TITLE

Discovery of intake biomarkers of lentils, chickpeas and white beans by untargeted LC-MS metabolomics in serum and urine

FULL AUTHOR NAMES

Mar Garcia-Aloy[1,2,3], Marynka Ulaszewska[3], Pietro Franceschi[4], Sheila Estruel-Amades[1], Christoph H. Weinert[5], Alba Tor-Roca[1,2], Mireia Urpi-Sarda[1,2], Fulvio Mattivi[3,6,*], Cristina Andres-Lacueva[1,2,*]

[1]Biomarkers and Nutrimetabolomics Laboratory, Department of Nutrition, Food Sciences and Gastronomy, XaRTA, INSA, Faculty of Pharmacy and Food Sciences, University of Barcelona, Barcelona, Spain.

[2]CIBER Fragilidad y Envejecimiento Saludable (CIBERFES), Instituto de Salud Carlos III, Barcelona 08028, Spain.

[3]Department of Food Quality and Nutrition, Research and Innovation Center, Fondazione Edmund Mach (FEM), San Michele all'Adige, Italy.

[4]Computational Biology Unit, Research and Innovation Center, Fondazione Edmund Mach, San Michele all'Adige, Italy.

[5]Department of Safety and Quality of Fruit and Vegetables, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Karlsruhe, Germany.

[6]Department of Physics, University of Trento, San Michele all'Adige, Italy.

KEYWORDS

biomarkers; legumes; metabolomics; dietary assessment; nutrimetabolomics

CORRESPONDENCE

Cristina Andres-Lacueva (Faculty of Pharmacy and Food Sciences, University of Barcelona: Avinguda Joan XXIII, 27–31, 08028 Barcelona, Spain): candres@ub.edu

Fulvio Mattivi (Department of Physics, University of Trento: Via E. Mach 1, 38010 San Michele all'Adige, Italy): fulvio.mattivi@unitn.it

ABBREVIATIONS

BFI, biomarkers of food intake; FoodBAll, Food Biomarker Alliance.

ABSTRACT

Scope: To identify reliable biomarkers of food intake (BFIs) of pulses.

Methods and results: A randomized crossover postprandial intervention study was conducted on 11 volunteers who consumed lentils, chickpeas and white beans. Urine and serum samples were collected at distinct postprandial time points up to 48 h, and analyzed by LC-HR-MS untargeted metabolomics. Hypaphorine, trigonelline, several small peptides and polyphenol-derived metabolites proved to be the most discriminating urinary metabolites. Two arginine-related compounds, dopamine sulfate and epicatechin metabolites, with their microbial derivatives, were identified only after intake of lentils, whereas protocatechuic acid was identified only after consumption of chickpeas. Urinary hydroxyjasmonic and hydroxydihydrojasmonic acids, as well as serum pipecolic acid and methylcysteine, were found after white bean consumption. Most of the metabolites identified in the postprandial study were replicated as discriminants in 24 h urine samples, demonstrating that in this case the use of a single, noninvasive sample was suitable for revealing the consumption of pulses.

Conclusions: The results of the present untargeted metabolomics work revealed a broad list of metabolites that are candidates for use as biomarkers of pulse intake. Further studies are needed to validate these BFIs and to find the best combinations of them to boost their specificity.

1. INTRODUCTION

Pulses refer to the edible nonoil dried seeds of legumes, such as lentils, chickpeas, dry beans and dry peas. They present a unique nutritional value characterized by a high content of protein, fiber and a variety of phytochemicals, as well as by a low glycemic index. Consumption of pulses has been associated with beneficial effects on human health. In fact, regular pulse consumption has been associated with lower body weight [1], blood pressure [2] and LDL cholesterol [3], as well as with an improvement in markers of glycemic control [4]. In recent years the promotion of their consumption has been prioritized by a wide range of public and private stakeholders culminating in the declaration of the year 2016 as the International Year of Pulses by the General Assembly of the United Nations, which was coordinated by the Food and Agriculture Organization of the United Nations.

To study in depth the effects of these foods and to gain more insight into the molecular mechanisms by which they act, a precise measurement of food intake is required. Here, biomarkers of food intake (BFIs) have emerged as a promising tool for correctly assessing food intake [5]. To date, only a limited number of foods have been associated with validated BFIs, but pulses are not among them, since only a few studies dealing with this topic have been recently published [6–11]. The first of them was focused on the quantification of kaempferol in urine after bean consumption [6]. However, this compound is present in a wide range of vegetables [12]. Results from another human study suggested serum pipecolic acid and S-methylcysteine as biomarkers of dry bean consumption [7]. Animal studies reported an increase in the urinary excretion of trigonelline, homoeridictyol chalcone and two peptides after a bean-based diet in dogs [8], and increased urinary excretions of different species of dipeptides, as well as arginine-related metabolites in hypertensive rats fed with lentils [9]. More recently our research group published two human studies applying untargeted NMR experiments, one in an observational

framework [10] while the other was a postprandial controlled intervention study [11]. The first study showed that pulse consumers had higher urinary excretions of different metabolites related to choline, protein and energy metabolism [10], whereas the results of the second study suggested trigonelline, 3-methylhistidine, dimethylglycine, TMA, glutamine, choline, lysine and histidine as candidate biomarkers of pulse intake [11]. However, as the authors pointed out, these metabolites are not highly specific to pulse consumption.

In light of this scarcity of knowledge regarding biomarkers of pulse consumption, the aim of the present study was to assess the metabolic fingerprint of urine and serum associated with their intake in order to further identify reliable BFIs of the most commonly consumed types of pulses (i.e. lentils, chickpeas and dry beans) using an LC-MS untargeted metabolomics approach. This technique enables the detection of a wide range of metabolites even at low concentrations while biosamples selected for analysis usually carry a large amount of dietary information. This research was developed within the same context of the NMR study mentioned before [11]. In addition to analyzing the nutrikinetic profiles in urine and serum, in this study we aimed to go one step further and develop a complementary experiment with pooled urinary samples. This was focused on a comparision of the data provided by the nutrikinetic profile with the 6 h and 24 h pooled urine samples to check the overlapping information. The aforementioned experiment was carried out in order (1) to get an overview of the postprandrial impact of the evaluated foods without the interference of any additional intake (6 h pools), and (2) to evaluate the information provided by a simpler, less expensive and more easily available type of sample such as the 24 h urine pool.

2. MATERIALS AND METHODS

2.1. Study design

The design of this study (Figure 1) has been defined within the frame of the European JPI-funded project "Food Biomarker Alliance" (FoodBAll) [13]. In fact, this is one of the seven standardized postprandial intervention studies designed to discover new potential food intake biomarkers for a broad range of common foods. Our study was focused on different types of the most commonly consumed kinds of pulses, i.e. lentils, chickpeas and white beans, using white pasta as the control food. The study was registered at the ISRCTN registry with the code ISRCTN17200423. Details of this randomized, controlled, crossover study have been reported previously [11] and are listed in the Supporting Information. The clinical intervention was approved by the Bioethical Committee of the University of Barcelona (ref: IRB00003099) and followed Helsinki Declaration guidelines.

