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Published in:

Journal of Chromatography. A

DOI:

[10.1016/j.chroma.2020.461807](https://doi.org/10.1016/j.chroma.2020.461807)

Publication date:

2021

License:

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Document Version:

Accepted author manuscript

[Link to publication](#)

Citation for published version (APA):

Iturraspe, E., Da Silva, K. M., Talavera Andújar, B., Cuykx, M., Boeckmans, J., Vanhaecke, T., Covaci, A., & van Nuijs, A. L. N. (2021). An exploratory approach for an oriented development of an untargeted hydrophilic interaction liquid chromatography-mass spectrometry platform for polar metabolites in biological matrices. *Journal of Chromatography. A*, 1637, [461807]. <https://doi.org/10.1016/j.chroma.2020.461807>

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

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27
28 **Highlights**

- 29 • Six HILIC and one RPLC columns are tested for polar metabolites.
- 30 • A decision tree-based univariate approach is used to optimize HILIC-MS methods.
- 31 • 99% of analytical panel standards can successfully be separated and detected.
- 32 • Human urine, plasma and liver cells show thousands of features with mRSD < 25%.

- 33 • Hundreds of metabolites were annotated in biological samples with L1 or L2
34 confirmation.
35

36 **Abstract**

37 The analysis of polar metabolites based on liquid chromatography-mass spectrometry (LC-MS)
38 methods should take into consideration the complexity of interactions in LC columns to be able
39 to cover a broad range of metabolites of key biological pathways. Therefore, in this study,
40 different chromatographic columns were tested for polar metabolites including reversed-phase
41 and hydrophilic interaction liquid chromatography (HILIC) columns. Based on a column
42 screening, two new generations of zwitterionic HILIC columns were selected for further
43 evaluation. A tree-based method optimization was applied to investigate the chromatographic
44 factors affecting the retention mechanisms of polar metabolites with zwitterionic stationary
45 phases. The results were evaluated based on a scoring system which was applied for more than
46 80 polar metabolites with a high coverage of key human metabolic pathways. The final
47 optimized methods showed high complementarity to analyze a wide range of metabolic classes
48 including amino acids, small peptides, sugars, amino sugars, phosphorylated sugars, organic
49 acids, nucleobases, nucleosides, nucleotides and acylcarnitines. Optimized methods were
50 applied to analyze different biological matrices, including human urine, plasma and liver cell
51 extracts using an untargeted approach. The number of high-quality features (< 30% median
52 relative standard deviation) ranged from 3,755 for urine to 5,402 for the intracellular
53 metabolome of liver cells, showing the potential of the methods for untargeted purposes.

54

55 **Keywords**

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

58 **1. Introduction**

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

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

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

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

100

101 **2. Materials and methods**

102 **2.1. Chemicals and materials**

103 Analytical standards were purchased from Sigma Aldrich (St. Louis, Missouri, USA), Merck
104 (Darmstadt, Germany) and Janssen Chimica (Beerse, Belgium). A total number of 72 panel
105 standards was used during the experiments covering a wide range of metabolic classes including
106 amino acids (19), amino acid metabolites (5), phosphorylated amino acid metabolites (2),
107 peptides (5), sugars (4), amino sugars (1), phosphorylated sugars (1), organic acids (6),
108 phosphorylated organic acids (2), tricarboxylic acid cycle (TCA) intermediates (6), nucleobases
109 or analogues (4), nucleosides (3), nucleotides (3), cofactors or -enzymes (6) and small chain
110 acylcarnitines (5). Using the final optimized methods, 13 additional analytical panel standards
111 (3 amino acids, 3 amino acid metabolites, 2 sugars, 1 organic acid, 1 nucleobase or analogue, 2
112 cofactors or -enzymes and 1 amine oxide) were analyzed, bringing the total number to 85 panel
113 standards. In Fig. 1, 65 out of 85 panel standards were used for pathway mapping to visualize
114 the metabolic coverage. All chemical standards used during method optimization are listed in
115 the supplementary information (S1). L-glutamic acid-2,3,3,4,4-D₅, L-leucine-5,5,5-D₃, L-
116 lysine-¹³C₆-¹⁵N₂, succinic acid-2,2,3,3-D₄, D-tryptophan-2,4,5,6,7,-D₅ and glucose-¹³C₆ were
117 used as internal standards during the sample preparation of biological samples. All internal
118 standards were purchased from Sigma Aldrich, except for D-tryptophan-2,4,5,6,7-D₅, which
119 was bought from CDN isotopes (Pointe-Claire, Quebec, Canada).

