

A split-range acquisition method for the non-targeted metabolomic profiling of human plasma with hydrophilic interaction chromatography - high-resolution mass spectrometry

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23 ABSTRACT

24

25 Untargeted metabolomics of human plasma with mass spectrometry is of particular interest in 26 medical research to explore pathophysiology, find disease biomarkers or for the 27 understanding of the response to pharmacotherapy. Since analytical performances may be 28 impacted by the laboratory environment and the acquisition method settings, the objectives of 29 this study were to assess the role of interfering compounds and to propose an acquisition 30 method to maximise the metabolome coverage for human plasma metabolomic analysis. 31 Human plasma samples were processed with liquid/liquid extraction then analysed with 32 HILIC-high resolution mass spectrometry. A method with a single m/z range was compared to 33 four methods with different split acquisition ranges and four sets of ionization source 34 parameters were compared. The data were analysed with the R software and on the 35 Worklow4Metabolomics online platform. 36 The major interfering compounds were identified in blank samples where they accounted for 37 up to 86% of the signal intensity. Splitting the acquisition range into 3 m/z ranges improved 38 the number of detected features, the number of features with proposed annotation in the Human Metabolome Database, as well as signal intensity throughout the whole m/z range. 39 40 The method performing best was the one using three m/z ranges of approximatively the same 41 extent. Ionization source parameters also strongly affected the number of detected features. 42 Splitting the acquisition range into 3 m/z ranges with optimised ionisation source parameters 43 allows a comprehensive analysis of the human plasma metabolome with perspectives for 44 applications to pathophysiological studies. 45

46 KEYWORDS: metabolomics; high-resolution mass spectrometry; hydrophilic interaction
47 liquid chromatography; human plasma

48 1. INTRODUCTION

49 Metabolomics is a phenotyping approach interested in molecules located downstream (in 50 biological terms) of those targeted by established genomics, transcriptomics and proteomics 51 approaches. It provides novel insights into mechanisms of disease pathogenesis and is useful 52 for the discovery of biomarkers for disease diagnosis, prognosis or for the prediction of the 53 response to pharmacotherapy in the era of precision medicine [1, 2]. As for the above-54 mentioned classical "omics" technologies, untargeted approaches are best suited for the 55 discovery of biomarkers and mass spectrometry (MS) has become the most convenient 56 analytical platform for the sensitive and comprehensive metabolomic profiling of human 57 tissues and fluids, especially with the expansion of high-resolution instruments. Plasma is the 58 biofluid of choice for human studies, it is easy to collect and reflects the systemic metabolism. 59 It contains thousands of metabolites of varying abundance, molecular weight, polarity, water 60 solubility and ionization states which may each require specific analytical conditions to be 61 detected. Therefore, the interest of combining hydrophilic interaction liquid chromatography 62 (HILIC) and reverse-phase (RP) liquid chromatography to improve the coverage of plasma 63 metabolome has already been reported [3-6]. Although the aim of the analysis is to focus only 64 on molecules present in the plasma sample, the performance of untargeted analysis may also 65 be impacted by interfering compounds from the laboratory environment, i.e. in the materials, 66 reagents or in the analytical instruments as a residue from previous analytical runs. Well-67 described interferences are proteins, solvents, polymers, plastics and additives such as 68 detergents or ion pairing reagents, which are known to affect MS analysis [7]. Optimal 69 acquisition parameters should therefore both maximize the detection of compounds of interest 70 and minimize the influence of contaminants. However, despite the widespread use of 71 metabolomic analysis, the influence of exogenous contaminants and the optimisation of mass 72 spectrometry parameters for the metabolomic analysis of human plasma has never been

investigated to date. The objectives of the present study were first to assess the main
interfering compounds in a laboratory setting then to propose an analytical strategy to
maximize the number of compounds detected in human plasma. HILIC was retained for
chromatography since it was described to have the widest serum metabolome coverage [3].