A total of 84 subjects expressed their interest in participating in this study, of whom 31 were screened. Twenty-six of them fulfilled the inclusion criteria and 14 were enrolled since they were the ones that finally fully agreed to participate in the study, with 11 (four men and seven women) completing the study in a crossover design with lentils, chickpeas and pasta (Figure S1). A subgroup of eight participants underwent the fourth intervention with white beans, which was scheduled later. The subjects were on average 28 ± 6 years old (range: 19–37) and had a BMI of 23.8 ± 3.6 kg/m^2 (range: 18.6–28.9).

2.2. Analysis of food samples

Test foods were freeze-dried in the study center, shipped to the analytical lab on dry ice and stored at -80 °C before further peparation. Twenty mg of each sample were extracted twice with methanol/tert-butyl methyl ether first and methanol/water afterwards. Aliquots of the

supernatants were then evaporated in a SpeedVac and subsequently derivatized (methoximated and trimethylsilylated). Untargeted analyses were performed by LC-MS (results previously published by Llorach *et al.*[14]) and also using a comprehensive two-dimensional gas chromatography mass spectrometry (GC×GC-MS) system described elsewhere [15]. Data processing was done using the SquareDance workflow developed for GC×GC-qMS data sets [16] . Further details of sample preparation and the analytical procedure are provided in the Supporting Information. Automated compound annotation was performed using an in-house spectral library with fatty acid methyl esters-based retention indices. Finally, the annotation was manually verified by matching against the National Institute of Standards and Technology v14 library, the Fiehn library and the Golm Metabolome Database. The compounds identified in the four food products through the analyses with comprehensive two-dimensional gas chromatography mass spectrometry are listed in Table S2.

2.3. Untargeted metabolomics experiments on biological samples

Untargeted metabolomics analyses were performed in accordance with previously validated methodologies [17]. Briefly, urine samples were diluted with a series of internal and external standards in pure methanol and in Milli-Q water, whereas serum samples were prepared using Ostro plates. Afterwards, all samples were subjected to high-throughput metabolomics analysis using a reversed-phase liquid chromatography coupled to a high-resolution Orbitrap mass spectrometer. Later, acquired data were processed using the XCMS package in the R platform. A detailed description of these procedures, together with an exhaustive explanation about statistical analysis and metabolite identification, is given in Supporting Information (section "Untargeted metabolomics experiments on biological samples").

3. RESULTS

3.1. Identification of metabolites associated with legume consumption in the nutrikinetic study

Data adquired in this study showed good analytical performance. In-depth information about the quality of acquired data, as well as verification of data processing, is provided in the section "Data acquisition quality" in Supporting Information. After the processing of the raw MS data, further filtration steps were applied to the obtained data sets in order to reduce the amount of noisy and/or irrelevant features. The number of features in both the original and filtered data sets can be found in Supporting Information ("Data filtering" section). Finally, before proceeding with the feature selection, principal component analysis (PCA) was applied on each data set to explore trends in the data. A complete description of these results and their interpretation is provided in the section "Unsupervised multivariate analysis" in Supporting Information.

Among all the features retained after data filtering, 320 met the first selection requirement, based on their nutrikinetic curve behavior (see Supporting Information for futher details). Of these, 265 were found to be statistically significant (adjusted p-value < 0.05 of AUCs), 151 of which were assigned to one of the 54 identified metabolites (Table 1), whereas the rest remained unknown. Out of the 54 identified metabolites, 12 were identified at level I, 29 at level II and 13 at level III. Identified compounds were categorized into several groups taking into consideration information on the chemical class of their precursors (metabolites) or their common pathways of origin (Figure S5): small peptides, flavan-3-ol-derived metabolites, other polyphenol-derived metabolites, fatty acids, other food-derived metabolites and endogenous metabolites. A detailed description of the identification of discriminant metabolites is provided in the section "Identification of discriminating metabolites" in the Supporting Information, and information regarding their fragmentation spectra is given in Table S5. The responses of a selected group of

characteristic metabolites are depicted in Figure 2, while Figure S6 includes the kinetic curves of all identified metabolites. All plots were constructed with the MS response (peak area) of the most intensive feature of each metabolite.

3.2. Experiment on pooled samples

In order to compare the data provided by nutrikinetic profiles and a simpler type of sample such as 24 h urine, an additional experiment was performed. This was done with the aim of checking the overlapping information between these types of samples. Additionally, 6 h urine pools were also analyzed since during the first 6 hours volunteers only ingested the test meal (i.e. this pool included samples collected 1h, 2h, 4h and 6h after consuming the study food, Figure 1). In both cases, most of the identified urinary metabolites in the nutrikinetic experiments were also observed as being discriminant in both types of pooled samples (Table S6 and Figure S7). This demonstrates that in this case using a simpler type of sample (in that it is not required for the study volunteer to collect the samples in different containers) is also useful for observing the BFIs of pulse consumption. This has important connotations since 24 h urinary samples are usually collected in long intervention and epidemiological studies (although unfortunately some of them only have available spot urine instead of 24 h urine samples). Furthermore, the HCA (Figure 3) demonstrated that, although not all statistically significant features were annotated, the information on those already assigned to one of the identified metabolites allowed a good sample clustering performance.

4. DISCUSSION

In this work, several metabolites were found to be associated with the intake of some of the most widely consumed types of pulses. They were identified using liquid chromatography

coupled to high-resolution mass spectrometry. Most of these metabolites are candidates as BFIs for this food group since they showed increased urinary and/or serum concentrations after the consumption of at least one type of pulse, while the values observed after the consumption of the control food (pasta) were negligible. It was difficult to find biomarkers exclusively for one type of pulse, as most of them were common to at least two or three pulse types. The exceptions were flavan-3-ol-derived metabolites (M27–M31), two arginine-related compounds (M25 and M26) and dopamine sulfate (M43), which appeared to be specific for lentil intake in urine samples, whereas protocatechuic acid glucoside (M37) and ascorbic acid (M42) were elevated after the consumption of chickpeas. Hydrojasmonic (M39) and two hydroxydihydrojasmonic (M40 and M41) acids were characteristic of white bean intake. In serum samples, methylcysteine (M45) and pipecolic acid (M46) also resulted in particular from white bean consumption, and in both types of biosamples hypaphorine (M44) was discriminant for chickpeas and lentils. Two of the identified BFIs (i.e. methylcysteine and pipecolic acid) were detected as such in the test food to which they were assigned as markers (Table S2). This observation indicates that they were most likely nonmetabolized or not fully metabolized, suggesting that they would not be subject to substantial interindividual differences.

