1	Full paper
2	Comparison of a GC-Orbitrap-MS with Parallel GC-FID Capabilities for
3	Metabolomics of Human Serum
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23	Short Title: GC-MS and GC-FID based metabolomics of human serum
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33 Abstract

34 Gas chromatography mass spectrometry (GC-MS) platforms for use in high throughput and discovery metabolomics have heavily relied on time of flight (ToF), and low resolution 35 quadrupole and ion trap mass spectrometers and are typically run in electron ionization (EI) 36 modes for matching spectral libraries. Traditionally, detectors such as flame ionization detection 37 38 (FID), have also helped in identification and quantification of compounds in complex samples for diverse clinical applications, i.e., fatty acids. We probed if combination of FID in line with a 39 40 high-resolution instrument like a GC-Orbitrap-MS may confer advantages over traditional mass 41 spectrometry using EI.

42 We used a commercially available human serum sample to enhance the chemical space of serum using an advanced high resolution mass spectrometry (HR-MS) platform (QExactive Orbitrap-43 44 MS) with an FID feature for confident metabolite identification to assess the suitability of the 45 platform for routine clinical metabolomics research. Using the EI mode, we quantified 294 metabolites in human serum using GC-Orbitrap-MS. These metabolites belonged to 89 46 biological pathways in KEGG. Following a sample split, using an in-line FID analysis, 1117 47 peaks were quantified. Moreover, representative peaks from FID and their corresponding MS 48 49 counterparts showed a good correspondence when compared for relative abundance.

50 Our study highlights the benefits of the use of a higher mass accuracy instrument for untargeted 51 GC-MS-based metabolomics not only with EI mode but also orthogonal detection method such 52 as FID, for robust and orthogonal quantification, in future studies addressing complex biological 53 samples in clinical set ups.

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Keywords: metabolomics; flame ionization detection; Orbitrap; electron ionization; GC-MS;
serum; high resolution mass spectrometry.

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61 **1. Introduction**

Metabolomics is the comprehensive study and systematic quantification of small molecules in 62 the molecular weight range of 50-2000 Daltons in biological samples (cells, tissues, organs, 63 64 biofluids, or whole organisms), and thus, complements efforts from other high throughput omics 65 platforms such as genomics, transcriptomics, and proteomics as an indispensable platform. Platforms for generating metabolomics data typically include gas and liquid chromatography, or 66 capillary electrophoresis linked with mass-spectrometry (GC-MS, LC-MS, and CE-MS), and 67 spectroscopy approaches [such as nuclear magnetic resonance (NMR), infrared (IR), Raman] that 68 69 have helped address diverse biological questions allowing to connect the genotype with molecular phenotype (1). Particularly, gas chromatography mass spectrometry (GC-MS) is very 70 amenable to polar primary metabolites (such as sugars, amino acids, amines, sugar phosphates, 71 72 or sugar alcohols) (2) and fatty acids, in addition to excellent chromatographic resolution, thus lending itself to routine quantitative metabolomic applications (2). Newer high resolution (HR) 73 74 instruments such as Orbitrap mass spectrometers are capable of providing sub-ppm mass accuracy at high mass resolutions (i.e., > 60,000), and hence allow calculation of predicted 75 molecular formulas based on the mass defect of a detected metabolite ion (3-5), and generate 76 mass spectral data at high resolving power with mass accuracies <1 ppm However, these HRGC-77 78 MS platforms have found limited applications till date, baring handful applications in microbial 79 metabolomics (6) and a recent use in non-human primate biofluid (i.e., baboon) serum 80 metabolomics (7).

