An exploratory approach for an oriented development of an untargeted hydrophilic interaction liquid chromatography-mass spectrometry platform for polar metabolites in biological matrices

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Highlights

- Six HILIC and one RPLC columns are tested for polar metabolites.
- A decision tree-based univariate approach is used to optimize HILIC-MS methods.
- 99% of analytical panel standards can successfully be separated and detected.
- Human urine, plasma and liver cells show thousands of features with mRSD < 25%.

- Hundreds of metabolites were annotated in biological samples with L1 or L2 confirmation.

Abstract

The analysis of polar metabolites based on liquid chromatography-mass spectrometry (LC-MS) methods should take into consideration the complexity of interactions in LC columns to be able to cover a broad range of metabolites of key biological pathways. Therefore, in this study, different chromatographic columns were tested for polar metabolites including reversed-phase and hydrophilic interaction liquid chromatography (HILIC) columns. Based on a column screening, two new generations of zwitterionic HILIC columns were selected for further evaluation. A tree-based method optimization was applied to investigate the chromatographic factors affecting the retention mechanisms of polar metabolites with zwitterionic stationary phases. The results were evaluated based on a scoring system which was applied for more than 80 polar metabolites with a high coverage of key human metabolic pathways. The final optimized methods showed high complementarity to analyze a wide range of metabolic classes including amino acids, small peptides, sugars, amino sugars, phosphorylated sugars, organic acids, nucleobases, nucleosides, nucleotides and acylcarnitines. Optimized methods were applied to analyze different biological matrices, including human urine, plasma and liver cell extracts using an untargeted approach. The number of high-quality features (< 30% median relative standard deviation) ranged from 3,755 for urine to 5,402 for the intracellular metabolome of liver cells, showing the potential of the methods for untargeted purposes.

Keywords

Metabolomics; Polar metabolites; Hydrophilic interaction chromatography; Method optimization; Liquid chromatography-high resolution mass spectrometry

1. Introduction

Metabolomics, a systems biology discipline representing the analysis of endogenous metabolites, has grown tremendously over the past 25 years [1]. Most experiments are performed using high-end techniques such as nuclear magnetic resonance spectroscopy (NMR) or gas/liquid chromatography (resp. GC, LC) coupled to mass spectrometry (MS) [1,2]. Metabolomics approaches comprise of targeted and untargeted methods that can be applied in many research fields, such as food quality, discovery of prognostic or diagnostic biomarkers and elucidation of pathophysiological mechanisms [2–5].

In untargeted metabolomics, thousands of features derived from hundreds of compounds can be detected, identified and quantified. A feature in MS-based approaches is commonly characterized by its mass-to-charge ratio (*m/z*) and chromatographic retention time (RT). Detected features need to be handled carefully to prevent false positive results (e.g. background signal wrongly interpreted as metabolite) or false negative results (e.g. considering the convolution of isomeric species). Despite the advanced mathematical approaches applied by recent software versions for annotation, as reference standards are not available for all features, the use of supplementary orthogonal techniques is imperative [6–8]. Among these techniques, liquid chromatography (LC) hyphenated to MS has been one of the most applied techniques for metabolic profiling [3,9,10].

Due to the heterogeneity of molecules in the metabolome, varying from small polar organic acids to apolar triglycerides, a single LC-MS method is not capable to capture this broad variety of metabolites [11]. Traditionally, reversed-phase liquid chromatography (RPLC) is used for the separation of apolar to slightly polar metabolites, resulting in a wide lipid coverage, useful for lipidomics research [12]. Hydrophilic interaction liquid chromatography (HILIC), a technique compatible with electrospray ionization (ESI)-MS, has become the separation technique of choice for polar compounds such as organic acids and sugars [13,14]. In HILIC, a polar stationary phase is used in combination with an aqueous-organic mobile phase, which creates a water-rich layer around the stationary phase, in which various hydrophilic interaction mechanisms occur [15,16]. HILIC methods have the potential to retain and separate polar metabolites that show no retention or co-elute in RPLC and can lead to an increased MS sensitivity for polar compounds. However, HILIC has some disadvantages (e.g. limited choice of mobile phase compositions, long equilibration times) and it cannot be used for an untargeted metabolomics experiment without proper method development and validation of its actual metabolome coverage [17].

In the present work, different HILIC-columns and chromatographic settings were tested for an untargeted approach that can be applied for the polar metabolite fraction of different biological matrices. By employing HILIC-quadrupole-time-of-flight-MS (HILIC-QToF-MS) in negative and positive electrospray ionization modes (ESI (-) and ESI (+) respectively) to analyze standard mixtures of polar metabolites and various biofluids (plasma, urine), the capabilities and limitations of HILIC chromatography were explored. In addition to plasma and urine, the HepaRG cell line, a hepatic cell line derived from a human hepatocellular carcinoma, was used to test the suitability of the developed metabolomics platform on intra- and extracellular cell extracts.

2. Materials and methods

2.1. Chemicals and materials

Analytical standards were purchased from Sigma Aldrich (St. Louis, Missouri, USA), Merck (Darmstadt, Germany) and Janssen Chimica (Beerse, Belgium). A total number of 72 panel standards was used during the experiments covering a wide range of metabolic classes including amino acids (19), amino acid metabolites (5), phosphorylated amino acid metabolites (2), peptides (5), sugars (4), amino sugars (1), phosphorylated sugars (1), organic acids (6), phosphorylated organic acids (2), tricarboxylic acid cycle (TCA) intermediates (6), nucleobases or analogues (4), nucleosides (3), nucleotides (3), cofactors or -enzymes (6) and small chain acylcarnitines (5). Using the final optimized methods, 13 additional analytical panel standards (3 amino acids, 3 amino acid metabolites, 2 sugars, 1 organic acid, 1 nucleobase or analogue, 2 cofactors or -enzymes and 1 amine oxide) were analyzed, bringing the total number to 85 panel standards. In Fig. 1, 65 out of 85 panel standards were used for pathway mapping to visualize the metabolic coverage. All chemical standards used during method optimization are listed in the supplementary information (S1). L-glutamic acid-2,3,3,4,4-D_5, L-leucine-5,5,5-D_3, L-lysine-$^{13}C_6$-$^{15}N_2$, succinic acid-2,2,3,3-D_4, D-tryptophan-2,4,5,6,7,-D_5 and glucose-$^{13}C_6$ were used as internal standards during the sample preparation of biological samples. All internal standards were purchased from Sigma Aldrich, except for D-tryptophan-2,4,5,6,7-D_5, which was bought from CDN isotopes (Pointe-Claire, Quebec, Canada).