78 2. MATERIALS AND METHODS

79 2.1.Reagents

80 LC-MS-grade ammonium formate and formic acid (98%) were supplied by Sigma Aldrich

81 (Saint Quentin Fallavier, France). LC-MS-grade methanol, acetonitrile, chloroform and water

82 were from Fisher Scientific (Illkirch, France). Deuterated internal standards (IS) were from

83 Bertin technologies (Montigny le Bretonneux, France) (anandamide-d₄, 25-

84 hydroxycholesterol-d₆, 2-arachidonoylglycerol-d₈) and LCG standards (St. Louis, MO, USA)

85 (hydrocortisone-d₄, glyburide-d₃, fludrocortisone-d₂, testosterone-d₃, propoxyphene-d₅,

86 chlorpromazine-d₃ and fluoxetine-d₆). Plasma was obtained from the local blood bank

87 (Etablissement Français du Sang, Rungis, France).

88

89 2.2.Sample preparation

90 One hundred microliters of plasma were spiked with $10 \,\mu$ L of the mix of deuterated internal

91 standards (anandamide-d₄, 25-hydroxycholesterol-d₆, 2-arachidonoylglycerol-d₈,

92 hydrocortisone- d_4 and glyburide- d_3 , 0.5 mg L⁻¹ and fludrocortisone- d_2 , testosterone- d_3 ,

93 propoxyphene- d_5 , chlorpromazine- d_3 and fluoxetine- d_6 , 0.05 mg L⁻¹) then subjected to

94 liquid/liquid extraction using 1 mL of methanol/chloroform/water (1:2:1 v/v/v) [8]. The

95 mixture was vortexed for 30 seconds, agitated for 20 mins and centrifuged at 10000 rpm for 5

96 mins. 200 µL of upper phase were collected and dried under vacuum. The dried extracts were

97 reconstituted with 75 μ L of ammonium formate/acetonitrile (20:80 ν/ν) and transferred into

98 injection vials for analysis. Plasma samples from 3 different healthy donors were analyzed in

99 triplicate in each experimental condition together with blank samples consisting in mobile

100 phase only. Samples were injected in random order.

101

102 **2.3.Chromatographic separation**

103 Chromatography was performed with an UltiMate 3000 system (Thermo Scientific Dionex,

- 104 Les Ulis, France). A SeQuant[®] 4.6 mm x 150 mm, 5 μm i.d. ZIC-pHILIC column (AIT
- 105 France, Houilles, France) was used for chromatography [9]. Separation was performed under
- 106 gradient elution using a mobile phase system consisting of ammonium formate 10 mM pH 3.8
- 107 (solvent A) and acetonitrile (solvent B). Starting conditions were 95% solvent B for 3 min
- 108 then reaching 8% at 25 min. This plateau was kept for 5 more minutes, then back to 95% and
- 109 equilibration for 10 minutes. Flow rate was 0.3 mL min⁻¹ and oven temperature 40°C.
- 110

111 **2.4.High-resolution mass spectrometry analysis**

112 Compounds were detected with a Q-Exactive mass spectrometer (Thermofisher) equipped

113 with a heated electrospray ionization (HESI) source. Nitrogen (N2-45 nitrogen generator,

114 VWR International, Fontenay sous bois, France) was employed as sheath and auxiliary gas.

115

116 2.4.1. Source parameters

The HESI source was set in the positive ionization mode with a capillary temperature of
275°C. Unless otherwise stated, sheath and auxiliary gas flow rate were set at 40 and 25
arbitrary units, respectively; S-lens RF level at 100, spray voltage at 4000 V and temperature
of vaporization at 100°C.

121

122 2.4.2. Acquisition parameters

In the first part of the study, samples were analyzed using a single full-scan m/z range (60-900) to identify the main analytical interferences. Then, four other acquisition methods were evaluated to assess the influence of splitting the scan range at masses of the main interfering compounds. Comparison was performed between the full-scan method (m/z 60-900, method M1) and different split methods (Figure 1): method M2 (3 m/z ranges, m/z 60-101; 105-213; 128 215-900), method M3 (3 *m/z* ranges, *m/z* 60-81; 85-213; 215-900), method M4 (3 *m/z* ranges,

129 *m/z* 60-82; 82-213; 213-900) and method M5 (3 *m/z* ranges, *m/z* 60-300; 300-600; 600-900).

