International Journal of Obesity
https://doi.org/10.1038/s41366-020-0628-1

TECHNICAL REPORT

Techniques and Methods

Quantifying the human diet in the crosstalk between nutrition and health by multi-targeted metabolomics of food and microbiota-derived metabolites

Raúl González-Domínguez[1,2] · Olga Jáuregui[2,3] · Pedro Mena[4] · Kati Hanhineva[5] · Francisco José Tinahones[6,7] · Donato Angelino[4] · Cristina Andrés-Lacueva[1,2]

Received: 30 December 2019 / Revised: 18 May 2020 / Accepted: 1 June 2020

Abstract

Background Metabolomics is a powerful tool for investigating the association between nutrition and health status. Although urine is commonly employed for studying the metabolism and transformation of food components, the use of blood samples could be preferable to gain new insights into the bioavailability of diet-derived compounds and their involvement in health. However, the chemical complexity of blood samples hinders the analysis of this biological fluid considerably, which makes the development of novel and comprehensive analytical methods mandatory.

Methods In this work, we optimized a multi-targeted metabolomics platform for the quantitative and simultaneous analysis of 450 food-derived metabolites by ultra-high performance liquid chromatography coupled to tandem mass spectrometry. To handle the chemical complexity of blood samples, three complementary extraction methods were assayed and compared in terms of recovery, sensitivity, precision and matrix effects with the aim of maximizing metabolomics coverage: protein precipitation, reversed solid-phase extraction, and hybrid protein precipitation with solid-phase extraction-mediated phospholipid removal.

Results After careful optimization of the extraction conditions, protein precipitation enabled the most efficient and high-throughput extraction of the food metabolome in plasma, although solid-phase extraction-based protocols provided complementary performance for the analysis of specific polyphenol classes. The developed method yielded accurate recovery rates with negligible matrix effects, and good linearity, as well as high sensitivity and precision for most of the analyzed metabolites.

Conclusions The multi-targeted metabolomics platform optimized in this work enables the simultaneous detection and quantitation of 450 dietary metabolites in short-run times using small volumes of biological sample, which facilitates its application to epidemiological studies.

Supplementary information The online version of this article (https://doi.org/10.1038/s41366-020-0628-1) contains supplementary material, which is available to authorized users.

✉ Cristina Andrés-Lacueva
candres@ub.edu

[1] Biomarkers and Nutrimetabolomics Laboratory; Department of Nutrition, Food Sciences and Gastronomy; Food Technology Reference Net (XaRTA); Nutrition and Food Safety Research Institute (INSA); Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain

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

[3] Scientific and Technological Center of University of Barcelona (CCiTUB), 08028 Barcelona, Spain

[4] Human Nutrition Unit, Department of Food and Drugs, University of Parma, Medical School Building C, Via Volturno, 39, 43125 Parma, Italy

[5] Institute of Public Health and Clinical Nutrition, University of Eastern Finland, Kuopio, Finland

[6] Department of Endocrinology and Nutrition, Virgen de la Victoria University Hospital, Institute of Biomedical Research in Malaga (IBIMA), Malaga, Spain

[7] CIBER Physiopathology of Obesity and Nutrition (CIBERobn), Institute of Health Carlos III, Madrid, Spain

Introduction

Metabolomics is nowadays one of the most powerful tools in nutrition research since metabolites can be used as direct and objective indicators of food intake, and they can also provide valuable information about multiple biological and lifestyle factors (e.g., genetic background, disease, microbiota, and xenobiotics) [1]. The potential applications of metabolomics in nutrition (i.e., nutrimetabolomics) and biomedical research include (i) the discovery of food intake biomarkers for dietary assessment, (ii) the identification of metabolic pathways altered because of dietary interventions, and (iii) the investigation of the association between nutrition and health status. The measurement of dietary biomarkers has demonstrated excellent performance in increasing the efficacy of dietary assessment, complementing traditional self-reported surveys [2]. Furthermore, metabolomics approaches are also of particular interest for studying diseases closely linked to nutritional and lifestyle factors, such as obesity and metabolic disorders. Indeed, numerous metabolomics-based works have been published in recent years investigating the interaction between diet, genes, and microbiota in obesity and related disorders, as well as developing precision nutrition recommendations [3, 4]. However, recent research emphasizes the need for novel tools for accurate measurement of food-derived metabolites to gain deeper insights into the association between nutrition and health in nutritional epidemiology, particularly in a quantitative manner to allow for cross-cohort comparisons [5–7].

The food metabolome is highly heterogeneous and complex, comprising nutrients, secondary bioactive metabolites, additives, and cooking-derived compounds [8]. After ingestion, these dietary components are extensively transformed by phase I/II reactions and/or gut microbiota, and are then rapidly excreted mostly in urine, but also in other matrixes such us feces and bile. Due to water reabsorption in the kidney, the concentration of food metabolites is usually higher in urine than in other biological samples, clearly reflecting the ADME (Absorption, Distribution, Metabolism, and Excretion) process [1]. For this reason, and because large volumes can be collected using non-invasive procedures, urine is normally the preferred biofluid in nutrimetabolomics for studying the metabolism and transformation of food components [1, 5]. On the other hand, plasma/serum samples are more likely to provide deeper insights into the bioavailability of nutrients and diet × health interactions, since blood is a rich source of metabolically active compounds that are in transit from one organ to another, whereas the major function of urine is only to dispose of unwanted compounds in the body [9]. Furthermore, the advantages of blood samples compared with urine include: (i) lower inter- and intra-individual variability [1]; (ii) the possibility of detecting lipophilic biomarkers, which usually have longer half-lives [10]; and (iii) the more common availability of blood samples in large-cohort studies.