120 Methanol ultrapure (MeOH), acetonitrile (ACN) and formic acid (99%) (HCOOH), all
121 ULC/MS-CC/CSF grade, were purchased from Biosolve (Valkenswaard, The Netherlands).
122 Ammonium formate (≥ 99%, LC-MS grade) (NH₄COOH), ammonium carbonate HPLC grade
123 ((NH₄)₂CO₃) and ammonium acetate LC-MS grade (NH₄COOCH₃) were obtained from Sigma
124 Aldrich. Acetic acid (100%) (HCOOCH₃), ammonia solution (25%) (NH_{3(aq)}) and ethanol

125 (EtOH), all LC-MS grade, isopropanol for analysis (ACS reagent) (IPA) and chloroform
126 (analytical grade) (CHCl₃) were purchased from Merck. Ultrapure water (H₂O) used throughout
127 the experiments was obtained from an Elga Pure Lab apparatus (Tienen, Belgium).

128 For the experiments with liver cells, differentiated HepaRG cells, Basal Hepatic Medium,
129 HepaRG Thaw, Seed and General-Purpose Supplement and HepaRG Maintenance and
130 Metabolism Supplement were acquired from Biopredic International (Rennes, France).
131 HepaRG cells were seeded in Permanox 2-well Lab-Tek chamber slides from Nunc, Thermo
132 Scientific (Rochester NY, USA) and incubated using a Galaxy 170 S incubator acquired from
133 Eppendorf (Hamburg, Germany). Rat tail collagen for coating was provided by Corning (New
134 York, USA). Eppendorf Safe-Lock tubes and 0.2 µm nylon centrifugal filters were acquired
135 from respectively Eppendorf and VWR (Pennsylvania, USA) and used during sample
136 preparation. Pure, dry nitrogen (AZOTE N28, N₂) used for solvent evaporation was obtained
137 from Air Liquide Belge (Liège, Belgium). 384 well plates (PS, small volume) were bought from
138 Greiner Bio-One (Vilvoorde, Belgium). Human blood was collected in sterile Vacuette
139 K₃EDTA premium tubes acquired from Greiner Bio-One and aseptic polypropylene urine
140 recipients from Disera (Izmir, Turkey) were used for urine collection.

141

142 **2.2. Preparation of analytical standards**

143 Analytical standard solutions were prepared by dissolving solids or diluting liquid standards in
144 H₂O, MeOH, EtOH or a combination of H₂O with MeOH or EtOH (50/50, v/v), depending on
145 the solubilization properties of the compounds, to obtain a stock solution of 50 µg/mL. All
146 panel standards were combined in a mixture, the solvent was evaporated to dryness using a
147 stream of N₂ at room temperature, and reconstituted in H₂O/ACN (35/65, v/v) to obtain a final
148 concentration of 1 µg/mL. The final mixtures were stored at -20 °C before injection.

149

150 **2.3. Sample preparation**

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

157 **2.3.1. Intracellular extracts of HepaRG cells**

158 Ethical approval for the use of HepaRG cells was provided by the Medical Ethics Committee
159 of the University Hospital Brussels (reference number 143201941214). Differentiated HepaRG
160 cells were incubated for 8 days at 37 °C, 5% CO₂ and saturated humidity.

161 In order to extract the intracellular metabolome of the cells, the sample preparation method of
162 Cuykx *et al.* [18] was slightly adapted. Briefly, cells were flash-frozen using liquid nitrogen
163 and scraped with a solution of 80% MeOH and 20% 10 mM NH₄COOCH₃ (v/v) at -80 °C. The
164 MeOH solution containing the cells was recovered to perform liquid-liquid extraction (LLE)
165 (3/2/2, v/v/v, MeOH/H₂O/CHCl₃). A mixture of internal standards was added (final
166 concentration 1 µg/mL). The lower fraction (MeOH/H₂O) was divided in two for the analysis
167 in ESI (+) and ESI (-) mode. After drying under N₂ stream, extracts were reconstituted in
168 ACN/H₂O (65/35, v/v). A detailed protocol of the sample preparation was added to the
169 supplementary information (S2).