130 Resolution was set at 70000, AGC target at 10^6 and maximum IT at 256 ms. Data acquisition

- 131 was performed using Chromeleon v 6.80 and Xcalibur v3.0.63 (Thermofisher).
- 132

148

133 2.5.Data analysis

134 XCalibur RAW files were first converted to mzML and centroidized with msConvert [10],

then processed using IPO [11] and XCMS (v1.50.1) [12] packages running under R. The

136 CentWave algorithm [13] was used for automatic peak picking: ppm = 10, snthresh = 10,

137 peakwidth = 15-70, mzdiff= 0.01 and prefilter = c(3, 5000), with the peakwidth parameter

138 optimized with IPO. Alignment step was performed using the group.density method (bw = 5,

139 mzwid = 0.01, minfrac = 0.5) and retcor.loess method (missing = $n_{blank+1}$, extra = 1). The

140 peak table obtained at this step therefore contained the total number of peaks in study

141 samples. Then, several filters were applied to exclude non-relevant peaks (based on analytical

142 criteria) detected by XCMS [14] (Supplementary Figure 1): first, robust peaks were defined as

143 peaks found in ≥ 2 of the samples of at least one condition; then all features with a retention

144 time outside the window 3-30 min were discarded (retention time filter) and features detected

145 in biological samples with a mean intensity less than 2-fold the intensity observed in blank

146 samples, or features detected in blank samples only were also filtered out (signal filter). This

147 group therefore constitutes the "robust, relevant peaks" group. The peak picking score (PPS)

149 each method. PPS was defined as the ratio of reliable peaks (RP) (which are peaks part of an

obtained with the IPO algorithm was used to assess the quality of the peak list obtained which

150 isotopologue consisting of 13 C isotope peaks) weighted by the exponential factor 2, to the

151 total number of peaks ((number of reliable peaks)²/(total number of peaks)). Therefore, if the

- 152 RP value and the total number of relevant peaks increase by the same amount, the PPS
- 153 increases. All these filtering steps and peak definitions are shown in Supplementary Figure 1.

155 3. RESULTS AND DISCUSSION

156 **3.1.Evaluation of interferences**

157 Interferences originating from the laboratory environment and unrelated to plasma samples 158 were assessed by the examination of ions present in blank samples (Figure 2). Three profiles 159 were found for the most abundant interfering ions: intermittent interferences occurring at a 160 specific retentions time (m/z 102.1274 and 104.1066); interferences present during a 161 significant part of the run (m/z 167.0123 and 214.0892) and interferences present during the 162 whole run (m/z 83.0602). The signal intensity for those interfering compounds was between 10^7 and 4.10^9 , which accounted up to a maximum of 85.7% of the total signal. The major 163 164 interferences were identified as being acetonitrile (m/z 83.0602), triethylamine (m/z 102.1274) 165 and n-butyl benzenesulfonamide (m/z 214.0892). Acetonitrile originates from mobile phase, 166 triethylamine was previously used on the same instrument and is well-described to have a 167 persistent memory effect in LC-MS [15], and n-butyl benzenesulfonamide is a widespread 168 plasticizer [7]. Considering the signal intensity observed for these molecules, the C-trap 169 should be filled faster with interferences, which in turn should decrease the sensitivity for 170 compounds of interest. Therefore, the effect of splitting the acquisition m/z range was 171 examined to avoid the acquisition of the main interfering compounds and possibly enhance 172 the detection of metabolites.

