified as 7-methylxantine. In this context, a loss of 43 Da was also detected in metabolite cluster 13, giving an ion at m/z 138.0659 from the ion at m/z 181.0720. This MS pattern had been previously proposed for theobromine.[45] After comparison with commercial standard, this metabolite was identified as theobromine.

Another example that addresses the importance of the characterization of ions is when the LC-MS behavior of metabolite clusters 17 and 19 was compared. Both metabolite clusters showed a daughter ion at m/z 209 (209.0814 and 209.0815, respectively. Table 2). Nevertheless, whereas the ion 209.0814 was assigned as a fragment caused by a loss of water in its quasi-molar ion (227.0936) (cluster 17), the ion

Table 2. Identification of Cluster Metabolites after Single Cocoa Intake from Human Volunteers

			*u*c*[1] (contribution)			*p*(corr)[1] (confidence)						
metabolite cluster	retention time	detected mass $[M+H]^+$	6 h	12 h	24 h	6 h	12 h	24	metabolite putative identification	assignation	theoretical mass $[M+H]^+$	mass difference (mDa)[a]
1	0.53	182.0805	0.05	-[b]	-	0.94	-	-	Tyrosine[c]	$[M+H]^+$	182.0811	0.6
2	0.67	140.0333	0.10	-	-	0.96	-	-	Hydroxynicotinic acid	$[M+H]^+$	140.0342	0.9
		122.0220	0.08	-	-	0.95	-	-		$[M+H-H_2O]^+$	122.0236	1.6
3	0.90	199.0829	0.14	0.14	0.15	0.97	0.98	0.98	AMMU[d]	$[M+H]^+$	199.0825	−0.4
		171.0882	0.12	0.12	0.13	0.97	0.99	0.98		$[M+H-CO]^+$	171.0876	−0.6
4	1.02	140.0705	0.04	-	-	0.65	-	-	Trigonelline	$[M+H]^+$	140.0706	0.1
5	1.25	199.0818	0.12	0.12	0.13	0.97	0.90	0.88	AMMU[d]	$[M+H]^+$	199.0825	0.7
		171.0872	0.11	0.12	0.13	0.97	0.98	0.98		$[M+H-CO]^+$	171.0876	0.4
6	1.83	183.0523	0.15	0.12	0.15	0.98	0.89	0.98	7-methyluric acid	$[M+H]^+$	183.0512	−1.1
7	2.18	167.0575	0.15	0.13	0.17	0.97	0.84	0.97	7-methylxanthine	$[M+H]^+$	167.0563	−1.2
		124.0491	0.06	0.06	0.07	0.97	0.93	0.97		$[M+H-CHNO]^+$	124.0505	1.4
8	2.32	183.0525	0.05	0.05	0.06	0.86	0.70	0.69	3-methyluric acid	$[M+H]^+$	183.0512	−1.3
9	2.62	167.0586	0.12	0.14	0.19	0.68	0.90	0.96	3-methylxanthine	$[M+H]^+$	167.0563	−2.3
10	3.02	151.1212	0.06	0.02	-	0.80	0.60	-	3,5-Diethyl-2-methylpyrazine	$[M+H]^+$	151.1229	1.7
11	3.05	197.0675	0.11	0.09	0.08	0.98	0.94	0.93	3,7-dimethyluric acid	$[M+H]^+$	197.0669	−0.6
12	3.60	137.0597	0.05	-		0.70	-		hydroxyacetophenone	$[M+H]^+$	137.0597	0
13	3.63	181.0720	0.17	0.16	0.18	0.98	0.98	0.96	Theobromine[c]	$[M+H]^+$	181.0719	−0.1
		138.0659	0.07	0.06	0.03	0.91	0.92	0.60		$[M+H-CHNO]^+$	138.0661	0.2
14	3.68	251.1007	0.05	0.04	0.04	0.90	0.92	0.86	Cyclo(Ser-Tyr)	$[M+H]^+$	251.1026	1.9
15	4.15	169.0496	0.06	-	-	0.92	-	-	Vanillic acid[c]	$[M+H]^+$	169.0495	−0.1
16	4.23	226.0725	0.12	-	-	0.93	-	-	Vanilloylglycine	$[M+H]^+$	226.0709	−1.6
		151.0397	0.11	-	-	0.92	-	-		$[M+H-\text{glycine}]^+$	151.0395	−0.2
17	4.63	227.0936	0.02	0.06	0.02	0.76	0.95	0.64	4-hydroxy-5-(3,4-dihydroxyphenyl)-valeric acid[e]	$[M+H]^+$	227.0913	−2.3
		209.0814	0.03	0.09	0.04	0.85	0.98	0.84		$[M+H-H_2O]^+$	209.0808	−0.6
18	4.75	195.0875	0.09	0.05	-	0.88	0.60	-	Caffeine[c]	$[M+H]^+$	195.0876	0.1
19	5.13	385.1134	0.03	0.08	0.04	0.83	0.98	0.77	5-(3′,4′-dihydroxyphenyl)-γ-valerolactone glucuronide[e]	$[M+H]^+$	385.1129	−0.5
		209.0815	0.09	0.15	0.09	0.92	0.98	0.82		$[M+H-\text{GlcA}^g]^+$	209.0808	−0.7
20	5.33	385.1131	0.10	0.14	0.10	0.97	0.99	0.82	5-(3′,4′-dihydroxyphenyl)-g-valerolactone glucuronide[e]	$[M+H]^+$	385.1129	−0.2
		209.0818	0.11	0.15	0.10	0.95	0.97	0.75		$[M+H-\text{GlcA}]^+$	209.0808	−1.0
		149.0603	0.05	0.06	0.05	0.85	0.87	0.79		$[M+H-\text{GlcA-}C_2H_3O_2]^+$	149.0597	−0.6
21	5.38	195.1111	0.04	-	-	0.82	-	-	Cyclo(Pro-Pro)	$[M+H]^+$	195.1127	1.6
22	5.47	399.1300	0.02	0.09	0.04	0.68	0.98	0.66	3′-methoxy-4′-hydroxyphenylvalerolactone glucuronide[e]	$[M+H]^+$	399.1285	−1.5
		223.0976	0.10	0.16	0.10	0.93	0.98	0.77		$[M+H-\text{GlcA}]^+$	223.0964	−1.2
		163.0758	0.03	0.08	0.04	0.84	0.98	0.72		$[M+H-\text{GlcA-}C_2H_3O_2]^+$	163.0753	−0.5
23	5.87	223.0981	0.02	0.07	0.03	0.69	0.97	0.80	3′-methoxy-4′-hydroxyphenylvalerolactone[e]	$[M+H]^+$	223.0964	−1.7
24	6.12	289.0386	-	0.04	-	-	0.84	-	5-(3′,4′-dihydroxyphenyl)-γ-valerolactone-sulfate[e]	$[M+H]^+$	289.0376	−1.0
25	6.93	371.0401	0.06	-	-	0.80	-	-	Epicatechin-*O*-sulfate	$[M+H]^+$	371.0431	3.0
26	7.00	289.0404	-	0.07	-	-	0.75	-	5-(3′,4′-dihydroxyphenyl)-γ-valerolactone-sulfate[e]	$[M+H]^+$	289.0376	−2.8
27	7.10	305.1028	0.09	-	-	0.79	-	-	*O*-Methylepicatechin	$[M+H]^+$	305.1019	−0.9

