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ABSTRACT

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

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

1. INTRODUCTION

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

73 investigated to date. The objectives of the present study were first to assess the main
74 interfering compounds in a laboratory setting then to propose an analytical strategy to
75 maximize the number of compounds detected in human plasma. HILIC was retained for
76 chromatography since it was described to have the widest serum metabolome coverage [3].
77

2. MATERIALS AND METHODS

2.1.Reagents

LC-MS-grade ammonium formate and formic acid (98%) were supplied by Sigma Aldrich (Saint Quentin Fallavier, France). LC-MS-grade methanol, acetonitrile, chloroform and water were from Fisher Scientific (Illkirch, France). Deuterated internal standards (IS) were from Bertin technologies (Montigny le Bretonneux, France) (anandamide-d_4, 25-hydroxycholesterol-d_6, 2-arachidonoylglycerol-d_8) and LCG standards (St. Louis, MO, USA) (hydrocortisone-d_4, glyburide-d_3, fludrocortisone-d_2, testosterone-d_3, propoxyphene-d_5, chlorpromazine-d_3 and fluoxetine-d_6). Plasma was obtained from the local blood bank (Etablissement Français du Sang, Rungis, France).

2.2.Sample preparation

One hundred microliters of plasma were spiked with 10 µL of the mix of deuterated internal standards (anandamide-d_4, 25-hydroxycholesterol-d_6, 2-arachidonoylglycerol-d_8, hydrocortisone-d_4 and glyburide-d_3, 0.5 mg L^{-1} and fludrocortisone-d_2, testosterone-d_3, propoxyphene-d_5, chlorpromazine-d_3 and fluoxetine-d_6, 0.05 mg L^{-1}) then subjected to liquid/liquid extraction using 1 mL of methanol/chloroform/water (1:2:1 *v/v/v*) [8]. The mixture was vortexed for 30 seconds, agitated for 20 mins and centrifuged at 10000 rpm for 5 mins. 200 µL of upper phase were collected and dried under vacuum. The dried extracts were reconstituted with 75 µL of ammonium formate/acetonitrile (20:80 *v/v*) and transferred into injection vials for analysis. Plasma samples from 3 different healthy donors were analyzed in triplicate in each experimental condition together with blank samples consisting in mobile phase only. Samples were injected in random order.

2.3.Chromatographic separation

Chromatography was performed with an UltiMate 3000 system (Thermo Scientific Dionex, Les Ulis, France). A SeQuant® 4.6 mm x 150 mm, 5 µm i.d. ZIC-pHILIC column (AIT France, Houilles, France) was used for chromatography [9]. Separation was performed under gradient elution using a mobile phase system consisting of ammonium formate 10 mM pH 3.8 (solvent A) and acetonitrile (solvent B). Starting conditions were 95% solvent B for 3 min then reaching 8% at 25 min. This plateau was kept for 5 more minutes, then back to 95% and equilibration for 10 minutes. Flow rate was 0.3 mL min^{-1} and oven temperature 40°C.

2.4.High-resolution mass spectrometry analysis

Compounds were detected with a Q-Exactive mass spectrometer (Thermofisher) equipped with a heated electrospray ionization (HESI) source. Nitrogen (N2-45 nitrogen generator, VWR International, Fontenay sous bois, France) was employed as sheath and auxiliary gas.

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.

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;

215-900), method M3 (3 *m/z* ranges, *m/z* 60-81; 85-213; 215-900), method M4 (3 *m/z* ranges, *m/z* 60-82; 82-213; 213-900) and method M5 (3 *m/z* ranges, *m/z* 60-300; 300-600; 600-900). Resolution was set at 70000, AGC target at 10^6 and maximum IT at 256 ms. Data acquisition was performed using Chromeleon v 6.80 and Xcalibur v3.0.63 (Thermofisher).