81 Most studies have used (GC)-high resolution accurate mass (HRAM) mass spectrometers only in electron ionization (EI) mode of operation. However, flame ionization detection (FID) is without 82 83 a doubt the most often used gas chromatography (GC) detection method, a technology which dates back to early 1960s and finds applications ranging from analysis of hydrocarbons to fatty 84 85 acids. When a full spectrum is recorded using mass selective detector (MSD) during a chromatographic run, sensitivity is often inferior in a MS detector when compared to a FID (8). 86 A combination of GC-MS and gas chromatography-flame-ionization detection (GC-FID) also is 87 an old idea, typically run independently and/ or in parallel, with its roots going back to the 1960s. 88 Then the chromatograms are manually aligned and then peaks were partitioned into bins 89 90 according to retention time values. Unfortunately, comparisons between chromatograms by MS

and by GC detectors are difficult since the results vary depending on the samples and the carbon atoms in the molecules being analyzed. FID is sensitive for compounds containing carbon, and its sensitivity is better than thermal conductivity detector (TCD). Previous studies have claimed that GC-FID is considered to be more reliable and sensitive for quantitative analysis than GC– MS, while GC–MS can provide more definite qualitative information and biomolecule identification (9). GC-FID is also considered more sensitive, more reproducible and covers a wider dynamic range when compared to GC-MS in full scan monitoring mode (10).

Recent studies have applied GC-FID in cataloging the human serum metabolomes as a 98 complimentary technique to GC-MS, LC-MS and NMR (11). However, GC-FID and GC-MS or 99 100 LC-MS as parallel methods have only been used in analysis of bacterial metabolites (12), for 101 targeted fatty acid and lipid characterization in human plasma (13), fecal volatile characterization (14), and transgenic rice metabolism (15) among others. However, none of these analyses were 102 103 performed using HRMS equipped with both FID and MS detectors that used the same samples at the same time. Combined TLC/GC-FID analysis when compared to GC-MS as the two methods 104 105 for analysis of human serum lipids, allowed identification and quantification of only eight 106 metabolites in common (arachidonic acid, eicosanoic acid, linoleic acid, oleic acid, palmitelaidic 107 acid, palmitic acid, stearic acid and tetradecanoic acid) (11), suggesting significant 108 complementarity of FID and MS analysis of the human metabolome. Previously, ethanol, 109 methanol, and formate concentrations were measured by headspace GC-FID analysis in vitreous 110 and blood samples collected postmortem (16).

111 As can be seen, most of these efforts used GC-FID and GC-MS as two independent approaches one after another and not in-line. To our knowledge, studies have not attempted to characterize 112 113 the complex biological matrixes of clinically relevant samples such as human serum, and to show their joint application in clinical metabolomics, and rather have only been used for targeted 114 chemical constituents such as drugs, pesticides, and organic exogenous chemicals. Our study is 115 the first attempt to identify and quantify serum metabolites using a high mass resolution gas 116 117 chromatography mass spectrometer (GC-Orbitrap-MS) with two detectors (FID and MS) on a 118 comparative basis. The principal aims of this study were to assess the capabilities of GC-FID 119 analysis in parallel to a GC-Orbitrap-MS analysis for quantification and identification of

- 120 metabolites in human serum as a test sample, in order to exploit the full capabilities of these two
- 121 detectors and instrument for untargeted clinical metabolomics.
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123 **2.Materials and Methods**

124 **2.1 Chemicals**

Solvents such as acetonitrile, isopropanol, and pyridine were of HPLC grade, and methoxyamine
hydrochloride (MeOX), 1% TMCS in *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA),
and adonitol, were obtained from Sigma-Aldrich, St. Louis, USA.

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129 2.2 Human serum sample

130 Human serum (Cat. No. H6914, from a male AB clotted whole blood, USA origin, sterile-

131 filtered) was obtained from Sigma-Aldrich, St. Louis, USA.

132 **2.3 Serum sample extraction and derivatization for GC-MS and GC-FID analysis**