173

174 **3.2.Effect of excluding interfering compounds from acquisition**

The m/z of the most intense intermittent interferences (m/z 102.1274 and 104.1066) as well as from n-butyl benzenesulfonamide (m/z 214.0892) were first excluded from the acquisition method (Figure 1; M2 acquisition method). The comparison of the triplicate analysis of plasma samples with the original M1 method and this modified M2 method resulted in the increase of the mean total number of peaks from 5,366 to 15,304 peaks, with a corresponding 180 increase of robust, relevant peaks from 2,892 to 8,340 peaks (Table 1). 368 peaks were 181 specific of the M1 method; 2,210 of the M2 method while 3,086 were common to both 182 methods (Supplementary Table 1). The peaks specific to the M2 method were equally 183 distributed over all the m/z range and mostly detected in the first 10 min or between 15-20 184 min of the analytical run (Figure 3A). 185 Then, the m/z ranges of the interference detected during the whole run (acetonitrile, m/z186 83.0602) as well as from n-butyl benzenesulfonamide (m/z 214.0892) were excluded (Figure 187 1, method M3). For this M3 method, the total number of peaks was 14,848, corresponding to 188 8,203 robust, relevant peaks. 2,019 peaks were specific of the M3 method (compared to M1; 189 Supplementary Table 1). As for method M2, these peaks were equally distributed over the m/z190 range and mostly detected in the first 10 min or between 15-20 min of the analytical run 191 (Figure 3B). Altogether, these results suggest that excluding interfering m/z from the 192 acquisition allows a better detection of the sample compounds all over the m/z -and time-

193 ranges.

194

195 **3.3.Effect of** *m*/*z* **range splitting**

196 Since splitting the m/z range systematically increased the number of peaks detected, we 197 addressed whether this observation was due to the exclusion of interferences or if it was the 198 consequence of splitting the acquisition method into several m/z ranges. The method M2 was 199 therefore compared to method M4 where the acquisition range was split into three continuous 200 ranges, without any gap between each range (Figure 1). As shown in Table 1, both methods 201 allowed the detection of an equivalent number of total and robust, relevant peaks and 5,441 202 features were common to both methods (Supplementary Table 1). This suggests that splitting 203 the scan range is more useful for increasing the number of detected compounds than 204 excluding specific interferences.

205

206 **3.4.Effect of** *m*/*z* **range size**

207 Finally, in order to fully assess the impact of m/z range splitting, a new M5 method was 208 designed, where the m/z interval of each of the 3 split ranges was between 240-300 units 209 (Figure 1). Optimizing the size of m/z ranges increased the number of robust, relevant peaks 210 from 8,272 to 11,005 (33% increase in comparison to the M4 method), as shown in Table 1. 211 4,781 peaks were common to both methods whereas 1,764 peaks were specific of the M5 212 method. The gain in the number of peaks detected is mainly in the m/z range from 300 to 900 213 (Supplementary Table 1 and Supplementary Figure 2). With this latest M5 method, the PPS 214 score was the highest obtained among the 5 methods. The mass spectrometric fundamentals 215 explaining this phenomenon for Q-Exactive instruments are related to the value of the voltage 216 applied to the focusing lenses (RF) of the C-trap. This voltage is automatically adapted to the 217 smallest mass value of the scan by the instrument and cannot be modified. For example, for a 218 scan of 60 to 900 m/z it is set to allow optimal extraction of small mass ions, and therefore the 219 extraction of high mass ions will not be optimal. By dividing the same mass range into 3 220 segments (60-300, 300-600 and 600-900 m/z), and thus into 3 consecutive scans, an RF value 221 is applied for each segment and depends on the smallest mass in the segment. A first value is 222 therefore optimized for masses close to 60, a second for masses close to 300 and a third for 223 masses close to 600. In addition, for scans from 300 to 600 and from 600 to 900 m/z, the 224 quadrupole filters all masses below 300 and 600 and therefore decreases the background noise generated by the mobile phase (or other contaminants), which allows the detection of more 225 226 peaks of interest. Thus, the performance of using several consecutive scans on complementary 227 mass ranges for methods intended to cover large mass ranges is better, because the value of 228 the RF applied to the C-trap will be optimized for each scan and will allow a better extraction 229 of the ions from each scan.