The aim of this work was to optimize a targeted metabolomics method for the analysis of diet-related metabolites in blood samples. Previous publications on this topic usually employ an enzymatic hydrolysis step of phase II metabolites [11–13], which significantly simplifies the metabolome complexity and consequently the analytical procedure, but hinders the performance of comprehensive metabolomics because optimal hydrolysis conditions depend on specific metabolite classes. Recent studies described the optimization of targeted methods focused on the analysis of specific biomarker classes [14–16]. However, the great complexity of the food metabolome makes mandatory the development of novel methods to increase the analytical comprehensiveness, allowing the simultaneous analysis of as many metabolites as possible in a single run to minimize costs and the consumption of valuable biological samples. Furthermore, high-throughput nutrimetabolomics approaches are also needed to explore the inter-individual variability in response to food consumption [17]. In this context, we have recently developed a metabolomics platform for the simultaneous quantitation of 350 food intake biomarkers in urine samples [18]. Nonetheless, the application of these methodologies to blood is hindered considerably by the chemical complexity of this biological fluid, characterized by high contents of proteins and lipids, and lower concentrations of dietary metabolites compared with urine. To overcome this hurdle, a multi-targeted metabolomics method has been optimized in the present work for the detection and quantification of a wide range of food-related metabolites and microbiota derivatives in plasma, paying special attention to the optimization of efficient extraction protocols.

Materials and methods

Extraction of plasma samples

For the optimization of the extraction conditions, blank plasma samples were collected from healthy volunteers after one week of a low-polyphenol diet, as previously described [19]. Furthermore, to look for potential food-derived metabolites for which standards are currently not available, healthy volunteers were asked to follow acute dietary interventions with several foods (orange, grapefruit, apple, banana, red wine, beer, green tea, coffee, soy sprouts, walnuts, wholegrain rye, and oat), as described elsewhere [18]. These foods were consumed at dinner, and then first-

morning-void urine samples were collected (i.e., 8–12 h after intake).

For all the tested extraction methods, plasma samples (100 µL) were first thawed in an ice bath and spiked with 10 µL of a set of isotopically labeled internal standards (ferulic acid-1,2,3-$^{13}C_3$, L-phenylalanine-^{15}N) dissolved in ultrapure water at 1 mg L^{-1}. For validation purposes, some samples were also spiked with known concentrations of 256 food-derived metabolites for which pure standards were available (see Supplementary Information). After the extraction as described below for the three compared methods, extracts were taken to dryness using a MaxiVac β vacuum concentrator (Daejeon, South Korea), and reconstituted with 100 µL of water:acetonitrile (80:20, v/v) containing 0.1% formic acid and internal standards for quantification (taxifolin and caffeine-$^{13}C_3$, 100 µg L^{-1}).

Protein precipitation (PPT)

Plasma samples were mixed with 500 µL of cold acetonitrile (−20 °C) containing 1.5 M formic acid and 10 mM ammonium formate in an Eppendorf tube, and then vigorously shaken using a vortex mixer. Samples were kept at −20 °C for 10 min to promote PPT, then centrifuged at 10,000 × *g* for 10 min at 4 °C, and supernatants were finally transferred to new tubes.

Hybrid PPT and solid-phase extraction (SPE)-mediated phospholipid removal (Ostro®)

Following a modification of the method previously developed by Tulipani et al. [20], plasma samples were pipetted into Ostro® 96-well plates (Waters, Milford, MA, USA) and mixed with 500 µL of cold acetonitrile (−20 °C) containing 1.5 M formic acid and 10 mM ammonium formate. Subsequently, plates were vortexed and kept at −20 °C for 10 min to promote in-well PPT. A Waters Positive Pressure-96 Processor was then employed to collect deproteinized extracts in a 96-well collection plate. Finally, 500 µL of cold acetonitrile (−20 °C) containing 0.5% ammonia (v/v) were added to wells containing the protein precipitates to perform a second extraction. After vortex shaking, positive pressure was again applied to collect the second extract in the same collection plate.

SPE (Oasis® HLB)

SPE was performed using Oasis® HLB 96-well plates, filled with 30 mg of sorbent (Waters, Milford, MA, USA), according to the method described by González-Domínguez et al. with some modifications [18]. Briefly, the sorbent was first conditioned with 1 mL of methanol and 1 mL of water containing 1.5 M formic acid and 10 mM ammonium formate. Then, a mixture of the plasma sample with 900 µL of 2% H_3PO_4 in water (v/v) was loaded onto the preconditioned plate. Plates were washed with 1 mL of water containing 1.5 M formic acid and 10 mM ammonium formate. Finally, retained metabolites were eluted with 1.5 mL of methanol containing 1.5 M formic acid and 10 mM ammonium formate.

