Analytica Chimica Acta 1033 (2018) 108–118

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

A validated multi-matrix platform for metabolomic fingerprinting of human urine, feces and plasma using ultra-high performance liquid-chromatography coupled to hybrid orbitrap high-resolution mass spectrometry

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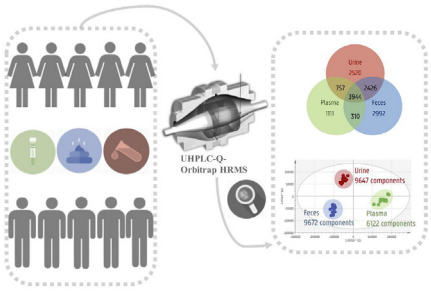
HIGHLIGHTS

- A non-targeted UHPLC-HRMS based platform for feces, plasma and urine is presented.
- Excellent validation parameters, including linearity, precision and recovery were achieved.
- Respectively 9 672, 9647 and 6122 components were retrieved for feces, urine and plasma.
- Feces appears to be equally informative as plasma, while the use of the multi-matrix platform improves the model's discriminative abilities.
- The multi-matrix platform provides unique opportunities for biomarker detection or pathway elucidation.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 26 March 2018
Received in revised form
20 June 2018
Accepted 23 June 2018
Available online 26 June 2018

Keywords:
Blood plasma
Feces
Urine
UHPLC-Q-exactive™ orbitrap HRMS

ABSTRACT

In recent years, metabolomics has surfaced as an innovative research strategy in human metabolism, whereby selection of the biological matrix and its inherent metabolome is of crucial importance. However, focusing on a single matrix may imply that relevant molecules of complementary physiological pathways, covered by other matrices, are missed. To address this problem, this study presents a unique multi-matrix platform for polar metabolic fingerprinting of feces, plasma and urine, applying ultra-high performance liquid-chromatography coupled to hybrid quadrupole-Orbitrap high-resolution mass spectrometry, that is able to achieve a significantly higher coverage of the system's metabolome and reveal more significant results and interesting correlations in comparison with single-matrix analyses. All three fingerprinting approaches were proven 'fit-for-purpose' through extensive validation in which a number of endogenous metabolites were measured in representative quality control samples. For targeted and untargeted validation of all three matrices, excellent linearity (coefficients of determination

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https://doi.org/10.1016/j.aca.2018.06.065
0003-2670/

Polar metabolomics
Metabolic fingerprinting

$R^2 \geq 0.99$ or 0.90 respectively), recovery and precision (coefficients of variance $\leq$ 15% or 30% respectively) were observed. The potential of the platform was demonstrated by subjecting fecal, urine and plasma samples (collected within one day) from ten healthy volunteers to metabolic fingerprinting, yielding respectively 9 672, 9 647, and 6122 components. Orthogonal partial least-squares discriminant analysis provided similar results for feces and plasma to discriminate according to gender (p-value, $R^2(X)$, $R^2(Y)$ and $Q^2(Y)$), suggesting feces as an excellent alternative biofluid to plasma. Moreover, combining the different matrices improved the model's predictivity, indicating the superiority of multi-matrix platforms for research purposes in biomarker detection or pathway elucidation and in the selection of the most optimal matrix for future clinical purposes.

1. Introduction

The metabolome comprises a diverse array of biomolecules and is regarded as the ultimate endpoint of the biological cascade in which transcription, translation, and protein functioning constitute the preceding phases. As such, the metabolome has been acknowledged as the best reflection of the biological phenotype and is therefore frequently targeted in life science research, thereby implementing a strategy of so-called metabolomics [1]. This holistic top-down analytical approach attempts to map the metabolome of the biological system under investigation and underpin the complex interactions between the host, its commensal microbial community, and other exposomal factors such as diet, stress, age, and lifestyle [2,3]. Moreover, as the metabolome captures metabolic-induced changes in a direct manner, metabolomics represents an ideal strategy to deepen the knowledge about metabolic anomalies and (patho)physiological pathways, which has for example been achieved for type 2 diabetes, Crohn's disease, cancer, atherosclerosis, cardiovascular disease, etc. [4–8]. An informed perspective will eventually provide clinicians and individuals alike with actionable information for managing health.

Unfortunately, due to the high chemical diversity among metabolites, it is currently not possible to measure the entire metabolome using a single analytical strategy [9]. In this context, one tends to segregate the non-polar (lipidome) and polar metabolome fraction when implementing metabolomics. Concurrent measurement of both fractions is of course most designated as complementary or interlinked metabolic pathways may be revealed upon completion of the metabolomics workflow. However, proposed mechanistic hypotheses in relation to the clinical condition may point towards the measurement of one particular metabolome fraction as the best starting point. Additionally, selection of a proper biological matrix in terms of practical (invasive or non-invasive) and/or clinical (close metabolic linkage to the clinical condition) fitness must be considered as well. Most commonly used specimens within routine clinical research are urine, blood plasma or serum, and to a lesser extent feces, each with its specific characteristics.

Blood transports chemicals to and from tissues, thus representing a valuable reservoir of endogenous and exogenous chemicals in the body. Moreover, as both polar and non-polar metabolites are present in this matrix, the range of biochemical processes that may be reflected by the blood metabolome is strongly extended. As such, implementing blood metabolomics is considered adequate to assess a plethora of metabolic diseases, as these have been listed in the review of Duarte, Diaz and Gil [10]. Moreover, blood metabolomics is highly suited for assessing and understanding acute illnesses such as sepsis [11]. Based on these elements, blood metabolomics has already rendered multiple marker molecules from various chemical classes and for diverse clinical conditions, contributing to a better understanding of the pathophysiological pathways involved [12–17]. When using blood plasma or serum, one of the major drawbacks, is the invasive nature of sample collection, which is especially problematic in (young) children, those in whom venous access is problematic, and those that are averse to blood sampling. In this context, urine represents an interesting alternative as it is easy to obtain in large volumes, sterile, relatively stable, largely free from interfering proteins or lipids, and chemically complex [3,18]. Urine typically contains metabolic breakdown products from a wide range of foods, drinks, drugs, environmental contaminants, endogenous waste metabolites and bacterial by-products. As these waste products are water-soluble, the diversity in chemical functionalities and biological pathways is somewhat limited compared to blood. In addition, urine is also more prone to the influence of diet and diurnal variation [8,19,20]. Nevertheless, also urinary metabolomics may reveal clinically valuable marker molecules, which is supported by the fact that dipstick tests are today routinely used to measure for example urinary glucose, bilirubin, ketone bodies, nitrates, leukocyte, esterase, hemoglobin, and urobilinogen. The review of Duarte, Diaz and Gil [10] provides a comprehensive overview of those disorders in which metabolic profiling or fingerprinting of urine yielded descriptive marker molecules. Eventually, the use of fecal samples in metabolomics is becoming increasingly popular, although not yet at the same level of blood or urine. A major motivation to consider fecal material relates to the fact that the inherent metabolome most efficiently captures the complex interactions between the gut microbiome, the host and the diet [4,21–24]. Indeed, there is an increasing awareness that the gut microbiota play a crucial role in the etiology of multiple gastrointestinal diseases by regulating various host metabolic pathways [22]. As such, dysbiosis of the gut microbial community has for example been correlated with obesity, rheumatoid arthritis, autism, food allergy, cardiovascular disease and type 2 diabetes. In this context, own work demonstrated significant differences in the fecal metabolome when comparing patients with type 2 diabetes or inflammatory bowel disease to healthy controls [22,25–27]. In contrast to blood, feces (and urine) are considered more suited to depict chronic illnesses, with the fecal metabolome providing valuable insights into the metabolic and biological activities that precede the sampling moment [28]. Typical targets that may serve as candidate fecal markers are short chain fatty acids, sterols and bile acids.