Serum samples (30 µL) were subjected to sequential solvent extraction once each with 1 mL of 133 acetonitrile: isopropanol: water (3:3:2, v/v) ratio and 500 μ L of acetonitrile: water (1:1, v/v) ratio 134 mixtures at 4 °C(17). Adonitol (5 µL from 10 mg/ml stock) was added to each aliquot as an 135 internal standard prior to solvent extraction. The pooled extracts (~ 1500μ L) from the two steps 136 were dried under vacuum at 4 °C and parallel extractions performed on empty microcentrifuge 137 tubes only served as extraction blanks to account for background (extraction conditions, 138 derivatization reagents) noise and other sources of contamination (septa, liner, column, vials, 139 140 handling among others). Blanks were intermittently used to see that no carryovers occurred 141 during randomized run orders and to manually filter out contaminating chemicals from the combined list of features obtained from the blanks. Samples were then sequentially derivatized 142 with methoxyamine hydrochloride (MeOX) and 1% TMCS in N-methyl-N-trimethylsilyl-143 trifluoroacetamide (MSTFA) as described elsewhere (7, 18, 19). Steps involved addition of 10 144 µL of MeOX (20 mg/mL) in pyridine, followed by incubation under shaking at 55 °C for 60 min 145 followed by trimethylsilylation at 60 °C for 60 min after adding 90 µL MSTFA as described (2, 146 147 7).

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149 **2.4 GC-Orbitrap-MS instrument parameters**

A robotic arm TriPlusTM RSH autosampler (Thermo Fisher ScientificTM, Bremen, Germany) 150 151 injected 1µL of derivatized sample into a Programmable Temperature Vaporizing (PTV) injector 152 at initial temp of 90 °C to a transfer temp of 290 °C on TRACETM 1310 gas chromatograph 153 (Thermo Fisher Scientific[™], Austin, TX). Helium carrier gas at a flow rate of 1.6 mL/min was 154 used for separation on a Thermo Fisher ScientificTM TG-5MS (60 m length \times 0.25 mm i.d. \times 0.25 155 µm film thickness) column. The initial oven temperature was held at 90 °C for 0.5 min, followed by an initial gradient of 10 °C/min ramp rate to 250 °C, where it was held for 5 min, and a 156 157 gradient of 5 °C/min ramp rate to 295 °C. The final temperature was 295 °C and was held for 35 min. Eluting peaks were transferred through an auxiliary transfer temperature of 250 °C into a Q 158 159 ExactiveTM-GC mass spectrometer (Thermo Fisher ScientificTM, Bremen, Germany). The mass spectrometer has a resolving power (RP) of 120,000 full width at half maximum (FWHM) at m/z 160 200 with EI or CI capabilities. From the ion source, an AQT quadrupole is used for precursor ion 161 isolation, which leads into the Orbitrap mass analyzer. Electron ionisation (EI) at 70 eV energy, 162 emission current of 50 µA with an ion source temperature of 230 °C was used in all experiments. 163 164 A filament delay of 5.3 min was selected to prevent excess reagents from being ionized. High resolution EI spectra were acquired using 60,000 resolution (FWHM at m/z 200) with a mass 165 range of *m*/*z* 50-650. 166

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168 **2.5 GC-FID analysis**

GC-FID (**Supplementary Figure S1**) analysis was accomplished on the TRACETM 1310 gas chromatograph (Thermo Fisher ScientificTM, Austin, TX). The detector temperature was set at $305 \,^{\circ}$ C where the ignition threshold was 0.5 Pa, airflow of 350 mL/min, hydrogen flow 35 mL/min., and makeup gas 30 mL/min. All other analytic conditions including the column type and column temperature, the injection temperature, splitless injection conditions, carrier gas and the linear velocity were the same as those of GC–MS analysis.

For both analyses, the acquisition sequence started with blank solvent (pyridine) injections, followed by randomized lists of extraction blanks (B), reagent blanks (R), solvent (pyridine-P), and samples (S), where sequences of B, R, and P were injected at scheduled intervals for monitoring shifts in retention indices (RI) as well as serving as system quality control (QC)checks.