170

171 2.3.2. Extracellular extracts of HepaRG cells

172 In order to analyze the extracellular metabolome of HepaRG cells, the used incubation medium
173 was collected at the same day as the extraction of the HepaRG cells. The sample preparation
174 was based on the method of Cuykx *et al.* [18] and Dettmer *et al.* [24]. Briefly, medium was
175 quenched using a solution of 80% MeOH and 20% 10 mM NH₄COOCH₃ (v/v) at -80 °C.
176 Quenched medium was used to perform LLE (3/2/2, v/v/v, MeOH/H₂O/CHCl₃). The
177 subsequent sample preparation steps were similar to the steps performed for the intracellular
178 extracts which are explained in detail in the supplementary information (S2).

179

180 2.3.3. Human plasma

181 Blood samples were collected from 6 healthy volunteers, 3 males and 3 females, aged 24-31
182 years. The sampling was approved by the Ethical Committee of the University Hospital
183 Antwerp (EC/PC/av1/2018.039). Extraction was performed using K₃EDTA tubes, which were
184 centrifuged within 15 min after collection. The sample preparation protocol was adapted from
185 Benito *et al.* [19], Chen *et al.* [20] and Bruce *et al.* [21] and is explained in detail in the
186 supplementary information (S3). Briefly, plasma samples were mixed with -80 °C MeOH/EtOH
187 (1/1, v/v) and centrifuged. The supernatant was evaporated to dryness under a stream of N₂ and
188 reconstituted using ACN/H₂O (65/35, v/v) spiked with a 1 µg/mL internal standard mixture.

189

190 2.3.4. Human urine

191 Urine samples were collected from 6 healthy volunteers, 3 males and 3 females aged 24-48
192 years. The donation was approved by the Ethical Committee of the University Hospital Antwerp
193 (18/31/357). Sample preparation was based on the method of Wu *et al.* [22] and the
194 recommendations of Fernández-Peralbo *et al.* [23] and is explained in detail in the
195 supplementary information (S4). Briefly, urine samples were diluted with ACN and
196 centrifuged. An internal standard mixture was added to the supernatant (final concentration 1
197 $\mu\text{g/mL}$).

198

199 **2.4. Mass spectrometry parameters**

200 Mass spectrometry detection was performed on an Agilent 6530 QToF-MS with Agilent Jet
201 Stream Electrospray Ionization (Agilent Technologies, Santa Clara, USA). In ESI (+) mode,
202 nitrogen was used as drying and sheath gas at 250 °C and 350 °C with flow rates of 8 L/min
203 and 11 L/min, respectively. The nebulizer gas pressure was set at 45 psig, the MS capillary
204 voltage at 2000 V, the nozzle voltage at 0 V and the fragmentor at 150 V. In ESI (-) mode, the
205 drying and sheath gas had a temperature of 250 °C and a flow of 10 L/min and a temperature
206 of 350 °C and a flow of 10 L/min respectively. The nebulizer gas pressure was set at 45 psig,
207 the MS capillary voltage at 2000 V, the nozzle voltage at 0 V and the fragmentor at 100 V. For
208 both ionization modes, data were acquired in 2 GHz extended dynamic mode with a scan range
209 of 60-1000 m/z and a scan rate of 2 spectra/s. Full scan data were stored in profile mode.
210 Calibration of the mass axis was performed within run using purine (m/z 121.0508 in ESI (+)
211 mode and m/z 119.0363 in ESI (-) mode) and hexakis (1H, 1H, 3H-tetrafluoropropoxy)
212 phosphazine (m/z 922.0097 in ESI (+) mode and m/z 980.0163 in ESI (-) mode). The calibrant
213 solution was constantly infused during the run with an additional isocratic pump (Agilent 1200
214 series G1310A) and mixed with the effluent using a T-piece connected to the ESI source. Data-
215 dependent MS/MS (auto-MS/MS) acquisition was obtained at collision energies of 10, 20 and
216 40 eV using a separate method with a scan rate of 2 spectra/s and 6.67 spectra/s for MS and
217 MS/MS spectra, respectively. The maximum precursors/scan cycle was set at 12.

