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1 **GC-MS BASED METABOLOMICS OF COLON CANCER CELLS USING**
2 **DIFFERENT EXTRACTION SOLVENTS**

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14 **ABSTRACT**

15 The increasing incidence of colorectal cancer enforces the development of novel
16 methodologies and protocols to deepen in the molecular mechanisms that govern disease
17 pathophysiological events. The aim of this work is to deepen in the optimum metabolite
18 extraction protocol from adherent mammalian cells of colon cancer for high throughput
19 metabolomics using gas chromatography coupled to mass spectrometry (GC-MS). GC-MS
20 results showed that metabolic information obtained from colon cancer cells was highly
21 dependent on metabolite extraction selection, which at the same time is extremely
22 influenced by the analytical platform. A further purpose of this investigation is to uncover
23 an unexplored portion of HT-29 colon cancer cells metabolome, complementary to other
24 already explored by CE-MS and LC-MS methods. At this respect, a total of a large number
25 of 150 metabolites were identified in HT-29 colon cancer cells by GC-MS. In addition,
26 metabolite extraction protocol was observed to be crucial for the determination of
27 potentially interesting clusters of metabolites. In summary, the extraction protocol with
28 acetonitrile involving ACN-isopropanol-water was shown to be the most appropriate
29 extraction procedure for fatty acids and/or related pathways analysis among the four
30 metabolic extraction procedures. Most of the metabolites involved in pathways of amino
31 acids, glutathione, amino sugars and other polar metabolites present higher performance of
32 extraction in the were better extracted with acidified water extract, although metabolic
33 species in water extraction showed the best overall reproducibility. Although pathways
34 involving nitrogenous bases could be investigated using organic or aqueous extracts, a
35 higher number of more metabolites involved in these pathways can be identified in
36 the aqueous extracts. In addition, metabolite extraction protocol was observed to be crucial
37 for the determination of potentially interesting clusters of metabolites.

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39 **Keywords:** Colon cancer, Sample treatment, Metabolomics, gas chromatography-mass
40 spectrometry, GC-MS.

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42 **Abbreviations:** CRC, colorectal cancer; MSEA, metabolite set enrichment analysis; MPA,
43 metabolic pathway analysis; MSTFA, N-methyl-N-(trimethylsilyl)-trifluoroacetamide;
44 ORA, overrepresentation analysis; PBS, phosphate buffered saline; PCA, principal
45 component analysis; RIM, internal retention index markers; TIC, total ion chromatogram

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9 46 1. INTRODUCTION

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11 47 In the past few years, cancer biology research has increasingly been focused on metabolism
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13 48 in cancer cells [1]. The complexity and diversity of biological alterations inherently linked
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15 49 to cancer metabolism is in accordance with the increasing unresolved issues to determine
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17 50 specific causes in cancer development, to assess the progression and to unravel molecules
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19 51 or pathways to target in cancer therapy and/or prevention. It is known that cancer cells
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21 52 show different metabolism from healthy cells. One of the well-known metabolic alterations
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23 53 in cancer cells is the glycolysis utilization over the oxidative phosphorylation. ~~Normal cells~~
24
25 ~~rely on glycolysis followed by oxidative phosphorylation to generate ATP molecules while~~
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27 ~~cancer cells use glycolytic pathway for rapid proliferation and biosynthesis. This~~
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29 ~~dependence upon aerobic glycolysis, also known as Warburg effect, is the best-accepted~~
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31 ~~alteration of tumour cells~~ [2, 3]. In the past years, other perturbations in specific metabolic
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33 57 pathways have been addressed. ~~Widespread alterations in the metabolism of cancer cell are~~
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35 58 linked to energy metabolism [4] such as pyruvate production [5] and tricarboxylic acid
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37 59 (TCA) cycle [6]. ~~Normal healthy cells suffer from complex biochemical changes to be~~
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39 60 ~~transformed into tumour cells, not well elucidated yet. Widespread cancer cells~~
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41 61 ~~characteristics include special needs such as high energy status or increased biomolecules~~
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43 62 ~~generation among others, suggesting that cancer disease encompasses heterogeneous and~~
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45 63 ~~complex parts of the metabolism [1].~~ The progress of Metabolomics offers valuable
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47 64 opportunity to better understand biochemical changes produced in cancer metabolism for
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49 65 improving early detection, progression and therapy monitoring of cancer disease [7].
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51 66 Metabolomics is able to meet the challenge of detecting hundreds of metabolites in short
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53 67 time analysis thanks to the evolution of analytical technologies and software tools for data
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69 processing and statistics [8]. With the evolution of metabolomics platforms, rapid and
70 comprehensive analyses of the metabolome complexity can be performed in a high-
71 throughput manner with improved sensitivity, accuracy and resolution [1]. Typical
72 metabolomic studies are based on mass spectrometry (MS) or nuclear magnetic resonance
73 (NMR) whose advantages and limitations have been discussed elsewhere [9, 10]. It is now
74 assumed that the coverage of the human metabolome is impossible to achieve with a single
75 analytical methodology and an increasingly common practice is the combination of
76 analytical techniques to achieve complementary information [11]. Metabolomics potential
77 has already been shown with the detection of novel biomarkers involved in different
78 metabolic pathways related to breast [12], liver [13], prostate [14], colon [15] or lung
79 cancer [16] among the vast amount of investigations. From those publications it can be
80 assumed that different cancer subtypes exhibit different phenotypes and therefore show
81 different metabolic alterations and biomarkers. This diversity enforces the investigation of
82 cancer with special attention to most worldwide harmful cancer types.

83 Colorectal cancer is the second leading cause of cancer mortality in the USA [17] and
84 Europe [18] with a continuous increasing incidence. Identifying soon the colon cancer
85 apparition and/or progression will increase our knowledge on efficient drug discovery
86 research and prevention. With this aim, in the last decade, Metabolomics has emerged to
87 understand pathophysiological processes related to colon cancer. From the about 30 works
88 related to colon cancer Metabolomics published so far, a minor part have been
89 accomplished using NMR [19-23] with a clear trend toward the use of MS-based analytical
90 platforms either alone [24-26] or in combination with separation techniques such as

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91 capillary electrophoresis (CE) [27-30], liquid chromatography (LC) [24, 27-29, 31-34] and
92 gas chromatography (GC) [15, 31-33, 35-44].

93 Metabolomics investigations of colorectal cancer by GC-MS are summarized in Table [S1](#).

94 As can be seen in Table [S1](#) a variety of samples including biofluids, tissues and cell culture
95 models have been under scrutiny. ~~The minimum invasive nature with relative ease of
96 sampling and less limited volumes result in a predominance of biofluids over the other
97 samples types for metabolomics studies. From those sample types, cell culture presents
98 unique advantages such as sample supply of live human cells, easy control of experimental
99 factors and much higher reproducibility in studies inter-laboratories.~~ The use of cell

100 cultures is included in common reference model systems and is considered an invaluable
101 biomedical research tool. However, the application of cell culture in metabolomics requires
102 further development and standardization of study design steps, metabolism quenching
103 method selection and optimization of metabolite extraction protocols, among others [45,
104 46]. Namely, quenching step at the time of harvesting aims to prevent metabolic content to
105 be altered before the analysis so that the metabolic state of the cell is preserved. There is a
106 great controversy and continuous research in the determination of the optimum quenching
107 method. Until date the most widely spread techniques to quench the metabolism in cells is a
108 shock maintaining the cells below -20 °C [47, 48], the addition of cold solvents such as cold
109 methanol [49], use of cold isotonic PBS [49-51] or the combination in a single step of
110 quenching and extraction procedures in mammalian adherent cells [45, 52].

111 As can be observed in Table [S1](#), three cell lines (namely SW, HT and Caco-2) have been
112 used to investigate colon cancer following metabolomics approaches using GC-MS. All
113 these cell lines are very well established for in-vitro studies of colorectal cancer and vary in

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114 phenotype, growth rate, differentiation and morphology among other characteristics [53,
115 54]. Metabolic fingerprinting by GC- MS from two SW cell lines (SW-1116 and SW-480)
116 was first reported by Zimmermann et al., [35]. More recently LC-MS and CE-MS based
117 metabolomics have been used to investigate HT-29 cell line revealing significant metabolic
118 information in colon cancer metabolism after treatment with different polyphenol-rich
119 extracts [28, 29, 55].

120 Considering that no single method is appropriate for the determination of all intracellular
121 metabolites, metabolic information obtained from cell cultures will depend on multiple
122 factors and metabolite extraction selection is highly influenced by the analytical platform
123 [56]. Optimization and selection of the protocol for metabolite extraction from HT-29 cells
124 [57] and evaluation of the subsequent cytosol sample treatment [30] for CE-MS
125 metabolomics have been previously carried out. CE-MS is particularly suited for the rapid
126 separation of ionic, weakly ionic, and/or highly polar metabolites and aqueous solvent was
127 the preferred for metabolite extraction of HT-29 cells [57]. On the contrary, GC-MS is
128 preferred for the analysis of less polar, volatile (or amenable to chemical derivatization)
129 compounds and requires thermal stability of the analytes. Considering that no single
130 analytical platform is able to determine all intracellular metabolites, in this work, a GC-MS
131 method has been developed due to the high complementarity nature of GC-MS used in this
132 work and the CE-MS [57] and LC-MS [28, 29] methods already developed by our group.
133 Further, taking into account all the above considerations, in this work GC-MS has been
134 used to evaluate the coverage and reproducibility of four metabolic extraction solvents
135 selected ~~attending to~~according to the physicochemical diversity of metabolites including the
136 large variation on solubility (in aqueous or organic solvents) and based on previous

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137 | published works [30, 57-63]. To our knowledge this is the first time that HT-29 colon
138 | cancer cells are examined using GC-MS Metabolomics which could be of special
139 | importance in future studies to detect biochemical alterations due to colon cancer or to
140 | explore new preventive interventions.

141 | **2. MATERIALS AND METHODS**

142 | **2.1. Chemicals**

143 | All reagents were of analytical grade. Phosphate buffered saline (PBS) was purchased from
144 | Lonza (Barcelona, Spain). For cell counting, trypan blue was purchased from Sigma
145 | Aldrich (St. Louis, MO, USA). Metabolite extraction solvents were of MS grade: formic
146 | acid and 2-propanol were from Riedel-de Haën (Seelze, Germany) while water and
147 | acetonitrile were from Labscan (Gliwice, Poland). Reagents for derivatization included
148 | methoxyamine hydrochloride from Sigma Aldrich, pyridine (silylation grade) and N-
149 | methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) purchased in 1 mL bottles from
150 | Pierce (Rockford IL USA).

151 | For GC-MS analysis, a mixture of internal retention index markers (RIM) was prepared
152 | using fatty acid methyl esters of C8, C9, C10, C12, C14, C16, C18, C20, C22, C24, C26,
153 | C28 and C30 linear chain length, dissolved in chloroform (from Mallinckrodt Baker Inc.,
154 | Phillipsburg, NJ, USA) at a concentration of 0.8 mg/mL (C8-C16) and 0.4 mg/mL (C18-
155 | C30). Fatty acid methyl esters were from Sigma Aldrich.

156 | FC43 (Perfluorotributylamine) was ~~purchased~~acquired from Leco (Stockport, Cheshire,
157 | UK) for MS mass calibration.

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158 **2.2. Cell culture**

159 The human HT-29 colorectal carcinoma cell line was purchased from the American Type
160 Culture Collection (ATCC- LGC, Promochem, UK). HT-29 cells were cultured in
161 McCoy's 5A medium supplemented with 10% heat-inactivated fetal calf serum, 50 U/mL
162 penicillin G, and 50 U/mL streptomycin, at 37 °C in humidified atmosphere with 5% CO₂.
163 Once cells reached about 50% confluency, they were trypsinized, neutralized with culture
164 medium and plated at 10000 cells/cm² density in different P150 culture dishes. Cell
165 cultures were allowed to adhere overnight at 37 °C and then plates were pooled. Next, cell
166 count was performed using the trypan blue exclusion test to examine the viability and to
167 estimate the number of cells. For that purpose, 5 µL cell suspension pool were further
168 diluted 1:1 in trypan blue stock solution (0.4%, w/v in sterile PBS) and counted in a
169 Neubauer counting chamber using a light microscope (ID3, Carl Zeiss, Jena, Germany).
170 The estimated volume to have 10 million cells from the culture suspension was added to 20
171 aliquots. After aliquots were centrifuged (300 xg, 10 min) and supernatant was discarded,
172 pellets containing 10 x 10⁶ cells were subjected to metabolite extraction.

173 **2.3. Metabolite extraction and derivatization**

174 The 20 aliquots were divided in four solvent extraction groups: A, ACN; B, ACN-IsopOH-
175 Water (3:3:1, v/v/v); C, Water; and D, 5% formic acid in water. Five independent
176 metabolite extractions (with 10 million cells each) were performed for each extraction
177 solvent group. Metabolic content extraction from HT-29 cells have been previously
178 optimized in our laboratory [57] and applied in this work with slight modifications. Briefly,
179 300 µL of extraction solvent and 0.3 g glass beads (212-300 µm) from Sigma-Aldrich were

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9 180 added to 10×10^6 cell pellets. Then metabolism quenching and cell disruption was
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11 181 accomplished by using three cycles of snap-freeze (liquid N₂, 1 min), thawing in
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13 182 ultrasound bath (50 Hz, 3 min) (ultrasonic cleaning bath Ultrasons from JP Selecta,
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15 183 Barcelona, Spain) and grinding using a mixer mill for 3 min at 30/s (MM 400, Retsch
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17 184 GmbH, Germany). After this, cell insoluble material and glass beads were removed by
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19 185 centrifugation (24000 xg for 10 min at 4°C) and the supernatant was collected, and 50 µL
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21 186 metabolic extracts were vacuum-dried and kept at -80°C until derivatization. The
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23 187 supernatants obtained in A and B extraction groups are protein-free considering organic
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25 188 extraction solvents lead to protein precipitation. On the contrary, supernatants obtained
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27 189 from aqueous-based extractions (C and D extraction groups) were submitted to
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29 190 ultrafiltration with 3 kDa centrifugal filters (Amicon Ultra 0.5 mL, Millipore, Billerica, MA,
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31 191 USA) to remove proteins according to the manufacturer's protocol. 50 µL of both metabolic
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33 192 extracts were vacuum-dried and kept at -80°C until derivatization.