Splitting into 2 or 3 m/z ranges was also compared. With a method consisting in 2 m/z ranges (60-200 and 200-900), the number of relevant, robust peaks was 5,854, which is about 3-fold lower than with the 3-scan range method.

233

3.5.Consequences on metabolite annotation

Although detecting a higher number of robust, reliable peaks demonstrates increased sensitivity, understanding pathophysiological mechanisms with metabolomic analysis makes it mandatory to annotate (i.e. identify) the compounds of interest. Therefore, the number of annotated compounds after HMDB database query was compared between the initial M1 and the final M5 methods. For the M1 method, compound identification was proposed for 1,633 of the relevant, robust peaks, raising to 4,397 for the M5 method *i.e.* a 169% increase in the number of peaks with proposed annotation.

242

243 **3.6.**Consequences on signal intensity for metabolites or deuterated standards

244 Finally, although splitting the m/z range obviously increases the number of detected features, 245 the impact of splitting on the signal intensity of known and unknown compounds was 246 assessed. For deuterated internal standards, mean peak area of triplicate analysis of 3 plasma 247 samples with the M5 method was $126 \pm 52\%$ [49-275] of the mean peak area observed with 248 the M1 method. When comparing the signal intensity of features detected with the initial M1 and final M5 method, a mean 1.6-fold increase in intensity was observed, with 25^{th} and 75^{th} 249 250 percentiles at 0.95 and 1.85, respectively (Figure 4). This increase occurred for all 3 m/z251 ranges and was even greater for the highest m/z, whereas splitting into 2 m/z ranges may 252 induce a loss of signal in the low m/z range [16].

253

3.7.Ionization source parameters

255 The parameters of the ESI strongly influence the number of detected features, as shown with 256 the metabolomic analysis of plant samples [17], and should therefore be optimized for each 257 experimental setting. To assess whether compound detection could be further improved in 258 human plasma with optimizing ionization source parameters, four sets of parameters were 259 compared with low, medium, high or intermediate values for S-Lens RF level, spray voltage, 260 sheath and auxiliary gas flow rate and temperature of vaporization (Table 2). Results are 261 shown in Table 3. The intermediate set of parameters provided the highest number of robust, 262 relevant peaks and highest PPS score.

264 CONCLUSIONS

265 Scientists employing high-resolution instruments for untargeted mass spectrometry analysis, 266 such as metabolomic studies, face new challenges with respect to the simultaneous recording 267 of a huge number of features in a given m/z range, which may be either background noise or 268 compounds of interest. Metabolites are considered to be molecules with a molecular weight 269 less than 1,000 Da, which implies to cover a mass range between about 50 and 1,000 Da to 270 ensure the widest coverage. In the vast majority of methods described to date, a single 271 acquisition range as well as generic ionization source parameters were used. Although a 272 strategy for splitting the acquisition range into a low and high m/z ranges was previously 273 investigated for the analysis of renal cell metabolome with reverse-phase chromatography 274 [16], no investigation with an interference-driven splitting strategy was reported to date for 275 the analysis of human plasma. The results of the present study show the interest of splitting 276 the m/z acquisition range into 3 segments for the metabolomic analysis of human plasma with 277 hydrophilic interaction liquid chromatography coupled to high-resolution mass spectrometry. 278 Since acquisition range splitting outperforms excluding the acquisition of the main 279 interferences, such strategy may be universally applied, regardless of the contaminants that 280 may be present in each laboratory environment. The performance gain affects both the 281 number of compounds detected and also the number of molecules for which an annotation can 282 be proposed by querying the databases, which is the final goal of the metabolomic analysis. In 283 addition, sensitivity was also improved throughout the entire m/z range whereas different 284 splitting strategies may induce a loss of signal [16]. In combination with the optimization of 285 the ionization source parameters, the proposed method therefore allows a more 286 comprehensive analysis of the human plasma metabolome which may be applied to 287 pathophysiological studies.