Although top-down systems biology by analysis of metabolic fingerprints has already rendered promising results in terms of biomarker revelation and pathway elucidation, the vast majority of these studies tend to focus on a single biological matrix. Obviously, in that way, crucial marker molecules of complementary physiological pathways – that are expressed more clearly or uniquely in an alternative biological matrix – can be missed [29]. Combining data from various matrices is presumed to reveal interesting correlations across matrices, which may aid in understanding metabolic mechanisms and qualify revealed biomarkers in a clinical

context. Therefore, we propose the strategy of multi-matrix metabolomics; analyzing multiple biological specimens (feces, blood plasma, and urine) from the same individual on a single analytical platform. So far, the use of a multi-matrix platform has only been reported by Zhao, Ni, Su, Li, Dong, Chen, Wei, Zhang, Guiraud, Martin, Rajani, Xie and Jia [30], thereby conducting metabolic profiling for feces, urine, and serum in a targeted microbiome metabolic study. However, to discover potential disease biomarkers, a truly untargeted fingerprinting strategy is more preferable to a profiling approach as the latter depends on a priori knowledge. As such, this study is the first to establish a multi-matrix strategy that is suited for untargeted fingerprinting of the polar metabolome fraction, and this for feces, urine, and blood plasma. Whereas extraction of each matrix type was separately optimized using a chemometrics strategy, full-scan analysis was performed by ultra-high performance liquid-chromatography (UHPLC) coupled to hybrid quadrupole-Orbitrap high-resolution mass spectrometry (HRMS) with a fixed hardware configuration. Indeed, an optimal and single setting with respect to the exchangeable hardware parts (i.e. LC-column, solvents, ionization source and position) allows to perform continuous and sequential analysis of samples from different matrix types, without any need to intervene. The strengths of multi-matrix fingerprinting compared to single-matrix analysis were demonstrated on a cohort of healthy volunteers, revealing gender-related metabolic differences.

2. Materials and methods

2.1. Reagents and chemicals

Analytical standards (Table S1) and internal standards (ISTD) (D-valine-d8, L-alanine-d3) were purchased from Sigma-Aldrich (St-Louis, Missouri, USA), ICN Biomedicals Inc. (Ohio, USA), TLC Pharmchem (Vaughan, Ontario, Canada) or Cambridge Isotope Laboraties Inc. (Tewksbury, Massachusetts, USA). Solvents were obtained from Fisher Scientific (Loughborough, UK) and VWR International (Merck, Darmstadt, Germany). Ultrapure water was obtained by usage of a purified-water system (Millipore, Brussels, Belgium).

2.2. Instrumentation

UHPLC-Quadrupole-Orbitrap HRMS analysis was based on the validated method of Vanden Bussche, Marzorati, Laukens and Vanhaecke [22]. In brief, chromatographic separation was achieved on a Dionex UltiMate 3000 XRS UHPLC system (Thermo Fisher Scientific, San José, CA, USA), equipped with an Acquity HSS T3 C18 column (1.8 μm, 150 × 2.1 mm) (Waters, Manchester, UK), kept at a constant temperature of 45 °C. A binary solvent system consisting of ultrapure water (A) and acetonitrile (B), both acidified with 0.1% formic acid, was used at a constant flow rate of 0.4 mL min^{-1}. A gradient profile with following proportions (v/v) of solvent A was applied: 0–1.5 min at 98%, 1.5–7.0 min from 98% to 75%, 7.0–8.0 min from 75% to 40%, 8.0–12.0 min from 40% to 5%, 12.0–14.0 min at 5%, 14.0–14.1 min from 5 to 98%, followed by 4.0 min of re-equilibration. A 10-μL aliquot was injected for each matrix sample. Detection was performed on a Q-Exactive™ stand-alone bench top quadrupole-Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, San José, CA, USA), which was preceded by heated electrospray ionization (HESI-II source) in polarity switching mode. The position of the ionization source remained unchanged for the three matrices, i.e. 0/B/1. Ionization source and instrumental parameters were a sheath, auxiliary and sweep gas flow rate of, respectively, 50, 25 and 3 arbitrary units (a.u.), a heater and capillary temperature of 350 °C and 250 °C, an S-lens RF level of 50 V, a spray voltage of ± 4.0 kV, an *m/z* scan range from 53.4 to 800 Da, and an automatic gain control target of $1e^6$ ions. The maximum injection time and mass resolution settings were set at 70 ms and 140 000 full width at half maximum (FWHM).

Prior to mass spectrometric detection, initial instrument calibration was achieved by infusing ready-to-use calibration mixtures (Thermo Fisher Scientific, San José, CA, USA). Before and after analysis of samples, a standard mixture of 291 target analytes (Table S1), with a concentration of 5 ng μL^{-1} was injected to check the operational conditions of the device. To adjust for instrumental fluctuations, quality control (QC) samples (a pool of samples made from the biological test samples to be studied) were included. They were implemented at the beginning of the analytical run to stabilize the system and at the end of the sequence run for signal corrections within analytical batches, and this for each matrix, starting with the fecal samples, followed by urine and plasma.

Targeted data processing was carried out with Xcalibur 3.0 software (Thermo Fisher Scientific, San José, CA, USA), whereby compounds were identified based on their *m/z*-value, C-isotope profile, and retention time relative to that of the internal standard. Statistical and pathway analyses were performed by means of SAS Enterprise Guide 7 (SAS Institute Inc., Cary, USA) and MetaboAnalyst 3.0 (Xia Lab, McGill University, Quebec, Canada), respectively. For untargeted data interpretation, the software package Sieve™ 2.2 (Thermo Fisher Scientific, San José, CA, USA) was used to achieve automated peak extraction, peak alignment, deconvolution, and noise removal. As major parameters, a minimum peak intensity of 500 000 a.u., retention time width of 0.3 min, and mass window of 6 ppm were employed for feature extraction, with retention time, *m/z*-value and signal intensity as main feature descriptors. Outputs of the targeted and untargeted data preprocessing were subjected to multivariate statistical, which was realized using Simca™ 14.1 software (Umetrics AB, Umea, Sweden). Principal component analysis (PCA) was performed for data exploration, allowing to reveal natural patterning of samples and potential outliers. This was followed by OPLS-DA to establish predictive models, which were validated by evaluating some quality parameters ($R^2(X)$ and $Q^2(Y)$, permutation testing ($n = 100$), and cross-validated ANOVA (CV-ANOVA) ($p\text{-value} < 0.05$).