Methanol ultrapure (MeOH), acetonitrile (ACN) and formic acid (99%) (HCOOH), all ULC/MS-CC/CSF grade, were purchased from Biosolve (Valkenswaard, The Netherlands). Ammonium formate (≥ 99%, LC-MS grade) (NH_4COOH), ammonium carbonate HPLC grade ($(NH_4)_2CO_3$) and ammonium acetate LC-MS grade (NH_4COOCH_3) were obtained from Sigma Aldrich. Acetic acid (100%) ($HCOOCH_3$), ammonia solution (25%) ($NH_{3(aq)}$) and ethanol

(EtOH), all LC-MS grade, isopropanol for analysis (ACS reagent) (IPA) and chloroform (analytical grade) ($CHCl_3$) were purchased from Merck. Ultrapure water (H_2O) used throughout the experiments was obtained from an Elga Pure Lab apparatus (Tienen, Belgium).

For the experiments with liver cells, differentiated HepaRG cells, Basal Hepatic Medium, HepaRG Thaw, Seed and General-Purpose Supplement and HepaRG Maintenance and Metabolism Supplement were acquired from Biopredic International (Rennes, France). HepaRG cells were seeded in Permanox 2-well Lab-Tek chamber slides from Nunc, Thermo Scientific (Rochester NY, USA) and incubated using a Galaxy 170 S incubator acquired from Eppendorf (Hamburg, Germany). Rat tail collagen for coating was provided by Corning (New York, USA). Eppendorf Safe-Lock tubes and 0.2 µm nylon centrifugal filters were acquired from respectively Eppendorf and VWR (Pennsylvania, USA) and used during sample preparation. Pure, dry nitrogen (AZOTE N28, N_2) used for solvent evaporation was obtained from Air Liquide Belge (Liège, Belgium). 384 well plates (PS, small volume) were bought from Greiner Bio-One (Vilvoorde, Belgium). Human blood was collected in sterile Vacuette K_3EDTA premium tubes acquired from Greiner Bio-One and aseptic polypropylene urine recipients from Disera (Izmir, Turkey) were used for urine collection.

2.2. Preparation of analytical standards

Analytical standard solutions were prepared by dissolving solids or diluting liquid standards in H_2O, MeOH, EtOH or a combination of H_2O with MeOH or EtOH (50/50, v/v), depending on the solubilization properties of the compounds, to obtain a stock solution of 50 µg/mL. All panel standards were combined in a mixture, the solvent was evaporated to dryness using a stream of N_2 at room temperature, and reconstituted in H_2O/ACN (35/65, v/v) to obtain a final concentration of 1 µg/mL. The final mixtures were stored at -20 °C before injection.

2.3. Sample preparation

The sample preparation method has an important influence on the metabolite coverage in biological samples [11]. Therefore, the extraction methods were carefully chosen. The sample preparation methods for the liver cell extracts were adapted from a previously in-house validated method [18]. The sample preparation methods for plasma and urine samples were chosen based on literature screening of validated methods which had a good coverage for polar metabolites [19–23].

2.3.1. Intracellular extracts of HepaRG cells

Ethical approval for the use of HepaRG cells was provided by the Medical Ethics Committee of the University Hospital Brussels (reference number 143201941214). Differentiated HepaRG cells were incubated for 8 days at 37 °C, 5% CO_2 and saturated humidity.

In order to extract the intracellular metabolome of the cells, the sample preparation method of Cuykx *et al.* [18] was slightly adapted. Briefly, cells were flash-frozen using liquid nitrogen and scraped with a solution of 80% MeOH and 20% 10 mM NH_4COOCH_3 (v/v) at -80 °C. The MeOH solution containing the cells was recovered to perform liquid-liquid extraction (LLE) (3/2/2, v/v/v, MeOH/H_2O/$CHCl_3$). A mixture of internal standards was added (final concentration 1 µg/mL). The lower fraction (MeOH/H_2O) was divided in two for the analysis in ESI (+) and ESI (-) mode. After drying under N_2 stream, extracts were reconstituted in ACN/H_2O (65/35, v/v). A detailed protocol of the sample preparation was added to the supplementary information (S2).

2.3.2. Extracellular extracts of HepaRG cells

In order to analyze the extracellular metabolome of HepaRG cells, the used incubation medium was collected at the same day as the extraction of the HepaRG cells. The sample preparation was based on the method of Cuykx *et al.* [18] and Dettmer *et al.* [24]. Briefly, medium was quenched using a solution of 80% MeOH and 20% 10 mM NH_4COOCH_3 (v/v) at -80 °C. Quenched medium was used to perform LLE (3/2/2, v/v/v, MeOH/H_2O/$CHCl_3$). The subsequent sample preparation steps were similar to the steps performed for the intracellular extracts which are explained in detail in the supplementary information (S2).

2.3.3. Human plasma

Blood samples were collected from 6 healthy volunteers, 3 males and 3 females, aged 24-31 years. The sampling was approved by the Ethical Committee of the University Hospital Antwerp (EC/PC/avl/2018.039). Extraction was performed using K_3EDTA tubes, which were centrifuged within 15 min after collection. The sample preparation protocol was adapted from Benito *et al*. [19], Chen *et al.* [20] and Bruce *et al.* [21] and is explained in detail in the supplementary information (S3). Briefly, plasma samples were mixed with -80 °C MeOH/EtOH (1/1, v/v) and centrifuged. The supernatant was evaporated to dryness under a stream of N_2 and reconstituted using ACN/H_2O (65/35, v/v) spiked with a 1 µg/mL internal standard mixture.

2.3.4. Human urine

Urine samples were collected from 6 healthy volunteers, 3 males and 3 females aged 24-48 years. The donation was approved by the Ethical Committee of the University Hospital Antwerp (18/31/357). Sample preparation was based on the method of Wu *et al.* [22] and the recommendations of Fernández-Peralbo *et al.* [23] and is explained in detail in the supplementary information (S4). Briefly, urine samples were diluted with ACN and centrifuged. An internal standard mixture was added to the supernatant (final concentration 1 µg/mL).