Quantitative metabolomic fingerprinting by UHPLC-MS/MS

Metabolomic analyses were conducted following the methodology developed by González-Domínguez et al. with modifications (Table S1) [18]. Analyses were performed on an Agilent 1290 Infinity UHPLC system (Santa Clara, CA, USA) coupled to a Sciex QTRAP 6500 mass spectrometer equipped with an Ion-Drive Turbo V ion source (Framingham, MA, USA). Chromatographic separations were performed on a Luna Omega Polar C18 column, 100 mm × 2.1 mm (i.d. 1.6 µm), equipped with a fully porous polar C18 security guard cartridge from Phenomenex (Torrance, CA, USA). Water containing 0.1% formic acid and 10 mM ammonium formate and acetonitrile were used as aqueous (A) and organic (B) mobile phases in the negative ion mode, applying the following gradient program: 0–8 min, 5–20% B; 8–10 min, 20–100% B; 10–12 min, 100% B; 12–12.1 min, 100-5% B; 12.1–14 min, 5% B. Under positive ionization, water and acetonitrile, both containing 0.5% formic acid, were used as mobile phases: 0–5 min, 5–50% B; 5–5.1 min, 50–100% B; 5.1–7 min, 100% B; 7–7.1 min, 100–5% B; 7.1–9 min, 5% B. Other chromatographic conditions were as follows: column temperature, 40 °C; autosampler temperature, 4 °C; injection volume, 2 µL; flow rate, 0.5 mL min^{-1}. On the other hand, MS detection was performed by using the scheduled multiple reaction monitoring (sMRM) mode, under positive and negative ionization in separate runs, applying the following parameters: ion spray voltage, +4500/−3500 V; source temperature, 600 °C; curtain gas, 30 psi; ion source gas 1 and gas 2, 50 psi each; collision-activated dissociation gas, 3 psi; entrance potential, (+/−) 10 V. The MRM transitions were optimized by infusing individual solutions of commercial standards dissolved in mobile phase (proportion A:B 1:1 (v/v), 500 µg L^{-1}) into the mass spectrometer using a syringe pump at a flow rate of 5 µL min^{-1}. The optimization of MRM conditions for those metabolites for which authentic standards were not available was performed as previously described [18]. Briefly, samples collected after acute dietary interventions were subjected to product ion scan experiments (MS^2) by using predicted nominal masses of expected metabolites, and those peaks showing neutral losses of 176 Da (i.e., glucuronide conjugates) or 80 Da (i.e., sulfate conjugates) were

Table 1 Summary of metabolites included in the multi-targeted metabolomics fingerprinting platform.

Class	Metabolites	Food
Phenolic acids		
Hydroxybenzoic acids ($N = 52$)	Hydroxy/dihydroxy-benzoic, hippuric, (iso) vanillic, syringic, gallic acids	Plant foods (fruits, vegetables, grains, legumes, nuts)
Hydroxyphenylacetic acids ($N = 16$)	Hydroxy/dihydroxy-phenylacetic, homovanillic acids	
Hydroxycinnamic acids ($N = 30$)	Hydroxy/dihydroxy-cinnamic, (iso)ferulic, sinapic acids	
Hydroxyphenylpropionic acids ($N = 19$)	Hydroxy/dihydroxy-propionic, dihydro(iso) ferulic acids	
Others ($N = 35$)	Dihydroxyphenylpentanoic acid, pyrogallol, syringol, catechol, hydroxybenzaldehydes	
Flavonoids		
Flavan-3-ols ($N = 31$)	Catechin, epicatechin	Tea, berry fruits, cocoa, apple
Flavanones ($N = 10$)	Naringenin, hesperetin	Citrus fruits
Isoflavones ($N = 23$)	Daidzein, genistein, equol, biochanin A, formononetin	Soy, legumes
Flavones ($N = 5$)	Apigenin, luteolin	Plant foods (fruits, vegetables, grains, legumes, nuts)
Flavonols ($N = 10$)	Quercetin, kaempferol, isorhamnetin	Plant foods (fruits, vegetables, grains, legumes, nuts)
Anthocyanins ($N = 6$)	Cyanidin, malvidin, delphinidin, pelargonidin, peonidin, petunidin	Berry fruits
Dihydrochalcones ($N = 2$)	Phloretin	Apple
Phenylethanoids ($N = 13$)	Hydroxytyrosol	Olive oil
Stilbenes ($N = 20$)	Resveratrol	Grapes, red wine
Coumarins ($N = 7$)	Bergaptol, umbelliferone	Fruits (*Rutaceae*), vegetables (*Umbelliferae*)
Curcuminoids ($N = 2$)	Curcumin	Curcuma
Lignans ($N = 14$)	Matairesinol, (i)lariciresorcinol, secoisolariciresorcinol, pinoresinol, syringaresinol, medioresinol	Fiber rich foods
Prenylflavonoids ($N = 1$)	Isoxanthohumol	Beer
Other phytochemicals		
Benzoxazinoids ($N = 20$)	BOA, HBOA, DIBOA, HMBOA, DIMBOA	Wholegrain wheat and rye
Hydroxycinnamic amides ($N = 6$)	Avenanthramides	Wholegrain oat
Steroid glycosides ($N = 2$)	Avenacosides	Wholegrain oat
Glucosinolates ($N = 5$)	Sulforaphane	Cruciferous vegetables (cabbage, broccoli)
Organosulfurated metabolites ($N = 2$)	Allylcysteine	Allium vegetables (garlic, onion)
Glycoalkaloids ($N = 4$)	Solanidine, tomatidine	Solanaceae vegetables (potato, tomato)
Diterpenes ($N = 1$)	Atractyligenin glucuronide	Coffee
Microbiota-derived metabolites		
Hydroxyphenyl-valerolactones ($N = 25$)	Hydroxy/dihydroxy/trihydroxy/hydroxy-methoxy-phenylvalerolactones	Flavan-3-ol rich foods (tea, berry fruits, cocoa, apple)
Urolithins ($N = 9$)	Urolithins A, B, C	Ellagitannin rich foods (berry fruits, nuts, pomegranate)
Enterolignans ($N = 6$)	Enterolactone, enterodiol	Fiber rich foods
Hydroxylated phenylacetamides ($N = 9$)	Hydroxyphenylacetamide	Wholegrain wheat and rye
Phenoxazinones ($N = 4$)	APO, AMPO, AAPO, AAMPO	Wholegrain wheat and rye
Miscellaneous		