180 **2.6 GC-Orbitrap-MS data processing**

Acquired data was processed using Thermo Fisher ScientificTM TraceFinderTM 4.1 (Thermo 181 Fisher Scientific, Bremen, Germany) software for untargeted analysis. Initial analysis of 182 collected spectra included baseline correction, peak filtering, quantification, assignment of a 183 unique mass and retention indices, signal-to-noise calculation, and compound identification 184 based on the mass spectral pattern as compared to EI spectral libraries. Spectral libraries 185 consulted included: NIST Mass Spectral Reference Library (NIST14/2014; National Institute of 186 Standards and Technology, USA), the Wiley Registry of Mass Spectra - 11th Edition, the MSRI 187 spectral libraries from Golm Metabolome Database (20) available from Max-Planck-Institute for 188 Plant Physiology, Golm, Germany (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html), 189 MassBank (21), MoNA (Mass Bank of North America, (http://mona.fiehnlab.ucdavis.edu/) and a 190 191 vendor supplied high resolution (HR)-MS mass spectral library for the GC-MS dataset using 192 proprietary TraceFinder[™] software (Thermo Fisher Scientific) and MS-DIAL software ver. 3.51 193 (22) for additional searches, visualization and spectral matching. Further, to filter out noise and 194 less confident compounds, we discarded all compounds with a CV > 30 %. Further, all *siloxane*, halogen-derivatives, phthalate, acrylate, and silvloxy, borane, dioxolan, and silan, silox, -195 196 derivative compounds were removed from the list manually. For the MS platform, metabolite 197 annotation and assignment followed the metabolomics standards initiative (MSI) guidelines for 198 metabolite identification (23), with Level 2 identification based on spectral database match 199 (match factor >80%) and Level 3 identification where only compound groups were known (specific ions and RT regions of metabolites). 200

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202 **2.7 Data sharing**

The raw datasets and the metadata obtained from both the platforms are deposited at the Metabolomics Workbench (Study ID: **ST001037**) which are available for download at this link: <u>https://bit.ly/2PIFIW9 (pending publication date)</u>.

- 206
- 207 2.8 Statistical analysis

208 Statistical processing of both GC-FID and GC-MS data sets were performed using statistical 209 software R (Version 3.5.1) (24). Imputed, outlier removed, and scaled peak areas representative 210 of relative metabolite amounts obtained using DeviumWeb (25) are presented. Univariate and multivariate analysis: Hierarchical clustering analysis (HCA) was performed on Pearson 211 212 distances using PermutMatrix (26). The raw metabolite abundance values were Z-score normalized, and the color scale represents +2 (high) to -2 (low) abundance in the heat map. 213 214 Correlations reported are Pearson correlations which were visualized as heat maps, based on Z-215 score normalized data ranging from +1 (positive, red), 0 (no correlation, black), and -1 (negative, 216 green) correlation of metabolite abundance across biological and technical replicates. Partial least squared discriminant analyses (PLSDA) were performed using MetaboAnalyst 3.0 (27) and 217 DeviumWeb (25) where the output displayed score plots to visualize the sample groups. The data 218 were scaled with unit variance without any transformation. 219

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221 **2.9 Pathway enrichment analysis**

222Pathway enrichment was performed using MetaboAnalyst 3.0 (www.Metaboanalyst.ca) (27). For223IDconversions,theChemicalTranslationService(CTS:224http://cts.fiehnlab.ucdavis.edu/conversion/batch) was used in batch mode to convert the common225chemical names into their KEGG, HMDB, Metlin, PubChem CID, and ChEBI identifiers.

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227 **3. Results and Discussion**

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3.1 Comparison of metabolites and peaks from MS and FID detectors

230 We previously reported on the analysis of non-human primate serum from a baboon using HR-231 GC-MS alone (7). Here, we expanded our metabolomics analysis to human serum, and compared 232 two orthogonal detection techniques attached to a GC, a MS and a FID detector. Quantitation 233 using a FID is simple, as FID is a mass-sensitive detector that provides a nearly equal molar response to the number of carbon atoms in a hydrocarbon where the detector is fast, and response 234 is linear over a wide dynamic range (~ 10^7 to 10^8) (28). A comparison of the FID-chromatogram 235 and total ion chromatogram (TIC) from MS analysis are provided (Figure 1). The extracted FID 236 data (filtered) (Supplementary Table S1) and the annotated MS-data (Supplementary Table 237