2.4. Mass spectrometry parameters

Mass spectrometry detection was performed on an Agilent 6530 QToF-MS with Agilent Jet Stream Electrospray Ionization (Agilent Technologies, Santa Clara, USA). In ESI (+) mode, nitrogen was used as drying and sheath gas at 250 °C and 350 °C with flow rates of 8 L/min and 11 L/min, respectively. The nebulizer gas pressure was set at 45 psig, the MS capillary voltage at 2000 V, the nozzle voltage at 0 V and the fragmentor at 150 V. In ESI (-) mode, the drying and sheath gas had a temperature of 250 °C and a flow of 10 L/min and a temperature of 350 °C and a flow of 10 L/min respectively. The nebulizer gas pressure was set at 45 psig, the MS capillary voltage at 2000 V, the nozzle voltage at 0 V and the fragmentor at 100 V. For both ionization modes, data were acquired in 2 GHz extended dynamic mode with a scan range of 60-1000 *m/z* and a scan rate of 2 spectra/s. Full scan data were stored in profile mode. Calibration of the mass axis was performed within run using purine (*m/z* 121.0508 in ESI (+) mode and *m/z* 119.0363 in ESI (-) mode) and hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine (*m/z* 922.0097 in ESI (+) mode and *m/z* 980.0163 in ESI (-) mode). The calibrant solution was constantly infused during the run with an additional isocratic pump (Agilent 1200 series G1310A) and mixed with the effluent using a T-piece connected to the ESI source. Data-dependent MS/MS (auto-MS/MS) acquisition was obtained at collision energies of 10, 20 and 40 eV using a separate method with a scan rate of 2 spectra/s and 6.67 spectra/s for MS and MS/MS spectra, respectively. The maximum precursors/scan cycle was set at 12.

3. Analytical method optimization

3.1. Liquid chromatography column screening

Liquid chromatography analyses were performed on an Agilent 1290 Infinity UPLC system. The injection volume was set at 3 µL and the thermostat of the autosampler at 4 °C. Firstly, several chromatographic columns were screened using generic HILIC methods based on the recommendations of the supplier and previous publications [10,17]. Details concerning these

generic methods are specified in the supplementary information (S5). In addition to HILIC-QToF-MS, one RPLC-QToF-MS method was tested in parallel to evaluate the coverage of a reversed-phase method with an Acquity UPLC HSS T3 column previously used for metabolomics applications [25]. Columns included Luna HILIC (100 x 3.0 mm, 3 µm, cross-linked diol, silica-based, Phenomenex, USA), iHILIC-Fusion (100 x 2.1 mm, 1.8 µm, zwitterionic, charge modulated amide, silica-based, HILICON AB, Sweden), iHILIC-Fusion(+) (100 x 2.1 mm, 1.8 µm, zwitterionic, permanent positive charge modulated amide, silica-based, HILICON AB, Sweden), iHILIC-Fusion(P) (100 x 2.1 mm, 5 µm, zwitterionic, charge modulated amide, polymer-based, HILICON AB, Sweden), HILICpak VT-50 2D (150 x 2.0 mm, 5 µm, quaternary ammonium, polymer-based, Shodex, Japan), HILICpak VG-50 2D (150 x 2.0 mm, 5 µm, amino, polymer-based, Shodex, Japan) and Acquity UPLC HSS T3 (100 x 2.1 mm, 1.8 µm, C18, silica-based, Waters, USA). A graphical representation of the stationary phases of the above-mentioned columns is shown in Fig. 2.

3.2. Liquid chromatography method optimization

HILIC interactions are highly dependent on the used stationary phase, mobile phase composition and several other factors [16,26,27]. A decision tree optimization was chosen based on the influence of these factors in the following order: stationary phase > mobile phase pH and modifier > modifier concentration > additional parameters (temperature, gradient, flow) [28,29]. After the selection of the most suitable stationary phases, the solvent and pH effects were tested with ACN, MeOH or a combination of both as organic eluent and buffered H_2O as aqueous eluent. LC-MS analysis was performed in both ESI (+) and ESI (-) modes. Generally, high pH values were tested to ionize compounds in ESI (-) mode and low pH values in ESI (+) mode. In the starting conditions in ESI (-) mode, $(NH_4)_2CO_3$ was added to the aqueous mobile phase until the maximum tolerable pH value for each column. Following the recommendations of the manufacturer, a maximum pH ≈ 8 was tested for the Waters Acquity UPLC HSS T3 column and pH ≈ 9 for HILIC columns in ESI (-) mode. In ESI (+) mode, aqueous mobile phases were buffered with 10 mM of NH_4COOH and 0.1% HCOOH (pH ≈ 3.5). In addition, the effect of the pH was tested by injecting the mixture of panel standards at a basic pH with the above-mentioned restrictions, an acidic pH and neutral pH in both polarities, adjusting the pH with $NH_{3(aq.)}$, $(NH_4)_2CO_3$ or HCOOH when necessary. Salt modifiers affect the eluent strength, causing a greater impact on columns with dominant ionic interactions [17]. The modifier effect was tested using different modifiers, including NH_4COOCH_3, NH_4COOH and

258 $(NH_4)_2CO_3$ in ESI (-) mode and NH_4COOCH_3, NH_4COOH in ESI (+) mode at different
259 concentrations ranging from 1 mM to 30 mM.
260 Gradient conditions in HILIC were optimized based on starting conditions of 95% organic
261 phase, slowly decreasing to 20% at 10 min and keeping this condition for 4 min before returning
262 to the initial conditions for a 5 min equilibration. In reversed-phase mode, starting conditions
263 of 80% aqueous phase were slowly decreased to 15% at 8 min and kept at this condition for 5
264 min before returning to the initial conditions for a 5 min equilibration. Subsequently, the initial
265 flow rate of 0.2 mL/min was increased to 0.25 mL/min and 0.3 mL/min. Higher flows were
266 avoided to not exceed the maximum tolerable backpressure for the used columns. During
267 previous runs, the column temperature was kept at 30 °C. The effect of the temperature was
268 tested in the range of 30 °C to 60 °C. For ESI (-) mode, the bypassing of certain metal parts,
269 such as the heat exchanger, inline filter and MS diverter valve was tested to determine the effect
270 on the detection of anionic compounds [30,31]. Due to the purpose of this work, the
271 optimization was performed by changing chromatographic parameters one by one. The results
272 of each method guided the next tier in the method optimization. A flow chart with the factors
273 explored for method development is shown in Fig. 3. Detailed information regarding tested LC
274 conditions for each column and ionization mode can be found in the supplementary information
275 (S7).
276
277 **3.3. Data analysis of analytical standards**
278 The structure-based predictions for chemical properties and abundances of different ion forms
279 at specific pH values were calculated using the online tool Chemicalize (ChemAxon, Hungary).
280 The *m/z* values of common ESI adducts were calculated for each compound using the Mass
281 Spectrometry Adduct Calculator of Fiehn Lab (UC Davis) [32]. To obtain reliable, high-quality
282 results and avoid peak misidentification, individual chromatograms originating from standard
283 mixtures were manually extracted using a 5 ppm mass tolerance in MassHunter Qualitative
284 Software 10.0 (Agilent Technologies). Each adduct ion of the standards was inspected in three
285 instrumental replicates. To eliminate false positive results, ion chromatograms were compared
286 to solvent blanks and the isotopic distribution of each adduct was investigated by manually
287 comparing the experimentally obtained distribution in MassHunter Qualitative Software 10.0
288 with the theoretical distribution, calculated with the Isotope Distribution Calculator B7024.0
289 (Agilent Technologies).
290 For ESI (-) mode, ions were extracted for deprotonated ions and chloride adducts. Depending
291 on the mobile phase composition, carbonate, formate and/or acetate adducts were additionally

considered. For ESI (+) mode, ions were extracted for protonated, sodium and potassium adducts. Likewise, depending on mobile phase modifiers, ammonium adducts were additionally considered. Neutral losses were investigated depending on the compound class, for instance, water and carbon dioxide losses for compounds with a carboxylic acid function or phosphate loss for compounds with a phosphate group [33].