Table 1 (continued)

Class	Metabolites	Food
Methylxanthines ($N = 16$)	Methylxanthines, methyluric acids	Coffee, tea, cocoa
Artificial sweeteners ($N = 4$)	Acesulfame K, sucralose, saccharin and cyclamate	Sweetened beverages
Fatty acids ($N = 4$)	Pentadecanoic, margaric, eicosapentaenoic, docosahexaenoic acids	Dairy products (odd chain fatty acids), fish (polyunsaturated fatty acids)
Maillard reaction products ($N = 5$)	Furan derivatives	Heat-treated foods (coffee, cocoa)
Diketopiperazines ($N = 2$)	Cyclo(leucyl-prolyl), cyclo(prolyl-valyl)	Heat-treated foods (coffee, cocoa)
Polycyclic compounds ($N = 2$)	1-hydroxypyrene glucuronide, PhIP-G	Heat-treated (meat, fish)
Betaines ($N = 13$)	Betainized amino acids, trigonelline, arsenobetaine, ergothioneine, hypaphorine	Wholegrains (amino acid betaines), citrus fruits (proline betaine), coffee (trigonelline), mushrooms (ergothioneine), fish (arsenobetaine), legumes (trigonelline, hypaphorine)
Histidine derivatives ($N = 4$)	1-methylhistidine, 3-methylhistidine, carnosine, anserine	Animal foods
Salsolinol ($N = 2$)	Derivatives of salsolinol	Banana
Alcohol and tobacco consumption ($N = 6$)	Ethyl-glucuronide/sulfate, derivatives of nicotine	Alcohol and tobacco
Others ($N = 4$)	Creatinine, TMAO, tartaric acid, pinitol,	Various

subjected to MS^3 fragmentation of the corresponding aglycone. Then, MRM transitions and fragmentation parameters were experimentally optimized to obtain the highest sensitivity. Optimized MRM transitions, declustering potentials (DPs), collision energies (CEs), cell exit potentials (CXPs), retention times (RTs), and RT windows are listed in Table S1. Analyst 1.6.2 and Sciex OS-Q software (ABSciex, Framingham, MA, USA) were used for data acquisition and data processing, respectively.

Method validation

The optimized methodology was validated according to the guidelines defined by the US Food and Drug Administration (FDA) for bioanalytical method validation [21]. Calibration curves were prepared in both solvent and blank plasma at 12 concentration levels ranging from 0.1 to 2000 $\mu g\,L^{-1}$ by diluting individual stock solutions of standards (1000 $mg\,L^{-1}$). Recoveries were determined in plasma samples spiked at three concentration levels (5, 100, 500 $\mu g\,L^{-1}$), which were analyzed in triplicate. Matrix effects (MEs) were measured by comparing the analyte response of standards dissolved in solvent and plasma at the same concentration level (5, 100, 500 $\mu g\,L^{-1}$). Intra-day and inter-day precisions were evaluated by analyzing spiked plasma samples at three concentration levels (5, 100, 500 $\mu g\,L^{-1}$) five times within the same day and on three consecutive days, respectively. The limits of quantification (LOQs) were estimated in spiked plasma as the lowest concentration that gives an average signal-to-noise (S/N) ratio above 10, with accuracies varying from 80% to 120% of the theoretical value. LOQs were calculated by subtracting the analyte response observed in non-spiked blank plasma.

Validation of putative biomarkers

Ten healthy volunteers (40.4 ± 4.1 years, 6/4 males/females) were enrolled in a 1-month intervention trial with a Mediterranean diet supplemented with red wine (270 $mL\,day^{-1}$). Fasting plasma samples were collected at baseline (free-living) and at the end of the intervention period, and were stored at −80 °C until analysis. The study was performed in accordance with the principles contained in the Declaration of Helsinki. The Bioethical Committee of the Hospital Virgen de la Victoria (Málaga, Spain) approved the study protocol, and all the participants provided written informed consent. The study was registered under ClinicalTrials.gov as NCT03101436. The metabolomics dataset obtained after analyzing plasma samples were subjected to *t*-test statistical analysis to look for altered metabolites because of the intervention.