2.3. Sample collection

Feces. The fecal extraction protocol was optimized and validated on a pool of freeze-dried fecal samples ($n = 6$). Fecal samples underwent 48 h of lyophilization to warrant the elimination of microbial activity and facilitate homogenization.

Blood plasma. Plasma was collected in heparin coated tubes. Method optimization and validation was performed on a pool of human blood plasma (H2B, ESTER Techopole – 6 allée Skylab – 87068 LIMOGES Cedex – France, Reference 20000P, Batch number: ED1505003).

Urine. Fasted morning urine was chosen to optimize and validate the extraction protocol. More specifically, a pool of morning urine samples ($n = 3$) was used.

Multi-matrix. To demonstrate the strengths of multi-matrix fingerprinting, fresh fecal, plasma and urine samples were obtained from healthy male ($n = 5$) and female ($n = 5$) volunteers (25–41 years old, normal body mass index) (Table S2), which were not subjected to any dietary restrictions or antibiotic treatment during at least 6 months prior to sample donation. Volunteers were recruited among the laboratory personnel through informal announcement. Blood samples were collected in heparin tubes and immediately centrifuged (3000 x g) after which the supernatans

was collected and stored. Subsequent to the donation of morning urine and fasted plasma samples, fecal samples were collected with a Fecotainer® (AT Medical B.V., Enschede, The Netherlands). A delay in the fecal metabolome on urine and plasma may be expected, which can be attributed to the gastrointestinal transition time. However, combining the different matrices will enable the analysis of the metabolic (patho)-physiologic state of the individual at the moment of and the period prior to sample collection. Indeed, plasma collection will provide real-time information, while urine and fecal samples can provide retrospective information on the individual's health. After sample collection, plasma and urine samples were immediately aliquoted and stored at −80 °C, awaiting analysis. Fecal samples were lyophilized first, which resulted in the average removal of 71.8% ± 5.8% water, and subsequently stored at −80 °C. All samples were de-identified and analyzed anonymously.

This study was approved by the ethical committee of Ghent University Hospital (EC 2016/0673).

2.4. Development and chemometric optimization of sample extraction

Development of an individual generic extraction procedure for each of the three biological matrices relied on a sequential strategy of experimental designs. Hereby, statistical evaluation by Modde 5.0 (Umetrics, Umea, Sweden) was performed to assess metabolite coverage based on the absolute number of detected features and extraction efficiency, deduced from the absolute signal intensity of a predefined group of endogenous metabolites, which were identified based on their accurate mass, C-isotope profile and retention time relative to that of the internal standard. Selection of these metabolites was executed per matrix type, whereby it was aimed to have compounds with high endogenous abundance and at least one representative for each of the considered compound classes, i.e. alcohols, polyols, phenols, monocarboxylic acids, hydroxylic acids, multicarboxylic acids, imidazoles, ethers, ketones, amino acids, amines, nitroso compounds, alkenes and steroids (Table S1). To account for additional metabolite (sub)classes, not included in our analytical standard database, an untargeted approach was implemented as well during method optimization and validation, thereby providing adequate metabolome coverage. To effectuate the chemometric optimization of the sample extraction protocols, different variables with a possible effect on extraction yield were evaluated by means of fractional factorial designs (FFD, D-optimal quadratic design), followed by response surface modelling (RSM) to further optimize the statistically significant quantitative variables.

2.5. Finalized extraction protocols

Feces. To extract the polar fecal metabolome [22], 200 mg of lyophilized homogenized feces was dissolved in 4 mL of ultrapure water, after the addition of 100 μL ISTD mixture (25 ng μL^{-1} of D-valine-d_8 and L-alanine-d_3). Subsequent to 30 s of thorough vortexing, 1 mL of an ice-cold methanol and ultrapure water (80:20, v/v) mixture was added. The supernatant of the solid-liquid extraction was collected after 1 min of vortexing and 10 min of rotation, followed by a 10-min centrifugation step (13 300 x g, at 4 °C). Next, the extract was passed over a polyamide filter (diameter of 25 mm and pore size of 0.45 μm) (Machery-Nagel, Düren, Germany), diluted (1:3) with ultrapure water and transferred to a glass HPLC-vial.

Blood plasma. Generic extraction of the polar blood plasma metabolites was initiated by pipetting 150 μL plasma into a 1.5-mL Eppendorf tube, after which 250 μL methanol and 8 μL ISTD solution (25 ng μL^{-1} D-valine-d8 and L-alanine-d3) was added. Subsequently, the sample was vortexed for 2 min, followed by protein precipitation for 30 min at 4 °C, and collection of the supernatant after centrifugation (15 min, 4 °C, and 15 000 × g). Next, the supernatant was evaporated to a droplet of about 20 μL, using a Gyrovap centrifugal evaporator (Howe, Banbury, UK) (35 °C, vacuum conditions). The residue was diluted in 180 μL ultrapure water, vortexed for 30 s, and eventually transferred to a glass HPLC-vial with insert.

Urine. Extraction of the urinary metabolome was initiated by pipetting 300 μL urine into a 1.5-mL Eppendorf tube, after which 30 μL of ISTD mixture (100 ng μL^{-1} D-valine-d8 and of L-alanine-d3) was added. Following this, the sample was centrifuged for 8 min (1 000 x g, 4 °C). Next, 100 μL supernatant was collected and diluted (1:10) with ultrapure water, after which the resulting extract was transferred to a glass HPLC-vial.

2.6. Validation

As specific guidelines for the validation of untargeted metabolomic methods are lacking at the time, the validation procedure was based on previously published research and was pursued in a targeted as well as an untargeted fashion [22,27,31,32]. Performance parameters included linearity, precision, and recovery, which were assessed based on the absolute area of the compounds detected through metabolic fingerprinting and/or the selected endogenous metabolites. In doing so, it was important to incorporate multiple metabolite classes, relevant for the specific matrix and the associated metabolic processes. As such, the analytical performance for the polar metabolome was evaluated based on 23 identified metabolites for feces (Table S3), 32 for blood plasma (Table S4) and 45 for urine (Table S5).

Linearity. QC extracts were diluted serially (1, 2, 5, 10, 20, 50, 100, 200 and 500 times) with ultrapure water and assessment of linearity was based on the obtained coefficients of determination (R^2). For the untargeted assessment of linearity, only components recovered in all samples from the dilution series were included [32].