4.1. BFI for lentils

The results for flavan-3-ol-derived metabolites are consistent with the analyses of test foods, which showed that catechins were particular to lentils (Table S2). They were observed only in urine and their presence is due to the metabolism of catechins and proanthocyanidins by host and by intestinal microbiota. The urine integrated intensity-time curves clearly show two distinct nutrikinetic patterns, as already reported in another independent postprandrial dietary intervention [17]. On the one hand, (epi)catechin sulfate (M27) reached its maximum urinary levels within the

first 2–4 h post-consumption followed by a rapid decrease within the next few hours. This behavior suggests that this compound is probably absorbed in the upper part of the gastrointestinal tract with little or no contribution from the microbiota. On the other hand, the four valerolactone-derived metabolites (M28–M31) were characterized by a delayed appearance in urine. Their levels started to increase 4 h after consumption of lentils, reaching the maximum levels after 12 h, still being significantly higher after 24 h and reaching the baseline values within 48 h. This performance suggests a prolonged metabolism throughout the large intestine with the involvement of the microbiota. These profiles are in good agreement with previous studies reporting the presence of (epi)catechin and valerolactone conjugates in urine [17]. This suggests that the former may only be useful as a BFI when 24 h urine is available, whereas the others could be more useful as BFIs when only spot urines are accessible. However, increases in urinary excretions after the intake of other catechin- and proanthocyanidin-rich foods such as tea, cocoa and grape products have also been observed in previous studies [18], thereby limiting their specificity as candidate BFIs of lentils. Additionally, hydroxy- and oxoarginine (M25 and M26) urinary levels also increased with the intake of lentils. Maximum excretions of these compounds were observed 4 and 6 hours, respectively, after intake of lentils, although differences were observed from the first few hours until 48 h post-consumption (Table 1). Hydroxyarginine is a free nonprotein amino acid particular to lentils [9], and oxoarginine is a metabolite of the arginine pathway recently associated with lentil consumption [9]. Therefore, they are interesting indicators of lentil intake. Additionally, it has been suggested that they could contribute to the blood pressure-lowering effects associated with the consumption of lentils through an increase in the production of nitric oxide [9]. Due to the well-known degradation of the guanidino group during trimethylsilylation prior to GC×GC-MS analysis, the presence (or abundance) of arginine and related compounds in the test foods could not be determined [19]. An increased urinary excretion

of dopamine sulfate (M43, the predominant form of dopamine in the circulation) was also particularly noticeable after the consumption of lentils. It has already been reported that it is largely affected by meal consumption, coming from the intake of dopamine, dopamine sulfate or L-dihydroxyphenylalanine (DOPA), the conversion of dietary tyramine to dopamine, the action of tyrosinase to produce L-DOPA in the gastrointestinal lumen, or an increased release and metabolism of endogenous dopamine in gastrointestinal lining cells [20]. Very recently, dopamine sulfate was found to be among the BFIs best predicting the intake of banana [21].

4.2. BFI for chickpeas

Ascorbic acid (M42) appeared as a discriminant compound for chickpea consumption during the period 4 h to 6 h post-intake. In line with that, its oxidation product, dehydroascorbic acid, was only detected in high amounts in chickpeas (Table S2). However, ascorbic acid cannot be considered a compound from chickpeas per se, since it is employed as a food additive. Furthermore, since it is widely used in the food industry as a stabilizing agent, it lacks the specificity to be used as a BFI and was not further investigated.

4.3. BFI for white beans

Methylcysteine (M45) and pipecolic acid (M46) appeared in serum samples particularly after white bean intake. Their levels were already significantly increased 1 h post-consumption and remained as such until the period 24 h–48 h (Table 1, Figure S7a). In accordance with this observation, the serum levels of pipecolic acid and methylcysteine have already been proposed as potential biomarkers of dry bean consumption [7]. They are common nonprotein nitrogen components of the *Phaseolus vulgaris* species [22]. Specifically, whereas methylcysteine is not influenced by microbial metabolism, pipecolic acid has been reported to be a product of

microbial metabolism derived from lysine and has been described as a precursor of microbial compounds with anti-inflammatory, antitumor and antibiotic properties [7]. On the other hand, three jasmonic derivatives (M39–M41) appeared to be particular to white bean intake. They are lipid-derived phytohormones generated in plants as a response to stress and their presence has been described in different varieties and cultivars of *Vicia faba* beans [23].

4.4. BFI for legumes

As legumes are an important food source of proteins, amino acid-derived metabolites could be indicators of their consumption. Indeed, we observed higher serum and/or urinary levels of several species of di- and tripeptides (M01–M17). When differences among the different types of legumes were observed, in most cases chickpeas were among the types that resulted in the highest concentrations. This could have been related to the fact that chickpeas could have a higher amount of protein in their composition, but this did not turn out to be the case when we consulted the nutritional composition of the foods used [11]. These compounds may indicate that they are directly excreted in urine after pulse intake, and/or may represent proteolytic breakdown products of larger proteins present in these food sources. Most of the peptides were aspartyl-containing compounds. Interestingly, in another study developed within the FoodBAll project but focused on meat biomarkers, a series of small peptides were also identified as BFIs. However, in that case these were hydroxyproline-containing di- and tripeptides [24] and none of them were in line with the present study where a completely different protein-based food was consumed.

Another interesting discriminant compound was hypaphorine (M44), which is also referred to as tryptophan betaine or lenticin. This last nomenclature was assigned since it is found in lentils [25,26], althougth it has also been reported in chickpeas [27]. It is an indole alkaloid composed of tryptophan and three methyls. In the present study, it has been observed as being discriminant for

the consumption of chickpeas and lentils both in serum and urine biofluids. In serum it has been observed as discriminant in the first hour after consumption, peaking 4–6 h after consumption and resulting in a prolonged disappearance in both biofluids, indicating a slow excretion or metabolism. However, the complete clearance of its metabolism could not be estimated since this metabolite was still detected during the 24–48 h period, when the last sample was collected. It has been correlated with nut intake in different cross-sectional studies [28–31], and it has also been associated with the Mediterranean diet [32] and the Dietary Approaches to Stop Hypertension (DASH) [33] dietary patterns. Additionally, a study focused on peanut consumption also reported its presence in the breast milk of lactating women [34]. Curiously, it has been positively associated with homocysteine levels after a Mediterranean-based dietary treatment in subjects with metabolic syndrome features [32]. Although it has been shown to be a compound with neurological and glucose-lowering effects in rodents, its potential functional role in humans remains to be addressed.

In parallel and in agreement with the results observed in our NMR experiments [11], urinary levels of trigonelline (M47) were also increased after intake of the three different types of pulses. In this case, urinary trigonelline achieved higher levels after the intake of white beans followed by chickpeas and lentils, reflecting the different amounts observed in their composition [11] and, therefore, suggesting a possible dose-response connection. Interestingly, this was also described after bean consumption in the two independent untargeted studies previously published [7,8]. However, this alkaloid has also been proposed as a BFI of coffee intake [35]. Therefore, this lack of specificity could limit its use as a single BFI, but we hypothesize that it could be considered within a multi-metabolite biomarker panel together with some of the other discriminating metabolites also observed in this study [18]. For example, combined with flavan-3-ol-derived

metabolites, arginine-related compounds and hypaphorine increase the specificity for monitoring lentil consumption.