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36 193 The best sensitivity was achieved with the following optimized derivatization protocol.
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38 194 First, the dried extracts were dissolved in 10 µL of a solution consisting of 40 mg/mL
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40 195 methoxyamine in pyridine and shaken for 15 min at 60 °C to protect aldehyde and ketone
41
42 196 groups. Then 50 µL of MSTFA with 1% RIM was added for trimethylsilylation of acidic
43
44 197 protons and shaken at 37 °C for 30 min. Derivatized samples were immediately transferred
45
46 198 to 2 mL clear glass autosampler vials with microinserts (Agilent, Santa Clara CA), closed
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48 199 by 11 mm T/S/T crimp caps (MicroLiter, Suwanee GA) and analyzed by GC-MS.
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200 **2.4. GC-TOF MS analysis**

201 An Agilent 6890 gas chromatograph (Santa Clara CA) and a Leco Pegasus IV time of flight
202 mass (TOF) spectrometer were controlled by the Leco ChromaTOF software vs. 2.32 (St.
203 Joseph, MI). A 30 m long, 0.25 mm ID Rtx-5Sil MS column with 0.25 μm 95% dimethyl
204 5% diphenyl polysiloxane film and additional 10 m integrated guard column was used
205 (Restek, Bellefonte PA). High purity helium with built-in purifier (Airgas, Radnor PA) was
206 set at constant flow of 1 mL/min. The oven temperature was held constant at 50°C for 1
207 min and then ramped at 20°C/min to 330°C at which it was held constant for 5 min. The
208 transfer line temperature between gas chromatograph and mass spectrometer was set to
209 280°C. A Gerstel automatic liner exchange system with multipurpose sample MPS2 dual
210 rail and two derivatization stations was used in conjunction with a Gerstel CIS cold
211 injection system (Gerstel, Muehlheim, Germany). The injector operated in splitless mode,
212 opening the split vent after 25 s. Electron impact ionization at 70V was applied maintaining
213 ion source temperature at 250°C. For every 10 samples, a fresh multibaffled liner was
214 inserted (Gerstel) using the Maestro1 Gerstel software vs. 1.1.4.18 (Gerstel). Before and
215 after each injection, the 10 μL injection syringe was washed three times with 10 μL ethyl
216 acetate. 1 μL sample was filled using 39 mm vial penetration at 1 $\mu\text{L}/\text{s}$ fill speed, injecting
217 0.5 μL at 10 $\mu\text{L}/\text{s}$ injection speed. GC oven temperature was maintained at 50°C during one
218 minute followed by a temperature ramp at 20°C/min until 330°C at which it was held
219 constant for 5 min.

220 TOF-MS analysis in the positive ion mode was accomplished applying 1775 V detector
221 voltage. Data was recorded in the 85-500 m/z range for 1200 s after 290 s solvent delay.
222 Mass resolving power was 600 and acquisition rate was set at 17 spectra/s. Daily quality

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223 controls were used to assure quality of data. The instrument performed autotuning for mass
224 calibration using FC43 before each sequence analysis using the following m/z values: 93,
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226 226, 264, 265, 314, 326, 352, 376, 414, 415, 426, 448, 464, 502 and 503.

227 **2.5. Data mining**

228 GC-TOF MS data were acquired as ChromaTOF specific .peg files, and stored also in .txt
229 and .cdf generic formats. Data mining was performed as already described [60]. Briefly,
230 absolute spectra intensities from all sample files were automatically processed and filtered
231 by BinBase algorithm using previously optimized parameters [64]. Then post-processing
232 module to replace missing values was carried out. The resulting report contained row
233 identifier, mass spectra ions, quantification by means of unique ion height, retention index
234 and identification using defined databases [65]. Additional mass signal filtering was
235 manually performed to remove not identified and low confident ions from the table. Then
236 MS signals belonging to the same ion were grouped and data was normalized based on total
237 ion height for each sample. The table with high-confident metabolite information from all
238 extraction replicates obtained with the four solvents (five replicates per extraction solvent)
239 was then examined in detail. Principal component analysis (PCA) and hierarchical
240 clustering was performed to explore possible clustering, similarities and differences in
241 metabolic coverage obtained among the four extraction protocols. Then comparison of
242 metabolome coverage among the four extracts was performed by metabolite set enrichment
243 analysis (MSEA) and metabolic pathway analysis (MPA). MSEA was performed by means
244 of overrepresentation analysis (ORA). ORA assess whether a set of metabolites is enriched
245 in a particular metabolic pathway compared to the total of annotated metabolites for that

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246 pathway [66] (i.e. if the metabolites identified in the study represent a high percentage of
247 the metabolites theoretically annotated in that pathway) [66]. Statistical analysis, MSEA
248 and MPA were performed using metaboanalyst [67]. MBRole [68] was used to assist in the
249 biological interpretation.

250 3. RESULTS AND DISCUSSION

251 3.1. Metabolic extraction assessment

252 Typical total ion chromatograms (TIC) for the cell extracts obtained using the four different
253 extraction solvents tested in this work are represented in Fig. 1. As can be observed, the
254 four metabolite profiles were very different in terms of intensity and distribution of the
255 peaks along the chromatogram. Although aqueous extracts (Fig. 1C and 1D) presented
256 greater overall intensity, peaks were mainly placed at the beginning of the chromatogram.

257 After data processing, 364 metabolic signals were detected. Signal grouping and filtering
258 processes (i.e. unidentified metabolites and metabolites not found in at least 3 out of 5
259 replicates of each extraction solvent) rendered a total of 290 metabolites from which a
260 group of 150 metabolites could be identified (see Table 21 and Table S2). Then, each of the
261 metabolic extracts were investigated in detail in terms of detected metabolites and
262 identified metabolites found in each extract as well as other parameters to evaluate
263 metabolite extraction performance and reproducibility. An average variation of the internal
264 standards of 15% RSD was observed. Given that value the number of identified metabolites
265 with RSD < 15% was also determined for the four extraction solvents.

266 Among the two aqueous based extractions C (water) and D (acidified water), C showed
267 higher overall metabolome coverage in terms of number of detected (172 vs. 168) and

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9 268 identified metabolites (100 vs. 94). However, deepening in the identified metabolites in
10 269 both aqueous extracts, D showed a vast gain over C with respect to metabolite recovery
11 270 performance (76 vs. 25 metabolites found at maximum intensity in D and C respectively)
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13 271 but the inverse was observed in terms of reproducibility (RSD <15% in 62 vs. 20
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15 272 metabolites in C and D, respectively) (see Table 21). As can be observed in Table 21, a
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17 273 more evident enhancement was observed in B metabolic coverage when compared with A
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19 274 extract among the two organic extracts. Namely, 96 metabolites were identified out of the
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21 275 182 detected in B, while for A only 82 metabolites could be identified out of the 141
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23 276 detected. Moreover, triple metabolite recovery performance (i.e. metabolites at maximum
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25 277 intensity) from cell culture was observed for B with respect to A. In addition, metabolic
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27 278 content in A was extracted with less reproducibility among the 5 extraction replicates
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29 279 analysed, including only 17 identified metabolites with RSD values lower than 15%. Thus,
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31 280 among the organic solvents, B extract clearly offered the best overall performance when
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33 281 compared to A.
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38 282 **3.2. Colon cancer cell metabolome identified by GC-MS**

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41 283 Metabolic information obtained from the four extraction protocols was then statistically
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43 284 investigated in order to find patterns and possible clustering in metabolic coverage obtained
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45 285 applying the four extraction solvents. Fig. 2 represents the unsupervised hierarchical
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47 286 clustering of the samples (i.e. metabolic extracts) ~~attending to~~according to the relative
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49 287 content of each metabolite in each sample (represented by the colour of the cell). As can be
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51 288 observed from the clustering information of Fig. 2, the four extracts were mainly
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53 289 differentiated ~~attending to~~according to the polarity of the solvent used in the extraction.
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55 290 Thus, as expected two clear clusters were observed corresponding to A with B (i.e. organic
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291 based solvents), and C with D (i.e. aqueous based solvents). This information was also
292 observed and corroborated by means of PCA (see Fig. S1) and Venn diagram (see Fig. 3).
293 In addition, PCA also showed that the metabolic information obtained from both organic
294 solvents was more similar (presented less variation in their distribution in the plane defined
295 by the main principal components from PC1 to PC5) than the one more heterogeneous
296 obtained from both aqueous based extracts (more dispersion of aqueous samples in the
297 plane defined by the PCs) (see Fig. S1).

298 Venn representation (Fig. 3) corroborated that the highest similarity of metabolic coverage
299 was ~~attending to~~according to the extraction solvent polarity. Namely, organic based extracts
300 (A and B) shared 72 metabolites, among which, 18 metabolites were exclusively detected
301 in A and B. Similarly 80 metabolic species were common in both aqueous based extracts (C
302 and D) from which 14 were only found in C and D. In addition, it could be observed that
303 ACN and water extraction solvents (A and C, respectively) lead to the most different
304 metabolic extracts with only 57 metabolites in common (from which 49 were found in all
305 the four extracts) showing high complementarity.

306 To deepen in the coverage of the different extracts ~~attending to~~according to the nature of
307 the extracted metabolites and pathways involved, two web servers were used. Information
308 about the biological role and nature of the identified metabolites was derived from MBRole
309 web server analysis. Thus, the total set of metabolites with available KEGG identifiers in
310 the four extracts (107 out of 150 identified metabolites) were submitted to analysis. Most
311 important groups of metabolites identified were amino acids and peptides followed by fatty
312 acids and lipids, nucleic acids and carbohydrates. Other groups of metabolites were
313 represented to a lesser extent such as vitamins and cofactors (data not shown). Main

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9 314 metabolic pathways involving metabolites preferentially extracted (i.e. showing maximum
10 315 levels) using the organic-based (A and B) and aqueous-based (C and D) protocols were
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12 316 analyzed using Metaboanalyst (Table S34 and S24 respectively). Fatty acid and nitrogenous
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14 317 bases (i.e. pyrimidine and purine) related pathways are well represented in organic based
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16 318 extracts. Among the two organic extracts, contribution of B extract is clearly superior to A
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18 319 whose metabolites are almost exclusively involved in nucleotide sugars metabolism. The
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20 320 main reason for this inequality could be explained for the lowest number of metabolites at
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22 321 highest intensity in A (see Table 21). On the other hand, pathways involving biosynthesis
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24 322 and metabolism of amino acids, glutathione, amino sugars and nitrogenous bases among
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26 323 others, are highly represented in aqueous extracts (C and D). Most of the metabolites
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28 324 involved in those pathways present higher performance of extraction using acidified water
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30 325 extraction (D) when compared with C (water extraction). Although in lower relative
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32 326 concentration, metabolic species in C showed the best reproducibility.
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36 327 Finally, identified metabolites were submitted to enrichment analysis (MSEA) by means of
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38 328 overrepresentation analysis (ORA) to determine the more represented metabolic pathways
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40 329 in the defined set of metabolites. As can be observed in Fig. S2, “protein biosynthesis” is
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42 330 the metabolic pathway in which a higher number of the metabolites are involved. However,
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44 331 more useful information can be obtained if solvents are subdivided regarding polarity.
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46 332 When each protocol for metabolic extraction is investigated in detail, different trends are
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48 333 observed. MSEA results of metabolites preferentially extracted (i.e. showing maximum
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50 334 levels) in organic and aqueous based extracts are shown in Fig. 4 and Fig. 5 respectively.
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52 335 As can be observed depending on the nature and polarity of the solvents used in the
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54 336 extraction process diverse metabolic pathways are more suitable to study due to the very
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337 different metabolic information. Some of the most relevant metabolic pathways that can be
338 studied with more efficiency in organic based solvents are “RNA transcription” and “beta
339 oxidation of very long fatty acids” (Fig. 4), while pathways most suitable to be evaluated in
340 aqueous based extracts include protein, sugars and amino acids biosynthesis and
341 metabolism (Fig. 5). Interestingly, pathways involving metabolism of glutathione and
342 nucleotides can be approached using both organic and/or aqueous extracts.

343 **CONCLUDING REMARKS**

344 To date it is not possible to study the whole metabolome of a biological system and this is
345 partially due to the selection of a certain sample treatment and analytical platform. In this
346 work, four reliable sample preparation protocols have been evaluated to extract metabolites
347 from HT-29 colon cancer cells and to uncover a portion of this cell line metabolome by
348 GC-MS.

349 The unsupervised statistical analysis showed that the four extracts evaluated were mainly
350 differentiated ~~attending to~~according to the polarity of the solvent used in the extraction
351 process and that the overall GC-MS variability of aqueous extracts was higher compared to
352 the organic ones.

353 In summary, the protocol involving ACN-Isopropanol-water (3:3:1, v/v/v) was shown to be
354 the most appropriate extraction procedure for fatty acid and/or related pathways analysis
355 among the four metabolic extraction procedures. Metabolic extraction with ACN yielded
356 the lowest reproducibility of extraction together with the smallest metabolite coverage. On
357 the other hand, metabolic coverage was similar in both aqueous extracts. However, overall
358 performance of extraction of individual metabolites seemed to be higher using acidified

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359 water (5% formic acid) compared to just water, which on the contrary showed better GC-
360 MS reproducibility. Most of the metabolites involved in pathways of amino acids,
361 glutathione, amino sugars and other polar metabolites present higher performance of
362 extraction in the acidified water extract, although metabolic species in water extract show
363 the best overall reproducibility. Although pathways involving nitrogenous bases could be
364 investigated using organic or aqueous extracts, more metabolites involved in those
365 pathways can be identified in the aqueous extracts.

366 In light of the results obtained, qualitative information about the metabolic coverage and
367 related pathways exhibited high complementarity among the extracts. This information is
368 useful for future hypothesis-driven studies for the evaluation of a certain metabolic pathway
369 or a group of metabolites (i.e. if they are thought to be altered in HT29 colorectal cells by
370 the action of a drug or a natural bioactive compound).

371 **Conflict of interest statement**

372 The authors declare no conflict of interest.

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380 **References**

381 [1] R.A. Cairns, I.S. Harris, T.W. Mak, Regulation of cancer cell metabolism, *Nat. Rev. Cancer* 11
382 (2011) 85-95.

383 [2] O. Warburg, On the origin of cancer cells, *Science* 123 (1956) 309-314.

384 [3] M.D. Williams, R. Reeves, L.S. Resar, H.H. Hill Jr, Metabolomics of colorectal cancer: past and
385 current analytical platforms, *Anal. Bioanal. Chem.* 405 (2013) 5013-5030.

386 [4] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, *Cell* 144 (2011) 646-
387 674.

388 [5] M.G. Vander Heiden, J.W. Locasale, K.D. Swanson, H. Sharfi, G.J. Heffron, D. Amador-
389 Noguez, H.R. Christofk, G. Wagner, J.D. Rabinowitz, J.M. Asara, L.C. Cantley, Evidence for an
390 alternative glycolytic pathway in rapidly proliferating cells, *Science* 329 (2010) 1492-1499.

391 [6] H. Kondoh, M.E. Leonart, J. Gil, J. Wang, P. Degan, G. Peters, D. Martinez, A. Carnero, D.
392 Beach, Glycolytic enzymes can modulate cellular life span, *Cancer Res.* 65 (2005) 177-185.

393 [7] N.J. Serkova, K. Glunde, Metabolomics of cancer, *Methods Mol. Biol.* 520 (2009) 273-295.

394 [8] R.D. Beger, A review of applications of metabolomics in cancer, *Metabolites* 3 (2013) 552-574.