[a] Obtained as (theoretical mass-detected mass). [b] Denotes the *p*(corr) values lower than cut-off 0.5. [c] Identification was confirmed by standard comparison. [d] AMMU corresponds with 6-amino-5-[*N*-methylformylamino]-1-methyluracil. [e] Structural identification agrees with [Urpí-Sardà et al.,[23] Fardet et al.,[24] Sang et al.[25]].

209.0815 was assigned as a fragment produced by a loss of glucuronide moiety from its quasi-molar ion (385.1134) (cluster 19). The LC-MS behavior of metabolite cluster 17 suggested that this compound could be putatively identified as 4-hydroxy-5-(3,4-dihydroxyphenyl)-valeric acid. On the other hand, the LC-MS behavior of metabolite cluster 19 suggested a postulated identification as 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone glucuronide. This in-source fragmentation behavior of glucuronide conjugates has been proposed as a powerful tool to postulate their identification.[46]

The proposed identifications revealed that observed differences were explained by the excretion of a complex profile of cocoa-derived phytochemicals. Identified markers related to cocoa intake could be grouped in 7 classes as follows: purine alkaloid metabolites (Table 2, metabolite clusters 3,

5−9, 11, 13 and 18), polyphenol host metabolites (metabolite clusters 15, 16, 25 and 27), polyphenol colonic microbiota metabolism (metabolite clusters 17, 19, 20, 22−24 and 26), cocoa flavor and taste compounds (metabolite clusters 10, 12, 14 and 21), nicotinic acid metabolites (metabolite clusters 2 and 4) and amino acids (metabolite cluster 1).