2.5.Data analysis

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 CentWave algorithm [13] was used for automatic peak picking: ppm = 10, snthresh = 10, peakwidth = 15-70, mzdiff= 0.01 and prefilter = c(3, 5000), with the peakwidth parameter optimized with IPO. Alignment step was performed using the group.density method (bw = 5, mzwid = 0.01, minfrac = 0.5) and retcor.loess method (missing = n_blank+1, extra = 1). The peak table obtained at this step therefore contained the total number of peaks in study samples. Then, several filters were applied to exclude non-relevant peaks (based on analytical criteria) detected by XCMS [14] (Supplementary Figure 1): first, robust peaks were defined as peaks found in ≥2 of the samples of at least one condition; then all features with a retention time outside the window 3-30 min were discarded (retention time filter) and features detected in biological samples with a mean intensity less than 2-fold the intensity observed in blank samples, or features detected in blank samples only were also filtered out (signal filter). This group therefore constitutes the “robust, relevant peaks” group. The peak picking score (PPS) obtained with the IPO algorithm was used to assess the quality of the peak list obtained which each method. PPS was defined as the ratio of reliable peaks (RP) (which are peaks part of an isotopologue consisting of ^{13}C isotope peaks) weighted by the exponential factor 2, to the total number of peaks ((number of reliable peaks)² /(total number of peaks)). Therefore, if the

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

3. RESULTS AND DISCUSSION

3.1.Evaluation of interferences

Interferences originating from the laboratory environment and unrelated to plasma samples were assessed by the examination of ions present in blank samples (Figure 2). Three profiles were found for the most abundant interfering ions: intermittent interferences occurring at a specific retentions time (*m/z* 102.1274 and 104.1066); interferences present during a significant part of the run (*m/z* 167.0123 and 214.0892) and interferences present during the 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 interferences were identified as being acetonitrile (*m/z* 83.0602), triethylamine (*m/z* 102.1274) and n-butyl benzenesulfonamide (*m/z* 214.0892). Acetonitrile originates from mobile phase, triethylamine was previously used on the same instrument and is well-described to have a persistent memory effect in LC-MS [15], and n-butyl benzenesulfonamide is a widespread plasticizer [7]. Considering the signal intensity observed for these molecules, the C-trap should be filled faster with interferences, which in turn should decrease the sensitivity for compounds of interest. Therefore, the effect of splitting the acquisition *m/z* range was examined to avoid the acquisition of the main interfering compounds and possibly enhance the detection of metabolites.

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/z*
186 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/z*
190 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
225 generated by the mobile phase (or other contaminants), which allows the detection of more
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.

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.

3.6.Consequences on signal intensity for metabolites or deuterated standards

Finally, although splitting the *m/z* range obviously increases the number of detected features, the impact of splitting on the signal intensity of known and unknown compounds was assessed. For deuterated internal standards, mean peak area of triplicate analysis of 3 plasma samples with the M5 method was 126 ± 52% [49-275] of the mean peak area observed with 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} percentiles at 0.95 and 1.85, respectively (Figure 4). This increase occurred for all 3 *m/z* ranges and was even greater for the highest *m/z*, whereas splitting into 2 *m/z* ranges may induce a loss of signal in the low *m/z* range [16].

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.
263

CONCLUSIONS

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

TABLES

Table 1: Number of peaks in each method before and after filtering.

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

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

295 **Table 2**: Sets of parameters of the ionization source

296

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

297

298

299 **Table 3**: Effect of ionization source parameters on peak detection

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

301

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

303

FIGURE LEGENDS

Figure 1: Schematic overview of the different acquisition methods. *m/z* splitting ranges are depicted for each method. SR = scan range.

Figure 2: Extracted ion chromatograms of interfering compounds. Each colour corresponds to a plasma sample from a different donor.

Figure 3: Comparison of features between the M1 and M2 (A) and M1 and M3 (B) acquisition methods. *m/z* and retention time are represented for each feature (after retention time and signal filtering), for features specific to each method and features common to both methods. The green, blue and red dots represent the 3 plasma samples which were analysed in triplicates.

Figure 4: Intensity ratio for features common to the M5 and M1 methods. The M5/M1 ratio (log scale) are shown as a function of *m/z*. The horizontal dashed gray line represents the identity line, the horizontal black line represents the mean, vertical grey lines represent the *m/z* ranges of the M5 method.

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375

COMPETING INTERESTS STATEMENT

The authors have nothing to declare.