238 **S2**) are provided. Furthermore, given that there was a solvent delay time used for MS detector, only peaks from 8 to 60 min. (i.e., total 52 min.) were considered for FID peak quantification as 239 240 well, to only compare peaks/metabolites in affixed retention time windows. We ran five individual serum aliquots (n=5) with three technical replicates each, generating 15 runs and 241 242 corresponding data files. For MS-based analysis, 2765 metabolites were detected at least once across all the samples (including blanks, reagent blanks, and solvent), which were reduced to 298 243 244 compounds that passed all the quality filters described above. However, the S/N criteria used for FID and MS analysis are not comparable as they are different detection methods, and hence, the 245 246 total number of confident peaks called were very different from both the analysis. For FIDanalysis, about 1117 peaks were quantified with retention times across all samples (with < 50%247 missing values). The median and mean CVs for FID were at 73.43 and 87.90%, whereas those 248 for MS were 43.77 and 51.44%. Thus, with stringent filtering criteria, such as retaining only 249 250 compounds/ peaks with < 30% RSD, we retained 298 metabolites in the MS analysis and 83 such 251 peaks in the FID analysis. A previous study using fatty acid methyl ester (FAME) analysis showed that 28 FAME standards tested provided similar results for the novel GC-EI-MS-SIM 252 253 method and GC-EI-MS in the full scan mode, both of which were slightly worse than GC-FID 254 analysis (29).

255 When we performed hierarchical clustering (HCA) analysis of the top 50 features (either peaks 256 from FID or metabolites abundances from MS) from the two platforms, the results reveal a 257 clearer separation of sample groups (blanks vs samples) for the FID analysis (Figure 2 A, B) 258 when compared to those obtained from MS analysis. Similarly, a metabolite-metabolite Pearson 259 correlation analysis for peak and metabolite abundances revealed clearer clusters (two such 260 modules) for the FID data (Figure 3 A, B) as opposed to the MS data where the clusters are 261 diffused. When performing a supervised PLS-DA analysis, the FID data explained the clusters 262 better [cumulative score for the first two PCs (PC1, PC2) = 71%] when compared to the MS-data [cumulative score for the first two PCs (PC1, PC2) = 23%] (Figure 3 C, D). 263

We further evaluated the linear correspondence of the quantified compounds based on FID and MS results. We obtained good correlations for randomly handpicked compounds such as glucose, alanine, citric acid, and an unknown, as an example, with correlation scores ranging from 0.99 to 0.89, and fitting linear regression models (**Figure 4 A-D**). Nonetheless, in 268 comparative analysis of volatile compounds in virgin olive oil, it was demonstrated that good 269 selectivity, linearity and higher upper values of the working range are the main advantages of 270 solid-phase microextraction (SPME)-GC-FID versus low bottom values of working ranges, better sensitivity and lower limits of detection and quantification of SPME-GC-MS (30). In 271 272 another study, no differences associated to particular functional groups were observed between GC-FID and GC-MS, except for the acids, for which working range is much better for GC-FID 273 274 (30). Also, one-dimensional GC using FID may be sufficient to define biomarker ratios; however, if the samples are too complex, interferences from coeluting compounds will 275 276 complicate the analysis (31) (Bai et al., 2018).

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278 **3.2 Analysis of human serum using FID and HR MS**