218

219 **3. Analytical method optimization**

220 **3.1. Liquid chromatography column screening**

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

225 generic methods are specified in the supplementary information (S5). In addition to HILIC-
226 QToF-MS, one RPLC-QToF-MS method was tested in parallel to evaluate the coverage of a
227 reversed-phase method with an Acquity UPLC HSS T3 column previously used for
228 metabolomics applications [25]. Columns included Luna HILIC (100 x 3.0 mm, 3 μ m, cross-
229 linked diol, silica-based, Phenomenex, USA), iHILIC-Fusion (100 x 2.1 mm, 1.8 μ m,
230 zwitterionic, charge modulated amide, silica-based, HILICON AB, Sweden), iHILIC-
231 Fusion(+) (100 x 2.1 mm, 1.8 μ m, zwitterionic, permanent positive charge modulated amide,
232 silica-based, HILICON AB, Sweden), iHILIC-Fusion(P) (100 x 2.1 mm, 5 μ m, zwitterionic,
233 charge modulated amide, polymer-based, HILICON AB, Sweden), HILICpak VT-50 2D (150
234 x 2.0 mm, 5 μ m, quaternary ammonium, polymer-based, Shodex, Japan), HILICpak VG-50
235 2D (150 x 2.0 mm, 5 μ m, amino, polymer-based, Shodex, Japan) and Acquity UPLC HSS T3
236 (100 x 2.1 mm, 1.8 μ m, C18, silica-based, Waters, USA). A graphical representation of the
237 stationary phases of the above-mentioned columns is shown in Fig. 2.

238

239 3.2. Liquid chromatography method optimization

240 HILIC interactions are highly dependent on the used stationary phase, mobile phase
241 composition and several other factors [16,26,27]. A decision tree optimization was chosen
242 based on the influence of these factors in the following order: stationary phase > mobile phase
243 pH and modifier > modifier concentration > additional parameters (temperature, gradient, flow)
244 [28,29]. After the selection of the most suitable stationary phases, the solvent and pH effects
245 were tested with ACN, MeOH or a combination of both as organic eluent and buffered H₂O as
246 aqueous eluent. LC-MS analysis was performed in both ESI (+) and ESI (-) modes. Generally,
247 high pH values were tested to ionize compounds in ESI (-) mode and low pH values in ESI (+)
248 mode. In the starting conditions in ESI (-) mode, (NH₄)₂CO₃ was added to the aqueous mobile
249 phase until the maximum tolerable pH value for each column. Following the recommendations
250 of the manufacturer, a maximum pH \approx 8 was tested for the Waters Acquity UPLC HSS T3
251 column and pH \approx 9 for HILIC columns in ESI (-) mode. In ESI (+) mode, aqueous mobile
252 phases were buffered with 10 mM of NH₄COOH and 0.1% HCOOH (pH \approx 3.5). In addition,
253 the effect of the pH was tested by injecting the mixture of panel standards at a basic pH with
254 the above-mentioned restrictions, an acidic pH and neutral pH in both polarities, adjusting the
255 pH with NH_{3(aq)}, (NH₄)₂CO₃ or HCOOH when necessary. Salt modifiers affect the eluent
256 strength, causing a greater impact on columns with dominant ionic interactions [17]. The
257 modifier effect was tested using different modifiers, including NH₄COOCH₃, NH₄COOH and

258 $(\text{NH}_4)_2\text{CO}_3$ in ESI (-) mode and $\text{NH}_4\text{COOCH}_3$, 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

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

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

303

$$304 \quad \text{Quality Score} = \sum_{i=1}^n \text{Peak shape score} + \sum_{i=1}^n \text{Peak intensity score} + \sum_{i=1}^n \text{Retention time score}$$

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

306

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

310

311 **3.4. Data analysis of biological samples**

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

325 filling. Subsequently, blank filtration (fold change of 3) was performed with the BlankCheckR
326 function of the MetaboMeeSeeks package. Features showing MS/MS spectra were annotated
327 by comparison of accurate mass, retention time and MS/MS fragmentation with in-house
328 libraries, using MS-DIAL (v.4.24) with the All Public MS/MS library (v.15) for ESI (+) and (-
329) modes [7], HMDB [39] and METLIN [40]. Annotated features assigned with a level 1 or 2
330 confirmation according to the recommendations of Schymanski *et al.*, were considered in detail
331 [41]. A general overview of the method performance was illustrated through the numbers and
332 chromatographic distribution of detected features, in combination with their respective data
333 quality represented by their peak width and relative standard deviation.