Results and discussion

Multi-targeted metabolomics platform

In the present work, a novel multi-targeted metabolomics fingerprinting approach was optimized for the analysis of plasmatic food-derived metabolites and microbiota derivatives, by using a modification of the recently published Quantitative Dietary Fingerprinting (QDF) approach [18]. The coverage of the new method was significantly enlarged

by including some novel dietary metabolites: fatty acids (dairy products, fish), benzoxazinoids and microbiota derivatives (wheat and rye), avenanthramides and avenacosides (oat), lignans (fiber-rich foods), and some others. The optimized method thus enables the simultaneous detection and quantitation of 450 food-derived metabolites in very short run times (9 min + 14 min, under positive and negative ionization, respectively), as summarized in Table 1. From this metabolomic library, pure standards were available for 256 metabolites (level I identification according to the Metabolomics Standards Initiative guidelines). The rest of the metabolites included in the method were identified in samples collected after dietary interventions (level II identification), accounting for 43.2% of the total number of metabolites assayed, which evidences the difficulty of performing comprehensive nutrimetabolomics because of the lack of commercial standards. The MRM parameters of these latter metabolites were optimized as previously described [18].

To create this method, we not only considered already validated food intake biomarkers but also a comprehensive number of food-related metabolites and microbiota derivatives, which could be of great interest for different purposes. First, it should be noted that, to date, research on food intake biomarkers has been mainly accomplished by using non-targeted metabolomics approaches, which show a great potential in "discovery studies" but present serious analytical limitations for validation purposes (e.g., a lack of absolute quantitation, problems associated with robustness/reproducibility). Thus, we strongly believe that the methodology described in the present work could have great potential to perform more robust validation studies, according to the guidelines recently described [7]. Furthermore, although many of the metabolites covered in this methodology probably lack the required specificity to be considered as food intake biomarkers (e.g., most phenolic acids can be indicative of the consumption of plant-derived foods, but cannot serve as biomarkers of specific foods), they can provide additional and complementary information about metabolism and biotransformation processes, e.g., in nutrikinetic studies.

Optimization of the plasma extraction method

Three extraction methods commonly employed in nutrimetabolomics were optimized and compared for the simultaneous recovery of food-related metabolites listed in Table 1: (i) protein precipitation, (ii) hybrid protein precipitation and SPE-mediated phospholipid removal (Ostro®), and (iii) reversed-phase SPE (Oasis® HLB).

For protein precipitation (PPT), 1% formic acid in acetonitrile was first tested as an extractant, and provided good recoveries for simple phenolic acids but failed to extract most phase II metabolites and flavonoids. Various organic solvents were then compared with maximize the extraction efficiency, but in general, acetonitrile provided better recoveries and more efficient protein removal. Two-step extraction procedures, based on solvent-mediated PPT and subsequent extraction of the protein pellet, were also assayed by combining solvents with different polarities (e.g., methanol, acetone, ethyl acetate). The application of a second extraction step with methanol slightly increased the extraction recovery for some specific polyphenol classes (e.g., anthocyanins), but the resulting extracts were more prone to be contaminated with particles in suspension from the protein precipitate. As an alternative, different additives were tested with the aim of reducing interactions with proteins and improving the extraction process. The acidity of the precipitation solvent was found to be critical, especially for flavonoid aglycones and phase II metabolites. Additionally, the use of ammonium formate also improved the extraction of anionic compounds (e.g., sulfate derivatives), as previously described [18]. Therefore, the use of acetonitrile containing 1.5 M formic acid and 10 mM ammonium formate was demonstrated to provide the most efficient extraction of the 450 food-related metabolites here analyzed by means of PPT, with extraction recoveries in the range of 80–120% for the majority of metabolites monitored (Table S2). However, worse results were observed for some flavonoids, especially in their aglycone form, due to their chromatographic co-elution with phospholipid species (experimentally checked), which may interact with minor metabolites and cause ion suppression [22]. For this reason, a second extraction protocol based on hybrid PPT and SPE-mediated phospholipid removal was also tested. A slight modification of the method developed by Tulipani et al. [20], employing acetonitrile with 1.5 M formic acid and 10 mM ammonium formate for in-plate PPT, provided excellent recoveries for most of the metabolites monitored by UHPLC-MS/MS, but the extraction of flavan-3-ol metabolites was considerably worse than with simple PPT. According to Khymenets et al. [23], the application of a second extraction step with basic acetonitrile significantly improved the elution of this polyphenol class, but the extraction efficiency was still lower than that obtained by PPT. Finally, we also tested the potential of reversed-phase SPE for the extraction of plasma samples, as the gold-standard technique for the cleanup of complex biological samples and the extraction of polyphenols [24]. Taking as a reference the SPE methodology previously optimized by González-Domínguez et al. [18], but taking into consideration the improvements found in this study to minimize protein interactions by adding 1.5 M formic acid and 10 mM ammonium formate to extraction solvents, an efficient recovery of the majority of polyphenol classes was achieved.

Another crucial factor to be considered was the minimum volume of plasma needed to obtain reliable results. Similar extraction recoveries and precision were found by using volumes in the range of 20–200 µL, but sensitivity was significantly reduced while decreasing the initial sample volume due to dilution effects. Furthermore, the suitability of applying a pre-concentration step was also assessed to increase the method sensitivity. For this purpose, extracts obtained by using the three extraction protocols previously described were taken to dryness using a vacuum concentrator before UHPLC-MS/MS analysis. As a compromise between the volume of sample to be employed and the method sensitivity and robustness, the best results were obtained by extracting 100 µL of plasma/serum and using a reconstitution volume of 100 µL.