Precision. Precision comprised instrumental, intra- and inter-day assay precision, which were all evaluated based on the calculated coefficients of variance (CV). Instrumental precision was determined by repeatedly injecting (n = 10) a QC sample. For the intra-assay precision, multiple QC samples (n = 10) were extracted in parallel under identical experimental conditions, whereas inter-day assay precision (n = 20) included within-laboratory variation such as different analysts, days, etc [33].

Recovery. Assessment of recovery was performed in a targeted manner only, in accordance with the protocol of Vanden Bussche, Marzorati, Laukens and Vanhaecke [22] and following the Food and Drugs Administrations (FDA) guidelines [34]. More specifically, a comparison was made between the concentrations that were retrieved for samples that were spiked either after or before extraction, thereby considering three different concentration levels in triplicate. The protocol for spiking after extraction concerned the addition of 20, 30, and 50 μL of an analytical standard mixture (5 ng μL^{-1}) to 50 μL of a QC sample extract and subsequently standardized with ultrapure water to reach a volume of 100 μL. The protocol for spiking before extraction was attuned to obtain theoretically identical standard concentrations in the final extract as when spiking after extraction.

3. Results and discussion

3.1. Metabolomic fingerprinting method

Development of the analytical detection method was initially

performed on an Exactive™ HRMS system (Thermo Fisher Scientific, San José, CA, USA) [22], with the incorporation of 110 known gastro-intestinal relevant metabolites [6,35–38], ranging widely in physico-chemical characteristics. This analytical standard mixture was also used to adapt the method towards a Q-Exactive™ instrumental setting, applying a resolution of 140 000 FWHM at 1 Hz. Later on, metabolites considered to be relevant for plasma and urine were added to this standard mixture [39]. This resulted in a final list of 291 compounds (Table S1), which were likely to be present in at least one of the investigated matrices. HRMS parameters were optimized based on peak intensity and signal-to-noise ratio.

3.2. Chemometric sample extraction optimization

The significance of different extraction parameters on the detection capability was evaluated through a sequential strategy of experimental designs and based on both the absolute peak areas of metabolites with high endogenous abundance, specific for each matrix (Table S1), as well as the number of components discovered after Sieve™ processing.

Feces. Fecal samples underwent 48 h of lyophilization to warrant the elimination of microbial activity and facilitate homogenization. Moreover, research of Aggio, Mayor, Coyle, Reade, Khalid, Ratcliffe and Probert [40] showed that the recovery of volatile compounds could be significantly improved by lyophilization, due to a change in volume of the liquid and the gas phase. During the FFD, various factors displayed significant effects (p-value < 0.05) on the extraction efficiency, including the mass of the fecal material, the extraction solvent (ultrapure water) and extraction volume. In the subsequent RSM design, further optimization was performed to determine the best combination of fecal mass (200 mg) and extraction volume (1 mL). The incorporation of a dilution factor (1:3) was able to circumvent matrix effects that may hinder detection of e.g. multicarboxylic acids in the FFD [22].

Blood plasma. Preliminary work was conducted to determine the best blood-based matrix (plasma or serum, heparin versus EDTA coated tubes) to perform untargeted metabolomics, with blood plasma providing superior results in terms of metabolome coverage. This was confirmed by Barri and Dragsted [41] and Ishikawa, Maekawa, Saito, Senoo, Urata, Murayama, Tajima, Kumagai and Saito [42], who stated that plasma is the best matrix for optimal metabolite coverage. Heparin is considered to be the best anticoagulants for both polar metabolomics as well as lipidomic analysis [41,43], which was also concluded in this study. An FFD was performed to reveal significant effects (p-value < 0.05) of the amount of starting material, the extraction solvent (methanol), extraction volume, and purification technique (30 min protein precipitation, followed by centrifugation, both at 4 °C). Next, several vortexing steps were evaluated and the effect of evaporation was assessed by comparing evaporation to complete dryness to evaporation to a remaining 20 μL extract aliquot, with the latter providing the best results [44–47]. Optimization of significant quantitative factors was performed by means of an RSM design (Tables S6 and S7). A high volume of extraction solvent (250 μL methanol) and starting material (150 μL plasma), combined with a 2-min vortexing step appeared to render the highest extraction yield. A dilution of 1:10 was implemented to avoid HRMS detector saturation.

Urine. Urine samples are prone to diurnal variation, caused by differences in hydration level, fasting and exercise [48]. Morning urine is considered to be more concentrated and to provide the most holistic view on the metabolome [49,50]. Therefore, fasted morning urine was chosen to optimize and validate the extraction protocol. The D-optimal design showed a significant effect (p-value < 0.05) of extraction solvent (ultrapure water), dilution (4:1) of the urine sample and a negative association between centrifugation time and speed, suggesting a negative influence of both a very long centrifugation time (15 min) as well as a high centrifugation speed (4 000 x g) (Tables S8 and S9). Additionally, an RSM model was created to determine the optimal combination between centrifugation time (8 min) and speed (1 000 x g) (Table S8). The optimal dilution for the extracted sample was evaluated based on a serial dilution of QC samples, with a large number of targeted metabolites displaying a linear range between 1/500 and 1/10 dilution (29 out of 45), and saturation of the HRMS instrument above 1/10. This phenomenon could also be observed in the untargeted data, rendering 1/10 the optimal dilution for the urinary samples.

3.3. Validation of the analytical methods

If the method is able to fulfill the performance criteria set out for targeted approaches, such as linearity, precision and recovery for the selected known metabolites (endogenously present in the QC sample), it can be presumed that the untargeted analytical method is 'fit-for-its-purpose' [4]. The endogenously present metabolites were identified based on their accurate mass, C-isotope profile and retention time relative to that of the internal standard. Next to the targeted validation, an additional untargeted validation was considered to ensure holistic metabolome coverage [27]. The FDA recommends a nominal coefficient of variance (CV) of 15% as acceptable for a single bioanalytical test and a CV of 20% when operating close to the limit of detection [34]. For the validation of a metabolic fingerprinting method, a CV below 30% is considered acceptable, based on the fact that ions with a higher CV are not eligible as candidate biomarkers [31]. The percentage of components displaying a CV-value below 30% for both the instrumental, inter-day and intra-assay precision, as well as the linearity is presented in Table 1. A total of 15 532, 3 006 and 8 090 were discovered for feces, plasma and urine, respectively.