334

335 **4. Results and Discussions**

336 **4.1. Screening of LC columns**

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

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

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

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

359 50 2D and 84% for iHILIC-Fusion(P), while in ESI (+) mode, the percentage varied between
360 55% for Acquity HSS T3 and 63% for iHILIC-Fusion. The limited detection rate can be
361 explained by the wide range of different properties of the analytes and the need of ionization
362 before MS detection, since some metabolites are more easily converted to cations than anions
363 (e.g. carnitines), while the opposite applies for other metabolites such as organic acids. Detailed
364 results can be found in the the supplementary information (S6).

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

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

389

390 **4.2. Method optimization of selected columns**

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

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

416

417 4.2.1. Mobile phase solvent composition

418 Using iHILIC-Fusion column in ESI (+) mode, a buffered aqueous mobile phase A (0.1% (v/v)
419 HCOOH + 10 mM NH₄COOH, pH 3.5) was initially combined with ACN as mobile phase B.
420 The addition of 10% (v/v) MeOH to the mobile phase A slightly increased the summed quality
421 score, increasing retention and slightly increasing the intensity of the chromatographic peaks.
422 Longer retention times can be explained by the lower elutropic strength of MeOH compared to
423 H₂O, while higher intensities are observed due to a higher ionization efficiency. Addition of
424 5% (v/v) MeOH to mobile phase B decreased the summed quality score by 8%. This latter
425 decrease in quality score was mainly due to a deteriorated peak shape of some amino acids,
426 such as L-arginine, L-leucine and L-isoleucine. In addition, most analytes showed a slightly

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

432 For the iHILIC-Fusion(P) in ESI (-) mode, H₂O with 5 mM (NH₄)₂CO₃ (pH 8.7) was initially
433 used as mobile phase A and ACN as mobile phase B. The addition of 10% (v/v) MeOH to
434 mobile phase B and addition of 20% (v/v) MeOH to mobile phase A caused a decrease of the
435 summed quality score of 14% and 10% respectively. The addition of MeOH to both mobile
436 phase A and B decreased the summed quality score by 19%. The addition of MeOH to mobile
437 phase B mainly influenced the start of the run, due to the applied HILIC gradient, starting at a
438 composition with a high organic content. MeOH caused peak splitting for peptides, such as
439 leucin enkephalin and methionine enkephalin and tailing for amino acids (e.g. L-threonine) and
440 small peptides (e.g. glycyl-L-tyrosine). The peak shape deterioration could partly be explained
441 by the mismatch of mobile phase and sample solvent, which could be solved by changing the
442 reconstitution solvent. In addition, alcohols such as MeOH can compete for active polar sites
443 on the stationary surface and analytes, forming hydrogen bonding interactions and interfering
444 with the retention mechanisms. After all, polar protic solvents can be both donors and acceptors
445 of hydrogen bonds, while aprotic solvents, such as ACN, can be only hydrogen bond acceptors.
446 Hydrogen-bonding interactions between MeOH and analytes may introduce extra resonance
447 structures and cause broad or tailing peaks [44]. A decrease in the intensity of several panel
448 standards was observed when MeOH was added to the mobile phase. The decrease was
449 especially significant for organic acids and amino acids. L-serine and phosphocreatine were not
450 detected, since the S/N ratio of their corresponding signal dropped below 3. The decrease in
451 peak intensity could be caused by a lower ionization efficiency, due to the higher content of
452 MeOH. In comparison to MeOH, ACN has a lower viscosity, facilitating a higher ionization
453 efficiency in ESI due to production of finer droplets [45]. In addition, the retention time of most
454 panel standards was reduced slightly, due to the stronger elutropic strength of MeOH compared
455 to ACN and to the MeOH-induced decrease of the polarity of the dynamically immobilized
456 aqueous layer on the stationary phase, impeding the retention of polar compounds [44]. Using
457 MeOH in both mobile phase A and B, no improvement was observed in peak shape or intensity
458 for a single panel standard. In addition, peak shape deterioration, such as tailing and peak
459 splitting, was observed for several panel standards. The decrease in intensity of organic acids
460 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 H₂O/MeOH (9/1, v/v). The addition of 10 mM of NH₄COOH 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 NH₄COOH 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 HCOOCH₃ in H₂O) in ESI (+) mode was
478 buffered with NH₄COOCH₃ with concentrations ranging from 10-30 mM and pH 4.6-5. The
479 use of NH₄COOCH₃ 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 NH₄COOH 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 (NH₄)₂CO₃ to the mobile phase (pH 8.4) increased the total score by 20%, since it