395 [9] K. Dettmer, P.A. Aronov, B.D. Hammock, Mass spectrometry-based metabolomics, *Mass*
396 *Spectrom. Rev.* 26 (2007) 51-78.

397 [10] A. Smolinska, L. Blanchet, L.M. Buydens, S.S. Wijmenga, NMR and pattern recognition
398 methods in metabolomics: from data acquisition to biomarker discovery: a review, *Anal. Chim.*
399 *Acta* 750 (2012) 82-97.

1
2
3
4
5
6
7
8
9 400 [11] W.B. Dunn, D.I. Ellis, *Metabolomics: current analytical platforms and methodologies*, *TrAC-*
10 401 *Trend. Anal. Chem.* 24 (2005) 285-294.
11
12
13 402 [12] C. Denkert, E. Bucher, M. Hilvo, R. Salek, M. Orešič, J. Griffin, S. Brockmüller, F. Klauschen,
14 403 S. Loibl, D.K. Barupal, *Metabolomics of human breast cancer: new approaches for tumor typing*
15 404 *and biomarker discovery*, *Genome Med.* 4 (2012) 37.
16
17
18
19 405 [13] Q. Huang, Y. Tan, P. Yin, G. Ye, P. Gao, X. Lu, H. Wang, G. Xu, *Metabolic characterization*
20 406 *of hepatocellular carcinoma using nontargeted tissue metabolomics*, *Cancer Res.* 73 (2013) 4992-
21 407 5002.
22
23
24
25 408 [14] T. Zhang, D.G. Watson, L. Wang, M. Abbas, L. Murdoch, L. Bashford, I. Ahmad, N. Lam,
26 409 A.C. Ng, H.Y. Leung, *Application of holistic liquid chromatography-high resolution mass*
27 410 *spectrometry based urinary metabolomics for prostate cancer detection and biomarker discovery*,
28 411 *PloS one* 8 (2013) e65880.
29
30
31
32
33
34 412 [15] S. Nishiumi, T. Kobayashi, A. Ikeda, T. Yoshie, M. Kibi, Y. Izumi, T. Okuno, N. Hayashi, S.
35 413 Kawano, T. Takenawa, *A novel serum metabolomics-based diagnostic approach for colorectal*
36 414 *cancer*, *PloS one* 7 (2012) e40459.
37
38
39
40 415 [16] S. Hori, S. Nishiumi, K. Kobayashi, M. Shinohara, Y. Hatakeyama, Y. Kotani, N. Hatano, Y.
41 416 Maniwa, W. Nishio, T. Bamba, *A metabolomic approach to lung cancer*, *Lung Cancer* 74 (2011)
42 417 284-292.
43
44
45
46
47 418 [17] American Cancer Society, *Colorectal Cancer Facts & Figures 2011-2013*, Atlanta: American
48 419 Cancer Society (2011).
49
50
51 420 [18] F. Bray, *The burden of cancer in Europe, Responding to the challenge of cancer in Europe.. in:*
52 421 *M.P. Coleman, D.M. Alexe, T. Albrecht, M. McKee (Eds.) Responding to the challenge of cancer in*
53 422 *Europe. Institute of Public Health of the Republic of Slovenia, Ljubljana, 2008, pp. 7-40.*
54
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423 [19] D. Monleon, J.M. Morales, A. Barrasa, J.A. Lopez, C. Vazquez, B. Celda, Metabolite profiling
424 of fecal water extracts from human colorectal cancer, *NMR Biomed.* 22 (2009) 342-348.

425 [20] M. Tessem, K.M. Selnæs, W. Sjørusen, G. Tranø, G.F. Giskeødegård, T.F. Bathen, I.S.
426 Gribbestad, E. Hofslie, Discrimination of patients with microsatellite instability colon cancer using
427 ¹H HR MAS MR spectroscopy and chemometric analysis, *J. Proteome Res.* 9 (2010) 3664-3670.

428 [21] H. Wang, D.E. Schiller, V. Tso, C. Slupsky, C.K. Wong, R.N. Fedorak, A novel highly
429 sensitive test for detecting colon cancer using spot urine metabolomics, *Gastroenterology* 140
430 (2011) S-40.

431 [22] I. Bertini, S. Cacciatore, B.V. Jensen, J.V. Schou, J.S. Johansen, M. Kruhoffer, C. Luchinat,
432 D.L. Nielsen, P. Turano, Metabolomic NMR fingerprinting to identify and predict survival of
433 patients with metastatic colorectal cancer, *Cancer Res.* 72 (2012) 356-364.

434 [23] B. Jiménez, R. Mirnezami, J. Kinross, O. Cloarec, H.C. Keun, E. Holmes, R.D. Goldin, P.
435 Ziprin, A. Darzi, J.K. Nicholson, ¹H HR-MAS NMR spectroscopy of tumor-induced local
436 metabolic “field-effects” enables colorectal cancer staging and prognostication, *J. Proteome Res.* 12
437 (2013) 959-968.

438 [24] S.A. Ritchie, P.W. Ahiahonu, D. Jayasinghe, D. Heath, J. Liu, Y. Lu, W. Jin, A. Kavianpour,
439 Y. Yamazaki, A.M. Khan, Reduced levels of hydroxylated, polyunsaturated ultra long-chain fatty
440 acids in the serum of colorectal cancer patients: implications for early screening and detection,
441 *BMC Med.* 8 (2010) 1.

442 [25] F. Li, X. Qin, H. Chen, L. Qiu, Y. Guo, H. Liu, G. Chen, G. Song, X. Wang, F. Li, Lipid
443 profiling for early diagnosis and progression of colorectal cancer using direct-infusion electrospray
444 ionization Fourier transform ion cyclotron resonance mass spectrometry, *Rapid Commun. Mass Sp.*
445 27 (2013) 24-34.

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2
3
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8
9 446 [26] E.G. Armitage, H.L. Kotze, J.S. Fletcher, A. Henderson, K.J. Williams, N.P. Lockyer, J.C.
10 447 Vickerman, Time-of-flight SIMS as a novel approach to unlocking the hypoxic properties of cancer,
11
12 448 Surf. Interface Anal. 45 (2013) 282-285.
13
14
15 449 [27] A. Hirayama, K. Kami, M. Sugimoto, M. Sugawara, N. Toki, H. Onozuka, T. Kinoshita, N.
16
17 450 Saito, A. Ochiai, M. Tomita, H. Esumi, T. Soga, Quantitative metabolome profiling of colon and
18
19 451 stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry,
20
21 452 Cancer Res. 69 (2009) 4918-4925.
22
23 453 [28] C. Ibáñez, C. Simó, V. García-Cañas, Á Gómez-Martínez, J.A. Ferragut, A. Cifuentes,
24
25 454 CE/LC-MS multiplatform for broad metabolomic analysis of dietary polyphenols effect on colon
26
27 455 cancer cells proliferation, Electrophoresis 33 (2012) 2328-2336.
28
29
30 456 [29] C. Ibáñez, A. Valdés, V. García-Cañas, C. Simó, M. Celebier, L. Rocamora-Reverte, Á
31
32 457 Gómez-Martínez, M. Herrero, M. Castro-Puyana, A. Segura-Carretero, Global Foodomics strategy
33
34 458 to investigate the health benefits of dietary constituents, J. Chromatogr A 1248 (2012) 139-153.
35
36 459 [30] C. Simó, C. Ibáñez, Á Gómez-Martínez, J.A. Ferragut, A. Cifuentes, Is metabolomics
37
38 460 reachable? Different purification strategies of human colon cancer cells provide different CE-MS
39
40 461 metabolite profiles, Electrophoresis 32 (2011) 1765-1777.
41
42
43 462 [31] B. Tan, Y. Qiu, X. Zou, T. Chen, G. Xie, Y. Cheng, T. Dong, L. Zhao, B. Feng, X. Hu,
44
45 463 Metabonomics identifies serum metabolite markers of colorectal cancer, J. Proteome Res. 12 (2013)
46
47 464 3000-3009.
48
49 465 [32] D.C. Montrose, X.K. Zhou, L. Kopelovich, R.K. Yantiss, E.D. Karoly, K. Subbaramaiah, A.J.
50
51 466 Dannenberg, Metabolic profiling, a noninvasive approach for the detection of experimental
52
53 467 colorectal neoplasia, Cancer. Prev. Res. (Phila) 5 (2012) 1358-1367.
54
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57
58
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468 [33] Y. Cheng, G. Xie, T. Chen, Y. Qiu, X. Zou, M. Zheng, B. Tan, B. Feng, T. Dong, P. He,
469 Distinct urinary metabolic profile of human colorectal cancer, *J. Proteome Res.* 11 (2011) 1354-
470 1363.

471 [34] Y. Ma, H. Qin, W. Liu, J. Peng, L. Huang, X. Zhao, Y. Cheng, Ultra-high performance liquid
472 chromatography–mass spectrometry for the metabolomic analysis of urine in colorectal cancer, *Dig.*
473 *Dis. Sci.* 54 (2009) 2655-2662.

474 [35] D. Zimmermann, M. Hartmann, M.P. Moyer, J. Nolte, J.I. Baumbach, Determination of
475 volatile products of human colon cell line metabolism by GC/MS analysis, *Metabolomics* 3 (2007)
476 13-17.

477 [36] E.C.Y. Chan, P.K. Koh, M. Mal, P.Y. Cheah, K.W. Eu, A. Backshall, R. Cavill, J.K.
478 Nicholson, H.C. Keun, Metabolic profiling of human colorectal cancer using high-resolution magic
479 angle spinning nuclear magnetic resonance (HR-MAS NMR) spectroscopy and gas chromatography
480 mass spectrometry (GC/MS), *J. Proteome Res.* 8 (2008) 352-361.

481 [37] C. Denkert, J. Budczies, W. Weichert, G. Wohlgemuth, M. Scholz, T. Kind, S. Niesporek, A.
482 Noske, A. Buckendahl, M. Dietel, Metabolite profiling of human colon carcinoma–deregulation of
483 TCA cycle and amino acid turnover, *Mol. Cancer* 7 (2008) 72.

484 [38] Y.L. Ma, W.J. Liu, J.Y. Peng, P. Zhang, H.Q. Chen, H.L. Qin, Study on specific metabonomic
485 profiling of serum from colorectal cancer patients by gas chromatography-mass spectrometry,
486 *Zhonghua Wei Chang Wai Ke Za Zhi* 12 (2009) 386-390.

487 [39] Y. Ma, W. Liu, J. Peng, L. Huang, P. Zhang, X. Zhao, Y. Cheng, H. Qin, A pilot study of gas
488 chromatograph/mass spectrometry-based serum metabolic profiling of colorectal cancer after
489 operation, *Mol. Biol. Rep.* 37 (2010) 1403-1411.

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56
57
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65

490 [40] Y. Qiu, G. Cai, M. Su, T. Chen, Y. Liu, Y. Xu, Y. Ni, A. Zhao, S. Cai, L.X. Xu, Urinary
491 metabonomic study on colorectal cancer, *J. Proteome Res.* 9 (2010) 1627-1634.

492 [41] Y. Kondo, S. Nishiumi, M. Shinohara, N. Hatano, A. Ikeda, T. Yoshie, T. Kobayashi, Y.
493 Shiomi, Y. Irino, T. Takenawa, Serum fatty acid profiling of colorectal cancer by gas
494 chromatography/mass spectrometry, *Biomark. Med.* 5 (2011) 451-460.

495 [42] M. Mal, P.K. Koh, P.Y. Cheah, E.C.Y. Chan, Metabotyping of human colorectal cancer using
496 two-dimensional gas chromatography mass spectrometry, *Anal. Bioanal. Chem.* 403 (2012) 483-
497 493.

498 [43] T. Yoshie, S. Nishiumi, Y. Izumi, A. Sakai, J. Inoue, T. Azuma, M. Yoshida, Regulation of the
499 metabolite profile by an APC gene mutation in colorectal cancer, *Cancer Sci.* 103 (2012) 1010-
500 1021.

501 [44] L.C. Phua, M. Mal, P.K. Koh, P.Y. Cheah, E.C.Y. Chan, H.K. Ho, Investigating the role of
502 nucleoside transporters in the resistance of colorectal cancer to 5-fluorouracil therapy, *Cancer*
503 *Chemother. Pharmacol.* 71 (2013) 817-823.

504 [45] M.A. Lorenz, C.F. Burant, R.T. Kennedy, Reducing time and increasing sensitivity in sample
505 preparation for adherent mammalian cell metabolomics, *Anal. Chem.* 83 (2011) 3406-3414.

506 [46] C.A. Sellick, D. Knight, A.S. Croxford, A.R. Maqsood, G.M. Stephens, R. Goodacre, A.J.
507 Dickson, Evaluation of extraction processes for intracellular metabolite profiling of mammalian
508 cells: matching extraction approaches to cell type and metabolite targets, *Metabolomics* 6 (2010)
509 427-438.

510 [47] B. Álvarez-Sánchez, F. Priego-Capote, M.L. de Castro, Metabolomics analysis II. Preparation
511 of biological samples prior to detection, *TrAC-Trend. Anal. Chem.* 29 (2010) 120-127.

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47
48
49
50
51
52
53
54
55
56
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59
60
61
62
63
64
65

512 [48] B. Gonzalez, J. François, M. Renaud, A rapid and reliable method for metabolite extraction in
513 yeast using boiling buffered ethanol, *Yeast* 13 (1997) 1347-1355.

514 [49] S. Dietmair, N.E. Timmins, P.P. Gray, L.K. Nielsen, J.O. Krömer, Towards quantitative
515 metabolomics of mammalian cells: Development of a metabolite extraction protocol, *Anal.*
516 *Biochem.* 404 (2010) 155-164.

517 [50] S. Dietmair, M.P. Hodson, L. Quek, N.E. Timmins, P. Chrysanthopoulos, S.S. Jacob, P. Gray,
518 L.K. Nielsen, Metabolite profiling of CHO cells with different growth characteristics, *Biotechnol.*
519 *Bioeng.* 109 (2012) 1404-1414.

520 [51] J. Kronthaler, G. Gstraunthaler, C. Heel, Optimizing high-throughput metabolomic biomarker
521 screening: a study of quenching solutions to freeze intracellular metabolism in CHO cells, *Omics* 16
522 (2012) 90-97.

523 [52] Q. Teng, W. Huang, T.W. Collette, D.R. Ekman, C. Tan, A direct cell quenching method for
524 cell-culture based metabolomics, *Metabolomics* 5 (2009) 199-208.

525 [53] I. Chantret, A. Barbat, E. Dussaulx, M.G. Brattain, A. Zweibaum, Epithelial polarity, villin
526 expression, and enterocytic differentiation of cultured human colon carcinoma cells: a survey of
527 twenty cell lines, *Cancer Res.* 48 (1988) 1936-1942.