During column screening and method optimization experiments, the best signal was selected out of the different detected adducts by applying a scoring system to evaluate the peak shape, retention time and peak intensity for each analytical panel standard, as explained in Table 1. The scores were calculated according to Equation 1 per analytical panel standard and per method. Furthermore, eluting compounds were confirmed by comparison of their MS/MS fragmentation spectra with MassHunter METLIN Metabolite PCDL (Agilent Technologies).

$$Quality\ Score = \sum_{i=1}^{n} Peak\ shape\ score + \sum_{i=1}^{n} Peak\ intensity\ score + \sum_{i=1}^{n} Retention\ time\ score$$

Equation 1. Quality score equation based on the quality score sum of Table 1.

Similar strategies were previously applied to evaluate different LC-MS conditions by assigning individual scores to metabolites and combining the values [34]. This highlights the applicability of the score system to the analytical method development and evaluation.

3.4. Data analysis of biological samples

During data acquisition of each dataset of biological samples, the injection order was randomized, and the QC sample was injected six times at regular intervals. Data acquired in profile mode was centroided using the vendor's algorithm and converted to mzML format with MSConvert [35]. The converted files were exported to R [36]. Untargeted data of biological samples was pre-processed with XCMS 3.11 and feature quality was inspected with the MetaboMeeSeeks package in R [37,38]. Internal standards were inspected for intensity, area, mass accuracy and peak width in order to guide the choice of XCMS parameters. Peak picking was performed using *CentWav*e with a peak width ranging from 5 to 60 s for HILIC-MS ESI (-) mode and 5 to 30 s for HILIC-MS ESI (+) mode, maximum tolerated *m/z* of 20 ppm, minimum difference in *m/z* of 0.01 for peaks with the same retention time, S/N threshold equal to 5 and noise set to 500 based on the noise signal of the Agilent 6530 QToF. Alignment was performed with the *Orbiwarp* algorithm using a pooled QC as center sample. Features were grouped with the *PeakDensity* algorithm, followed by integration of missing peaks with chromatogram

filling. Subsequently, blank filtration (fold change of 3) was performed with the BlankCheckR function of the MetaboMeeSeeks package. Features showing MS/MS spectra were annotated by comparison of accurate mass, retention time and MS/MS fragmentation with in-house libraries, using MS-DIAL (v.4.24) with the All Public MS/MS library (v.15) for ESI (+) and (-) modes [7], HMDB [39] and METLIN [40]. Annotated features assigned with a level 1 or 2 confirmation according to the recommendations of Schymanski *et al.*, were considered in detail [41]. A general overview of the method performance was illustrated through the numbers and chromatographic distribution of detected features, in combination with their respective data quality represented by their peak width and relative standard deviation.

4. Results and Discussions

4.1. Screening of LC columns

The selection of the appropriate LC column is a critical step in the method development of LC-MS based metabolomics platforms. The tested columns in this study varied in terms of the chemistry of the stationary phase and the column dimensions. For this latter reason, the columns were not compared based on their full potential, but rather on the results obtained during a first screening experiment (*cf. infra*) based on the column chemistry in the scope of further method optimization.

The stationary phase of the iHILIC-Fusion column contains negatively charged sulfate and phosphate groups, and a charged quaternary amine, resulting in a slightly negative net surface charge. For this reason, this column was only used in ESI (+) mode, since anionic compounds, such as organic acids, are not expected to be retained efficiently on this stationary phase due to repulsive effects. The opposite applies for the iHILIC-Fusion(+) column, which was tested only in ESI (-) mode due to its permanent positive net surface charge which would repel protonated basic metabolites [10,42,43]. The iHILIC-Fusion(P) column was tested in ESI (-) mode due to its polymeric material stable at high pH (≈ 10), which might improve the retention and ionization of acidic metabolites.

Fig. 4 summarizes the panel of analytical standards used for the column screening with their summed scores based on peak shape, intensity and retention time. Using the Acquity HSS T3 column, 34% of detected analytes eluted close to the void time ($t_0 \leq RT \leq 1.1\ t_0$) in ESI (-) mode. For the HILIC methods, there were no analytical standards eluting close to the void time, highlighting the applicability of HILIC for the retention and separation of polar compounds.

The number of detected analytes was dependent on the used LC column and ionization mode. In ESI (-) mode, the percentage of detected compounds varied between 40% for HILICpak VT-

50 2D and 84% for iHILIC-Fusion(P), while in ESI (+) mode, the percentage varied between 55% for Acquity HSS T3 and 63% for iHILIC-Fusion. The limited detection rate can be explained by the wide range of different properties of the analytes and the need of ionization before MS detection, since some metabolites are more easily converted to cations than anions (e.g. carnitines), while the opposite applies for other metabolites such as organic acids. Detailed results can be found in the the supplementary information (S6).

The Acquity HSS T3 column showed a substantial degree of co-elution between 1.5-4 minutes, both in ESI (+) and (-) modes. Broad peaks were observed, especially for basic compounds, due to secondary interactions with residual silanol groups of the stationary phase. The HILICpak VG-50 2D column showed good separation for most analytes, but broad peaks for amino acids e.g. L-aspartic acid and L-histidine, while some small organic acids, such as fumaric acid and maleic acid, could not be detected. Both the Luna HILIC column and the HILICpak VT-50 2D column showed a poor quality for eluting panel standards with substantial tailing. For example, L-arginine, L-aspartic acid, L-histidine and quinolinic acid had a peak tail of over four minutes using the Luna HILIC column. Broad peak shapes are a common issue in HILIC mode, due to the complex interactions such as proton donor and/or acceptor interactions. In addition, significant tailing for positively charged metabolites can be induced by their adsorption on the negatively charged silica. This effect can be anticipated through adjustment of the eluent strength according to the type of interaction of the stationary phase; or by using polymeric columns. During the column screening, the iHILIC-Fusion(+) and iHILIC-Fusion showed the least tailing.