495 keeps a more stable pH over injections compared to NH_3 , due to a lower volatility. Different
496 modifiers (NH_4COOH , $\text{NH}_4\text{COOCH}_3$) and concentrations (2-10 mM) were tested. Higher
497 concentrations of modifiers are generally used to increase the polar eluent strength and improve
498 the peak shape, but it also can suppress the electrostatic interactions by titrating the stationary
499 phase ions [29,47]. Anionic compounds, especially organic acids could benefit from higher
500 concentrations of buffers to decrease repulsive effects with the column stationary phase which
501 has a net charge varying between 0 and -1. However, chromatographic runs tested with higher
502 amounts of salts in the mobile phase (>20 mM) caused significant signal decrease over
503 approximately ten injections. The combination of 2 mM $\text{NH}_4\text{COOCH}_3$ and 2 mM $(\text{NH}_4)_2\text{CO}_3$
504 as mobile phase A and ACN as mobile phase B showed the highest total quality score with a
505 chromatographic signal for 85% of the analyzed panel standards. After optimization of the pH
506 and the modifier concentration, additional analytical runs were performed using 10% (v/v)
507 MeOH in mobile phase B. Addition of MeOH enabled the detection of a higher number of
508 organic acids with a better peak shape. The summed quality score increased by 16%,
509 highlighting the complexity of interactions between the chosen modifier and mobile phase and
510 its influence on retention of analytes. Most undetected compounds, such as caffeine and
511 acylcarnitines, could be detected in ESI (+) mode, pointing out the power of method
512 complementarity.

513

514 4.2.3. Column temperature

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

518 In ESI (+) mode, no significant differences were observed when the column temperature was
519 increased from 30 °C to 40 °C. However, the summed quality score increased slightly at 50 °C
520 and 60 °C with 3% and 4%, respectively. The increase in the temperature reduced tailing and
521 FWHM for amino acids, sugars, and small chain acylcarnitines. This latter effect can be
522 explained by the temperature induced increase of the diffusion coefficient, resulting in narrower
523 peaks [44]. Additionally, butyric acid was only detected using a high column temperature of 50
524 °C or above, which might be due to enhancement of electrostatic interactions between the anion
525 and the charged stationary phase at higher temperatures [48]. No significant differences were
526 observed regarding signal intensity.

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

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

537 In ESI (-) mode, elevating the column temperature from 30 °C to 40 °C, 50 °C and 60 °C
538 increased the summed quality score slightly by 2%, 4%, and 1%, respectively. As with the
539 iHILIC-Fusion in ESI (+) mode, no significant temperature dependent differences were
540 observed regarding signal intensity and the peak shape improved with increasing temperature.
541 However, a column temperature of 60 °C caused peak splitting for several organic acids, such
542 as pyruvic acid, L-ascorbic acid and α -ketoglutaric acid and fronting for kynurenic acid. These
543 latter effects were not observed at lower temperatures and were reproducible. The true reason
544 behind the peak shape deterioration of organic acids is unknown but might be affected by the
545 lower thermal conductivity of the PEEK column material compared to stainless steel. At high
546 temperatures, the lower thermal conductivity might cause a temperature gradient within the
547 column, causing peak shape deterioration. Increasing the column temperature from 30 °C to 60
548 °C slightly shortened the retention time for the panel standards with a median reduction of 0.1
549 min. The decrease in retention time showed a maximum at 0.46 min. Based on the column
550 temperature experiment, 50 °C was selected as optimal temperature.