528 [54] D. Ahmed, P. Eide, I. Eilertsen, S. Danielsen, M. Eknæs, M. Hektoen, G. Lind, R. Lothe,
529 Epigenetic and genetic features of 24 colon cancer cell lines, *Oncogenesis* 2 (2013) e71.

530 [55] S. Fernández-Arroyo, A. Gómez-Martínez, L. Rocamora-Reverte, R. Quirantes-Piné, A.
531 Segura-Carretero, A. Fernández-Gutiérrez, J. Ferragut, Application of nanoLC-ESI-TOF-MS for the
532 metabolomic analysis of phenolic compounds from extra-virgin olive oil in treated colon-cancer
533 cells, *J. Pharm. Biomed. Anal.* 63 (2012) 128-134.

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534 [56] M. Čuperlović-Culf, D.A. Barnett, A.S. Culf, I. Chute, Cell culture metabolomics: applications
535 and future directions, *Drug Discov. Today* 15 (2010) 610-621.

536 [57] C. Ibáñez, C. Simó, A. Valdés, L. Campone, A.L. Piccinelli, V. García-Cañas, A. Cifuentes,
537 Metabolomics of adherent mammalian cells by capillary electrophoresis-mass spectrometry: HT-29
538 cells as case study, *J. Pharm. Biomed. Anal.* 110 (2015) 83-92.

539 [58] H. Meyer, H. Weidmann, M. Lalk, Methodological approaches to help unravel the intracellular
540 metabolome of *Bacillus subtilis*, *Microb. Cell. Fact.* 12 (2013) 69.

541 [59] A.D. Patterson, H. Li, G.S. Eichler, K.W. Krausz, J.N. Weinstein, A.J. Fornace, F.J. Gonzalez,
542 J.R. Idle, UPLC-ESI-TOFMS-based metabolomics and gene expression dynamics inspector self-
543 organizing metabolomic maps as tools for understanding the cellular response to ionizing radiation,
544 *Anal. Chem.* 80 (2008) 665-674.

545 [60] D.Y. Lee, O. Fiehn, High quality metabolomic data for *Chlamydomonas reinhardtii*, *Plant*
546 *Methods* 4 (2008) 7.

547 [61] J.L. Au, M.H. Su, M.G. Wientjes, Extraction of intracellular nucleosides and nucleotides with
548 acetonitrile, *Clin. Chem.* 35 (1989) 48-51.

549 [62] L. von Stechow, A. Ruiz-Aracama, B. van de Water, A. Peijnenburg, E. Danen, A. Lommen,
550 Identification of cisplatin-regulated metabolic pathways in pluripotent stem cells, *PloS one* 8 (2013)
551 e76476.

552 [63] O. Fiehn, T. Kind, Metabolite profiling in blood plasma, *Method. Mol. Biol.* 358 (2007) 3-17.

553 [64] O. Fiehn, G. Wohlgemuth, M. Scholz, Setup and Annotation of Metabolomic Experiments by
554 Integrating Biological and Mass Spectrometric Metadata. In: Ludäscher B., Raschid L. (eds) *Data*
555 *Integration in the Life Sciences. DILS 2005. Lecture Notes in Computer Science*, vol 3615.
556 Springer, Berlin, Heidelberg.

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557 [65] M. Scholz, O. Fiehn, SetupX--a public study design database for metabolomic projects, Pac.
558 Symp. Biocomput. (2007) 169-180.

559 [66] M. Chagoyen, F. Pazos, Tools for the functional interpretation of metabolomic experiments,
560 Brief Bioinform. 14 (2013) 737-744.

561 [67] J. Xia, N. Psychogios, N. Young, D.S. Wishart, MetaboAnalyst: a web server for metabolomic
562 data analysis and interpretation, Nucleic Acids Res. 37 (2009) W652-60.

563 [68] M. Chagoyen, F. Pazos, MBRole: enrichment analysis of metabolomic data, Bioinformatics 27
564 (2011) 730-731.

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565 **FIGURE CAPTIONS**

566 **Figure 1.** GC-MS total ion chromatograms (TIC) of metabolite extracts obtained using
567 ACN (A), ACN-IsopOH-water (3:3:1, v/v/v) (B), water (C), and 5% formic acid in water
568 (D). GC-MS conditions are described in the text.

569 **Figure 2.** Hierarchical clustering and heat map of total identified metabolites (rows) in all
570 samples (columns). A (ACN), B (ACN-IsopOH-water (3:3:1, v/v/v)), C (water) and D (5%
571 formic acid) extracts are represented in red, green, dark blue and light blue respectively.
572 Cell color represents metabolite relative content.

573 **Figure 3.** Venn diagram representation of number of HT-29 metabolites identified in A, B,
574 C and D extracts.

575 **Figure 4.** Summary plot for Metabolite Set Enrichment Analysis (MSEA) of metabolites
576 identified in the organic extracts (A and B) from HT-29 cells.

577 **Figure 5.** Summary plot for Metabolite Set Enrichment Analysis (MSEA) of metabolites
578 identified in the aqueous extracts (C and D) from HT-29 cells.

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Table 1. Summary of colorectal cancer (CRC) studies via GC-MS metabolomics published at Web of Science (December 2016) sorted by year of publication:

Matrix	Samples	Metabolomic approach	Analytical platform	Reference
Cell culture	Two colon cancer cell lines (SW1116 y SW480) versus a control cell line (NCM460)	Metabolic fingerprinting	GC-TOF-MS	[35]
Tissue	Tissue from patients with primary CRC (n=27) and control tissue (n=18)	Metabolic fingerprinting	GC-TOF-MS	[37]
Tissue	Tissue from tumour and control area from CRC patients (n=34)	Metabolic fingerprinting	HR-MAS-NMR GC-TOF-MS	[36]
Serum	CRC patients (n=31) and healthy controls (n=8)	Metabolic fingerprinting	GC-TOF-MS	[38]
Serum	CRC patients (n=30) before and after surgery	Metabolic fingerprinting	GC-TOF-MS	[39]
Human and rat urine	Human: CRC patients (n=60) and healthy controls (n=63) Rats before and after surgery treated with DMH (n=8) and control rats (n=8)	Metabolic fingerprinting DMH ⁺ metabonomic analysis	GC-TOF-MS	[40]
Serum	CRC patients (n=42) and healthy controls (n=8)	Fatty acids profiling	GC-TOF-MS	[41]
Urine	CRC patients (n=61) and healthy controls (n=62) Validation group: CRC patients (n=40) and healthy controls (n=41)	Metabolic fingerprinting	GC-TOF-MS UHPLC-Q/TOF-MS	[33]
Tissue	Tissue from tumour and control area from CRC patients (n=34)	Metabolic fingerprinting	GC-x-GC-TOF-MS	[42]
Mice tissue, plasma and feces	Samples collected at 3, 5 and 7 weeks after azoxymethane treatment (n=40) and placebo (n=35)	Metabolic fingerprinting	UHPLC-MS/MS GC-TOF-MS	[32]
Serum	CRC patients (n= 60) divided in four cancer stages (12 patient/stage) and healthy controls (n=60) Validation group: CRC patients (n=59) and healthy controls (n=63)	Metabolic profiling	GC-TOF-MS	[15]

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Mice tissue and serum Cell culture	Mice tissue (n=131) with and without gen-APC mutation Mice serum (n=126) with and without gen-APC mutation Cell line SW480 (n=75) with partial/total expression of gen APC	Metabolic fingerprinting	GC-TOF-MS	[43]
Tissue	Patients with recurrent (n=4) and non-recurrent (n=3) CRC after treatment with 5-fluorouracil	Nucleotides and derivatives profiling	GC x GC-TOF-MS	[44]
Serum	CRC patients (n=101) and healthy controls (n=102)	Metabolic fingerprinting	GC-TOF-MS UHPLC-Q/TOF-MS	[31]
Cell culture	Caco-2 cells versus other cancer cells (breast, neuroblastoma) and non cancer cell line	Metabolic profiling	GC-TOF-MS	[61]
Tissue and feces	Feces and tissue from tumour and control area from CRC patients (n=17)	Metabolic fingerprinting	UHPLC-MS/MS GC-TOF-MS	[62]
Plasma	Plasma from CRC patients (n=15) and healthy controls (n=15)	Metabolic fingerprinting	GC-TOF-MS	[63]
Serum	Serum from patients suffering adenoma (n=31), various stages of CRC (320) and healthy controls (n=254)	Metabolic fingerprinting	GC-TOF-MS	[64]

TABLES

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Table 21. Summary of metabolite extraction performance of the four extraction solvents used in this work.

Metabolic extract	Extraction solvent	Detected metabolites	Identified metabolites	Metabolites at maximum intensity ^a	Metabolites RSD<15% ^a
A	ACN	141	82	13	17
B	ACN-IsopOH-H ₂ O (3:3:1, v/v/v)	182	96	36	51
C	H ₂ O	172	100	25	62
D	5% HCOOH	168	94	76	20

^aMetabolites at maximum intensity (5th column) and with RSD <15% (6th column) out of the total identified metabolites.

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SUPPORTING INFORMATION

Supporting Figures

Figure S1. Principal component analysis (PCA) of the samples represented in the plane defined by the main principal components (PC) from PC1 to PC5. A, B, C and D extracts are represented in red (Δ), green (+), dark blue (X) and light blue (\diamond) respectively.

Figure S2. Summary plot for metabolite set enrichment analysis (MSEA) of metabolites identified in HT-29 cells. Top 50 metabolic pathways are shown.

Supporting Tables

Table S1. Summary of colorectal cancer (CRC) studies via GC-MS metabolomics published at Web of Science (December 2016) sorted by year of publication.

<u>Matrix</u>	<u>Samples</u>	<u>Metabolomic approach</u>	<u>Analytical platform</u>	<u>Reference</u>
<u>Cell culture</u>	<u>Two colon cancer cell lines (SW1116 y SW480) versus a control cell line (NCM460)</u>	<u>Metabolic fingerprinting</u>	<u>GC-TOF MS</u>	<u>[35]</u>
<u>Tissue</u>	<u>Tissue from patients with primary CRC (n=27) and control tissue (n=18)</u>	<u>Metabolic fingerprinting</u>	<u>GC-TOF MS</u>	<u>[37]</u>
<u>Tissue</u>	<u>Tissue from tumour and control area from CRC patients (n=31)</u>	<u>Metabolic fingerprinting</u>	<u>HR-MAS NMR GC-TOF MS</u>	<u>[36]</u>
<u>Serum</u>	<u>CRC patients (n=31) and healthy controls (n=8)</u>	<u>Metabolic fingerprinting</u>	<u>GC-TOF MS</u>	<u>[38]</u>
<u>Serum</u>	<u>CRC patients (n=30) before and after surgery</u>	<u>Metabolic fingerprinting</u>	<u>GC-TOF MS</u>	<u>[39]</u>
<u>Human and rat urine</u>	<u>Human: CRC patients (n=60) and healthy controls (n=63) Rats before and after surgery treated with DMH (n=8) and control rats (n=8)</u>	<u>Metabolic fingerprinting DMH^b metabonomic analysis</u>	<u>GC-TOF MS</u>	<u>[40]</u>

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Serum	<u>CRC patients (n=42) and healthy controls (n=8)</u>	<u>Fatty acids profiling</u>	<u>GC-TOF MS</u>	[41]
Urine	<u>CRC patients (n=61) and healthy controls (n=62)</u> <u>Validation group: CRC patients (n=40) and healthy controls (n=41)</u>	<u>Metabolic fingerprinting</u>	<u>GC-TOF MS</u> <u>UHPLC-Q/TOF MS</u>	[33]
Tissue	<u>Tissue from tumour and control area from CRC patients (n=31)</u>	<u>Metabolic fingerprinting</u>	<u>GC x GC-TOF MS</u>	[42]
Mice tissue, plasma and feces	<u>Samples collected at 3, 5 and 7 weeks after azoxymethane treatment (n=40) and placebo (n=35)</u>	<u>Metabolic fingerprinting</u>	<u>UHPLC-MS/MS</u> <u>GC-TOF MS</u>	[32]
Serum	<u>CRC patients (n= 60) divided in four cancer stages (12 patient/stage)) and healthy controls (n=60)</u> <u>Validation group: CRC patients (n=59) and healthy controls (n=63)</u>	<u>Metabolic profiling</u>	<u>GC-TOF MS</u>	[15]
Mice tissue and serum Cell culture	<u>Mice tissue (n=131) with and without gen APC mutation</u> <u>Mice serum (n=126) with and without gen APC mutation</u> <u>Cell line SW480 (n=75) with partial/total expression of gen APC</u>	<u>Metabolic fingerprinting</u>	<u>GC-TOF MS</u>	[43]
Tissue	<u>Patients with recurrent (n=4) and non-recurrent (n=3) CRC after treatment with 5-fluorouracil</u>	<u>Nucleotides and derivatives profiling</u>	<u>GC x GC-TOF MS</u>	[44]
Serum	<u>CRC patients (n=101) and healthy controls (n=102)</u>	<u>Metabolic fingerprinting</u>	<u>GC-TOF MS</u> <u>UHPLC-Q/TOF MS</u>	[31]
Cell culture	<u>Caco-2 cells versus other cancer cells (breast, neuroblastoma) and non cancer cell line</u>	<u>Metabolic profiling</u>	<u>GC-TOF MS</u>	[61]
Tissue and feces	<u>Feces and tissue from tumour and control area from CRC patients (n=17)</u>	<u>Metabolic fingerprinting</u>	<u>UHPLC-MS/MS</u> <u>GC-TOF MS</u>	[62]
Plasma	<u>Plasma from CRC patients (n=15) and healthy controls (n=15)</u>	<u>Metabolic fingerprinting</u>	<u>GC-TOF MS</u>	[63]

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Serum Serum from patients suffering
adenoma (n=31), various
stages of CRC (320) and
healthy controls (n=254) Metabolic fingerprinting GC-TOF MS [64]

Table S2. Identification of metabolites including KEGG and PubChem identifiers sorted by retention index.