The interaction mechanisms in HILIC, especially zwitterionic columns, are extremely diverse. They involve physical, intermolecular and chemical interactions between analyte and eluent and analyte and stationary phase [17]. Due to the complementarity of ESI (+) and (-) modes, some compounds were only detected in ESI (+) mode (e.g. acylcarnitines, caffeine) and others only in ESI (-) mode (e.g. small organic acids, nucleotides). Based on the summed quality scores, the number of detected standards and the method complementarity showed in Fig. 4, the iHILIC-Fusion (ESI (+) mode), Acquity UPLC HSS T3 (ESI (+) and (-) mode), iHILIC-Fusion(+) (ESI (-) mode) and iHILIC-Fusion(P) (ESI (-) mode) were selected for further method optimization.

4.2. Method optimization of selected columns

Full factorial designs are extremely useful to investigate the main effects on the response, covering all possible combinations of the investigated factors at the selected levels. However,

a decision tree-based univariate method optimization was chosen due to its time-saving properties and straightforward interpretation compared to a multiple-response full factorial approach. Furthermore, the factors that most significantly affect analysis were already known (i.e., solvent polarity and pH). In comparison, approximately 10 to 40 standard injections were needed with each LC column using the knowledge-based univariate method optimization, while a full-factorial design with 6 factors at only 3 levels (3^6) would correspond with 729 standard injections per LC column resulting in approximately 292 hours of data acquisition per column (not including blank injections, replicates, column equilibration and cleaning). Response surface designs were also considered, but due to the number of factors to be considered (> 4), lack of information about quadratic effects, and the exploratory goal of this work, the decision tree was still more adequate. The decision tree procedure allowed to change the chromatographic settings based on metabolite-stationary phase-mobile phase interactions in real time. In addition, this approach assisted in the decision to stop the optimization for one given column at a certain moment, allowing more time to optimize methods for more promising columns, when results are more dependent on the column chemistry than on the chromatographic settings. The results of iHILIC-Fusion (ESI (+) mode), Acquity UPLC HSS T3 (ESI (+) and (-) mode), iHILIC-Fusion (+) (ESI (-) mode) and iHILIC-Fusion(P) (ESI (-) mode) after optimization are shown in Fig. 4. Based on these results, two zwitterionic columns were selected as the best fit for polar metabolites, the iHILIC-Fusion(P) in ESI (-) mode and the iHILIC-Fusion in ESI (+) mode (Fig. 5). The effect of different factors, such as solvent, modifiers and temperature, is discussed in detail for the columns selected for the final methods in the following paragraphs. The results of the method optimization for the iHILIC-Fusion(+) column and RPLC column can be consulted in the supplementary information (S8 and S9).

4.2.1. Mobile phase solvent composition

Using iHILIC-Fusion column in ESI (+) mode, a buffered aqueous mobile phase A (0.1% (v/v) HCOOH + 10 mM NH_4COOH, pH 3.5) was initially combined with ACN as mobile phase B. The addition of 10% (v/v) MeOH to the mobile phase A slightly increased the summed quality score, increasing retention and slightly increasing the intensity of the chromatographic peaks. Longer retention times can be explained by the lower elutropic strength of MeOH compared to H_2O, while higher intensities are observed due to a higher ionization efficiency. Addition of 5% (v/v) MeOH to mobile phase B decreased the summed quality score by 8%. This latter decrease in quality score was mainly due to a deteriorated peak shape of some amino acids, such as L-arginine, L-leucine and L-isoleucine. In addition, most analytes showed a slightly

earlier retention time when MeOH was added to mobile phase B, due to the higher elutropic strength of MeOH, compared to ACN [17]. By adding MeOH to the mobile phase A and B, the summed quality score decreased further by 14%. Next to the deteriorated peak shape for the previously mentioned amino acids, a similar effect was observed for nucleosides, such as adenosine and inosine.

For the iHILIC-Fusion(P) in ESI (-) mode, H_2O with 5 mM $(NH_4)_2CO_3$ (pH 8.7) was initially used as mobile phase A and ACN as mobile phase B. The addition of 10% (v/v) MeOH to mobile phase B and addition of 20% (v/v) MeOH to mobile phase A caused a decrease of the summed quality score of 14% and 10% respectively. The addition of MeOH to both mobile phase A and B decreased the summed quality score by 19%. The addition of MeOH to mobile phase B mainly influenced the start of the run, due to the applied HILIC gradient, starting at a composition with a high organic content. MeOH caused peak splitting for peptides, such as leucin enkephalin and methionine enkephalin and tailing for amino acids (e.g. L-threonine) and small peptides (e.g. glycyl-L-tyrosine). The peak shape deterioration could partly be explained by the mismatch of mobile phase and sample solvent, which could be solved by changing the reconstitution solvent. In addition, alcohols such as MeOH can compete for active polar sites on the stationary surface and analytes, forming hydrogen bonding interactions and interfering with the retention mechanisms. After all, polar protic solvents can be both donors and acceptors of hydrogen bonds, while aprotic solvents, such as ACN, can be only hydrogen bond acceptors. Hydrogen-bonding interactions between MeOH and analytes may introduce extra resonance structures and cause broad or tailing peaks [44]. A decrease in the intensity of several panel standards was observed when MeOH was added to the mobile phase. The decrease was especially significant for organic acids and amino acids. L-serine and phosphocreatine were not detected, since the S/N ratio of their corresponding signal dropped below 3. The decrease in peak intensity could be caused by a lower ionization efficiency, due to the higher content of MeOH. In comparison to MeOH, ACN has a lower viscosity, facilitating a higher ionization efficiency in ESI due to production of finer droplets [45]. In addition, the retention time of most panel standards was reduced slightly, due to the stronger elutropic strength of MeOH compared to ACN and to the MeOH-induced decrease of the polarity of the dynamically immobilized aqueous layer on the stationary phase, impeding the retention of polar compounds [44]. Using MeOH in both mobile phase A and B, no improvement was observed in peak shape or intensity for a single panel standard. In addition, peak shape deterioration, such as tailing and peak splitting, was observed for several panel standards. The decrease in intensity of organic acids and amino acids became more intense with an increasing content of MeOH.

461

462 4.2.2. Mobile phase pH and modifiers

463 A significant number of metabolites in important metabolic pathways are ionizable compounds,
464 such as amino acids and TCA cycle intermediates. Salts present in the mobile phase, such as
465 ammonium acetate, can precipitate in some organic-aqueous compositions, depending on their
466 solubility and concentration, causing drastic damage to mass spectrometers [46]. Therefore, the
467 concentration of salts and its combination with organic solvents during the chromatographic
468 run were taken into account during the development of the methods.