Validation of the method

The quantitative multi-targeted platform developed in this work was validated in terms of linearity, extraction efficiency, matrix effects, sensitivity, and both intra- and inter-day precision for each one of the three extraction methods optimized, as summarized in Table 2 (detailed information can be found in Supplemental Tables S2–S5).

As shown in Fig. 1, the three protocols provided excellent extraction efficiencies for most phenolic acids and related phase II metabolites, but significant differences were observed concerning flavonoid derivatives. In general, Ostro® plates were best suited to the extraction of flavonoid aglycones, while HLB provided the lowest recoveries for these dietary markers. On the other hand, excellent recovery yields were obtained for phase II derivatives of flavonoids regardless of the extraction method, with the exception of some diglucuronide and sulfoglucuronide species of isoflavones, for which the use of HLB provided the best results. A different behavior was particularly observed for flavan-3-ols and some microbiota-derived hydroxyphenylvalerolactones, which were only successfully extracted by PPT. This could be due to the occurrence of strong interactions between these metabolites and the SPE sorbents, as previously described [25]. Furthermore, it is also noteworthy that maximum recovery rates for anthocyanin species were around 80%, in line with previous works reporting the difficulty of extracting and analyzing these flavonoids because of their susceptibility to undergo degradation and structural rearrangements [26]. Another notorious difference among the three optimized protocols is the inability of the HLB method to recover polar metabolites not retained in the SPE sorbent (Table S2). Similarly, HLB also provided lower extraction recoveries for some medium-polarity metabolites, such as hydroxytyrosol derivatives and glucosinolates. Finally, it should also be noted that some metabolites (e.g., benzoic acid) were not quantifiable by using SPE-based procedures (i.e., Ostro® and HLB) due to the release of some interfering compounds (checked in blank extracts).

Table 2 Validation parameters for diet-related metabolites with authentic standards ($n = 256$) using the three extraction methods optimized: solid phase extraction (Oasis® HLB), hybrid PPT, and SPE-mediated removal of phospholipids (Ostro®), and protein precipitation (PPT).

Oasis® HLB	Ostro®	PPT
Recovery		
80–120% (53.9%)	80–120% (75.8%)	80–120% (81.6%)
60–80% (12.1%)	60–80% (12.5%)	60–80% (12.1%)
40–60% (6.6%)	40–60% (7.4%)	40–60% (5.1%)
<40% (25.4%)	<40% (3.5%)	<40% (1.2%)
>120% (2.0%)	>120% (0.8%)	
Matrix effect		
75–125% (62.1%)	75–125% (80.1%)	75–125% (80.5%)
40–75% (11.7%)	40–75% (13.3%)	40–75% (12.9%)
<40% (26.2%)	<40% (4.3%)	<40% (1.5%)
	>125% (2.3%)	>125% (5.1%)
Limit of Quantification		
<1 µg L^{-1} (11.0%)	<1 µg L^{-1} (10.3%)	<1 µg L^{-1} (10.2%)
1–10 µg L^{-1} (30.5%)	1–10 µg L^{-1} (32.7%)	1–10 µg L^{-1} (32.5%)
10–50 µg L^{-1} (41.0%)	10–50 µg L^{-1} (33.4%)	10–50 µg L^{-1} (33.3%)
50–100 µg L^{-1} (7.5%)	50–100 µg L^{-1} (11.2%)	50–100 µg L^{-1} (12.2%)
>100 µg L^{-1} (10.0%)	>100 µg L^{-1} (12.4%)	>100 µg L^{-1} (11.8%)
Intraday precision		
<15% (99.0%)	<15% (98.4%)	<15% (98.8%)
15–20% (1.0%)	15–20% (1.6%)	15–20% (1.2%)
Interday precision		
<15% (91.5%)	<15% (91.7%)	<15% (86.7%)
15–20% (8.5%)	15–20% (8.3%)	15–20% (13.3%)

Results are summarized in ranges for each validation parameter evaluated: recovery rates, matrix effects, limits of quantification, intra- and inter-day precisions (in brackets, the percentage of metabolites found in each range).

Calibration curves, prepared both in solvent and in plasma matrix, showed high linearity over 3–4 orders of magnitude, within the concentration range 0.1–2000 µg L^{-1}. The MS responses obtained for each metabolite standard dissolved in solvent and in plasma at the same concentration level were compared with assess the matrix effects (MEs). Matrix effects were negligible for almost all compounds quantified (ME: 75–125%), with the exception of those metabolites not successfully extracted by using each of the three extraction methods assayed. Among polyphenol species, only flavan-3-ols (ME: 60–70% for PPT, 40–60% for Ostro®) and anthocyanins (ME: 40–70%) showed lower ME percentages. Furthermore, some very polar metabolites