Fecal samples. After serial dilution of the QC samples, an excellent linearity ($R^2 \geq 0.99$) was obtained for 20 of the 23 selected endogenous compounds. Good recoveries were obtained for all compounds, varying between 84.1 ± 28.3% and 108.0 ± 16.0% with an average CV of 9.4%. The instrumental precision had CVs from 3.4 to 14.8%. Evaluation of the repeatability and inter-day precision (n = 20) led to CVs ranging from 2.6 to 12.4% and 6.1–13.1%, respectively (Table S3). Results of the metabolic fingerprinting method are presented in Table 1, with 42.9% of the detected components having an R^2 above 0.90. Respectively 90.0, 89.4 and 84.7%

Table 1
The analytical performance criteria (precision and linearity), expressed in percentages, of the fingerprinting method for urine, plasma and feces, in which a total of 15 532, 3006 and 8090 components were discovered, respectively.

	Instrumental precision (%) (CV<30%)	Intra-assay precision (%) (CV<30%)	Inter-day precision (%) (CV<30%)	Linearity (%) (R^2 > 0.90)
Feces	90.0	89.4	84.7	42.9
Plasma	95.3	93.9	87.8	41.5
Urine	98.9	98.9	98.0	85.7

of the components showed a CV below 30% for instrumental precision, repeatability (intra-assay precision) and reproducibility (inter-day precision).

Plasma samples. Excellent linearities were achieved for 29 of the 32 selected metabolites ($R^2 \geq 0.99$). Good recoveries were obtained, ranging between 70.6 ± 23.0% and 125.6 ± 13.3%, with an average CV of 9.7%. Instrumental precision was situated between 3.2 and 17.1%, with an average precision of 8.6%. With the exception of six compounds, CVs for inter-day precision varied between 4.4 and 14.5% and those for the intra-assay precision between 3.6 and 19.3% (Table S4). The results of the validation on the fingerprinting data are presented in Table 1, with respectively 98.9%, 98.9% and 98.0% of the components showing a CV below 30% for instrumental precision, repeatability and reproducibility. 85.2% of the detected components displayed an R^2 above 0.90.

Urine samples. For 39 out of 45 selected target metabolites, a good linear trend was observed ($R^2 \geq 0.99$). Excellent recoveries were obtained for all metabolites, ranging between 90.8 ± 6.8% and 105.9 ± 7.2%, with an average CV of 7.1%. An excellent instrumental precision was observed (<15%) for all but three metabolites. The CVs for the intra-assay and inter-day repeatability varied from 2.4 to 20.1% and 3.5–17.6%, respectively. Five metabolites had a CV > 20%, these phenomena appeared to be metabolite class related (ethers, ketones) (Table S5). A large CV for intra-assay precision in specific classes could be explained by a difference in extraction efficiency [51]. This indicates that an extraction method cannot be optimal for all endogenously present metabolites originating from different physico-chemical classes. Results of the untargeted validation are presented in Table 1, with respectively 95.3%, 93.9% and 87.8% of the components showing a CV < 30% for instrumental precision, repeatability and reproducibility. 41.5% of the detected components displayed an $R^2 > 0.90$.

In comparison to other protocols that have been developed and validated for the concerned biological matrices [52–55], similar performance for the various method characteristics was achieved with our multi-matrix method. However, it should be noted that the cited protocols were specifically validated for only a limited number of targeted metabolites. As with our methodology excellent performance characteristics were obtained for a broad range of metabolite classes, assessed in both a targeted and untargeted fashion, a promising platform for multi-matrix fingerprinting was concluded.

3.4. Applicability of multi-matrix fingerprinting in biomarker studies

The validated multi-matrix metabolomics strategy was employed to register the metabolic state of 10 healthy volunteers, which allowed to define the assets of analyzing multiple biological matrices in a connective manner. Acquired full-scan data were assessed by untargeted fingerprinting and/or targeted profiling. With respect to the latter, the in-house constructed database of 291 polar metabolites was consulted, thereby having a positive hit in case that the *m/z*-value (allowed mass deviation ≤ 3 ppm), relative retention time (allowed deviation ≤ 2.5%), and isotope pattern ($^{13}C/^{12}C$ ratio compliant with CD 2002/657/EC requirements) matched that of the analytical standard.

Metabolome coverage by matrices. Within a clinical context, metabolomics has the primary intention to increase our knowledge on the metabolic mechanisms that are involved in health and disease, often making the link with exposomal or genetic influential factors. In this regard, a high coverage of the system's metabolome by the method(s) applied is of critical importance as this evidently aids in understanding the complex interactive biological pathways and discovery of fit-for-purpose biomarkers. This study demonstrated that the metabolomic fingerprint of feces is the most comprehensive across all biological matrices in terms of number of recovered components (likely to be metabolite features). Considering the matrix-specific QC samples, the metabolomic fingerprint of feces comprised 9 672 components (of which 68.4% were obtained in positive ionization mode), whereas urine recorded 9 647 components (60.9% in positive ionization mode) and plasma 6 122 components (67.3% in positive ionization mode) (Fig. 1).

Coverage complementarity of matrices. The complementarity of the different matrices was assessed through a Venn diagram (Fig. 2), covering 14 060 unique components. Hereby, 2 992, 2 520 and 1 111 components were noted to be uniquely present in the fecal, urinary, or plasma fingerprint, respectively. A total of 3 944 metabolite features was detected in all three matrices, and can therefore be considered the most informative and eligible as potential biomarkers, as they may reflect matrix-overlapping or complementary (patho)physiological pathways. Moreover, these metabolite features are of particular interest for metabolic and physiological flux analysis at a system's level. The major part of the plasma metabolome was also present in fecal (69.5%) and urinary

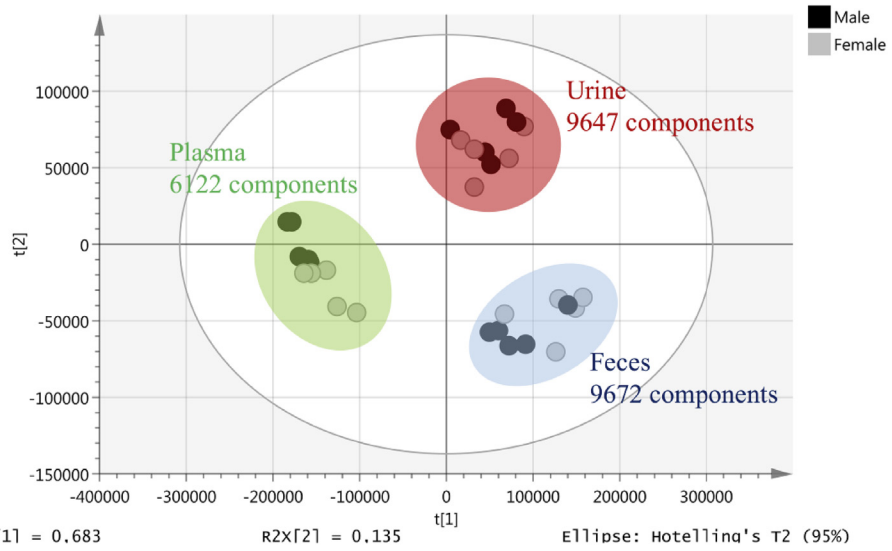

Fig. 1. PCA-X score plot based on the fecal, urinary, and plasma fingerprints of 10 healthy volunteers with clear clustering according to matrix.