469 Using the iHILIC-Fusion in ESI (+) mode, the aqueous mobile phase was firstly modified with
470 0.1% HCOOH in H_2O/MeOH (9/1, v/v). The addition of 10 mM of NH_4COOH increased the
471 total summed quality score by 20%, reducing peak splitting for compounds such as 3-OH-DL-
472 kynurenine, maltose, fructose and other panel standards with hydroxyl groups. This can be
473 explained by the stationary phase of iHILIC-Fusion which contains hydrogen bond acceptors
474 (S=O and P=O) and buffering with NH_4COOH can affect hydrogen bond interactions
475 positively. However, an increase in its concentration to 20 and 30 mM did not improve the total
476 score, on the contrary, the peak shape score decreased by 30% and 35%, respectively.
477 Additionally, an aqueous mobile phase (0.1% of $HCOOCH_3$ in H_2O) in ESI (+) mode was
478 buffered with NH_4COOCH_3 with concentrations ranging from 10-30 mM and pH 4.6-5. The
479 use of NH_4COOCH_3 decreased the quality score by approximately 19%, due to the increase of
480 alkalinity of the mobile phase which can deprotonate acidic compounds. The iHILIC-Fusion
481 column has a net charge varying between 0 and -1 depending on the pH of the mobile phase. A
482 negative net charge can cause repulsive interaction between deprotonated compounds and the
483 stationary phase, which can explain the poor peak shape at pH > 4. Therefore, 10 mM
484 NH_4COOH with 0.1% HCOOH (v/v) at pH 3.5 was chosen to proceed as the buffer solution in
485 ESI (+) mode.

486 Using the iHILIC-Fusion(P) in ESI (-) mode, the starting conditions included a mobile phase A
487 at pH 8.7, pH 7.6 or pH 2.9, adjusted with $NH_{3(aq.)}$ and HCOOH. The acidic pH resulted in a
488 34% decrease of the total summed quality score, in contrast with the results for pH 7.6, where
489 the quality score increased by 10%. The effect at low pH can be explained by the protons in the
490 mobile phase that can protonate anionic compounds, giving them a more hydrophobic character,
491 reducing the interactions with the iHILIC-Fusion(P) column. At low pH, the intensity score
492 decreased, especially for L-valine and L-phenylalanine that would be positively charged. They
493 were not detected due to poor ionization efficiency in ESI (-) mode at acidic pH. The addition
494 of 2 mM of $(NH_4)_2CO_3$ to the mobile phase (pH 8.4) increased the total score by 20%, since it

keeps a more stable pH over injections compared to NH_3, due to a lower volatility. Different modifiers (NH_4COOH, NH_4COOCH_3) and concentrations (2-10 mM) were tested. Higher concentrations of modifiers are generally used to increase the polar eluent strength and improve the peak shape, but it also can suppress the electrostatic interactions by titrating the stationary phase ions [29,47]. Anionic compounds, especially organic acids could benefit from higher concentrations of buffers to decrease repulsive effects with the column stationary phase which has a net charge varying between 0 and -1. However, chromatographic runs tested with higher amounts of salts in the mobile phase (>20 mM) caused significant signal decrease over approximately ten injections. The combination of 2 mM NH_4COOCH_3 and 2 mM $(NH_4)_2CO_3$ as mobile phase A and ACN as mobile phase B showed the highest total quality score with a chromatographic signal for 85% of the analyzed panel standards. After optimization of the pH and the modifier concentration, additional analytical runs were performed using 10% (v/v) MeOH in mobile phase B. Addition of MeOH enabled the detection of a higher number of organic acids with a better peak shape. The summed quality score increased by 16%, highlighting the complexity of interactions between the chosen modifier and mobile phase and its influence on retention of analytes. Most undetected compounds, such as caffeine and acylcarnitines, could be detected in ESI (+) mode, pointing out the power of method complementarity.

4.2.3. Column temperature

Column temperature is an important parameter to optimize because of its influence on mobile phase viscosity, analyte diffusivity and amount of energy for the analyte partitioning between the mobile phase and the aqueous layer on the stationary phase within HILIC [48].

In ESI (+) mode, no significant differences were observed when the column temperature was increased from 30 °C to 40 °C. However, the summed quality score increased slightly at 50 °C and 60 °C with 3% and 4%, respectively. The increase in the temperature reduced tailing and FWHM for amino acids, sugars, and small chain acylcarnitines. This latter effect can be explained by the temperature induced increase of the diffusion coefficient, resulting in narrower peaks [44]. Additionally, butyric acid was only detected using a high column temperature of 50 °C or above, which might be due to enhancement of electrostatic interactions between the anion and the charged stationary phase at higher temperatures [48]. No significant differences were observed regarding signal intensity.

Increasing temperature can decrease the retention time of neutral molecules due to the exothermic nature of partitioning of analytes between the organic mobile phase with a high

ACN content and the hydrophilic aqueous layer. For charged analytes separated on a charged surface, a temperature increase can result in stronger retention due to strong electrostatic interactions. Depending on the analyte and the contribution of partitioning or electrostatic interactions, column temperature could change the elution order and selectivity [44,48]. For the zwitterionic iHILIC-Fusion column in ESI (+) mode, the retention of the panel standards was more or less independent of temperature, with a negligible median reduction in retention time of 0.06 min at 60 °C. This behavior suggests a low enthalpic contribution and a high entropic contribution to the retention.

In ESI (-) mode, elevating the column temperature from 30 °C to 40 °C, 50 °C and 60 °C increased the summed quality score slightly by 2%, 4%, and 1%, respectively. As with the iHILIC-Fusion in ESI (+) mode, no significant temperature dependent differences were observed regarding signal intensity and the peak shape improved with increasing temperature. However, a column temperature of 60 °C caused peak splitting for several organic acids, such as pyruvic acid, L-ascorbic acid and α-ketoglutaric acid and fronting for kynurenic acid. These latter effects were not observed at lower temperatures and were reproducible. The true reason behind the peak shape deterioration of organic acids is unknown but might be affected by the lower thermal conductivity of the PEEK column material compared to stainless steel. At high temperatures, the lower thermal conductivity might cause a temperature gradient within the column, causing peak shape deterioration. Increasing the column temperature from 30 °C to 60 °C slightly shortened the retention time for the panel standards with a median reduction of 0.1 min. The decrease in retention time showed a maximum at 0.46 min. Based on the column temperature experiment, 50 °C was selected as optimal temperature.

In addition, the method using a temperature of 50 °C was compared to the same method at room temperature (25 °C), bypassing the heat exchanger and/or the inline filter and MS diverter valve. Bypassing the heat exchanger decreased summed peak shape score by 10% but increased the summed intensity score by 7%. Bypassing the heat exchanger, inline filter and MS diverter valve decreased the summed intensity score further by 16% and increased the summed intensity score with 12%. For example, for carbamoyl phosphate and α-ketoglutaric acid, the S/N value increased with 103% and 121%, respectively, when the heat exchanger was bypassed and with 135% and 162% when the heat exchanger, inline filter and MS diverter valve were bypassed. Bypassing the heat exchanger did negatively impact the peak shape through elimination of the increased diffusion coefficients induced by high column temperatures. This latter effect was seen as e.g. tailing for some amino acids (L-tryptophan, L-serine) and L-carnitine.