<u>Identification</u>	<u>Retention Index</u>	<u>Quantmass</u>	<u>KEGG ID</u>	<u>PubChem ID</u>
<u>lactic acid</u>	<u>217657</u>	<u>191</u>	<u>C01432</u>	<u>19789253</u>
<u>glycolic acid</u>	<u>227636</u>	<u>177</u>	<u>C03547</u>	<u>3698251</u>
<u>alanine</u>	<u>244189</u>	<u>116</u>	<u>C00041</u>	<u>5950</u>
<u>maleimide</u>	<u>245118</u>	<u>154</u>	<u>C07272</u>	<u>10935</u>
<u>butylamine</u>	<u>249493</u>	<u>174</u>	-	<u>8007</u>
<u>hydroxylamine</u>	<u>253158</u>	<u>146</u>	<u>C00192</u>	<u>787</u>
<u>butyrolactam</u>	<u>277199</u>	<u>142</u>	-	<u>12025</u>
<u>methanolphosphate</u>	<u>289520</u>	<u>241</u>	-	<u>13130</u>
<u>methylmalonic acid</u>	<u>311617</u>	<u>147</u>	<u>C02170</u>	<u>487</u>
<u>valine</u>	<u>313502</u>	<u>144</u>	<u>C00183</u>	<u>6287</u>
<u>hydroxycarbamate</u>	<u>325948</u>	<u>278</u>	-	-
<u>pyrophosphate</u>	<u>327517</u>	<u>110</u>	-	-
<u>urea</u>	<u>332913</u>	<u>189</u>	<u>C00086</u>	<u>1176</u>
<u>benzoic acid</u>	<u>339067</u>	<u>179</u>	<u>C00539</u>	<u>20144841</u>
<u>caprylic acid</u>	<u>343457</u>	<u>201</u>	<u>C06423</u>	<u>379</u>
<u>ethanolamine</u>	<u>343800</u>	<u>174</u>	<u>C00189</u>	<u>700</u>
<u>glycerol</u>	<u>344466</u>	<u>205</u>	<u>C00116</u>	<u>753</u>
<u>phosphate</u>	<u>345365</u>	<u>314</u>	-	<u>1061</u>
<u>leucine</u>	<u>346101</u>	<u>158</u>	<u>C00123</u>	<u>6106</u>
<u>2-deoxyerythritol</u>	<u>355045</u>	<u>117</u>	-	-
<u>1-deoxyerythritol</u>	<u>357129</u>	<u>117</u>	-	-
<u>isoleucine</u>	<u>361332</u>	<u>158</u>	<u>C00407</u>	<u>6306</u>
<u>proline</u>	<u>366784</u>	<u>142</u>	<u>C00148</u>	<u>145742</u>
<u>glycine</u>	<u>368707</u>	<u>248</u>	<u>C00037</u>	<u>5257127</u>
<u>succinic acid</u>	<u>370608</u>	<u>247</u>	<u>C00042</u>	<u>1110</u>
<u>glyceric acid</u>	<u>379720</u>	<u>189</u>	-	<u>752</u>
<u>uracil</u>	<u>385735</u>	<u>241</u>	<u>C00106</u>	<u>1174</u>
<u>fumaric acid</u>	<u>390016</u>	<u>245</u>	<u>C00122</u>	<u>21883788</u>

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<u>serine</u>	<u>395020</u>	<u>218</u>	<u>C00065</u>	<u>5951</u>
<u>pelargonic acid</u>	<u>399229</u>	<u>117</u>	<u>C01601</u>	<u>8158</u>
<u>threonine</u>	<u>409568</u>	<u>218</u>	<u>C00188</u>	<u>6288</u>
<u>thymine</u>	<u>420133</u>	<u>255</u>	<u>C00178</u>	<u>1135</u>
<u>glutaric acid</u>	<u>421260</u>	<u>261</u>	<u>C00489</u>	<u>23322899</u>
<u>beta-alanine</u>	<u>435564</u>	<u>248</u>	<u>C00099</u>	<u>239</u>
<u>homoserine</u>	<u>443878</u>	<u>218</u>	<u>C00263</u>	<u>12647</u>
<u>capric acid</u>	<u>451790</u>	<u>229</u>	<u>C01571</u>	<u>2969</u>
<u>3-aminoisobutyric acid</u>	<u>452655</u>	<u>248</u>	<u>C05145</u>	<u>25201103</u>
<u>aminomalonnate</u>	<u>455754</u>	<u>218</u>	<u>C00872</u>	<u>100714</u>
<u>malic acid</u>	<u>463180</u>	<u>233</u>	<u>C00711</u>	<u>20130941</u>
<u>parabanic acid</u>	<u>464991</u>	<u>100</u>	<u>-</u>	<u>67126</u>
<u>threitol</u>	<u>467595</u>	<u>217</u>	<u>C16884</u>	<u>169019</u>
<u>nicotinamide</u>	<u>469827</u>	<u>179</u>	<u>C00153</u>	<u>936</u>
<u>erythritol</u>	<u>471922</u>	<u>217</u>	<u>C00503</u>	<u>222285</u>
<u>n-acetylglutamate</u>	<u>477776</u>	<u>158</u>	<u>C00624</u>	<u>70914</u>
<u>1,5-anhydroglucitol</u>	<u>478275</u>	<u>101</u>	<u>C07326</u>	<u>64960</u>
<u>aspartic acid</u>	<u>480387</u>	<u>232</u>	<u>C00049</u>	<u>44367445</u>
<u>methionine</u>	<u>483560</u>	<u>176</u>	<u>C00073</u>	<u>6137</u>
<u>trans-4-hydroxyproline</u>	<u>484934</u>	<u>140</u>	<u>C01157</u>	<u>5810</u>
<u>oxoproline</u>	<u>485935</u>	<u>156</u>	<u>C01879</u>	<u>7405</u>
<u>4-aminobutyric acid</u>	<u>488730</u>	<u>304</u>	<u>C00334</u>	<u>119</u>
<u>isothreonic acid</u>	<u>489385</u>	<u>292</u>	<u>-</u>	<u>-</u>
<u>cysteine</u>	<u>500158</u>	<u>220</u>	<u>C00097</u>	<u>5862</u>
<u>creatinine</u>	<u>502599</u>	<u>115</u>	<u>C00791</u>	<u>588</u>
<u>2-hydroxyglutaric acid</u>	<u>506306</u>	<u>247</u>	<u>C02630</u>	<u>43</u>
<u>dodecanol</u>	<u>507619</u>	<u>243</u>	<u>C02277</u>	<u>8193</u>
<u>2-(4-hydroxyphenyl)ethanol</u>	<u>509853</u>	<u>179</u>	<u>C06044</u>	<u>10393</u>
<u>erythronic acid</u>	<u>512029</u>	<u>217</u>	<u>-</u>	<u>2781043</u>
<u>digitoxose</u>	<u>521798</u>	<u>117</u>	<u>-</u>	<u>-</u>
<u>ornithine</u>	<u>527113</u>	<u>142</u>	<u>C00077</u>	<u>6262</u>
<u>glutamic acid</u>	<u>529100</u>	<u>246</u>	<u>C00025</u>	<u>33032</u>
<u>5-aminovaleric acid</u>	<u>536657</u>	<u>174</u>	<u>C00431</u>	<u>138</u>
<u>phenylalanine</u>	<u>537804</u>	<u>218</u>	<u>C00079</u>	<u>6140</u>
<u>4-hydroxyphenylacetic acid</u>	<u>542795</u>	<u>179</u>	<u>C00642</u>	<u>127</u>
<u>xylose</u>	<u>546699</u>	<u>307</u>	<u>-</u>	<u>-</u>
<u>lauric acid</u>	<u>547906</u>	<u>117</u>	<u>C02679</u>	<u>3893</u>
<u>N-acetylaspartic acid</u>	<u>548028</u>	<u>158</u>	<u>C01042</u>	<u>65065</u>
<u>asparagine</u>	<u>553078</u>	<u>188</u>	<u>C00152</u>	<u>6267</u>
<u>ribose</u>	<u>553135</u>	<u>217</u>	<u>C00121</u>	<u>5779</u>

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taurine	558857	326	C00245	1123
xylitol	567437	217	-	6912
levoglucosan	569637	204	-	2724705
lyxitol	573587	217	C01904	94154
beta-glycerolphosphate	574470	243	-	126740
fucose	578299	160	-	-
UDP-glucuronic acid	587601	217	C00167	17473
putrescine	588872	174	C00134	1045
glycerol-alpha-phosphate	590747	357	C03189	754
diglycerol	591074	103	-	42953
glucose-1-phosphate	594647	217	-	439165
ribonic acid	599680	292	-	-
glutamine	600315	156	C00064	5961
phosphoethanolamine	603912	299	C00346	1015
3-phosphoglycerate	610734	227	C00597	724
citric acid	617342	273	C00158	19782904
hypoxanthine	619128	265	C00262	790
citrulline	621404	157	C00327	6992098
pinitol	622466	260	-	164619
UDP-N-acetylglucosamine	623789	226	C00043	445675
UDP GlcNAc	627437	226	-	-
dehydroascorbic acid	632888	173	-	-
myristic acid	634414	117	C06424	11005
fructose	639442	307	C02336	439709
mannose	645856	205	C00159	18950
adenine	646534	264	C00147	190
galactose	648756	319	C00984	439357
glucose	650867	319	C00221	64689
lysine	663483	156	C00047	5962
histidine	663790	154	C00135	6274
tyramine	664737	174	C00483	5610
hexuronic acid	667373	160	-	18845
sorbitol	667922	217	C00794	5780
tyrosine	671252	218	C00082	6057
pentadecanoic acid	674647	117	C16537	13849
1-hexadecanol	679596	299	C00823	2682
pantothenic acid	690887	291	C12276	11306073
xanthine	701688	353	C00385	1188
hexitol	704741	217	C00392	453
palmitoleic acid	706508	117	C08362	445638

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palmitic acid	710061	313	C00249	985
cysteine-glycine	715335	220	-	174707
N-acetylmannosamine	722897	129	C00645	439281
N-acetyl-D-mannosamine	726375	319	-	-
myo-inositol	730022	305	-	892
heptadecanoic acid	751309	117	-	10465
octadecanol	755409	327	-	8221
glutathione	761296	213	C02471	124886
tryptophan	780482	202	C00078	6305
oleic acid	781527	129	-	-
stearic acid	787622	117	C01530	5281
spermidine	792924	144	C00315	1102
fructose-6-phosphate 1	804279	315	-	-
cystine	804619	218	C01420	24798687
glycerol-3-galactoside	805227	204	-	-
hexose-6-phosphate 1	806282	387	-	-
pseudo uridine	813899	217	C02067	15047
glucose-6-phosphate	818275	387	C00092	5958
nonadecanoic acid	822782	117	C16535	12591
inositol-4-monophosphate	846510	315	-	161368
phosphogluconic acid	847013	299	-	-
oleamide	849710	144	C19670	5283387
arachidic acid	856421	117	C06425	10467
uridine	861508	217	C00299	6029
5-methoxytryptamine	864466	174	C05659	1833
inosine	897184	230	C00294	6021
1-monopalmitin	901749	129	-	14900
sucrose	915139	271	C00089	5988
adenosine	918039	236	C00212	60961
behenic acid	920648	117	C08281	8215
cellobiose	932179	204	C01971	6255
lactose	935640	191	-	-
1-monolein	952623	129	-	5283468
guanosine	954962	324	C00387	6802
1-monostearin	959214	129	-	24699
5'-deoxy-5'-methylthioadenosine	967036	236	C00170	439176
cytidine-5-monophosphate	974825	169	-	-
uridine-5-monophosphate	977896	169	-	-
inosine 5'-monophosphate	1016811	169	C00130	8582
adenosine-5-monophosphate	1041375	315	C00020	6083

cholesterol	1078536	129	C00187	5997
cholestan-3-ol	1082070	215	-	3240

Table S31. Main metabolic pathways involving metabolites preferentially extracted using the organic based solvents (A and B).

Pathway	Total	Expected	Hits	Raw p	-log(p)	Holm p	FDR	Impact
Fatty acid biosynthesis	49	0.7939	8	6.24E-07	14.287	4.99E-05	4.99E-05	0
Fatty acid metabolism	50	0.8101	3	0.0456	3.0869	1	1	0.0296
Pyrimidine metabolism	60	0.9722	3	0.0714	2.6396	1	1	0.1104
Purine metabolism	92	1.4907	3	0.1851	1.6867	1	1	0.1193
Galactose metabolism	41	0.6643	3	0.0274	3.5964	1	1	0.1174
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	14	0.2268	1	0.2049	1.5851	1	1	0
Citrate cycle (TCA cycle)	20	0.3241	1	0.2797	1.2742	1	1	0.0633
Sphingolipid metabolism	25	0.4051	1	0.3367	1.0887	1	1	0.0129
Arginine and proline metabolism	77	1.2476	2	0.3566	1.0312	1	1	0.0264
Fatty acid elongation in mitochondria	27	0.4375	1	0.3582	1.0267	1	1	0
Glutathione metabolism	38	0.6157	1	0.4651	0.7656	1	1	0.2374
Nitrogen metabolism	39	0.6319	1	0.4739	0.7468	1	1	0.0606
Glycerophospholipid metabolism	39	0.6319	1	0.4739	0.7468	1	1	0.0713
Inositol phosphate metabolism	39	0.6319	1	0.4739	0.7468	1	1	0.137
Ascorbate and aldarate metabolism	45	0.7291	1	0.5238	0.6466	1	1	0
Primary bile acid biosynthesis	47	0.7615	1	0.5394	0.6173	1	1	0.0552
Lysine degradation	47	0.7615	1	0.5394	0.6173	1	1	0.0651
Glyoxylate and dicarboxylate metabolism	50	0.8101	1	0.5619	0.5765	1	1	0.0033
Cysteine and methionine metabolism	56	0.9074	1	0.6037	0.5047	1	1	0.0074
Amino sugar and nucleotide sugar metabolism	88	1.4258	1	0.7688	0.263	1	1	0.0259
Steroid hormone biosynthesis	99	1.6041	1	0.8082	0.2129	1	1	0.0039

Table S24. Main metabolic pathways involving metabolites preferentially extracted using the aqueous-based solvents (C and D).