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Figure 1

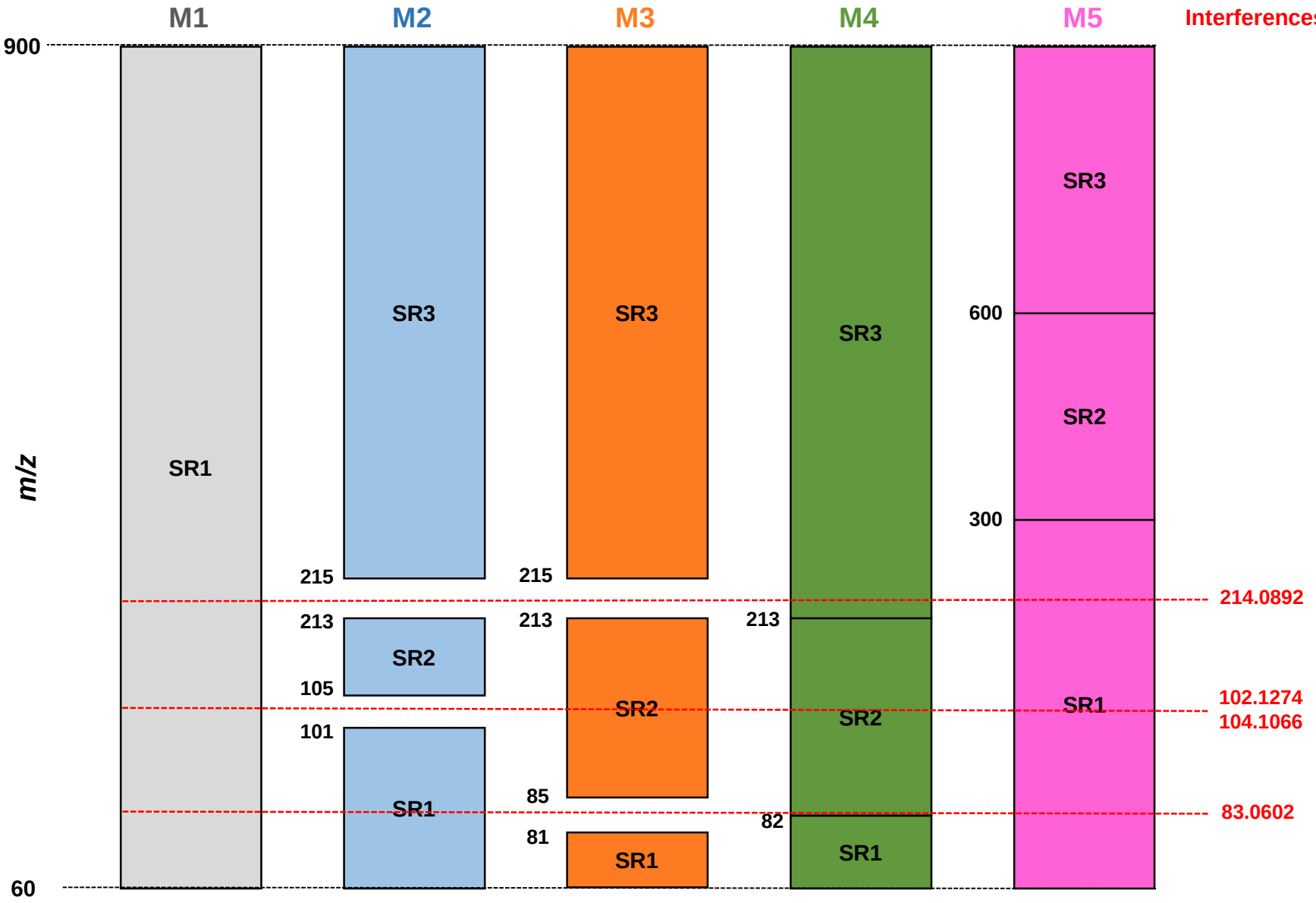

M1
M2
M3
M4
M5
Interferences:
900
60
m/z
SR1
SR3
215
213
SR2
105
101
SR1
SR3
215
213
SR2
85
81
SR1
SR3
213
SR2
82
SR1
SR3
600
SR2
300
SR1
214.0892
102.1274
104.1066
83.0602