279 We analyzed the HRGC-MS data from human serum for its relevance to both clinical and biological analysis. Of the 294 metabolites quantified, we obtained 56 metabolites as 280 281 trimethylsilylated derivatives and 238 others that were not derivatized (Supplementary Table **S2**). Of these, 133 metabolites were assigned KEGG IDs belonging to various human metabolic 282 pathways. These metabolites included S-adenosyl-L-methionine, adenosine monophosphate, S-283 adenosyl-L-homocysteine, glucose, alanine, lysine, formic acid, arginine, serine, tryptophan, 284 285 phenylalanine, urea, 5-phosphorylribose 1-pyrophosphate, biotin, histidine, proline, citric acid, 286 benzoic acid, valine, and threonine. The significantly higher number of metabolites detected in our current efforts, compared to our earlier analysis of a baboon serum sample (7) is attributed to 287 288 a longer run time of 60 minutes as opposed to the shorter protocol of 23 minutes in the earlier 289 study. Moreover, there are species specific metabolite differences among primate tissues (32) 290 and biofluids. Another 16 metabolites matched KEGG IDs belonging to drugs (i.e., lisinopril, 291 atazanavir, amisulpride, metergoline phenylmethyl ester, alfuzosin decanedioic acid, dibutyl 292 ester, aliskiren, zopiclone, bezafibrate, sulpiride, carbachol, risperidone, ranitidine, indapamide, 293 droperidol). The list also included 41 metabolites that were assigned a LIPIDMAPS ID. These quantified metabolites belonged to 89 various metabolic pathways (and 35 pathways with at least 294 295 3 metabolites mapped onto each of them), such as methylhistidine metabolism, thiamine 296 metabolism, glycine and serine metabolism, glucose-alanine cycle, biotin metabolism, carnitine 297 synthesis, transfer of acetyl groups into mitochondria, urea cycle, methionine metabolism,

homocysteine degradation, alanine metabolism among others (Supplementary Table S3). 298 299 Recently, GC-FID combined with precolumn derivatization with isobutyl chloroformate was 300 used for confident determination of nucleobases guanine, adenine, cytosine, and thymine from DNA samples (33). Other studies have focused on detection of the food chemicals, i.e., caffeine 301 302 in coffee grains using GC-FID as well (34). A very recent analysis of a reference material, NIST Standard Reference Material (SRM) 2378 fatty acids in frozen human serum using methods 303 304 NIST-1 and NIST-2 that use GC-FID and GC-MS platforms, respectively, revealed expanded 305 uncertainties for 12 fatty acids and reference values with expanded uncertainties for an additional 306 18 fatty acids (35).

307 Conversely, one cannot map peaks obtained from FID analysis for pathway mapping analysis or 308 enrichment analysis, without access to individual chemical/metabolite standards. However, as mentioned in the previous section, the robust quantification obtained from FID data is 309 310 advantageous for better quantification when compared to MS-based analysis only. Given that the past FID analysis efforts relied on FAME analysis for metabolite profiling, future analysis can 311 312 potentially expand on this detection method to use such integrated workflows as the one 313 described in this manuscript. However, robust software tools and analysis workflows that can 314 seamlessly integrate FID and MS-data in real time or offline, are clearly missing.

315 Nonetheless, both detectors represent a flexible tool for explorative studies and, if supported by 316 appropriate data-processing tools, would appear to be useful in any metabolic profiling study, as 317 was shown using 28 standard compounds including 17 amino acid standards and in CSF samples 318 with simultaneous acquisition with both MS and FID detectors (36). It was also reported that limit of detection (LOD) and limit of quantification (LOQ) are significantly lower for GC-319 320 APCI/ToF-MS than for GC-FID. Moreover, the quantitative response of the FID detector is free from ionization bias and those biases introduced by the type of mass analyzer or the instrumental 321 design of a mass spectrometer. Consequently, FID gives a better overall quantitative 322 representation in complex biological samples where traditional MS analysis often is challenged 323 324 by ion interference effects (36). Further, in a comparison of the non-esterified or free fatty acids 325 quantitative results between the TLC/GC-FID and the GC-MS platforms demonstrated that the 326 GC-MS concentrations of palmitic acid, vaccinic acid, oleic acid, linoleic acid, dihomo-ylinolenic acid and docosapenta-(4,7,10,13,16)-enoic acid are generally higher than those 327

measured by TLC/GC-FID (11) indicating higher sensitivity and detector-bias as far as MS is concerned.

Our study suffers from several limitations that we clearly recognize, esp. with lower sample size for this proof-of-concept study to demonstrate the applicability of the dual-detector platform for clinical metabolomics studies. Secondly, there are other biofluids such as plasma, saliva and even tissue or cell samples from humans that could be informative for further screening for comparison of those datasets on this new platform. Other complimentary approaches such as high resolution LC-MS/MS or even other detectors such as thermal conductivity detector (TCD) and electron capture detector (ECD) among a host of others would be worth exploring.