562 The rationale behind the bypassing of the heat exchanger, inline filter and MS diverter valve
563 was based on the chelating interaction of anionic metabolites, such as carboxylic acids and
564 phosphorylated anions with trace metals from the concerned hardware, resulting in a negative
565 impact on the peak shape and lower peak intensities impairing sensitivity [30,31]. For the same
566 reason, a polyether ether ketone (PEEK) iHILIC-Fusion(P) column was used instead of a
567 stainless-steel column. Alternatively, addition of a strong metal chelator such as EDTA as a
568 mobile phase additive could enhance the detection of acidic metabolites and phosphorylated
569 analytes. However, EDTA is highly ionizable and can cause substantial ion suppression [30].
570 Based on the results of the temperature experiment, bypassing the heat exchanger at room
571 temperature was selected for the final method due to the sensitivity improvement. Despite the
572 higher signal intensities acquired when the heat exchanger, inline filter and MS diverter valve
573 were bypassed, the inline filter and MS diverter valve were retained in the method in order to
574 avoid system contamination during analysis of complex biological samples.
575
576 4.2.4. Gradient and flow
577 Several gradients were tested to result in an evenly distribution of the analytical panel standards
578 in the retention time dimension. Generic HILIC gradients started with a high amount of organic
579 solvent, which was kept for 1-4 min depending on the column length, followed by a gradual
580 increase in the amount of polar solvent (water) to a maximum of 80%. The re-equilibration step
581 is crucial for HILIC columns to allow its return to the initial layer conditions in the entire
582 column. The generic and optimized gradients are described in Table S5.1 and Table 2,
583 respectively.
584 In parallel with gradient optimization, flow rates of 0.2–0.3 mL/min were tested; higher flow
585 rates were not considered taking maximal tolerable backpressures into account. For the iHILIC-
586 Fusion(P) and the iHILIC-Fusion column, flow rates of 0.2 mL/min and 0.25 mL/min were
587 selected respectively, based on the balance between analytical speed and chromatographic
588 resolution, which increased and decreased respectively when higher flow rates were applied.
589 The larger decrease of chromatographic resolution with increasing flow rate for the iHILIC-
590 Fusion(P) column compared to the iHILIC-Fusion column can be explained by the smaller
591 particle size of the latter.
592 For studies with complex samples, it is recommended to optimize the gradient and flow based
593 on untargeted strategies rather than using a mixture of standards. Fig. S10.1 shows an example
594 of this strategy using the dataset of HepaRG cells in ESI (+) mode to optimize gradient and
595 flow based on two-dimension feature distribution, distribution of peak width and gaussian peak

shape visualization. Gradient and flow can be optimized targeting a broad distribution of the features over *m/z*-time space, sharp chromatographic peaks and values of *egauss* as low as possible for the most intense peaks. The above-mentioned parameters were obtained from the *XCMSnExp* object during the pre-processing of biological samples.

4.3. Optimized methods

The optimized methods, which are described in Table 2, increased the number of detected analytes and the overall quality score of chromatographic peaks compared to the column screening experiment (Fig. 4). In ESI (+) mode, the optimized methods enabled the detection of 14 and 3 additional panel standards, using the iHILIC-Fusion and the Acquity HSS T3 column respectively. In addition, the quality score increased with 46% and 32% respectively. In ESI (-) mode, the optimized methods enabled the detection of 9, 19 and 11 additional panel standards, using the iHILIC-Fusion(P), the iHILIC-Fusion(+) and the Acquity HSS T3 column respectively, while the quality score increased with 20%, 58% and 21% respectively. The circular heatmap in Fig. 5 shows the coverage of the standard mixture using the final two methods, which are described in Table 2 in column A and F. Using the iHILIC-Fusion(P) column in ESI (-) mode, 80 out of 85 panel standards could be separated and detected, while using the iHILIC-Fusion column in ESI (+) mode, 73 standards could be separated and detected. Combining both ionization modes, 84 out of 85 panel standards could be separated and detected, highlighting the complementarity of ESI (+) and (-) ionization modes. The method using ESI (-) mode clearly showed better results for organic acids, such as lactic acid, fumaric acid and L-ascorbic acid, which were undetected in ESI (+) mode. On the other hand, the method using ESI (+) mode enabled the separation and detection of acylcarnitines, such as trans-2-octanoyl-L-carnitine, and amine oxides, such as trimethylamine N-oxide, which were undetected by ESI (-) mode. Succinyl-co-enzyme A was the only standard which was undetected using either method. A single standard of succinyl-co-enzyme A was prepared in a concentration of 1 µg/mL. Subsequent analysis showed the presence of co-enzyme A, suggesting degradation of succinyl-co-enzyme A, which could be caused by compound hydrolysis [49].

Fig. 6 shows chromatographic peak shapes for metabolites from various metabolic classes using the final two selected methods. For the optimized method applying the iHILIC-Fusion(P) column in ESI (-) mode, kynurenic acid has a negative charge at the alkaline pH of the mobile phase. Consequently, negatively charged compounds showed short retention time due to repulsive effects with the stationary phase. Amino acids such as L-isoleucine, L-leucine and L-lysine are zwitterionic at the mobile phase pH ≈ 8, thus their quadrupolar electrostatic

interactions with the stationary phase became significant. An increasing number of nitrogen atoms in zwitterionic amino acids resulted in an increased retention. As a result, the retention time of L-lysine was approximately 10 min longer than the retention time of L-leucine. Neutral compounds, such as guanine, showed retention mechanisms based on hydrogen bond interactions and hydrophilic partition, eluting close to the region of most amino acids.

Using the optimized method with the iHILIC-Fusion column in ESI (+) mode, the acidic mobile phase conditions caused nicotinic acid to be neutral or partially positively charged, showing stronger interactions with the stationary phase and a better peak shape compared to the alkaline conditions using the iHILIC-Fusion(P) in ESI (-) mode. Trimethylamine N-oxide and isovaleryl-L-carnitine are positively charged compounds at low pH, thus ion exchange mechanisms will be dominant. In addition, the higher the carbon chain, the shorter the retention time due to hydrophobicity, for instance, the retention time of butyryl-L-carnitine is approximately 1 min longer than isovaleryl-L-carnitine (Fig. S11.2). Additional chromatograms for all optimized methods can be consulted in the supplementary information (S11).