551 In addition, the method using a temperature of 50 °C was compared to the same method at room
552 temperature (25 °C), bypassing the heat exchanger and/or the inline filter and MS diverter valve.
553 Bypassing the heat exchanger decreased summed peak shape score by 10% but increased the
554 summed intensity score by 7%. Bypassing the heat exchanger, inline filter and MS diverter
555 valve decreased the summed intensity score further by 16% and increased the summed intensity
556 score with 12%. For example, for carbamoyl phosphate and α -ketoglutaric acid, the S/N value
557 increased with 103% and 121%, respectively, when the heat exchanger was bypassed and with
558 135% and 162% when the heat exchanger, inline filter and MS diverter valve were bypassed.
559 Bypassing the heat exchanger did negatively impact the peak shape through elimination of the
560 increased diffusion coefficients induced by high column temperatures. This latter effect was
561 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

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

600

601 **4.3. Optimized methods**

602 The optimized methods, which are described in Table 2, increased the number of detected
603 analytes and the overall quality score of chromatographic peaks compared to the column
604 screening experiment (Fig. 4). In ESI (+) mode, the optimized methods enabled the detection
605 of 14 and 3 additional panel standards, using the iHILIC-Fusion and the Acquity HSS T3
606 column respectively. In addition, the quality score increased with 46% and 32% respectively.
607 In ESI (-) mode, the optimized methods enabled the detection of 9, 19 and 11 additional panel
608 standards, using the iHILIC-Fusion(P), the iHILIC-Fusion(+) and the Acquity HSS T3 column
609 respectively, while the quality score increased with 20%, 58% and 21% respectively. The
610 circular heatmap in Fig. 5 shows the coverage of the standard mixture using the final two
611 methods, which are described in Table 2 in column A and F. Using the iHILIC-Fusion(P)
612 column in ESI (-) mode, 80 out of 85 panel standards could be separated and detected, while
613 using the iHILIC-Fusion column in ESI (+) mode, 73 standards could be separated and detected.
614 Combining both ionization modes, 84 out of 85 panel standards could be separated and detected,
615 highlighting the complementarity of ESI (+) and (-) ionization modes. The method using ESI
616 (-) mode clearly showed better results for organic acids, such as lactic acid, fumaric acid and
617 L-ascorbic acid, which were undetected in ESI (+) mode. On the other hand, the method using
618 ESI (+) mode enabled the separation and detection of acylcarnitines, such as trans-2-octanoyl-
619 L-carnitine, and amine oxides, such as trimethylamine N-oxide, which were undetected by ESI
620 (-) mode. Succinyl-co-enzyme A was the only standard which was undetected using either
621 method. A single standard of succinyl-co-enzyme A was prepared in a concentration of 1
622 $\mu\text{g/mL}$. Subsequent analysis showed the presence of co-enzyme A, suggesting degradation of
623 succinyl-co-enzyme A, which could be caused by compound hydrolysis [49].

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

630 interactions with the stationary phase became significant. An increasing number of nitrogen
631 atoms in zwitterionic amino acids resulted in an increased retention. As a result, the retention
632 time of L-lysine was approximately 10 min longer than the retention time of L-leucine. Neutral
633 compounds, such as guanine, showed retention mechanisms based on hydrogen bond
634 interactions and hydrophilic partition, eluting close to the region of most amino acids.
635 Using the optimized method with the iHILIC-Fusion column in ESI (+) mode, the acidic mobile
636 phase conditions caused nicotinic acid to be neutral or partially positively charged, showing
637 stronger interactions with the stationary phase and a better peak shape compared to the alkaline
638 conditions using the iHILIC-Fusion(P) in ESI (-) mode. Trimethylamine N-oxide and
639 isovaleryl-L-carnitine are positively charged compounds at low pH, thus ion exchange
640 mechanisms will be dominant. In addition, the higher the carbon chain, the shorter the retention
641 time due to hydrophobicity, for instance, the retention time of butyryl-L-carnitine is
642 approximately 1 min longer than isovaleryl-L-carnitine (Fig. S11.2). Additional
643 chromatograms for all optimized methods can be consulted in the supplementary information
644 (S11).

645

646 **4.4. Untargeted Analysis of biological samples**

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

655 The mRSD of the six analytical replicates of the pooled QC sample was 15.2% for extracellular
656 extracts of HepaRG cells (HepaRG EC) in ESI (+) mode and 16.7% in ESI (-) mode. For
657 intracellular extracts (HepaRG IC), the mRSD was approximately 16.0% for both ionization
658 modes. For urine, the mRSDs were 23.2% in ESI (+) mode and 11.6% in ESI (-) mode while
659 12.1% and 11.3% for plasma in ESI (+) and ESI (-) modes, respectively. Relative standard
660 deviations values below 30% define a high-quality dataset for untargeted analysis which reflects
661 a good method stability over runs with different matrices [10,50]. The number of features in the
662 QC pooled sample after blank subtraction were always higher in ESI (+) mode compared to
663 ESI (-) mode. For HepaRG IC, HepaRG EC, plasma and urine, the number of detected features

664 amounted 3652, 2570, 3565 and 3178 respectively in ESI (+) mode, while 1749, 1622, 917 and
665 577 features were detected in ESI (-) mode. In addition to mRSD and number of features, data-
666 dependent (auto-MS/MS) acquisition was included during the analysis of each matrix to support
667 annotations.