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338 **4. Conclusions**

We demonstrated the advantages of a combined GC-FID and HRGC-MS analyses when compared to results obtained from the individual platforms, and how this can boost analytical biochemistry and downstream metabolomics applications. It remains a challenge, like any other untargeted metabolomics platform, to consolidate and align features detected using FID and MS for reliable quantification. We also propose that such instruments which lend the capabilities of detectors that work on different principles would be helpful for correct identification of compounds, especially when standards are available.

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347 Author contributions

BBM, EB, and MO designed the research; BBM and EB performed the experiments; MO provided essential reagents and materials, BBM and EB analyzed the data, BBM and MO wrote the manuscript, and BBM interpreted the data and has the primary responsibility for the final content and edits. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

353 Human and Animal Rights and Informed Consent

- 354 This article does not contain any studies with human or animal subjects performed by any of the
- authors, but only a de-identified commercially available human serum sample.

356 **Research funding**

357 None declared.

358

359 **Employment or leadership**

360 None declared.

361

362 Honorarium

363 None declared.

364

365 **Conflict of interest**

366 The authors wish to confirm that there are no known conflicts of interest associated with this

- 367 publication and there has been no significant financial support for this work that could have
- 368 influenced its outcome.

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469 Figure Legends

470

471 Figure 1. Comparison of chromatograms for human serum sample and representative HR-

472 GC-MS spectra. Chromatograms derived from FID analysis, and a total ion chromatogram

obtained from MS analysis, and XIC (m/z 353.17774; unknown) at 5 ppm accuracy are shown.

474 The HRGC-EI-MS spectra of six representative compounds are (A) 2-deoxytetronic acid, (B)

475 methionine, (C) glutamic acid, (D) phenylalanine, (E) lauric acid, and (F) aminomalonic acid.

Figure 2. A two-way hierarchical clustering heat map of the serum metabolome (top 50 peaks as obtained from PLS-DA analysis) data for (A) MS detector and (B) FID detector. Each column displays the metabolic pattern of individual samples (extraction blanks, solvents, reagent blanks, and samples). Amount of each peak in individual samples is expressed as relative value obtained by Z-normalization and is represented by the color scheme, in which red and blue indicate high and low concentrations of metabolites, respectively. Rows: samples; Columns: metabolites.

Figure 3. Metabolite-metabolite Pearson correlation map for peak areas for (A) MS analysis and (B) FID-detected peaks. Amount of each peak in individual samples is expressed as relative value obtained by Z-normalization and is represented by the color scheme, in which red and blue indicate high and low values for respectively, for peaks (FID) and metabolites (MS). Supervised PLS-DA analysis for (C) MS analyzed compounds and (D) FID-detected peaks.

489 Figure 4. Scatter plots (fitting linear regression models) for FID (x-axis) and MS data (yaxis) for showing linearity in their response factors for all the samples. (A) Glucose 490 491 [Correlation coefficient (r): 0.9999; Sample size: 13; Intercept (a): -363496920.727; Slope (b): 1.08; Regression line equation: y=1.08x-363496920.727], (B) Alanine (Correlation coefficient 492 493 (r): 0.958; Sample size 15, Intercept (a): 65761895.608, Slope (b): 0.232, Regression line equation: y=65761895.608+0.232x], (C) Citric acid [Correlation coefficient (r): 0.993; Intercept 494 (a): 229891680.564, Slope (b): 2.059, Regression line equation: v=229891680.564+2.059x) (D) 495 Unknown (Correlation coefficient (r): 0.8954) ; Sample size 15; Intercept (a): -37477621.483; 496 Slope (b): 13462.916, Regression line equation: y=13462.916x-37477621.483]. 497

- **Supplementary Table S1.** Peak lists obtained for GC-FID analysis.
- **Supplementary Table S2.** List of metabolites captured using MS data.
- **Supplementary Table S3.** Pathway enrichment analysis for the MS quantified metabolites.
- 501 Supplementary Figure S1. The GC-FID detector (Thermo Fisher Scientific).