4.4. Untargeted Analysis of biological samples

Biological matrices containing high amounts of salts (e.g., urine and cellular extracts) and high amounts of lipids (e.g., plasma) were analyzed in order to test the analytical performance and the coverage of small polar metabolites with key biological functions. The precision of the dataset was defined by calculating the relative standard deviation (RSD) of the intensity of the features in each matrix and for each ionization mode (Fig. 7). The median RSD (mRSD) is often used to evaluate the overall quality of the features for untargeted data analysis [10]. The mRSD of the QC pooled samples in Fig. 7 was used to assess the repeatability of the analytical method in the matrices.

The mRSD of the six analytical replicates of the pooled QC sample was 15.2% for extracellular extracts of HepaRG cells (HepaRG EC) in ESI (+) mode and 16.7% in ESI (-) mode. For intracellular extracts (HepaRG IC), the mRSD was approximately 16.0% for both ionization modes. For urine, the mRSDs were 23.2% in ESI (+) mode and 11.6% in ESI (-) mode while 12.1% and 11.3% for plasma in ESI (+) and ESI (-) modes, respectively. Relative standard deviations values below 30% define a high-quality dataset for untargeted analysis which reflects a good method stability over runs with different matrices [10,50]. The number of features in the QC pooled sample after blank subtraction were always higher in ESI (+) mode compared to ESI (-) mode. For HepaRG IC, HepaRG EC, plasma and urine, the number of detected features

amounted 3652, 2570, 3565 and 3178 respectively in ESI (+) mode, while 1749, 1622, 917 and 577 features were detected in ESI (-) mode. In addition to mRSD and number of features, data-dependent (auto-MS/MS) acquisition was included during the analysis of each matrix to support annotations.

Features with MS/MS spectra were matched against METLIN, MS-DIAL and HMDB databases. Urine samples showed the highest number of matches with polar metabolites. As a proof of concept, in urine samples, 90 compounds were identified with level 1 or 2, including metabolites such as adenosine, L-proline, citric acid, taurine, uric acid, L-glutamine, estrone and a small number of exogenous compounds, such as bisphenol A, caffeine and vanillin.

In intra- and extracellular extracts of HepaRG cells, several amino acids, acylcarnitines and organic acids were identified, but no exogenous compounds. This was expected based on the origin of the samples and highlights a rigorous sample preparation and column cleaning, avoiding cross-contamination.

For plasma samples, in addition to amino acids such as L-tyrosine, L-proline, L-histidine, some phospholipids were detected, such as lysophosphatidylcholine 18:2 and lysophosphatidyl-ethanolamine 18:1. This demonstrates that the sample preparation method should be optimized in order to remove all lipids for matrices such as plasma, since they can cause ion suppression for small molecules.

The table with the annotations for each biological matrix with their HMDB identifier and database used for MS/MS spectra matching can be found in the supplementary information as a proof of concept (S12).

5. Conclusions

This study handled the optimization of HILIC-MS methods using a decision tree-based univariate method optimization approach, with the objective of developing a platform that can be used to investigate polar metabolites during untargeted metabolomics applicable for different biological matrices. During method optimization, the mix-mode interaction mechanisms of two generations of HILIC columns were investigated using 85 representative standards for polar metabolites from various metabolic pathways. Combining the final optimized HILIC-MS method in ESI (+) and the HILIC-MS method in ESI (-), almost 100% of polar standards could be separated and detected, covering key pathways of the polar human metabolome. The methods were successfully applied using different biological matrices of human origin, including urine, plasma and extracts of hepatic cells. Further optimization of sample preparation

techniques can improve the coverage of polar metabolites, given the fact that the authors used methods published elsewhere.

6. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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9. Legend to Figures

Fig. 1. Pathway coverage of the analytical panel standards used for method development. Standards were selected based on their polarity to cover the polar side of the pathway map. ATP: Adenosine triphosphate. ADP: Adenosine diphosphate. AMP: Adenosine monophosphate. β-NADPH: β-Nicotinamide adenine dinucleotide phosphate. SAMe: S-adenosyl-L-methionine. NADH: Nicotinamide adenine dinucleotide.

Fig. 2. Stationary phases of the LC columns used for screening. The text highlighted in red refers to polymer-based columns and the one in grey refers to silica-based columns.
HILIC: Hydrophilic interaction liquid chromatography. PEEK: Polyether ether ketone. HSS: High strength silica.

Fig. 3. Method optimization flowchart.
LC: Liquid chromatography. ACN: Acetonitrile. MeOH: Methanol.

Fig. 4. Heatmap showing the coverage of the standard mixture during column screening and acquisition with the final optimized methods. Scores are based on peak shape, peak intensity and retention time. Note that the Luna, VG-50 and VT-50 column were only used during the column screening experiment. Column's legend: Fusion: iHILIC-Fusion, HSST3: Acquity UPLC HSS T3, Luna: Luna HILIC, VG-50: HILICpak VG-50 2D, VT-50: HILICpak VT-50 2D, Fusion(+) : iHILIC-Fusion(+), Fusion(P): iHILIC-Fusion(P). HILIC: Hydrophilic interaction liquid chromatography. RPLC: Reversed-phase liquid chromatography. SAMe: S-adenosyl-L-methionine. NADH: Nicotinamide adenine dinucleotide. β-NADPH: β-Nicotinamide adenine dinucleotide phosphate.

Fig. 5. Circular heatmap showing the coverage of the standard mixture with the final optimized methods. Positive ionization mode is shown in a blue-based color pallet and negative ionization mode in a green-based color pallet.
SAMe: S-adenosyl-L-methionine. NADH: Nicotinamide adenine dinucleotide. β-NADPH: β-Nicotinamide adenine dinucleotide phosphate.

Fig. 6. Extracted ion chromatograms of panel standards from various metabolic classes. The standards were detected using the optimized methods in negative electrospray ionization

mode (left) and positive electrospray ionization mode (right).

Fig. 7. Boxplot with relative standard deviation (RSD) for QC pooled of *in vitro* (HepaRG extracellular (EC) and intracellular (IC) extracts) and *in vivo* (human plasma and urine) samples.
mRSD: Median relative standard deviation. ESI: Electrospray ionization.

10. Legend to Tables

Table 1. Scoring system to evaluate peak shape, peak intensity and retention time. The scoring system was used for each analytical panel standard to guide the choice of LC column and the method optimization.
LC: Liquid chromatography. FWHM: Full width at half maximum. S/N: Signal-to-noise ratio. RT: Retention time.

Table 2. Final liquid chromatography conditions obtained after method optimization. U(H)PLC: Ultra (high) performance liquid chromatography. HSS: High strength silica. HILIC: Hydrophilic interaction liquid chromatography. RPLC: Reversed phase liquid chromatography. SS: Stainless steel. PEEK: Polyether ether ketone. ACN: Acetonitrile. MeOH: Methanol. MS: Mass spectrometry. ESI: Electrospray ionization.