Pathway	Total	Expected	Hits	Raw p	-log(p)	Holm p	FDR	Impact
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10	Aminoacyl-tRNA biosynthesis	75	2.7732	14	2.85E-07	15.07	2.28E-05	2.28E-05	0.169	Formatted: English (United States)	
11	Alanine aspartate and glutamate metabolism	24	0.8874	8	1.16E-06	13.671	9.12E-05	4.62E-05	0.5575	Formatted: Font: Bold, Font color: Text 1, English (United States)	
12	Arginine and proline metabolism	77	2.8471	13	2.65E-06	12.839	0.000207	7.08E-05	0.4744	Formatted: English (United States)	
13	Nitrogen metabolism	39	1.442	9	7.33E-06	11.824	0.0005641	0.0001465	0.0076	Formatted: Font color: Text 1, English (United States)	
14	Starch and sucrose metabolism	50	1.8488	8	0.0003822	7.8695	0.02905	0.0056231	0.3363	Formatted: English (United States)	
15	beta-Alanine metabolism	28	1.0353	6	0.0004217	7.7711	0.03163	0.0056231	0.3232	Formatted: English (United States)	
16	Pyrimidine metabolism	60	2.2185	8	0.0013446	6.6117	0.099501	0.015367	0.135	Formatted: Font color: Text 1, English (United States)	
17	Cyanoamino acid metabolism	16	0.5916	4	0.0022609	6.092	0.16505	0.02039	0	Formatted: English (United States)	
18	Glutathione metabolism	38	1.4051	6	0.0022938	6.0775	0.16516	0.02039	0.0642	Formatted: English (United States)	
19	Galactose metabolism	41	1.516	6	0.0034173	5.6789	0.24263	0.027338	0.0485	Formatted: Font color: Text 1, English (United States)	
20	Amino sugar and nucleotide sugar metabolism	88	3.2538	9	0.004433	5.4187	0.31031	0.03224	0.2075	Formatted: English (United States)	
21	Phenylalanine metabolism	45	1.6639	6	0.0054974	5.2035	0.37932	0.03665	0.1665	Formatted: Font color: Text 1, English (United States)	
22	Propanoate metabolism	35	1.2941	5	0.0083368	4.7871	0.56691	0.051304	0.0866	Formatted: English (United States)	
23	Pentose and glucuronate interconversions	53	1.9597	6	0.012229	4.4039	0.81935	0.06988	0.0685	Formatted: English (United States)	
24	Pantothenate and CoA biosynthesis	27	0.9983	4	0.015981	4.1364	1	0.085232	0.253	Formatted: Font color: Text 1, English (United States)	
25	Purine metabolism	92	3.4017	8	0.018586	3.9853	1	0.092931	0.1436	Formatted: English (United States)	
26	Pentose phosphate pathway	32	1.1832	4	0.028469	3.5589	1	0.13397	0.1574	Formatted: Font color: Text 1, English (United States)	
27	Glycine serine and threonine metabolism	48	1.7748	5	0.030202	3.4998	1	0.13423	0.4927	Formatted: English (United States)	
28	Cysteine and methionine metabolism	56	2.0706	5	0.053576	2.9267	1	0.21767	0.1344	Formatted: English (United States)	
29	Butanoate metabolism	40	1.479	4	0.057939	2.8484	1	0.21767	0.0461	Formatted: English (United States)	
30	Tyrosine metabolism	76	2.8101	6	0.059324	2.8247	1	0.21767	0.1258	Formatted: English (United States)	
31	D-Glutamine and D-glutamate metabolism	11	0.4067	2	0.05986	2.8157	1	0.21767	0.139	Formatted: English (United States)	
32	Valine leucine and isoleucine biosynthesis	27	0.9983	3	0.075419	2.5847	1	0.26233	0.0265	Formatted: English (United States)	
33	Glycolysis or Gluconeogenesis	31	1.1462	3	0.10445	2.2591	1	0.34815	0	Formatted: English (United States)	
34	Methane metabolism	34	1.2572	3	0.12868	2.0505	1	0.41176	0.0175	Formatted: English (United States)	
35	Sulfur metabolism	18	0.6656	2	0.14121	1.9575	1	0.43448	0.0378	Formatted: English (United States)	
36	Citrate cycle (TCA cycle)	20	0.7395	2	0.1674	1.7874	1	0.49599	0.0313	Formatted: English (United States)	
37	Valine leucine and isoleucine degradation	40	1.479	3	0.18221	1.7026	1	0.5206	0.0223	Formatted: English (United States)	
38	Thiamine metabolism	24	0.8874	2	0.2218	1.506	1	0.61187	0	Formatted: English (United States)	
39	Lysine degradation	47	1.7378	3	0.25063	1.3838	1	0.63876	0.2262	Formatted: English (United States)	
40	D-Arginine and D-ornithine metabolism	8	0.2958	1	0.26056	1.3449	1	0.63876	0	Formatted: English (United States)	
41	Fructose and mannose metabolism	48	1.7748	3	0.26074	1.3442	1	0.63876	0.07	Formatted: English (United States)	
42	Phenylalanine tyrosine and tryptophan biosynthesis	27	0.9983	2	0.26349	1.3337	1	0.63876	0.008	Formatted: English (United States)	
43	Glyoxylate and dicarboxylate metabolism	50	1.8488	3	0.28111	1.269	1	0.66144	0.0398	Formatted: English (United States)	
44	Lysine biosynthesis	32	1.1832	2	0.33288	1.1	1	0.73486	0.0999	Formatted: English (United States)	
45	Glycerolipid metabolism	32	1.1832	2	0.33288	1.1	1	0.73486	0.2091	Formatted: English (United States)	
46	Biotin metabolism	11	0.4067	1	0.33987	1.0792	1	0.73486	0	Formatted: English (United States)	
47	Inositol phosphate metabolism	39	1.442	2	0.42658	0.85196	1	0.89806	0.012	Formatted: English (United States)	
48	Nicotinate and nicotinamide	44	1.6269	2	0.48927	0.71483	1	0.97855	0.0383	Formatted: English (United States)	

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10	metabolism								
11	Histidine metabolism	44	1.6269	2	0.48927	0.71483	1	0.97855	0.1404
12	Primary bile acid biosynthesis	47	1.7378	2	0.52482	0.64469	1	1	0.0164
13	Taurine and hypotaurine metabolism	20	0.7395	1	0.53073	0.6335	1	1	0.3309
14	Caffeine metabolism	21	0.7765	1	0.54823	0.60107	1	1	0.0305
15	Sphingolipid metabolism	25	0.9244	1	0.61199	0.49104	1	1	0
16	Pyruvate metabolism	32	1.1832	1	0.70288	0.35257	1	1	0.1376
17	Ubiquinone and other terpenoid-quinone biosynthesis	36	1.3311	1	0.745	0.29437	1	1	0
18	Porphyrin and chlorophyll metabolism	104	3.8455	3	0.75021	0.2874	1	1	0
19	Glycerophospholipid metabolism	39	1.442	1	0.77266	0.25791	1	1	0.0556
20	Ascorbate and aldarate metabolism	45	1.6639	1	0.81939	0.19919	1	1	0.008
21	Tryptophan metabolism	79	2.9211	1	0.95152	0.04969	1	1	0

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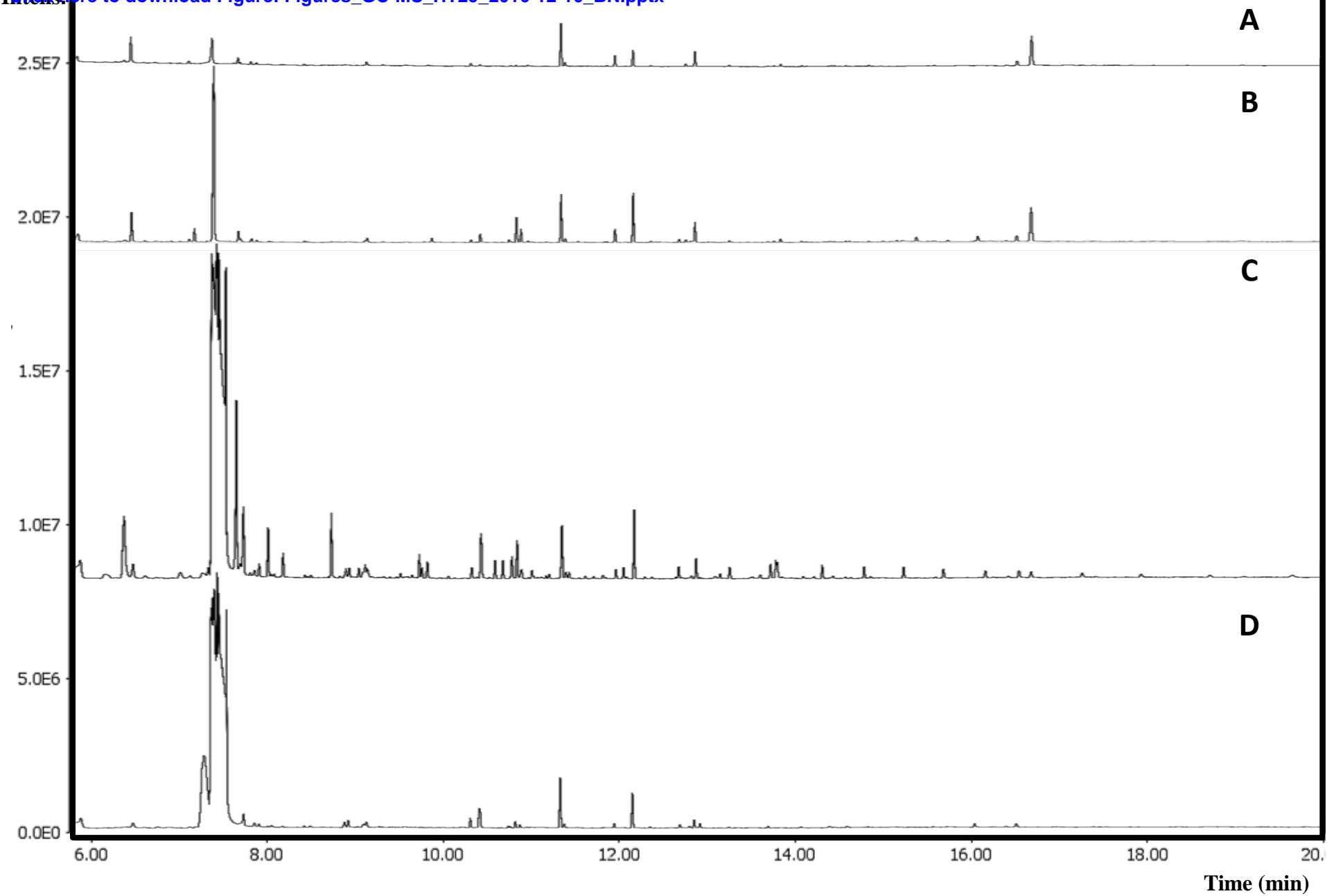


Figure 1.

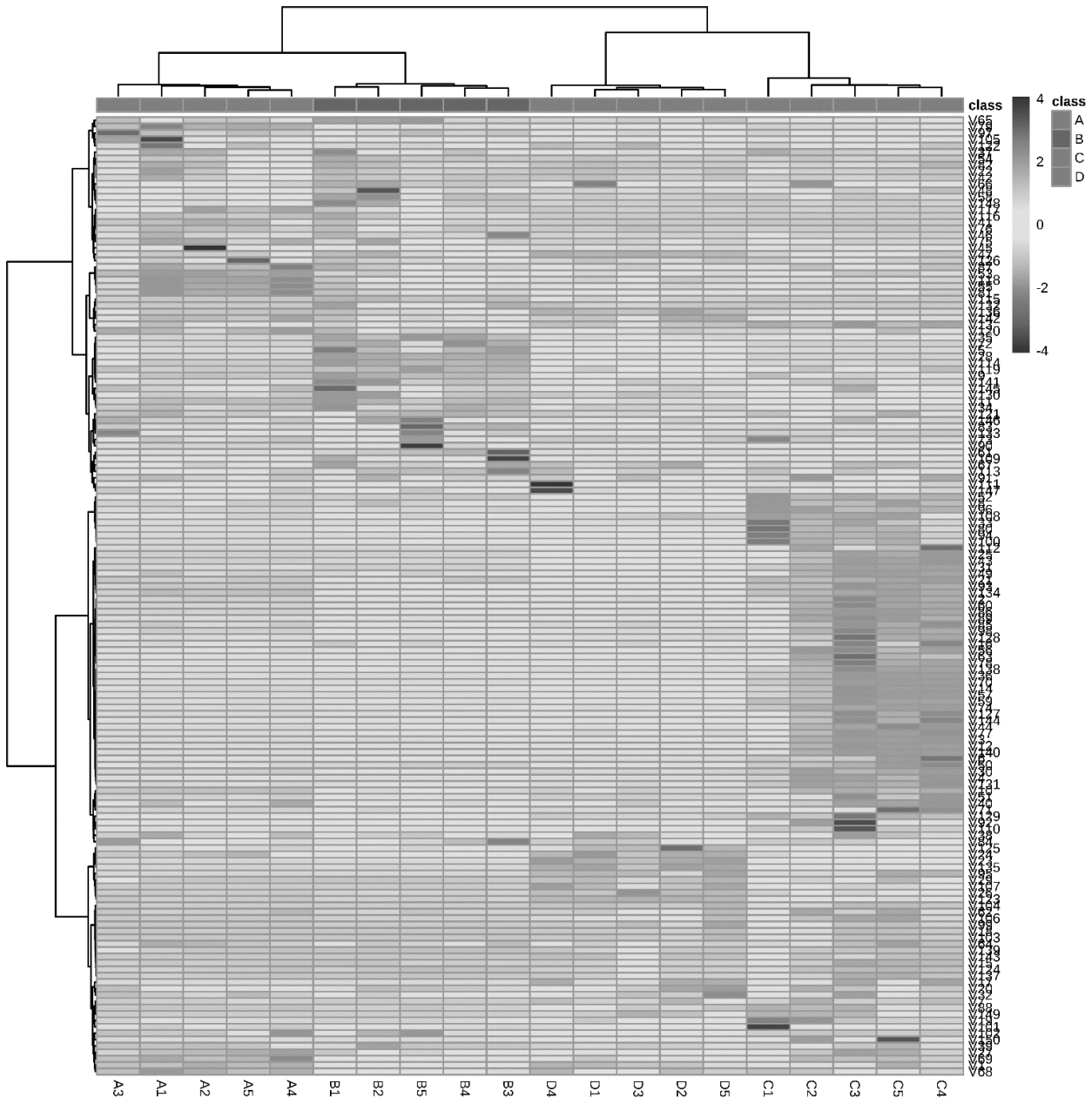


Figure 2.