Other metabolites whose amounts were increased in urine after pulse intake were tentatively identified as phloroglucinol glucuronide (M32) and phloroglucinol sulfate (M33). Both are metabolites containing benzoyl groups, probably generated by microbial degradation of polyphenols contained in pulses, and therefore reflecting polyphenol metabolite cleavage products of gut microbiota action.

4.5. Endogenous metabolites

Major increases in glucuronidated forms of dicarboxylic fatty acids (i.e. dodecanedioic acid glucuronide, M51, and tretradecanedioic acid glucuronide, M53) and hydroxy fatty acids (i.e. dihydroxydecanoic acid glucuronide, M50, and hydroxydodecadienoic acid glucuronide, M52) were observed after pasta intake (used as control food) when compared with the consumption of the other types of pulses. Given the type of metabolites (glucuronidated medium-chain fatty acids), these metabolites probably reflect a differential endogenous response rather than being a metabolite from an exogenous food compound (also bearing in mind that pasta was not the food with the highest fat content [11]). Therefore, this could indicate a different response related to lipid metabolism. For example, in the case of dicarboxylic fatty acids, they probably reflect a shift from a reduced β-oxidation toward an enhanced ω-oxidation of fatty acids since dicarboxylic fatty acids are enhanced when there is a deficiency in fatty acid oxidation or when β-oxidation is overwhelmed [36]. Additionally, dihydroxydecanoic acid is an intermediate from the ω-oxidation of hydroxydecanoic acid to hydroxydecanedioic acid [37], and 4-hydroxydodecadienoic acid has also been previously detected in human urine and measured in higher amounts in situations characterized by the presence of oxidative stress such as that due to aging and diabetes [38]. Given

that insulin resistance is mediated by a dysregulation of fatty acid metabolism [39] and that pulse consumption has been associated with both better glycemic control [4] and healthier blood lipid levels [3], the differential urinary levels of these metabolites could be related to an alteration in the corresponding pathways manifested early postprandially that could mediate these beneficial effects already observed in the aforementioned clinical outputs. However, given the design of the current study, this is only a hypothesis of the underlying mechanisms, which should be verified in further studies designed specifically for this purpose.

4.6. Strengths and weaknesses

Most of the BFIs identified in urinary samples from the kinetic study were replicated as discriminant metabolites in 24 h pooled urine. The collection of this type of sample is much simpler than the methodology that has to be used for performing a nutrikinetic study. Additionally, in several epidemiological and intervention studies this type of sample is the one collected by the researchers. Therefore, our observation has important connotations for the future, since it demonstrates that with this type of sample we can reach the same conclusions. Additionally, further analyses of 24 h urine samples available in biobanks from previous studies with legumes will be able to be carried out in order to see which of these candidate BFIs for legumes are replicated in independent studies.

Given that in the already available scientific bibliography none of the identified metabolites were highly specific, further research focused on their combination is required in order to evaluate their robustness and usefulness as candidate BFIs of these foods.

The crossover design of the present study allowed comparisons between the different meals on a within-participant basis. This allowed a better evaluation of the treatment effect since each participant served as its own control [40]. However, it was not the case with white beans, which

were administered in the last phase for all volunteers. Further, an additional untargeted analysis of the study foods helped us to find out whether the detected marker candidates were original constituents of the test foods or resulted from host or microbial metabolism.

The study also has some additional weaknesses. Firstly, a full-confidence identification level could not be reached for most of the differential metabolites as the corresponding chemical standards were commercially unavailable. However, identifications at level II and III have also provided interesting information that has allowed us to infer a biological interpretation of the observed results of the present study. On the other hand, the highly controlled environment in which the participants were involved throughout the study together with the high quantity of food provided would have favored the observation of significant candidate BFIs. Therefore, some of the candidate BFIs deciphered in the current study could not be replicated in other studies with lower amounts of ingested food and/or where the background diet is not as controlled as in our case [40]. Therefore, further studies are needed to validate the results deciphered by the present analyses. Lastly, in this case, pasta was chosen as a control food since it is a low-polyphenol food product, although other foods could also have been chosen instead of it.

4.7. Concluding remarks

In conclusion, the results of the present untargeted metabolomics work revealed a long list of metabolites that are candidates to be used as biomarkers of pulse intake. Flavan-3-ol-derived metabolites and arginine-related compounds were found to be specific for lentils, whereas protocatechuic acid glucoside was particular to chickpeas; methylcysteine and pipecolic, hydroxyjasmonic and hydroxydihydrojasmoinic acids to white beans; and hypaphorine to chickpeas and lentils. On the other hand, a wide range of peptides, trigonelline and some polyphenol-derived metabolites appeared to be more generic indicators of pulse consumption.

Most of the BFIs identified in urinary samples were replicated as discriminant metabolites in 24 h pooled urine, demonstrating that with 24 h urine pools we can reach almost the same conclusions. The next step would be to develop and apply a quantitative method for the compounds identified in this untargeted metabolomics analysis. Next, biological validation will be required to test these candidate biomarkers in other populations with more individuals and under different experimental conditions and even under free-living conditions.

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AUTHOR CONTRIBUTIONS

The authors' responsibilities were as follows: MGA, CAL and FM designed the research; MGA, SEA, MUS and CAL designed and conducted the clinical trial; MGA and AT ran the food sample preparation; CHW conducted the GC×GC-MS metabolomics analyses; MGA, MU and FM conducted the LC-MS metabolomics analyses; MGA, MU, PF and FM conduted data processing and statistical analysis; MGA drafted the manuscript; MGA, CAL and FM have

primary responsibility for the final content of the manuscript; and all authors provided critical intellectual input, and revised and approved the final manuscript.

CONFLICT OF INTEREST

None of the authors have declared a conflict of interest.

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FIGURE LEGENDS

Figure 1. Schematic representation of the design of the randomized, controlled, crossover study. This scheme was repeated in each treatment session. The top panel (above the dashed line) refers to the days that each of the interventions lasted, while the one below includes the information from the samples collected on the day of the intervention. Participants were assigned randomly to the test food in a crossover design. Urine samples were collected before the ingestion of the test product and during the indicated time intervals, whereas blood samples were also collected before the administration of the assigned meal and at the specified time points. After a one-week washout period, volunteers followed a two-day restricted diet before the intervention day. Standardized meals were provided to the volunteers from the dinner of the day before the intervention day, as well as from 6 h up to 48 h after the administration of the test food.

Figure 2. Selected metabolite kinetic curves in urine and serum samples. X axis: time point; Y axis: peak intensity (MS response). Lines represent medians and bars IQR range. Abbreviations: C: control; B: white beans; L: lentils; P: chickpeas.

Figure 3. Heatmaps of identified metabolites after intake of lentils (L), chickpeas (P), beans (B) or control (C) in 6 h pools (A) and 24 h pools (B). Blue and orange cells correspond to low- and high-metabolite levels, respectively. Columns are samples, and rows are metabolites colored by treatment and class of compound, respectively.