Figure 2

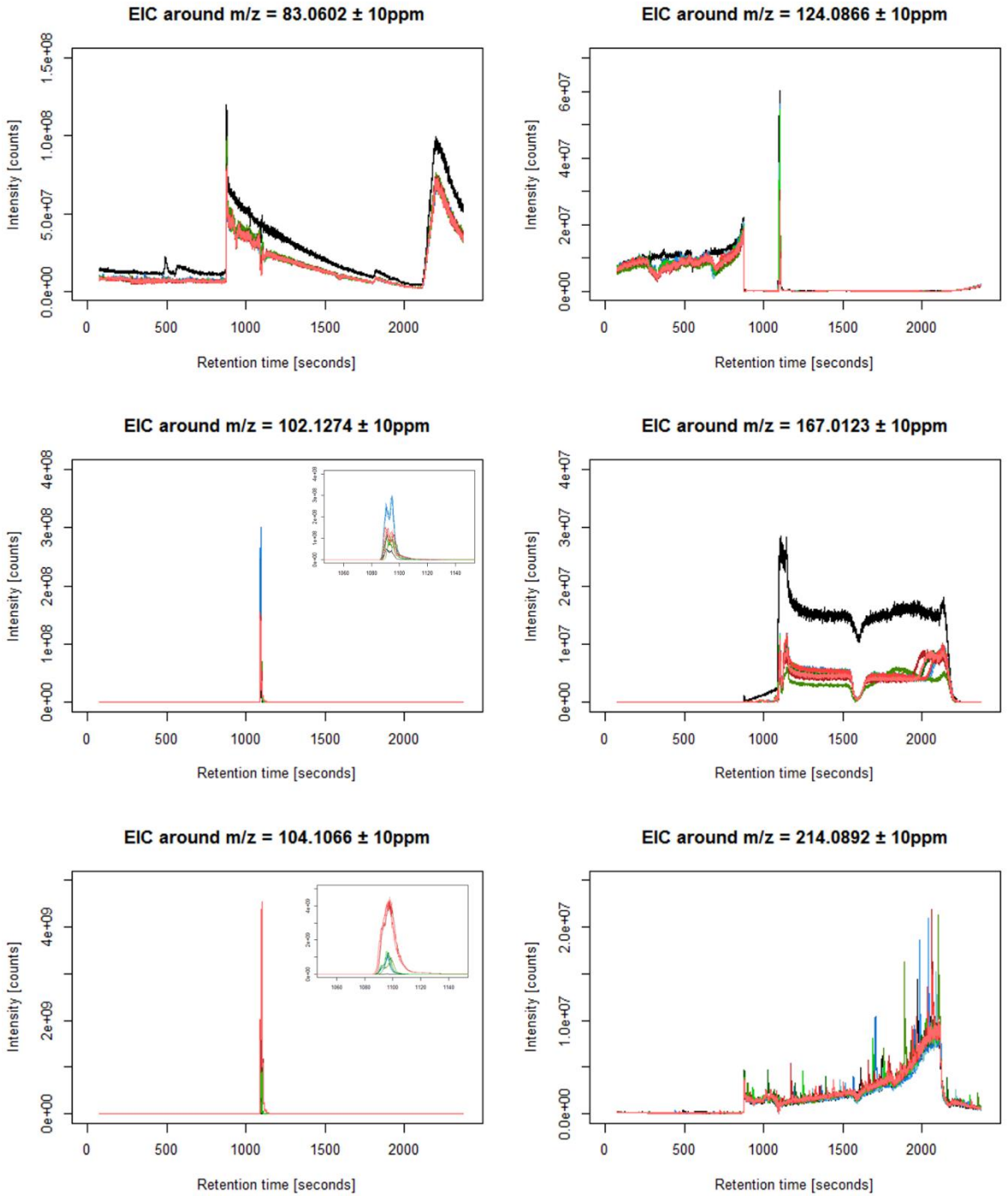

EIC around m/z = 83.0602 ± 10ppm
Intensity [counts]
0.0e+00
5.0e+07
1.0e+08
1.5e+08
0
500
1000
1500
2000
Retention time [seconds]
EIC around m/z = 124.0866 ± 10ppm
Intensity [counts]
0e+00
2e+07
4e+07
6e+07
0
500
1000
1500
2000
Retention time [seconds]
EIC around m/z = 102.1274 ± 10ppm
Intensity [counts]
0e+00
1e+08
2e+08
3e+08
4e+08
0
500
1000
1500
2000
Retention time [seconds]
EIC around m/z = 167.0123 ± 10ppm
Intensity [counts]
0e+00
1e+07
2e+07
3e+07
4e+07
0
500
1000
1500
2000
Retention time [seconds]
EIC around m/z = 104.1066 ± 10ppm
Intensity [counts]
0e+00
2e+09
4e+09
0
500
1000
1500
2000
Retention time [seconds]
EIC around m/z = 214.0892 ± 10ppm
Intensity [counts]
0.0e+00
1.0e+07
2.0e+07
0
500
1000
1500
2000
Retention time [seconds]