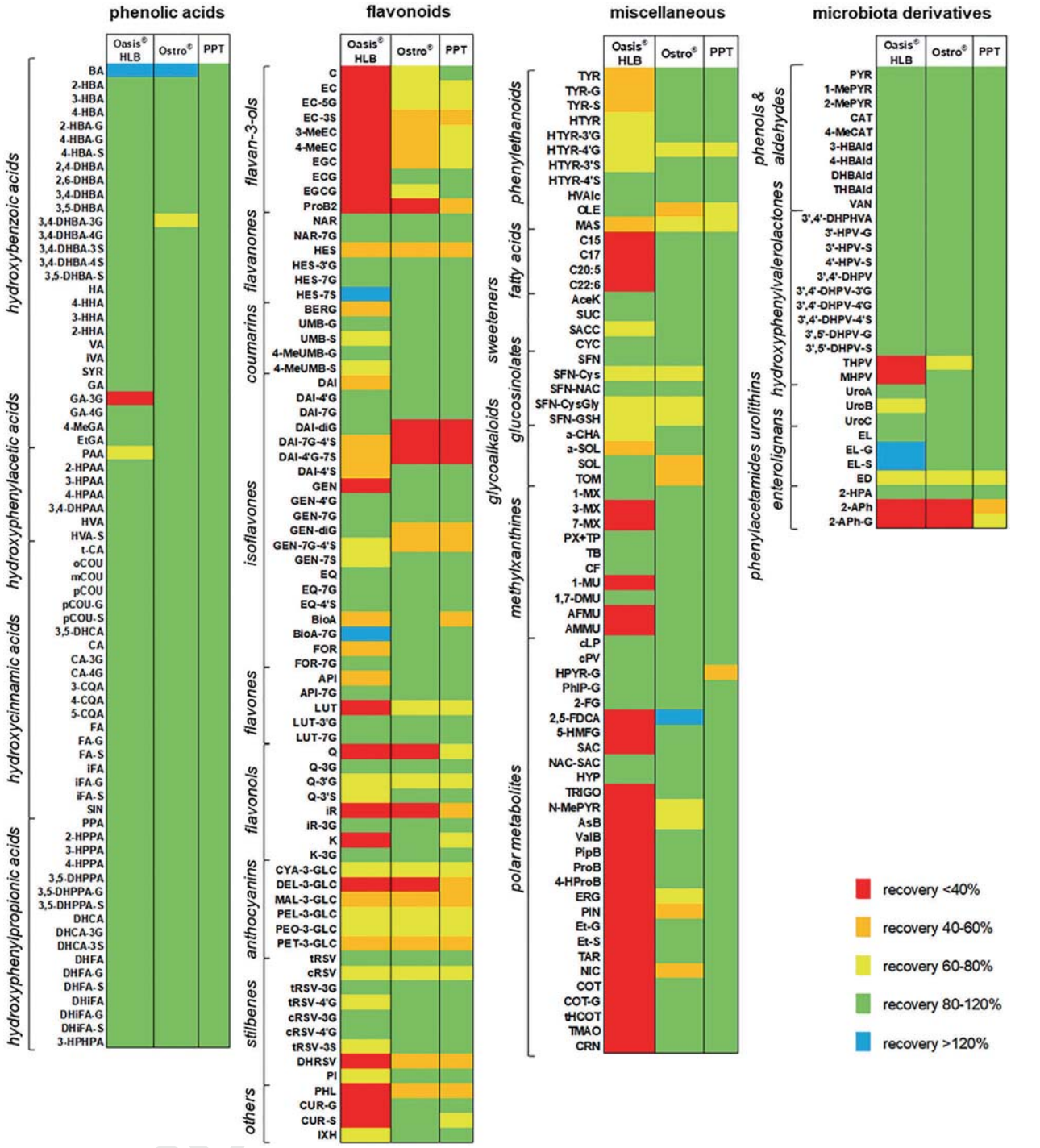

Fig. 1 Heat maps representing the recovery rates for dietary metabolites with authentic standards validated using the three extraction methods: solid phase extraction (Oasis® HLB), hybrid PPT and SPE-mediated removal of phospholipids (Ostro®) and protein precipitation (PPT). Information about abbreviations of metabolite names can be found in Table S1.

analyzed in the void volume of the chromatographic method were also slightly affected by ion suppression or ion enhancement effects (ME: 60–70% and 125–140%, respectively). Therefore, this shows that calibration curves prepared in solvent can be used for plasma quantification without the need for a matrix-matched calibration, thereby considerably simplifying the analytical workflow.

The method sensitivity was estimated by calculating the limits of quantification (LOQs) in spiked plasma samples for each metabolite. For polyphenolic metabolites, lower LOQs were generally obtained by applying HLB, followed

by PPT and finally Ostro®. These were below 50 μg L^{-1} (0.5–5 μmol L^{-1}) for most compounds (with the exception of some phenolic acids) and in the range 0.1–10 μg L^{-1} (0.01–1 μmol L^{-1}) for less polar species. Higher sensitivity was obtained for metabolites analyzed under positive polarity, with LOQs not surpassing 10 μg L^{-1} (0.1–1 μmol L^{-1}) for almost any of the compounds. Finally, instrumental precision was shown to be reproducible over a minimum period of three days, with intra- and inter-day precisions below 15% for most metabolites, except for those with higher LOQs, which were in the range 15–20%.

To sum up, it is noteworthy that the three extraction methods optimized here have their own strengths and weaknesses, with complementary analytical performance. Protein precipitation stands out as the most suitable extraction method for comprehensive metabolomics fingerprinting. On the other hand, SPE-based procedures could also be of great interest for analyzing specific polyphenol classes (e.g., Oasis® HLB for phase II metabolites of isoflavones, Ostro® for flavonoid aglycones). In general, PPT could be considered the gold-standard extraction method given its broad analytical coverage. Furthermore, the technical simplicity and cost-efficiency of this protocol facilitate its implementation in large-scale epidemiological studies. As a counterpart, the application of SPE-based procedures would be recommended in studies with a particular interest in those polyphenol classes previously described, or as a complement to PPT.