Biological Interpretation of Cocoa Powder Intake Biomarkers. Cocoa food products are sources of purine alkaloids such as caffeine and, mainly, theobromine.[5] Here, the most important metabolites derived from the theobromine metabolism were identified (Table 2). The excretion pattern of detected theobromine in this study agrees with the metabolic pathway proposed by the KEGG database (KEGG map00232). These compounds represent the main contributors in all three OSC-PLS-DA models (Figure 3), reflecting that for 24 h after punctual consumption of cocoa powder the excretion of methylxanthines is still elevated. Our results are in accordance with Rodopoulus et al.[5] who detected theobromine, 7-mehtylxanthine, 3-methylxanthine, 7-methyluric acid and 6-AMMU in urine 36 h after theobromine administration to healthy adult volunteers. Theobromine and caffeine are the most important alkaloids in cocoa, while theophylline is only present as traces.[47]

Other important markers of cocoa consumption are metabolites linked to cocoa polyphenol metabolism. These metabolites follow two different patterns of excretion. Some of these are clearly associated with the first 6 h after cocoa intake (metabolite clusters 15, 16, 25 and 27), while others are associated with the period between the 6 and 12 h after cocoa ingestion (metabolite clusters 17, 19, 20, 22−24 and 26). The identification results revealed that the metabolites associated with the first 6 h were polyphenol host metabolites such as epicatechin sulfate or *O*-methylepicatechin as well as vanillic acid, previously identified as markers of cocoa or chocolate polyphenol consumption.[48,49] Associated with this pattern, the metabolite vanilloylglycine was tentatively identified. This metabolite has been related to the metabolism of phenolic acids[50] and also with vanillin, which is an important food additive of cocoa products[48] including cocoa powder. To our knowledge, this is the first time that this metabolite has been related to cocoa consumption. Regarding metabolites associated with the period between 6 and 12 h, the identification has revealed that these metabolites were phenylvaleric acid or phenylvalerolactone derivatives. These compounds, derived from ring fission of epicatechin, are formed in the large intestine by the colonic microbiota metabolism.[51] A similar excretion profile of epicatechin colonic microbiota metabolites has been previously reported.[25,52] In addition, a recent metabolomic study about the metabolic effect of catechin supplementation in rats fed a high-fat diet showed the colonic microbiota polyphenol metabolites as important markers of catechin consumption.[24]

Regarding cocoa flavor, 3,5-diethyl-2-methylpyrazine (metabolite cluster 10) and hydroxyacetophenone (metabolite cluster 12) were identified. These compounds have been included in a list of major odor compounds of cocoa powder.[6] Cocoa diketopiperazines (metabolite clusters 14 and 21) are compounds generated during the roasting of fermented cocoa beans from hydrophobic amino acids that contribute to the bitter taste of roasted cocoa.[7,46]

Trigonelline and hydroxynicotinic acid, metabolites of nicotinic acid, were identified only in samples collected 6 h after cocoa intake. Nicotinic acid, also called niacin or vitamin B3, is a water-soluble vitamin with an important role in energy metabolism that is present in cocoa (http://www.ars-grin.gov/duke/plants.html). Trigonelline (*N*-methylnicotinic acid) is a pyridine alkaloid synthesized from nicotinic acid that has been detected in cocoa, and mainly in coffee, and also in mammalian urine after administration of nicotinic acid.[53] Transformation of nicotinic acid to hydroxynicotinic acid is catalyzed by the enzyme nicotinate hydroxylase (KEGG map00760).

Tyrosine was identified as a metabolite related to amino acids. This metabolite was an important contributor in the model at 6 h. The phytochemical database of Dr. Duke shows that *Theobroma cacao* L. seeds provide a important amount of tyrosine (http://www.ars-grin.gov/duke/plants.html).

Conclusions

Dietary intervention studies carried out in both human and animal models revealed distinct urinary metabolome changes resulting from the diet. Here, an important effect on urinary metabolome during the 24 h after a punctual intake of cocoa powder was detected. Moreover, these changes were not influenced by matrix as no global differences were found between cocoa powder consumption with milk or with water. Overall, 27 metabolites responsible for urinary metabolome modifications have been identified either putatively or confirmed with authentic standards showing a complex profile including alkaloids, polyphenols (host and colonic microbiota metabolites) and flavor components of cocoa power. To our knowledge, this is the first time that several of these metabolites, such as vanilloylglycine or diketopiperazine, have been related to cocoa consumption. Metabolomic strategy is a powerful tool for identifying new markers of exposure and is useful for confirming the robustness of some expected metabolites such as polyphenol metabolites. However, more databases focused on food metabolome will be needed to attempt their full identification. The obtained results reinforce the hypothesis that metabolites derived from dietary compounds (food metabolome) could be an important part of urinary metabolome, and their full identification is necessary to relate their consumption to their expected effects on health.

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