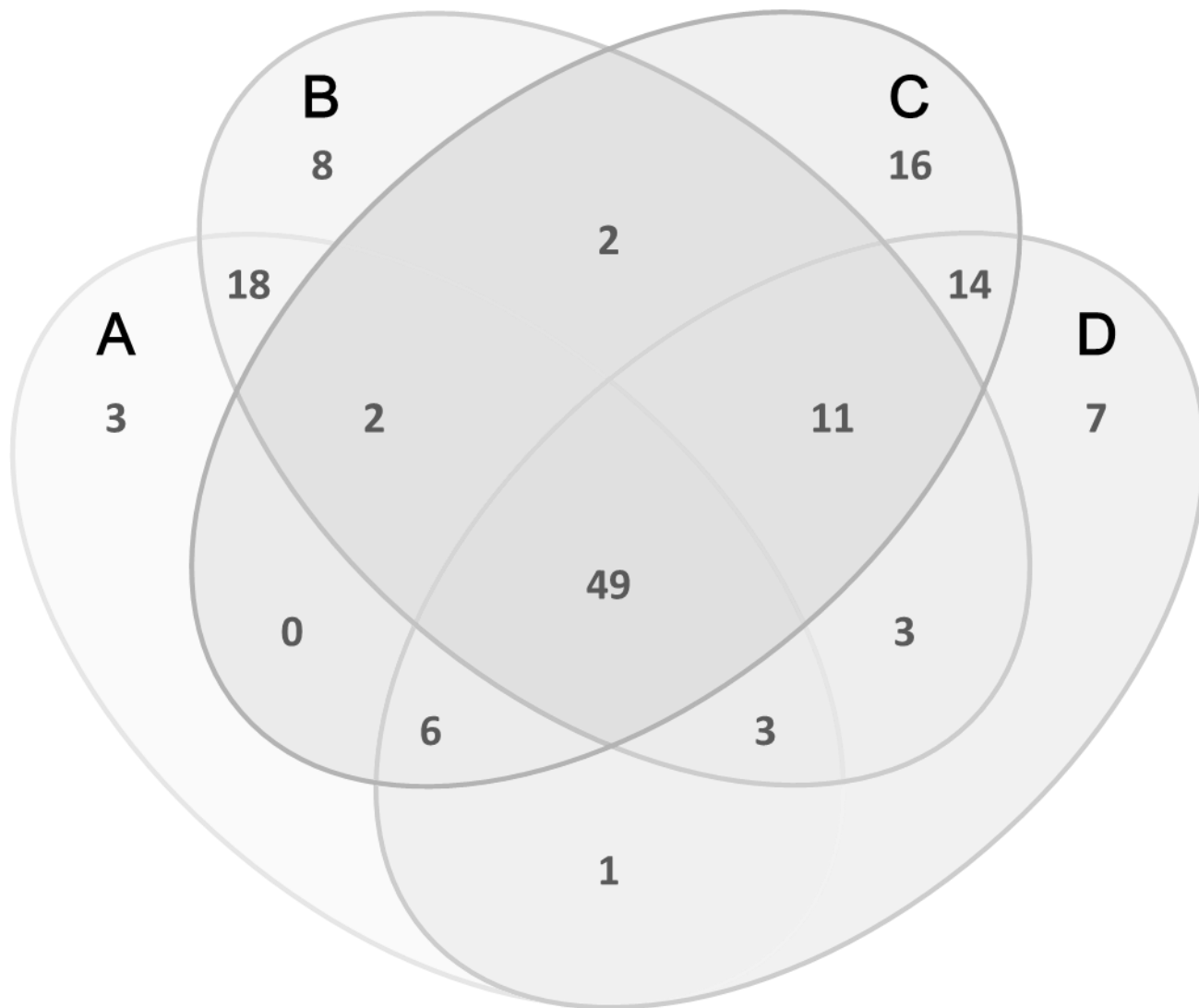


Figure 3.

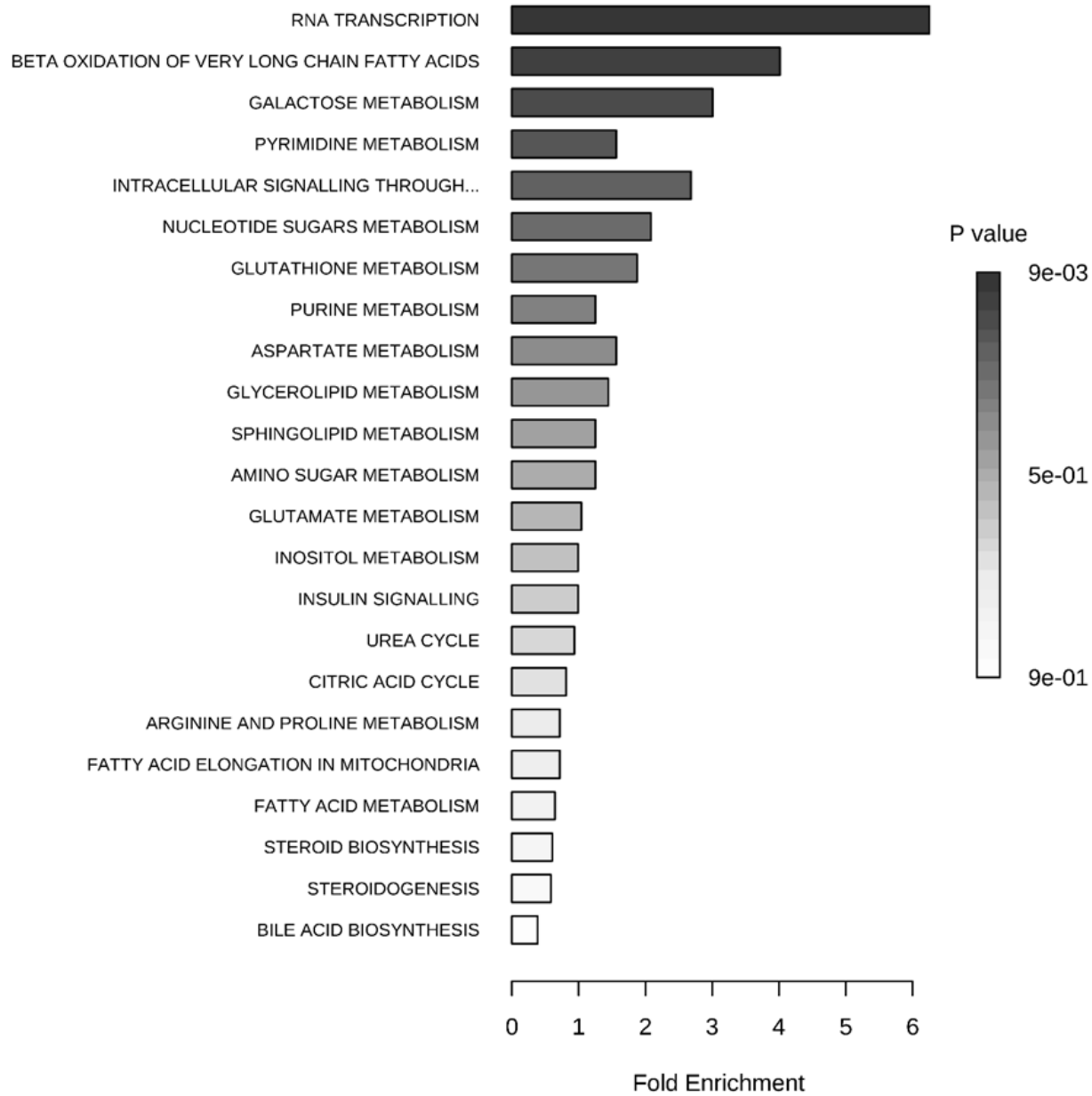


Figure 4.

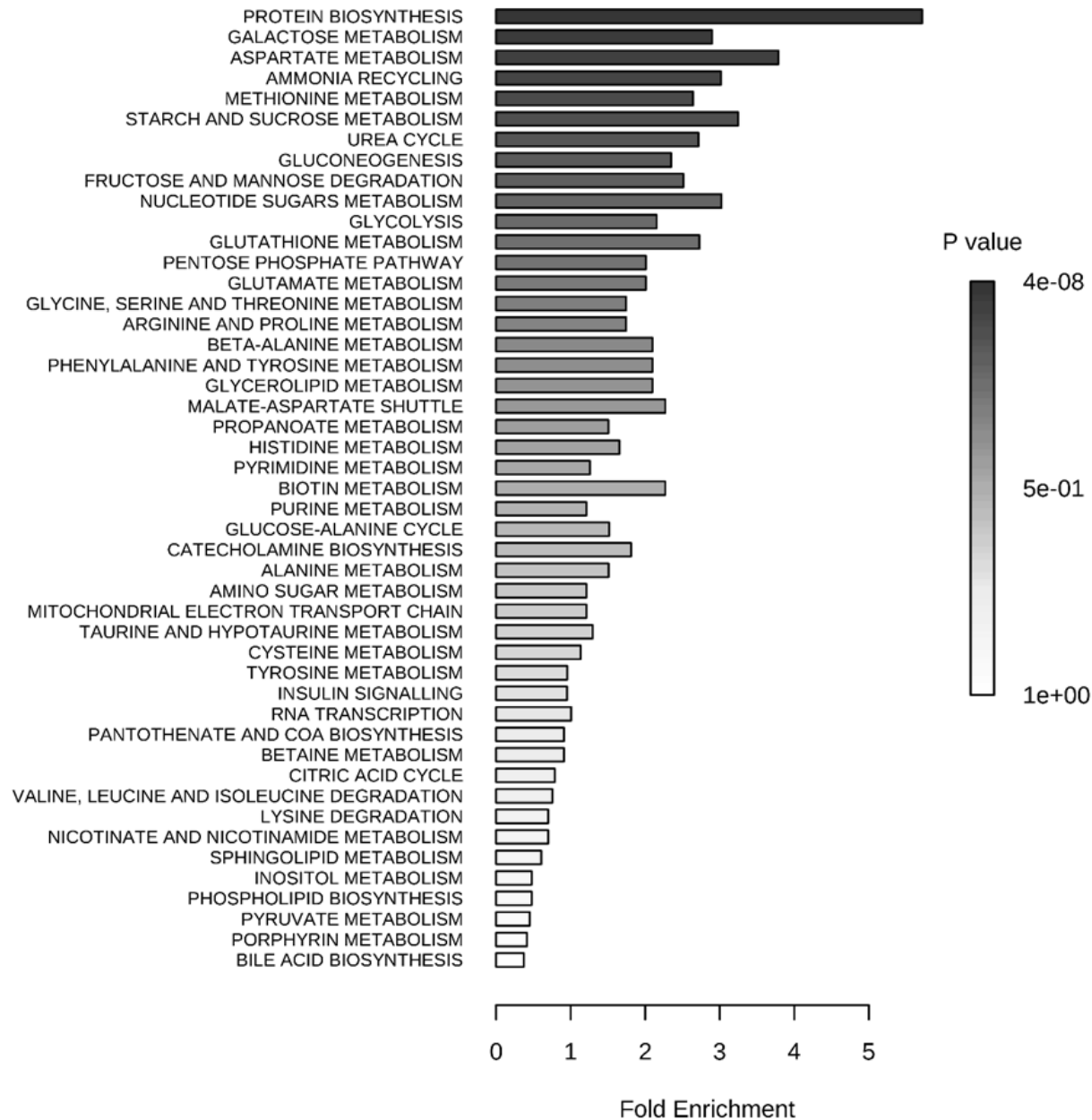


Figure 5.

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1 **GC-MS BASED METABOLOMICS OF COLON CANCER CELLS USING**
2 **DIFFERENT EXTRACTION SOLVENTS**

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4 **15 ABSTRACT**

5
6 16 The increasing incidence of colorectal cancer enforces the development of novel
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8 17 methodologies and protocols to deepen in the molecular mechanisms that govern disease
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10 18 pathophysiological events. The aim of this work is to deepen in the optimum metabolite
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12 19 extraction protocol from adherent mammalian cells of colon cancer for high throughput
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14 20 metabolomics using gas chromatography coupled to mass spectrometry (GC-MS). GC-MS
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16 21 results showed that metabolic information obtained from colon cancer cells was highly
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18 22 dependent on metabolite extraction selection, which at the same time is extremely
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20 23 influenced by the analytical platform. A further purpose of this investigation is to uncover
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22 24 an unexplored portion of HT-29 colon cancer cells metabolome, complementary to other
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24 25 already explored by CE-MS and LC-MS methods. At this respect, a total of 150
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26 26 metabolites were identified in HT-29 colon cancer cells by GC-MS. The extraction protocol
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28 27 with acetonitrile-isopropanol-water was the most appropriate for fatty acids and related
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30 28 pathways analysis. Most of the metabolites involved in pathways of amino acids,
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32 29 glutathione, amino sugars and other polar metabolites were better extracted with acidified
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34 30 water, although water extraction showed the best overall reproducibility. Although
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36 31 pathways involving nitrogenous bases could be investigated using organic or aqueous
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38 32 extracts, a higher number of metabolites involved in these pathways were identified in the
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40 33 aqueous extracts. In addition, metabolite extraction protocol was observed to be crucial for
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42 34 the determination of potentially interesting clusters of metabolites.
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55 36 **Keywords:** Colon cancer, Sample treatment, Metabolomics, gas chromatography-mass
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57 37 spectrometry, GC-MS.
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39 **Abbreviations:** CRC, colorectal cancer; MSEA, metabolite set enrichment analysis; MPA,
40 metabolic pathway analysis; MSTFA, N-methyl-N-(trimethylsilyl)-trifluoroacetamide;
41 ORA, overrepresentation analysis; PBS, phosphate buffered saline; PCA, principal
42 component analysis; RIM, internal retention index markers; TIC, total ion chromatogram

1. INTRODUCTION

In the past few years, cancer biology research has increasingly been focused on metabolism in cancer cells [1]. The complexity and diversity of biological alterations inherently linked to cancer metabolism is in accordance with the increasing unresolved issues to determine specific causes in cancer development, to assess the progression and to unravel molecules or pathways to target in cancer therapy and/or prevention. It is known that cancer cells show different metabolism from healthy cells. One of the well-known metabolic alterations in cancer cells is the glycolysis utilization over the oxidative phosphorylation [2, 3]. In the past years, other perturbations in specific metabolic pathways have been addressed linked to energy metabolism [4] such as pyruvate production [5] and tricarboxylic acid (TCA) cycle [6]. The progress of Metabolomics offers valuable opportunity to better understand biochemical changes produced in cancer metabolism for improving early detection, progression and therapy monitoring of cancer disease [7]. Metabolomics is able to meet the challenge of detecting hundreds of metabolites in short time analysis thanks to the evolution of analytical technologies and software tools for data processing and statistics [8]. With the evolution of metabolomics platforms, rapid and comprehensive analyses of the metabolome complexity can be performed in a high-throughput manner with improved sensitivity, accuracy and resolution [1]. Typical metabolomic studies are based on mass spectrometry (MS) or nuclear magnetic resonance (NMR) whose advantages and limitations have been discussed elsewhere [9, 10]. It is now assumed that the coverage of the human metabolome is impossible to achieve with a single analytical methodology and an increasingly common practice is the combination of analytical techniques to achieve complementary information [11]. Metabolomics potential has already been shown with the

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4 66 detection of novel biomarkers involved in different metabolic pathways related to breast
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7 67 [12], liver [13], prostate [14], colon [15] or lung cancer [16] among the vast amount of
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9 68 investigations. From those publications it can be assumed that different cancer subtypes
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11 69 exhibit different phenotypes and therefore show different metabolic alterations and
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14 70 biomarkers. This diversity enforces the investigation of cancer with special attention to
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16 71 most worldwide harmful cancer types.