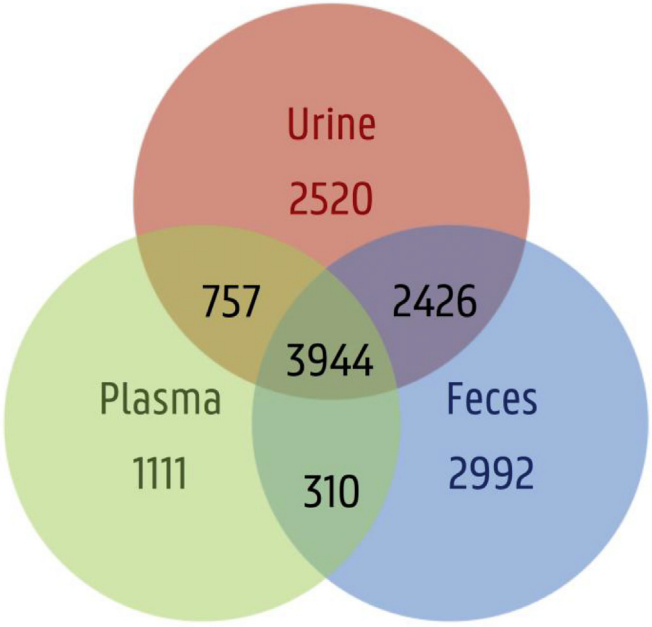

Fig. 2. Venn diagram to indicate the complementarity in terms of metabolome coverage across the different biological matrices. In total, 14 060 unique metabolites were discovered in the human metabolome.

samples (76.8%). As such, these matrices are considered equally informative as compared to plasma.

Single versus multi-matrix fingerprinting to differentiate according to metabolic state. A typical step within the metabolomics workflow concerns the multivariate data analysis, which intends to reveal significant differences between the metabolomic fingerprints from the study cohorts under investigation. As mentioned before, up to date, this was predominantly attempted on the basis of a single biological matrix. In this work, as no specific disease conditions or age differences were present among the participants, it was opted to determine the metabolic discrepancies between males and females. The extent to which this was possible, was compared for the single versus multi-matrix fingerprinting strategy. Data for multivariate statistical analysis were pareto-scaled (using the square root of the standard deviation as scaling factor) and log-transformed to standardize the range of signal intensities and induce normality, respectively. For each matrix type, a PCA-X model was constructed using the combined data from the positive and negative ionization mode. The associated score plots suggested good instrumental precision as good clustering of subsequent QC samples was observed (Figure S1 – S3). In addition, clustering according to gender was noted for the fecal and plasma samples, which indicated that the dominant fraction of variance in the associated metabolomes could be assigned to gender or gender-related factors (Figure S1 – S3). Based on this exploratory modelling step, urine samples turn out to be less promising in unfolding metabolic discrepancies that are function of gender. The subsequent OPLS-DA modelling strategy confirmed these findings, suggesting that plasma and feces are equally suited to assess gender-dependent metabolic differences, being reflected by an excellent $Q^2(Y)$ (> 0.8), acceptable CV-ANOVA p-value (< 0.035), and good permutation testing (Table 2). To our knowledge, this is the first study to demonstrate gender discriminating abilities of fecal samples, which appear to be equally informative as plasma in this particular context [50,56,57]. Even though only a minority of the microbiome is characterized by gender-related differences [58], feces displayed excellent discriminative abilities. Therefore, it is considered an even more informative biofluid compared to plasma for microbiome associated conditions. Until now, there is still no accepted approach for sample size determination for metabolic phenotyping, as the statistical power not only relates to sample size, but also to effect size and significance level, which cannot be anticipated [59]. Indeed, despite the limited number of participants included, excellent validation parameters ($R^2(X)$, $R^2(Y)$, $Q^2(Y)$ and CV-ANOVA) for feces and plasma were achieved, while for urine, the high p-value pointed towards insignificant OPLS-DA models, which could be improved by increasing the number of participants [50,56,57,60]. Only for $R^2(X)$ different values were observed, which could be merely contributed to the higher number of detected components in fecal samples over plasma (respectively 9 672 and 6 122).

Subsequently, the discriminating performance was also assessed by combining the fingerprint data from the various matrices, exploiting the value of multi-matrix fingerprinting. The established OPLS-DA models were able to discriminate according to gender, being endorsed by the performance of the various validation parameters (Table 3) and good permutation testing. This with the exception of the model that was based on the urinary and fecal fingerprints, whereby the obtained CV-ANOVA p-value suggested borderline-significance (Fig. S4). These data indicate that the combination of invasive and non-invasive sampling offers an excellent alternative for the single-matrix studies. This is endorsed by the fact that it can provide a multitude of information regarding the current and passed metabolic (patho)physiologic state of the individual.

Based on all of the above-cited OPLS-DA models, components with differentiating potential were defined. To this end, the VIP-score (≥ 2), Jack-knifed confidence interval (not across zero) and the S-plot descriptors (|covariance| and |correlation|) were taken into consideration as selection parameters. For the Pearson's correlation coefficient, a value of 0.6021 was adopted as cutoff, with a significance level of 0.05 [61,62]. For the |covariance|, it was opted to implement an adjusted threshold for each model, i.e. only those metabolites with an absolute covariance belonging to the highest quarter of the S-plot were retained, due to the varying number of X-variables (metabolites) and its associated impact on the covariance profile. As such, in a single-matrix context, 24 discriminative metabolites could be retrieved for feces and 19 for plasma. When considering the validated two-matrix models, 49 metabolites were retained for urine/plasma and 41 for feces/plasma. Eventually, the model that was based on the metabolic fingerprints of all three matrices resulted in the discovery of 69 discriminating metabolites,

Table 2
Validation parameters of the OPLS-DA model for the single matrices in discriminating between genders.

	$R^2(X)$	$R^2(Y)$	$Q^2(Y)$	p-value
Feces	0.45	0.99	0.83	0.035
Plasma	0.60	0.97	0.84	0.030
Urine	0.52	0.92	0.71	0.120

Table 3
Validation parameters for the OPLS-DA models that were constructed based on the fingerprints of multiple biological matrices and aiming for discrimination according to gender.