Table 1. Identified metabolites that were significantly different in the nutrikinetics experiment after intake of lentils, chickpeas, white beans or control.

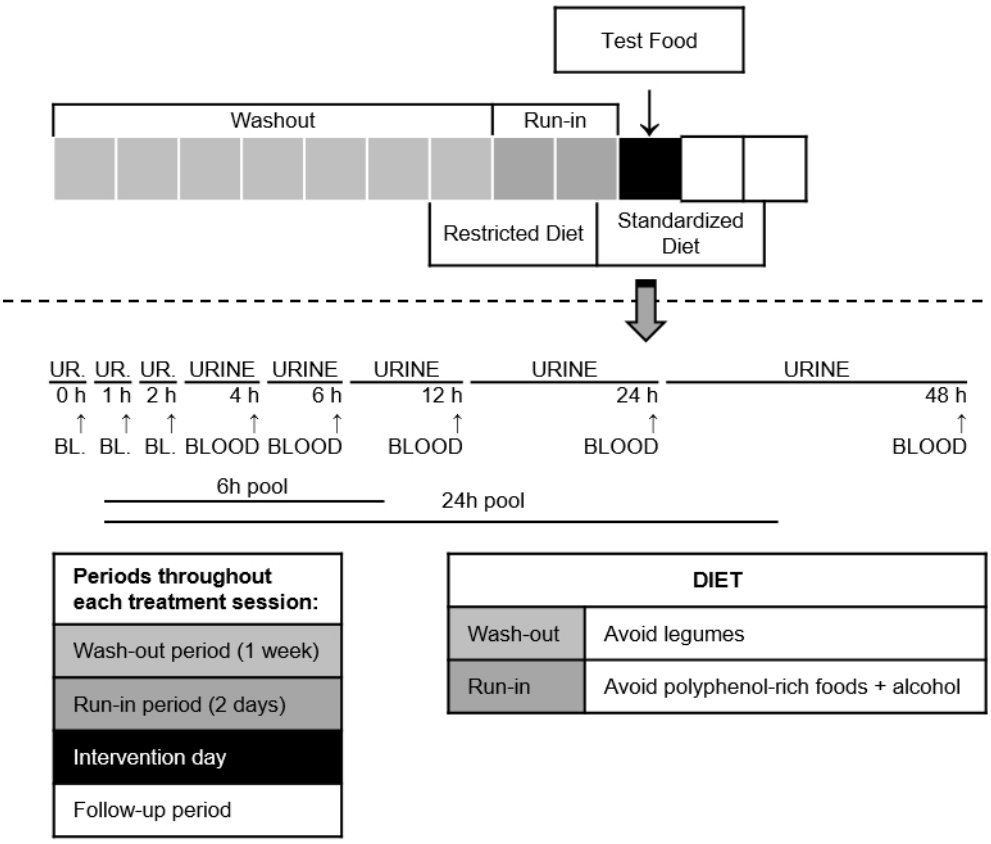
M	Metabolite	Formula (m/z)	RT	Annotations	LI (ref)	Behavior	Fluid: time	p-adjusted
M01	Asp-Ala	$C_7H_{12}N_2O_5$ (204.07461)	0.92	205.0819 $[M+H]^+$	2 (MyCompoundID)	(L & P & B) > C	U: 02h-12h	$4.47x10^{-5}$
M02	Asp-Gly / Gly-Asp	$C_6H_{10}N_2O_5$ (190.05896)	0.90	191.0662 $[M+H]^+$	1 (std)	(L & P & B) > C	U: 04h-24h	$7.97x10^{-5}$
M03	Asp-Leu	$C_{10}H_{18}N_2O_5$ (246.12156)	U: 2.98 S: 3.03	U: 247.1289 $[M+H]^+$; 248.1321 $^{13}C[M+H]^+$; 245.1145 $[M-H]^-$; 343.0913 $[M-H+C_2H_3O_2K]^-$; 227.1039 $[M-H-H_2O]^-$ S: 247.1287 $[M+H]^+$; 245.1142 $[M-H]^-$	1 (std)	(L & P) > B > C	U: 04h-12h S: 02h-06h	U: $1.96x10^{-5}$ S: $1.09x10^{-5}$
M04	Asp-Met	$C_9H_{16}N_2O_5S$ (264.07798)	1.43	265.0853 $[M+H]^+$	2 (std)	P > (L & B & C)	U: 02h-12h	$7.60x10^{-4}$
M05	Asp-Phe	$C_{13}H_{16}N_2O_5$ (280.10591)	3.77	281.1132 $[M+H]^+$; 282.1165 $^{13}C[M+H]^+$; 279.0987 $[M-H]^-$	2 (std)	(L & P & B) > C	U: 02h-06h	$1.12x10^{-5}$
M06	Asp-Ser / Ser-Asp	$C_7H_{12}N_2O_6$ (220.06952)	0.91	221.0769 $[M+H]^+$	3	(L & P & B) > C	U: 04h-12h	$5.15x10^{-5}$
M07	Asp-Tyr	$C_{13}H_{16}N_2O_6$ (296.10082)	U: 1.86 S: 1.67	U: 297.1082 $[M+H]^+$; 279.0976 $[M+H-H_2O]^+$ S: 297.1081 $[M+H]^+$	2 (std)	(L & P & B) > C	U: 02h-06h S: 02h-06h	U: $1.09x10^{-5}$ S: $1.91x10^{-5}$
M08	Asp-Val	$C_9H_{16}N_2O_5$ (232.10591)	U: 1.33 S: 1.35	U: 233.1132 $[M+H]^+$ S: 233.1131 $[M+H]^+$	2 (std)	(L & P & B) > C	U: 02h-12h S: 04h-06h	U: $1.48x10^{-5}$ S: $4.66x10^{-4}$
M09	Asp-(i)Leu-(i)Leu / (i)Leu-Asp-(i)Leu	$C_{16}H_{29}N_3O_6$ (359.20561)	5.46	360.2127 $[M+H]^+$	2 (MyCompoundID)	(L & P) > B > C	S: 02h-06h	$9.81x10^{-6}$
M10	Asp-(i)Leu-Pro / (i)Leu-Asp-Pro	$C_{15}H_{25}N_3O_6$ (343.17432)	4.38	344.1818 $[M+H]^+$	3	P > L > (B & C)	U: 06h-12h	$9.55x10^{-6}$
M11	Asp-Ala-(i)Leu / Ala-Asp-(i)Leu	$C_{13}H_{23}N_3O_6$ (317.15869)	3.64	318.1660 $[M+H]^+$	2 (MyCompoundID)	P > (L & B & C)	U: 04h-12h	$5.52x10^{-5}$
M12	Asp-Asn-Val / Asn-Asp-Val	$C_{13}H_{22}N_4O_7$ (346.14883)	1.31	347.1563 $[M+H]^+$	1 (std)	(L & P) > B > C	U: 02h-12h	$1.03x10^{-5}$
M13	Asp-Gly-(i)Leu	$C_{12}H_{21}N_3O_6$ (303.14302)	U: 3.36 S: 3.57	U: 304.1504 $[M+H]^+$; 302.1359 $[M-H]^-$ S: 304.1503 $[M+H]^+$	1 (std)	(L & P & B) > C	U: 04h-06h S: 02h-06h	U: $4.28x10^{-4}$ S: $1.81x10^{-5}$
M14	Asp-Gly-Tyr / Gly-Asp-Tyr	$C_{15}H_{19}N_3O_7$ (353.12230)	1.86	354.1297 $[M+H]^+$	3	P > L > (B & C)	U: 02h-12h	$1.85x10^{-5}$
M15	Asp-Gly-Val	$C_{11}H_{19}N_3O_6$ (289.12737)	1.50	291.1380 $^{13}C[M+H]^+$	1 (std)	(L & P) > (B & C)	U: 04h-06h	$2.03x10^{-2}$
M16	Asp-Thr-Pro / Thr-Asp-Pro	$C_{13}H_{21}N_3O_7$ (331.13793)	1.23	332.1453 $[M+H]^+$	3	P > L > (B & C)	U: 04h-24h	$8.52x10^{-6}$