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

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

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

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

685

686 **5. Conclusions**

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

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

699

700 **6. Declaration of Competing Interest**

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

703

704 **7. Acknowledgements**

705 Elias Iturraspe was funded by the Research Scientific Foundation-Flanders (FWO) - project
706 number 1161620N. Katyeny Manuela da Silva acknowledges a doctoral fellowship BOF
707 DOCPRO 4 from the University of Antwerp. Begoña Talavera Andújar was funded by Erasmus
708 + Internship (Program 999/030P, University of Castilla La Mancha, Spain). The authors thank
709 Anja Heymans of the Vrije Universiteit Brussel for her help during the incubation of HepaRG
710 cells.

711

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- 887

888 9. Legend to Figures

889

890 Fig. 1. Pathway coverage of the analytical panel standards used for method development.

891 Standards were selected based on their polarity to cover the polar side of the pathway map.

892 ATP: Adenosine triphosphate. ADP: Adenosine diphosphate. AMP: Adenosine

893 monophosphate. β -NADPH: β -Nicotinamide adenine dinucleotide phosphate. SAME: S-

894 adenosyl-L-methionine. NADH: Nicotinamide adenine dinucleotide.

895

896 Fig. 2. Stationary phases of the LC columns used for screening. The text highlighted in red

897 refers to polymer-based columns and the one in grey refers to silica-based columns.

898 HILIC: Hydrophilic interaction liquid chromatography. PEEK: Polyether ether ketone.

899 HSS: High strength silica.

900

901 Fig. 3. Method optimization flowchart.

902 LC: Liquid chromatography. ACN: Acetonitrile. MeOH: Methanol.

903

904 Fig. 4. Heatmap showing the coverage of the standard mixture during column screening and

905 acquisition with the final optimized methods. Scores are based on peak shape, peak intensity

906 and retention time. Note that the Luna, VG-50 and VT-50 column were only used during

907 the column screening experiment. Column's legend: Fusion: iHILIC-Fusion, HSST3:

908 Acquity UPLC HSS T3, Luna: Luna HILIC, VG-50: HILICpak VG-50 2D, VT-50:

909 HILICpak VT-50 2D, Fusion(+) : iHILIC-Fusion(+), Fusion(P): iHILIC-Fusion(P). HILIC:

910 Hydrophilic interaction liquid chromatography. RPLC: Reversed-phase liquid

911 chromatography. SAME: S-adenosyl-L-methionine. NADH: Nicotinamide adenine

912 dinucleotide. β -NADPH: β -Nicotinamide adenine dinucleotide phosphate.

913

914 Fig. 5. Circular heatmap showing the coverage of the standard mixture with the final

915 optimized methods. Positive ionization mode is shown in a blue-based color pallet and

916 negative ionization mode in a green-based color pallet.

917 SAME: S-adenosyl-L-methionine. NADH: Nicotinamide adenine dinucleotide. β -NADPH:

918 β -Nicotinamide adenine dinucleotide phosphate.

919

920 Fig. 6. Extracted ion chromatograms of panel standards from various metabolic classes. The

921 standards were detected using the optimized methods in negative electrospray ionization

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

923

924 Fig. 7. Boxplot with relative standard deviation (RSD) for QC pooled of *in vitro* (HepaRG
925 extracellular (EC) and intracellular (IC) extracts) and *in vivo* (human plasma and urine)
926 samples.

927 mRSD: Median relative standard deviation. ESI: Electrospray ionization.

928 **10. Legend to Tables**

929

930 Table 1. Scoring system to evaluate peak shape, peak intensity and retention time. The scoring
931 system was used for each analytical panel standard to guide the choice of LC column and the
932 method optimization.

933 LC: Liquid chromatography. FWHM: Full width at half maximum. S/N: Signal-to-noise ratio.

934 RT: Retention time.

935

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