Validation of putative biomarkers

The PPT-based method previously optimized was applied to plasma samples from a clinical trial (NCT03101436) with the aim of testing its suitability for detecting dietary metabolites in samples obtained from free-living subjects, where these metabolites are usually found at low concentrations. Furthermore, we also analyzed samples collected after a one-month intervention with a Mediterranean diet supplemented with red wine as a case study to demonstrate the utility of plasmatic metabolites as markers of specific food intake.

Some microbiota-derived metabolites were regularly detected in more than 80% of the plasma samples analyzed from free-living subjects, including phenolic acids (around 15% of the total number of metabolites assayed), hydroxyphenyl-valerolactones (e.g., 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone) and enterolignans (e.g., enterolactone), which were predominantly found in the form of sulfate conjugates. Similarly, methylxanthines, fatty acids, and amino acid derivatives were also quantified in most of these samples. In contrast, the detection rate of the rest of metabolites assayed was much lower, which is indicative of their higher specificity as food-intake biomarkers. Thus, the consumption of particular foods was reflected in the detection of specific metabolites classes, e.g., flavanones were associated with citrus intake (phase II derivatives of naringenin and hesperitin), isoflavones with soy (phase II derivatives of daidzein and genistein), stilbenes with red wine (phase II derivatives of resveratrol and microbiota-derived dihydroresveratrol), and glucosinolates with cruciferous vegetables (sulforaphane N-acetylcysteine). In a second validation step, the methodology was applied to plasma samples from subjects adhered to a Mediterranean diet supplemented with red wine. Statistical analysis evidenced a significant increase in plasmatic levels of cis-resveratrol 4′-sulfate, dihydroresveratrol 3-sulfate, and ethyl sulfate, which are known biomarkers of red wine intake, after this long-term intervention period (Table 1) [27]. This, therefore, demonstrates the potential of the metabolomics platform developed here to quantify important aspects of the human diet, although future studies are needed to investigate other food groups.

Comparison with other metabolomics platforms

In general, the methodology optimized in the present work provided a similar analytical performance to that shown by other validated methods based on targeted nutrimetabolomic analysis of plasma/serum samples found in literature [28–30]. However, most of these previously published methods provide biased analytical coverage towards specific biomarker classes, which makes the application of several complementary analyses mandatory in order to obtain a comprehensive overview of the food metabolome. Conversely, the metabolomics approach developed here allows the simultaneous quantitation of 450 food-related metabolites and microbiota derivatives in a single and short run, thereby minimizing costs and the consumption of valuable biological samples. Furthermore, this multi-targeted metabolomics method represents an excellent complement to other platforms usually employed in the metabolomics research field (e.g., Metabolon, Biocrates), which are mainly focused on the endogenous metabolome.

Conclusions

Metabolomics nowadays plays a prominent role in nutrition epidemiology for deciphering the association between nutrition and health. However, various authors have emphasized in recent years that one of the major challenges currently faced by nutrimetabolomics researchers is the need for novel methods for large-scale quantitative metabolomics to allow for cross-cohort comparisons and the pooling of data [6]. The present work clearly demonstrates the crucial importance of the extraction method for

analyzing the circulating food and microbiota-derived metabolome in plasma/serum samples. We have optimized three complementary extraction procedures based on PPT, SPE, and hybrid PPT with SPE-mediated removal of phospholipids, each one having their own strengths and weaknesses. In general, PPT provides the most comprehensive metabolomic fingerprints, although SPE-based protocols could also be of interest in studies focused on specific polyphenol metabolites. The combination of these novel extraction methods with a multi-targeted UHPLC-MS/MS platform enables the simultaneous detection and quantitation of 450 dietary metabolites in very short-run times and using low volumes of biological sample, which facilitates its application to epidemiological studies. Furthermore, the use of simple and high-throughput extraction and analytical methods considerably minimizes the use of chemicals, and consequently costs. This methodology was tested in plasma samples collected from free-living subjects and after a one-month intervention with a Mediterranean diet supplemented with red wine, demonstrating its utility in the clinical practice.

Another research gap in nutrimetabolomics is the lack of robust validation studies of putative food intake biomarkers [31], which could be overcome by applying the method optimized here. Therefore, future studies are needed to test this methodology in acute/long-term controlled food intervention trials with the aim of checking the frequency of detection and kinetics of these food-related metabolites, especially considering inter-individual variability factors, and assessing their correlation with food intake. Evaluation of the strengths and weaknesses of using plasma or urine matrices for analyzing food intake biomarkers is also of critical importance.

Acknowledgements This work has received funding from the Spanish Ministry of Economy and Competitiveness (MINECO, PCIN-2015-229, PCIN-2015-238, PCIN-2017-076) under the umbrella of the European Joint Programming Initiative "A Healthy Diet for a Healthy Life" (JPI HDHL, http://www.healthydietforhealthylife.eu), the CIBERFES and CIBEROBN (co-funded by the FEDER Program from EU), and from the Generalitat de Catalunya's Agency AGAUR (2017SGR1546). RGD thanks "Juan de la Cierva" program MINECO (FJCI-2015-26590) and CAL the ICREA Academia award 2018. Authors thank to Paul Needs and Paul Kroon (Quadram Institute Bioscience) for kindly providing in-house synthesized standards.

Compliance with ethical standards

Conflict of interest xxx.

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