M17	Pro-HPro-Gly / HPro-Pro-Gly	$C_{12}H_{19}N_3O_5$ (285.13245)	3.74	286.1398 $[M+H]^+$	2 (Metlin)	(L & P & B) > C	U: 04h-06h	$1.71x10^{-2}$
M18	Cyclo((i)Leu-Phe)	$C_{15}H_{20}N_2O_2$ (260.15246)	6.75	261.1596 $[M+H]^+$	2 (Yamamoto 2016 / MetFrag)	P > (L & B & C)	S: 01h-06h	$4.47x10^{-5}$
M19	Cyclo(His-Pro)	$C_{11}H_{14}N_4O_2$ (234.11166)	1.17	235.1191 $[M+H]^+$	2 (Yamamoto 2016 / MetFrag)	(L & P) > B > C	U: 01h-04h	$3.60x10^{-3}$
M20	(i)Leucine derivative (I)	$C_{10}H_{18}N_2O_5$ (246.12156)	3.64	247.1288 $[M+H]^+$	3	(L & P) > B > C	S: 02h-06h	$2.70x10^{-5}$
M21	(i)Leucine derivative (II)	$C_{12}H_{20}N_2O_6$ (288.13212)	U: 4.64 S: 4.89	U: 289.1394 $[M+H]^+$ S: 289.1392 $[M+H]^+$	3	(L & P & B) > C	U: 06h-12h S: 04h-06h	U: $1.96x10^{-4}$ S: $2.45x10^{-4}$
M22	Glycine derivative	$C_9H_{17}N_3O_4$ (231.12191)	1.40	232.1292 $[M+H]^+$; 215.1026 $[M+H-NH_3]^+$	3	(L & B) > (P & C)	U: 02h-06h	$1.09x10^{-3}$
M23	Phenylalanine derivative	$C_{21}H_{25}N_3O_5$ (399.17940)	5.34	400.1867 $[M+H]^+$	3	(P & B) > (L & C)	U: 01h-12h	$1.03x10^{-5}$
M24	Proline derivative	$C_{10}H_{16}N_2O_4$ (228.11099)	U: 3.59 S: 3.98	U: 229.1183 $[M+H]^+$ S: 229.1181 $[M+H]^+$	3	(L & P & B) > C	U: 04h-06h S: 02h-06h	U: $4.39x10^{-2}$ S: $5.15x10^{-3}$
M25	Hydroxyarginine	$C_6H_{14}N_4O_3$ (190.10658)	0.80	191.1139 $[M+H]^+$	2 (MetFrag)	L > (P & B & C)	U: 01h-48h	$6.28x10^{-5}$
M26	Oxoarginine	$C_6H_{11}N_3O_3$ (173.08003)	0.93	174.0873 $[M+H]^+$	2 (MetFrag)	L > (P & B & C)	U: 02h-48h	$1.61x10^{-4}$
M27	(Epi)catechin sulfate	$C_{15}H_{14}O_9S$ (370.03584)	4.33	369.0287 $[M-H]^-$	2 (van der Hoof 2012)	L > (P & B & C)	U: 01h-24h	$4.37x10^{-5}$
M28	4-Hydroxy-5-(dihydroxyphenyl)-valeric acid-O-sulfate	$C_{11}H_{14}O_8S$ (306.04092)	4.23	305.0337 $[M-H]^-$	2 (van der Hoof 2012)	L > (P & B & C)	U: 06h-12h	$3.37x10^{-5}$
M29	5-(3',4'-Dihydroxyphenyl)-y-valerolactone-3-O-sulfate	$C_{11}H_{12}O_7S$ (288.03036)	4.70	306.0643 $[M+NH_4]^+$; 287.0232 $[M-H]^-$; 288.0265 $^{13}C[M-H]^-$; 207.0664 $[M-H-sulf]^-$	2 (van der Hoof 2012)	L > (P & B & C)	U: 04h-48h	$7.31x10^{-5}$
M30	5-(3',4'-Dihydroxyphenyl)-y-valerolactone-O-glucuronide	$C_{17}H_{20}O_{10}$ (384.10563)	4.58	385.1129 $[M+H]^+$; 402.1395 $[M+NH_4]^+$; 209.0808 $[M+H-gluc]^+$	2 (van der Hoof 2012)	L > (P & B & C)	U: 04h-24h	$1.63x10^{-5}$
M31	5-(3',5'-Dihydroxyphenyl)-y-valerolactone-methyl-glucuronide	$C_{18}H_{22}O_{10}$ (398.12128)	4.76	416.1551 $[M+NH_4]^+$; 223.0964 $[M+H-gluc]^+$; 397.1141 $[M-H]^-$	2 (van der Hoof 2012)	L > (P & B & C)	U: 04h-48h	$8.09x10^{-6}$
M32	Phloroglucinol glucuronide	$C_{12}H_{14}O_9$ (302.06377)	U: 1.85 S: 1.79	U: 303.0711 $[M+H]^+$; 304.0745 $^{13}C[M+H]^+$; 325.0531 $[M+Na]^+$; 341.027 $[M+K]^+$; 468.1498 [+]; 301.0566 $[M-H]^-$; 302.0597 $^{13}C[M-H]^-$; 399.0336 $[M-H+C_2H_3O_2K]^-$ S: 303.0710 $[M+H]^+$	2 (std)	(L & P & B) > C	U: 01h-12h S: 01h-04h	U: $4.50x10^{-5}$ S: $4.24x10^{-4}$
M33	Phloroglucinol sulfate	$C_6H_6O_6S$ (205.98850)	U: 1.35 S: 1.43	U: 206.9954 $[M+H]^+$; 224.0228 $[M+NH4]^+$; 244.9517 $[M+K]^+$; 127.0388 $[M+H-sulf]^+$; 204.9814 $[M-H]^-$; 205.9847 $^{13}C[M-H]^-$; 206.9772 $2^{13}C[M-H]^-$; 302.9583 $[M-H+C_2H_3O_2K]^-$; 125.0248 $[M-H-sulf]^-$ S: 204.9814 $[M-H]^-$	1 (std)	(L & P & B) > C	U: 01h-12h S: 01h-04h	U: $7.31x10^{-5}$ S: $3.37x10^{-5}$
M34	2-Hydroxyhippuric acid	$C_9H_9NO_4$ (195.05315)	5.28	196.0604 $[M+H]^+$; 241.1048 $[M+C_2H_8N]^+$; 239.0925 $[M+C_2H_8N]^-$	1 (std)	B > (L & P & C)	U: 01h-06h	$8.72x10^{-3}$