	$R^2(X)$	$R^2(Y)$	$Q^2(Y)$	p-value
3 matrices	0.41	0.98	0.83	0.036
Urine - Feces	0.38	0.99	0.80	0.056
Urine - Plasma	0.43	0.98	0.84	0.031
Feces - Plasma	0.47	0.99	0.83	0.035

with 20 originating from urine, 28 from feces, and 21 from plasma (Fig. S5). These metabolites are considered most descriptive as they were retrieved across the various biofluids. The applicability of feces as a study matrix was highlighted by the high number of discriminating metabolites in both the single-matrix as well as the multi-matrix methodology. The developed multi-matrix platform proved to be very promising as it combines information from different matrices, thereby increasing the number of recovered metabolites, having the potential to reveal more significant results compared to single-matrix studies, including the discovery of biomarkers and unraveling mechanistic information. Moreover, performing a multi-matrix pilot study can aid tremendously in determining the optimal experimental setup, including optimal biofluid selection and sample size determination.

Multi-matrix targeted profiling to differentiate according to metabolic state. A targeted profiling strategy was utilized to evaluate the discrimination of study groups, using our in house database comprising 291 known metabolites, more particularly focusing on the 73 metabolites that were detected in all three matrices. The intensity profiles of these metabolites across the various matrices is visualized in a heat map (Fig. 3), thereby applying signal correction based on the sample's average intensity of the internal standard D-valine-d_8. Hierarchical cluster analysis (one minus Pearson correlation, MetaboAnalyst 3.0) enabled to

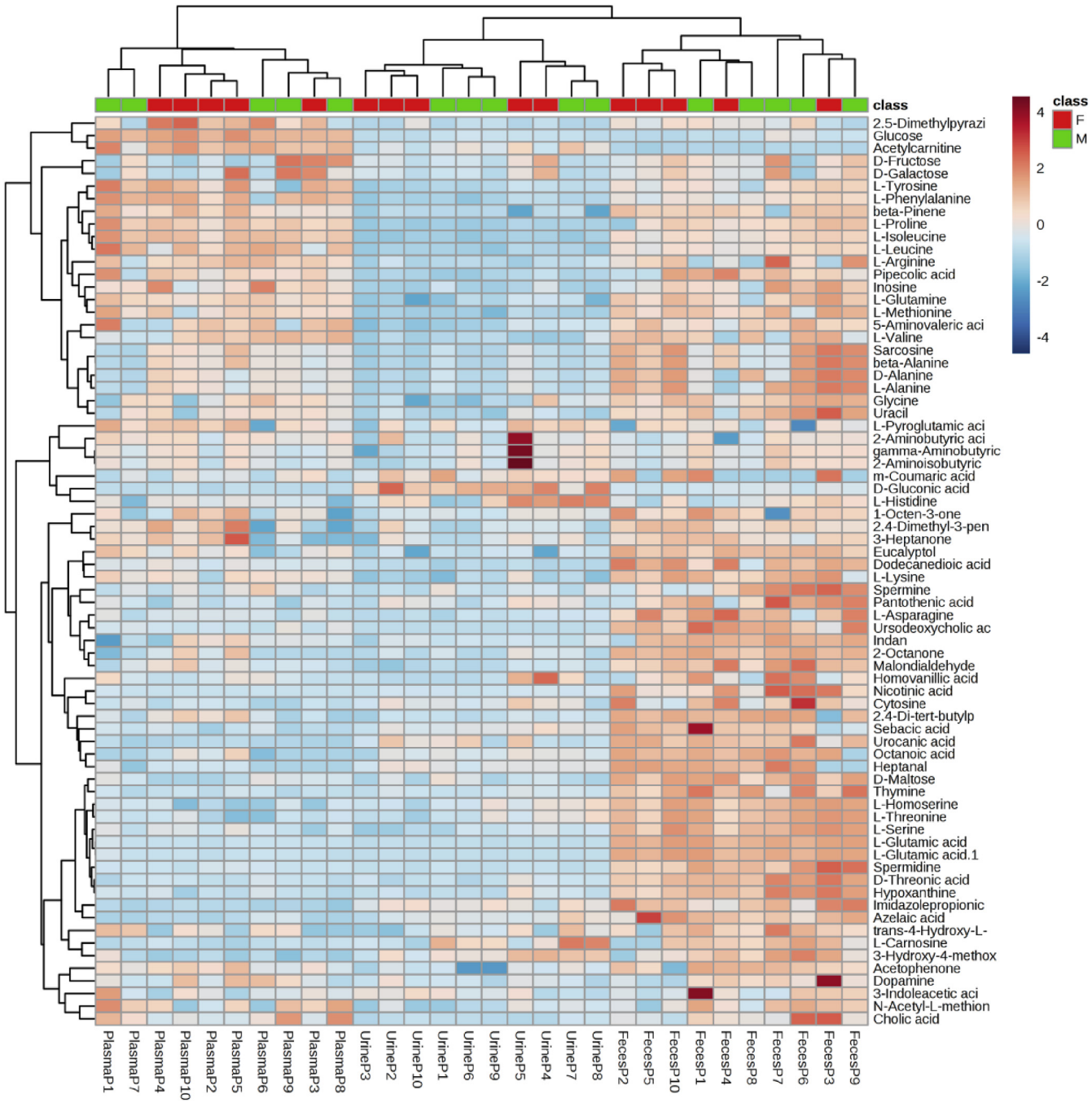

Fig. 3. Heat map of 73 identified metabolites, that were detected in all three matrices and evaluated in light of gender-specific metabolic shifts. This heat map was constructed based on signal intensities, corrected by the internal standard.

discriminate between matrices, with fecal samples displaying in general the highest metabolite signal intensities. However, a perfect discrimination between the study groups could not be obtained, due to the limited power of the targeted metabolites (n = 291) in comparison to untargeted screening. This points to the superiority of holistic untargeted polar metabolomic fingerprinting for classifying according to metabolic state.