Figure 3

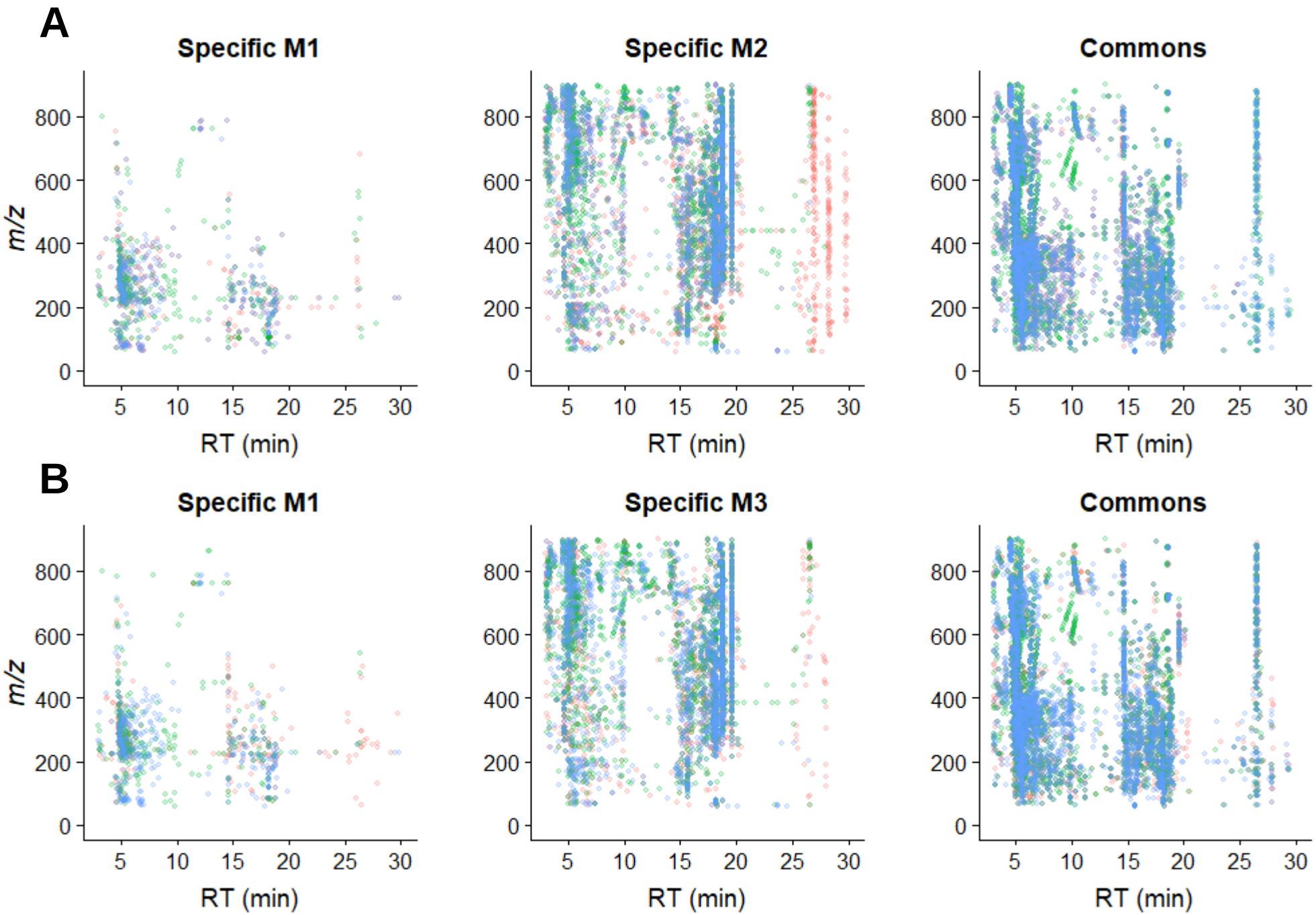
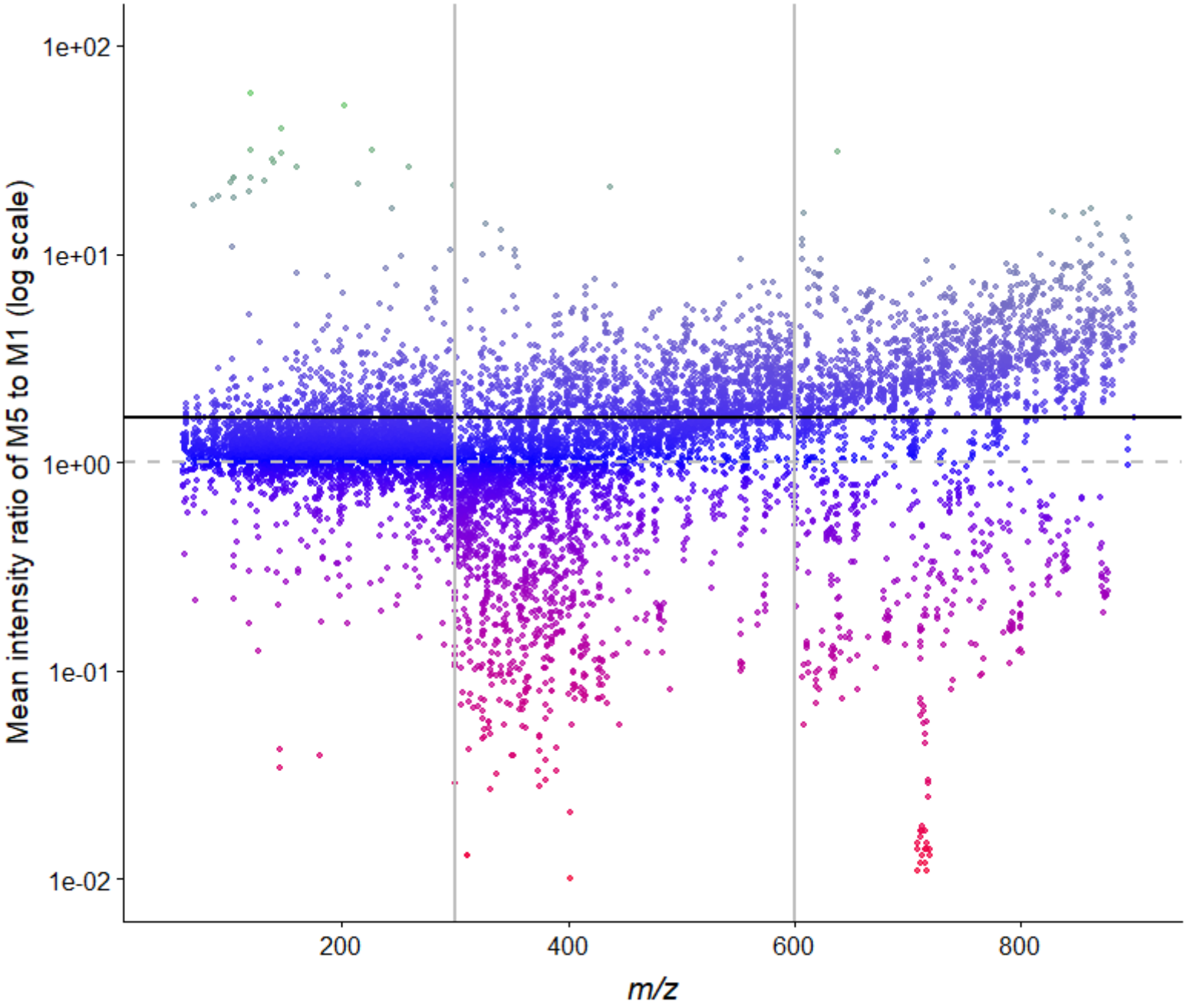

A
Specific M1
Specific M2
Commons
B
Specific M1
Specific M3
Commons
m/z
RT (min)
0
200
400
600
800
5
10
15
20
25
30

Figure 4


1e+02
1e+01
1e+00
1e-01
1e-02
Mean intensity ratio of M5 to M1 (log scale)
200
400
600
800
m/z