M35	Aminosalicyluric acid	$C_9H_{10}N_2O_4$ (210.06405)	1.78	211.0713 $[M+H]^+$; 209.0569 $[M-H]^-$	3 (in silico)	(L & P & B) > C	U: 02h-24h	$6.37x10^{-6}$
M36	Muconic acid	$C_6H_6O_4$ (142.02660)	1.64	141.0197 $[M-H]^-$	2 (mzCloud)	(L & P & B) > C	U: 01h-24h	$5.52x10^{-5}$
M37	Protocatechuic acid glucoside	$C_{13}H_{16}O_9$ (316.07942)	2.62	315.0722 $[M-H]^-$; 413.0493 $[M-H+C_2H_3O_2K]^-$	2 (Cuparencu 2016 / mzCloud)	P > L > (B & C)	U: 02h-04h	$6.37x10^{-6}$
M38	Vanillic acid sulfate	C8H8O7S (247.99906)	2.91	246.9920 $[M-H]^-$	2 (mzCloud)	L > (P & B & C)	U: 04h-12h	$7.49x10^{-4}$
M39	Hydroxyjasmonic acid	C12H18O4 (226.12049)	5.18	227.1278 $[M+H]^+$; 225.1134 $[M-H]^-$	2 (Valente 2018)	B > (L & P & C)	U: 01h-24h	$6.96x10^{-4}$
M40	Hydroxydihydrojasmonic acid (I)	C12H20O4 (228.13614)	4.81	229.1434 $[M+H]^+$; 211.1328 $[M+H-H_2O]^+$; 193.1223 $[M+H-2(H_2O)]^+$; 227.1290 $[M-H]^-$	3 (in silico)	B > (L & P & C)	U: 01h-12h	$3.98x10^{-4}$
M41	Hydroxydihydrojasmonic acid (II)	C12H20O4 (228.13614)	5.37	211.1328 $[M+H-H_2O]^+$; 227.1290 $[M-H]^-$	3 (in silico)	B > (L & P & C)	U: 01h-48h	$8.99x10^{-4}$
M42	Ascorbic acid	C6H8O6 (176.03208)	0.95	177.0393 $[M+H]^+$; 175.0251 $[M-H]^-$; 115.0041 $[M-H-C_2H_4O_2]^-$	1 (std)	P > (L & B & C)	U: 04h-06h	$1.23x10^{-3}$
M43	Dopamine sulfate	C8H11NO5S (233.03578)	1.31	234.0430 $[M+H]^+$; 232.0287 $[M-H]^-$	2 (std)	L > (P & B & C)	U: 01h-12h	$1.20x10^{-4}$
M44	Hypaphorine	C14H18N2O2 (246.13681)	U: 4.07 S: 4.36	U: 247.1441 $[M+H]^+$; 188.0706 $[M+H-(CH_3)_3N]^+$ S: 247.1440 $[M+H]^+$; 248.1473 $^{13}C[M+H]^+$; 188.0704 $[M+H-(CH_3)_3N]^+$; 189.0737 $^{13}C[M+H-(CH_3)_3N]^+$; 146.0598 $[M+H-(CH3)_3N-C_2H_2O]^+$; 144.0805 $[M+H-C_3H_9N-CO_2]^+$; 245.1295 $[M-H]^-$; 246.1328 $^{13}C[M-H]^-$; 291.1349 $[M+FA-H]^-$; 292.1382 $^{13}C[M+FA-H]^-$; 186.0561 $[M-H-(CH_3)_3N]^-$	1 (std)	(L & P) > (B & C)	U: 02h-48h S: 01h-48h	U: $2.42x10^{-5}$ S: $1.02x10^{-5}$
M45	Methylcysteine	C4H9NO2S (135.03539)	1.10	136.0425 $[M+H]^+$	1 (std)	B > (L & P & C)	S: 01h-48h	$4.86x10^{-4}$
M46	Pipecolic acid	C6H11NO2 (129.07897)	1.12	130.0861 $[M+H]^+$; 131.0894 $^{13}C[M+H]^+$; 152.0681 $[M+Na]^+$	1 (std)	B > (L & P & C)	S: 01h-24h	$4.51x10^{-4}$
M47	Trigonelline	C7H7NO2 (137.04767)	0.88	138.0549 $[M+H]^+$; 139.0582 13C$[M+H]^+$; 94.0650 $[M+H-CO_2]^+$	1 (std)	B > P > L > C	U: 02h-06h	$6.37x10^{-6}$
M48	y-CEHC glucose	C21H30O9 (426.18896)	6.28	444.2225 $[M+NH_4]^+$	2 (std)	(L & P & B) > C	U: 06h-12h	$8.46x10^{-4}$
M49	y-CEHC glucuronide	C21H28O10 (440.16822)	6.39	441.1753 $[M+H]^+$; 458.2016 $[M+NH_4]^+$; 459.2051 $^{13}C[M+NH_4]^+$; 463.1569 $[M+Na]^+$; 501.2073 $[M+H+C_2H_4O_2]^+$; 265.1434 $[M+H-gluc]^+$; 266.1467 $^{13}C[M+H-gluc]^+$; 151.0752 $[M+H-gluc-H_2O-C_4H_6-COCH_2]^+$; 537.1381 $[M-H+C_2H_3O_2K]^-$	2 (std)	(L & P & B) > C	U: 04h-12h	$1.89x10^{-4}$
M50	Dihydroxydecanoic acid glucuronide	C16H28O10 (380.16822)	U: 5.52 S: 5.60	U: 381.1755 $[M+H]^+$; 398.2020 $[M+NH_4]^+$; 379.1611 $[M-H]^-$ S: 379.1608 $[M-H]^-$	2 (in silico)	C > (L & P) > B	U: 04h-06h S: 02h-06h	U: $1.34x10^{-2}$ S: $3.35x10^{-4}$
M51	Dodecanedioic acid glucuronide	C18H30O10 (406.18387)	U: 6.14 S: 6.11	U: 407.1915 $[M+H]^+$; 424.2175 $[M+NH_4]^+$; 425.2211 $^{13}C[M+NH_4]^+$; 429.1737 $[M+Na]^+$; 467.2230 $[M+H+C_2H_4O_2]^+$; 389.1805 $[M+H-H_2O]^+$; 390.1839 $^{13}C[M+H-H_2O]^+$; 231.1591 $[M+H-gluc]^+$; 405.1767 $[M-H]^-$; 406.1802 $^{13}C[M-H]^-$; 427.1588 $[M+Na]^-$; 503.1539 $[M-H+C_2H_3O_2K]^-$ S: 405.1765 $[M-H]^-$	2 (std)	C > (L & P) > B	U: 02h-06h S: 01h-06h	U: $4.26x10^{-3}$ S: $2.11x10^{-4}$