Next, the ability of each known metabolite to discriminate between genders was statistically evaluated by means of a Wilcoxon two-sample test. Although only 19 metabolites contributed significantly (p-value < 0.05) to the male *vs.* female participant discrimination, a majority of the targeted metabolites displayed a difference of at least 30% between genders (Tables S9–S11), as such providing useful information on trends related to gender differentiation. Multiple papers have reported differences in concentration levels of individual metabolites or metabolite classes in urine and plasma [56,60,63–68]. For feces, however, no gender differentiating studies have been reported yet. As can be seen from Table S10, amino acids and amines were more concentrated in feces from male participants, which can be explained by a generally higher protein uptake by men [63] and an altered metabolisation of the ingested proteins due to differences in gastrointestinal microbiome [58] or cell metabolism between men and women. The latter is endorsed by the strong dependency of specific disease characteristics and their treatment efficacy according to gender [64]. For plasma, a variety of studies reported higher concentrations of amino acids in males, including L-valine, L-leucine, L-isoleucine, L-glutamine, L-proline, L-tyrosine, whereas for glycine and L-serine higher concentrations have been observed in females [56,60,63–65]. These observations were confirmed in this study (Table S11). Indeed, free fatty acids (FFAs) (monocarboxylic acids) and glucose were more abundant in plasma samples from females, which is in line with observations of Soeters, Sauerwein, Groener, Aerts, Ackermans, Glatz, Fliers and Serlie [66] (Table S11), demonstrating higher plasma FFA concentrations upon short-term fasting, leading to higher glucose levels in females. Finally, in parallel with observations of Saito, Maekawa, Pappan, Urata, Ishikawa, Kumagai and Saito [67], bile acids were more concentrated in male samples. With respect to urinary metabolomics, only a few studies have assessed gender differences. However, higher concentrations for ketoglutaric acid, D-fructose and urinary nucleosides have been reported in women [60,68], which was confirmed in this study as well (Table S12). The advantage of using a multi-matrix platform relates to the possibility of identifying complementary physiological pathways. To demonstrate this, pathway analysis (MetaboAnalyst 3.0) was performed for the targeted metabolites that were detected in all three matrices. This revealed noticeable differences (i.e. minimum 30% difference) in the beta-alanine pathway between male and female participants (Fig. 4), in line with Budczies, Brockmoller, Muller, Barupal, Richter-Ehrenstein, Kleine-Tebbe, Griffin, Oresic, Dietel, Denkert and Fiehn [69], who reported estrogen mediated alterations in the beta-alanine pathway. In male fecal samples, higher concentrations of metabolites associated with the beta-alanine pathway (i.e. gamma-aminobutyric acid, spermine, and spermidine) were observed. The elevated levels of these metabolites can be ascribed to the higher nutrient intake by men [63,70], or altered gastrointestinal metabolisation due to differences in microbiome or cell metabolism [58,64], with a higher production or less efficient absorption of spermine and spermidine (Table S10). Based on our multi-matrix approach, we can deduce that the differences in absorption rate between females and males is the main reason as the latter metabolites were retrieved in higher concentration levels in female plasma except for gamma-aminoburyic acid, for which no difference could be observed [71]. This may relate to the fact that spermine and spermidine are involved in the menstrual cycle and implicated in numerous cellular functions, including the synthesis of nuleic acids (Table S11) [72], which was also reflected in the higher observed concentrations of pantothenic acid, uracil and other nucleic acids (Table S12) in the urine of women. Urinary L-carnosine, on the other hand, was observed in higher concentrations in the male participants. As L-carnosine is a dipeptide specific for mammalian skeletal muscle, it has been suggested to be higher in men as a result of their higher muscle metabolism [60,73,74].

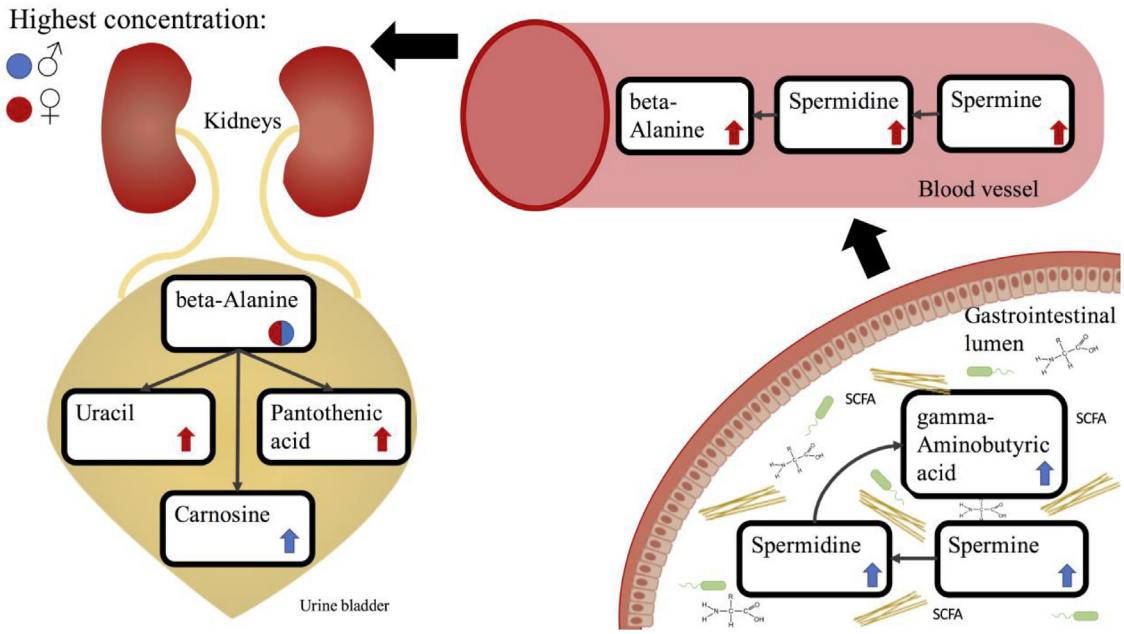

Fig. 4. The beta-alanine pathway show noticeable changes between male and female participants. Increase in male samples are indicated by blue arrows and increases in female samples are indicated in red (SCFA = Short Chain Fatty Acids). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusions

This study presents a unique multi-matrix metabolomics fingerprinting strategy for feces, blood plasma and urine, thereby using a single analytical platform, i.e. reversed phase UHPLC-quadrupole-Orbitrap HRMS. It may be noted that the typical exchangeable hardware parts (i.e. ionization source, analytical column, and solvents) were identical for all matrices, which allows a continuous analysis of samples along the different matrix types. This may greatly contribute towards an efficient gathering of full-scan data that is matrix-transcending and easy to integrate. As such, a multi-matrix approach perfectly fits in the concept of systems biology and may enclose significant value to elucidate intricate metabolic pathways. In this regard, acceptable performance criteria were obtained for the three metabolic fingerprinting methods, taking into account the fact that compound specific optimization is not feasible. These findings indicate that the analytical methods were fit-for-purpose and suitable to achieve high metabolome coverage, along diverse chemical classes. Indeed, evaluation of the metabolic fingerprints of feces, blood plasma, and urine revealed hundreds of metabolites with representatives for almost all of the considered classes. Using an intensity threshold of 500 000, the fecal metabolome had the highest coverage (9 672 metabolite features), followed by urine (9 647 metabolite features) and plasma (6 122 metabolite features). As such, due to its discriminative abilities, non-invasive nature of sample collection, and excellent coverage, feces proved itself as an excellent alternative to plasma, which is typically most frequently targeted in clinical omics studies. The differences in coverage in relation to the matrix type highlight the merits of performing a multi-matrix platform for disease related biomarker detection or potential pathway elucidation. Especially when performing a pilot study, implementing a multi-matrix platform will aid tremendously in determining the optimal experimental setup, including sample size and optimal biofluid.

Notes

The authors declare no competing financial interests.

Acknowledgements

Ellen De Paepe is supported by Research Foundation - Flanders (FWO, FWO18-SBB-042). The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. The authors wish to thank the 10 healthy volunteers in their participation to this study. Also many thanks to S. Bos, J. Goedgebuer and B. Pomian for their contributions to this work.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.06.065.

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