TABLES

290	Table 1: Number	of peaks	in each	method	before	and after	filtering.
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Method	Total peaks in blank sample (n)	Total peaks in study samples (mean [min-max])	Robust peaks (mean [min-max])	RT-filtered robust peaks (mean [min-max])	Robust, relevant peaks (signal- and RT-filtered robust peaks) (mean [min-max])	PPS
M1	1,879	5,366 [5,060-5,669]	4,689 [4,528-4,982]	3,970 [3,758-4292]	2,892 [2,615-3,315]	3,283
M2	6,876	15,304 [14,557-15,762]	12,858 [11,832-13,751]	10,612 [9,745-11,362]	8,340 [7,472-9,128]	6,707
M3	6,960	14,848 [13,932-15,445]	12,515 [11,620-13,132]	10,357 [9,535-10,920]	8,203 [7,443-8,827]	6,874
M4	7,983	14,721 [14,130-15,197]	12,473 [11,531-12,963]	10,287 [9,386-10,769]	8,272 [7,387-8,851]	6,847
M5	9,068	18,697 [17,695-19,296]	16,076 [15,098-15,592]	12,874 [11,933-13,377]	11,005 [10,146-11,542]	8,162

293 RT: retention time; PPS: peak picking score (the highest the better)

Table 2: Sets of parameters of the ionization source

Parameter	Low	Medium	Intermediate	High
S-lens RF level	50	75	100	100
Spray voltage	1,500	3,000	4,000	5,000
Sheath gas flow rate	10	35	40	60
Aux gas flow rate	5	15	25	30
Temperature of vaporization	100	200	100	300

Method	Total peaks in blank sample (n)	Total peaks in study samples (n)	Robust peaks (n)	RT-filtered robust peaks (n)	Robust, relevant peaks (signal- and RT-filtered robust peaks) (n)	PPS
Low	2,938	7,211	5,836	5,199	3,220	2,629
Medium	11,807	20,259	12,755	9,184	5,272	3,576
Intermediate	7,010	16,889	13,625	10,314	6,588	4,153
High	8,376	19,187	14,510	11,026	5,895	2,678

302 RT: retention time; PPS: peak picking score (the highest the better)

FIGURE LEGENDS

306	Figure 1: Schematic overview of the different acquisition methods. <i>m/z</i> splitting ranges
307	are depicted for each method. $SR = scan range$.
308	
309	Figure 2: Extracted ion chromatograms of interfering compounds. Each colour
310	corresponds to a plasma sample from a different donor.
311	
312	Figure 3: Comparison of features between the M1 and M2 (A) and M1 and M3 (B)
313	acquisition methods. m/z and retention time are represented for each feature (after retention
314	time and signal filtering), for features specific to each method and features common to both
315	methods. The green, blue and red dots represent the 3 plasma samples which were analysed in
316	triplicates.
317	
318	Figure 4: Intensity ratio for features common to the M5 and M1 methods. The M5/M1
319	ratio (log scale) are shown as a function of m/z . The horizontal dashed gray line represents the
320	identity line, the horizontal black line represents the mean, vertical grey lines represent the
321	m/z ranges of the M5 method.

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376 COMPETING INTERESTS STATEMENT

- The authors have nothing to declare.
- 378

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Figure 1



Figure 2

EIC around m/z = 83.0602 ± 10ppm EIC around m/z = 124.0866 ± 10ppm 1.5e+08 6e+07 1.0e+08 Intensity [counts] Intensity [counts] 4e+07 5.0e+07 2e+07 0.0e+00 ę 0e 2000 0 500 1000 1500 500 1000 1500 2000 0 Retention time [seconds] Retention time [seconds] EIC around m/z = 102.1274 ± 10ppm EIC around m/z = 167.0123 ± 10ppm 4e+08 4e+07 3e+08 3e+07 Intensity [counts] Intensity [counts] 2e+08 2e+07 1080 1100 1120 1e+08 404 1e-0e+00 ş 0 0 500 1000 1500 2000 500 1000 1500 2000 0 Retention time [seconds] Retention time [seconds]

EIC around m/z = 104.1066 ± 10ppm











Figure 4



m/z