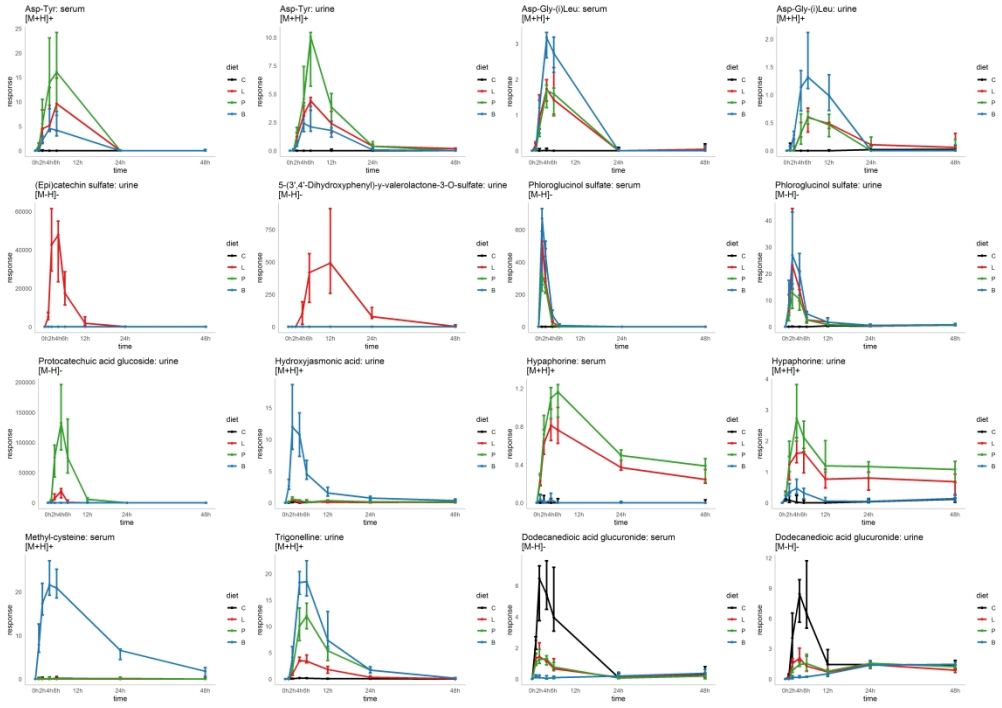
M52	Hydroxydodecadienoic acid glucuronide	C18H28O9 (388.17331)	7.07	195.1379 [M+H-gluc-H_2O]$^+$; 387.1661 [M-H]$^-$	2 (Metlin)	C > (L & P & B)	U: 02h-06h	$1.43x10^{-2}$
M53	Tetradecanedioic acid glucuronide	C20H34O10 (434.21517)	U: 6.89 S: 6.73	U: 435.2222 [M+H]$^+$; 457.2039 [M+Na]$^+$; 417.2118 [M+H-H_2O]$^+$; 223.1692 [M+H-gluc-2(H_2O)]$^+$; 433.2080 [M-H]$^-$; 434.2114 ^{13}C[M-H]$^-$; 455.1903 [M+Na]$^-$ S: 433.2077 [M-H]$^-$	2 (in silico)	C > (L & P) > B	U: 04h-06h S: 01h-06h	U: $2.39x10^{-2}$ S: $4.20x10^{-4}$
M54	Dicarboxylic fatty acid C12:1, dihydroxy	C12H20O6 (260.12597)	5.05	261.1333 [M+H]$^+$	3	C > (L & P) > B	U: 02h-12h	$2.38x10^{-2}$

Abbreviations: C: control; B: white beans; gluc, glucuronide; L: lentils; LI, level of identification; M, metabolite; P: chickpeas; ref, reference; RT, retention time; S, serum; sulf, sulfate; U, urine.



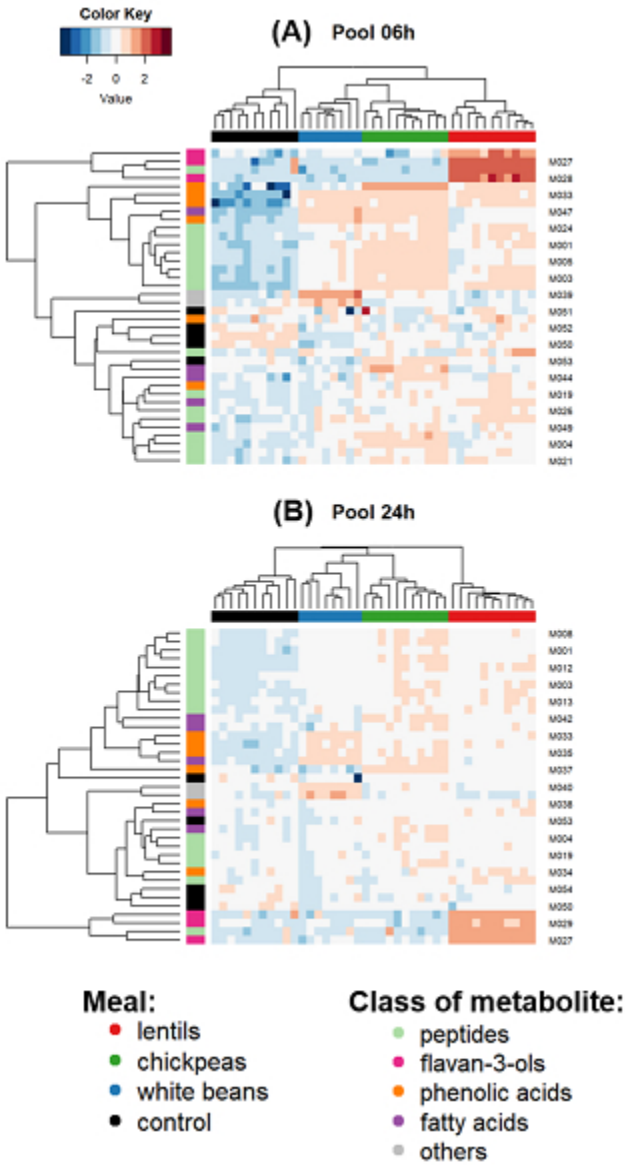
Study design

132x113mm (150 x 150 DPI)



Kinetic curves

533x381mm (300 x 300 DPI)



HCA

93x154mm (96 x 96 DPI)