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20 72 Colorectal cancer is the second leading cause of cancer mortality in the USA [17] and
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22 73 Europe [18] with a continuous increasing incidence. Identifying soon the colon cancer
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24 74 apparition and/or progression will increase our knowledge on efficient drug discovery
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26 75 research and prevention. With this aim, in the last decade, Metabolomics has emerged to
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28 76 understand pathophysiological processes related to colon cancer. From the about 30 works
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30 77 related to colon cancer Metabolomics published so far, a minor part have been
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33 78 accomplished using NMR [19-23] with a clear trend toward the use of MS-based analytical
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35 79 platforms either alone [24-26] or in combination with separation techniques such as
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38 80 capillary electrophoresis (CE) [27-30], liquid chromatography (LC) [24, 27-29, 31-34] and
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40 81 gas chromatography (GC) [15, 31-33, 35-44].
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45 82 Metabolomics investigations of colorectal cancer by GC-MS are summarized in Table S1.
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47 83 As can be seen in Table S1 a variety of samples including biofluids, tissues and cell culture
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49 84 models have been under scrutiny. The use of cell cultures is included in common reference
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51 85 model systems and is considered an invaluable biomedical research tool. However, the
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53 86 application of cell culture in metabolomics requires further development and
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55 87 standardization of study design steps, metabolism quenching method selection and
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57 88 optimization of metabolite extraction protocols, among others [45, 46]. Namely, quenching
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89 step at the time of harvesting aims to prevent metabolic content to be altered before the
90 analysis so that the metabolic state of the cell is preserved. There is a great controversy and
91 continuous research in the determination of the optimum quenching method. Until date the
92 most widely spread techniques to quench the metabolism in cells is a shock maintaining the
93 cells below -20 °C [47, 48], the addition of cold solvents such as cold methanol [49], use of
94 cold isotonic PBS [49-51] or the combination in a single step of quenching and extraction
95 procedures in mammalian adherent cells [45, 52].

96 As can be observed in Table S1, three cell lines (namely SW, HT and Caco-2) have been
97 used to investigate colon cancer following metabolomics approaches using GC-MS. All
98 these cell lines are very well established for in-vitro studies of colorectal cancer and vary in
99 phenotype, growth rate, differentiation and morphology among other characteristics [53,
100 54]. Metabolic fingerprinting by GC- MS from two SW cell lines (SW-1116 and SW-480)
101 was first reported by Zimmermann et al. [35]. More recently LC-MS and CE-MS based
102 metabolomics have been used to investigate HT-29 cell line revealing significant metabolic
103 information in colon cancer metabolism after treatment with different polyphenol-rich
104 extracts [28, 29, 55].

105 Considering that no single method is appropriate for the determination of all intracellular
106 metabolites, metabolic information obtained from cell cultures will depend on multiple
107 factors and metabolite extraction selection is highly influenced by the analytical platform
108 [56]. Optimization and selection of the protocol for metabolite extraction from HT-29 cells
109 [57] and evaluation of the subsequent cytosol sample treatment [30] for CE-MS
110 metabolomics have been previously carried out. CE-MS is particularly suited for the rapid
111 separation of ionic, weakly ionic, and/or highly polar metabolites and aqueous solvent was

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4 112 the preferred for metabolite extraction of HT-29 cells [57]. On the contrary, GC-MS is
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6 113 preferred for the analysis of less polar, volatile (or amenable to chemical derivatization)
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9 114 compounds and requires thermal stability of the analytes. Considering that no single
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11 115 analytical platform is able to determine all intracellular metabolites, in this work, a GC-MS
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14 116 method has been developed due to the high complementarity nature of GC-MS used in this
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16 117 work and the CE-MS [57] and LC-MS [28, 29] methods already developed by our group.
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19 118 Further, taking into account all the above considerations, in this work GC-MS has been
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21 119 used to evaluate the coverage and reproducibility of four metabolic extraction solvents
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24 120 selected according to the physicochemical diversity of metabolites including the large
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26 121 variation on solubility (in aqueous or organic solvents) and based on previous published
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29 122 works [30, 57-63]. To our knowledge this is the first time that HT-29 colon cancer cells are
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31 123 examined using GC-MS metabolomics which could be of special importance in future
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34 124 studies to detect biochemical alterations due to colon cancer or to explore new preventive
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36 125 interventions.

37 38 39 126 **2. MATERIALS AND METHODS**

40 41 42 127 **2.1. Chemicals**

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45 128 All reagents were of analytical grade. Phosphate buffered saline (PBS) was purchased from
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48 129 Lonza (Barcelona, Spain). For cell counting, trypan blue was purchased from Sigma
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51 130 Aldrich (St. Louis, MO, USA). Metabolite extraction solvents were of MS grade: formic
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53 131 acid and 2-propanol were from Riedel-de Haën (Seelze, Germany) while water and
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55 132 acetonitrile were from Labscan (Gliwice, Poland). Reagents for derivatization included
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58 133 methoxyamine hydrochloride from Sigma Aldrich, pyridine (silylation grade) and N-

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134 methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) purchased in 1 mL bottles from
135 Pierce (Rockford IL USA).

136 For GC-MS analysis, a mixture of internal retention index markers (RIM) was prepared
137 using fatty acid methyl esters of C8, C9, C10, C12, C14, C16, C18, C20, C22, C24, C26,
138 C28 and C30 linear chain length, dissolved in chloroform (from Mallinckrodt Baker Inc.,
139 Phillipsburg, NJ, USA) at a concentration of 0.8 mg/mL (C8-C16) and 0.4 mg/mL (C18-
140 C30). Fatty acid methyl esters were from Sigma Aldrich. FC43 (Perfluorotributylamine)
141 was acquired from Leco (Stockport, Cheshire, UK) for MS mass calibration.

142 **2.2. Cell culture**

143 The human HT-29 colorectal carcinoma cell line was purchased from the American Type
144 Culture Collection (ATCC- LGC, Promochem, UK). HT-29 cells were cultured in
145 McCoy's 5A medium supplemented with 10% heat-inactivated fetal calf serum, 50 U/mL
146 penicillin G, and 50 U/mL streptomycin, at 37 °C in humidified atmosphere with 5% CO₂.
147 Once cells reached about 50% confluency, they were trypsinized, neutralized with culture
148 medium and plated at 10000 cells/cm² density in different P150 culture dishes. Cell
149 cultures were allowed to adhere overnight at 37 °C and then plates were pooled. Next, cell
150 count was performed using the trypan blue exclusion test to examine the viability and to
151 estimate the number of cells. For that purpose, 5 µL cell suspension pool were further
152 diluted 1:1 in trypan blue stock solution (0.4%, w/v in sterile PBS) and counted in a
153 Neubauer counting chamber using a light microscope (ID3, Carl Zeiss, Jena, Germany).
154 The estimated volume to have 10 million cells from the culture suspension was added to 20
155 aliquots. After aliquots were centrifuged (300 xg, 10 min) and supernatant was discarded,
156 pellets containing 10 x 10⁶ cells were subjected to metabolite extraction.

157 **2.3. Metabolite extraction and derivatization**

158 The 20 aliquots were divided in four solvent extraction groups: A, ACN; B, ACN-IsopOH-
159 Water (3:3:1, v/v/v); C, Water; and D, 5% formic acid in water. Five independent
160 metabolite extractions (with 10 million cells each) were performed for each extraction
161 solvent group. Metabolic content extraction from HT-29 cells have been previously
162 optimized in our laboratory [57] and applied in this work with slight modifications. Briefly,
163 300 μL of extraction solvent and 0.3 g glass beads (212-300 μm) from Sigma-Aldrich were
164 added to 10×10^6 cell pellets. Then metabolism quenching and cell disruption was
165 accomplished by using three cycles of snap-freeze (liquid N₂, 1 min), thawing in
166 ultrasound bath (50 Hz, 3 min) (ultrasonic cleaning bath Ultrasons from JP Selecta,
167 Barcelona, Spain) and grinding using a mixer mill for 3 min at 30/s (MM 400, Retsch
168 GmbH, Germany). After this, cell insoluble material and glass beads were removed by
169 centrifugation (24000 xg for 10 min at 4°C) and the supernatant was collected, and 50 μL
170 metabolic extracts were vacuum-dried and kept at -80°C until derivatization. The
171 supernatants obtained in A and B extraction groups are protein-free considering organic
172 extraction solvents lead to protein precipitation. On the contrary, supernatants obtained
173 from aqueous-based extractions (C and D extraction groups) were submitted to
174 ultrafiltration with 3 kDa centrifugal filters (Amicon Ultra 0.5 mL, Millipore, Billerica, MA,
175 USA) to remove proteins according to the manufacturer's protocol. 50 μL of both metabolic
176 extracts were vacuum-dried and kept at -80°C until derivatization.

177 The best sensitivity was achieved with the following optimized derivatization protocol.

178 First, the dried extracts were dissolved in 10 μL of a solution consisting of 40 mg/mL
179 methoxyamine in pyridine and shaken for 15 min at 60 °C to protect aldehyde and ketone

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180 groups. Then 50 μ L of MSTFA with 1% RIM was added for trimethylsilylation of acidic
181 protons and shaken at 37 °C for 30 min. Derivatized samples were immediately transferred
182 to 2 mL clear glass autosampler vials with microinserts (Agilent, Santa Clara CA), closed
183 by 11 mm T/S/T crimp caps (MicroLiter, Suwanee GA) and analyzed by GC-MS.

184 **2.4. GC-TOF MS analysis**

185 An Agilent 6890 gas chromatograph (Santa Clara CA) and a Leco Pegasus IV time of flight
186 mass (TOF) spectrometer were controlled by the Leco ChromaTOF software vs. 2.32 (St.
187 Joseph, MI). A 30 m long, 0.25 mm ID Rtx-5Sil MS column with 0.25 μ m 95% dimethyl
188 5% diphenyl polysiloxane film and additional 10 m integrated guard column was used
189 (Restek, Bellefonte PA). High purity helium with built-in purifier (Airgas, Radnor PA) was
190 set at constant flow of 1 mL/min. The oven temperature was held constant at 50°C for 1
191 min and then ramped at 20°C/min to 330°C at which it was held constant for 5 min. The
192 transfer line temperature between gas chromatograph and mass spectrometer was set to
193 280°C. A Gerstel automatic liner exchange system with multipurpose sample MPS2 dual
194 rail and two derivatization stations was used in conjunction with a Gerstel CIS cold
195 injection system (Gerstel, Muehlheim, Germany). The injector operated in splitless mode,
196 opening the split vent after 25 s. Electron impact ionization at 70V was applied maintaining
197 ion source temperature at 250°C. For every 10 samples, a fresh multibaffled liner was
198 inserted (Gerstel) using the Maestro1 Gerstel software vs. 1.1.4.18 (Gerstel). Before and
199 after each injection, the 10 μ L injection syringe was washed three times with 10 μ L ethyl
200 acetate. 1 μ L sample was filled using 39 mm vial penetration at 1 μ L/s fill speed, injecting
201 0.5 μ l at 10 μ L/s injection speed. GC oven temperature was maintained at 50°C during one

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4 202 minute followed by a temperature ramp at 20°C/min until 330°C at which it was held
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6 203 constant for 5 min.
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10 204 TOF-MS analysis in the positive ion mode was accomplished applying 1775 V detector
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12 205 voltage. Data was recorded in the 85-500 m/z range for 1200 s after 290 s solvent delay.
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14 206 Mass resolving power was 600 and acquisition rate was set at 17 spectra/s. Daily quality
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16 207 controls were used to assure quality of data. The instrument performed autotuning for mass
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18 208 calibration using FC43 before each sequence analysis using the following m/z values: 93,
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20 209 95,100, 101, 112, 113, 114, 119, 131, 132, 145, 150, 164, 169, 176, 181, 214, 219, 220,
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22 210 226, 264, 265, 314, 326, 352, 376, 414, 415, 426, 448, 464, 502 and 503.
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28 211 **2.5. Data mining**

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31 212 GC-TOF MS data were acquired as ChromaTOF specific .peg files, and stored also in .txt
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33 213 and .cdf generic formats. Data mining was performed as already described [60]. Briefly,
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35 214 absolute spectra intensities from all sample files were automatically processed and filtered
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37 215 by BinBase algorithm using previously optimized parameters [64]. Then post-processing
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39 216 module to replace missing values was carried out. The resulting report contained row
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41 217 identifier, mass spectra ions, quantification by means of unique ion height, retention index
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43 218 and identification using defined databases [65]. Additional mass signal filtering was
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45 219 manually performed to remove not identified and low confident ions from the table. Then
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47 220 MS signals belonging to the same ion were grouped and data was normalized based on total
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49 221 ion height for each sample. The table with high-confident metabolite information from all
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51 222 extraction replicates obtained with the four solvents (five replicates per extraction solvent)
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58 223 was then examined in detail. Principal component analysis (PCA) and hierarchical
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224 clustering was performed to explore possible clustering, similarities and differences in
225 metabolic coverage obtained among the four extraction protocols. Then comparison of
226 metabolome coverage among the four extracts was performed by metabolite set enrichment
227 analysis (MSEA) and metabolic pathway analysis (MPA). MSEA was performed by means
228 of overrepresentation analysis (ORA). ORA assess whether a set of metabolites is enriched
229 in a particular metabolic pathway compared to the total of annotated metabolites for that
230 pathway (i.e. if the metabolites identified in the study represent a high percentage of the
231 metabolites theoretically annotated in that pathway) [66]. Statistical analysis, MSEA and
232 MPA were performed using metaboanalyst [67]. MBRole [68] was used to assist in the
233 biological interpretation.

234 **3. RESULTS AND DISCUSSION**

235 **3.1. Metabolic extraction assessment**

236 Typical total ion chromatograms (TIC) for the cell extracts obtained using the four different
237 extraction solvents tested in this work are represented in Fig. 1. As can be observed, the
238 four metabolite profiles were very different in terms of intensity and distribution of the
239 peaks along the chromatogram. Although aqueous extracts (Fig. 1C and 1D) presented
240 greater overall intensity, peaks were mainly placed at the beginning of the chromatogram.
241 After data processing, 364 metabolic signals were detected. Signal grouping and filtering
242 processes (i.e. unidentified metabolites and metabolites not found in at least 3 out of 5
243 replicates of each extraction solvent) rendered a total of 290 metabolites from which a
244 group of 150 metabolites could be identified (see Table 1 and Table S2). Then, each of the
245 metabolic extracts were investigated in detail in terms of detected metabolites and

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246 identified metabolites found in each extract as well as other parameters to evaluate
247 metabolite extraction performance and reproducibility. An average variation of the internal
248 standards of 15% RSD was observed. Given that value the number of identified metabolites
249 with RSD < 15% was also determined for the four extraction solvents.

250 Among the two aqueous based extractions C (water) and D (acidified water), C showed
251 higher overall metabolome coverage in terms of number of detected (172 vs. 168) and
252 identified metabolites (100 vs. 94). However, deepening in the identified metabolites in
253 both aqueous extracts, D showed a vast gain over C with respect to metabolite recovery
254 performance (76 vs. 25 metabolites found at maximum intensity in D and C respectively)
255 but the inverse was observed in terms of reproducibility (RSD <15% in 62 vs. 20
256 metabolites in C and D, respectively) (see Table 1). As can be observed in Table 1, a more
257 evident enhancement was observed in B metabolic coverage when compared with A extract
258 among the two organic extracts. Namely, 96 metabolites were identified out of the 182
259 detected in B, while for A only 82 metabolites could be identified out of the 141 detected.
260 Moreover, triple metabolite recovery performance (i.e. metabolites at maximum intensity)
261 from cell culture was observed for B with respect to A. In addition, metabolic content in A
262 was extracted with less reproducibility among the 5 extraction replicates analysed,
263 including only 17 identified metabolites with RSD values lower than 15%. Thus, among the
264 organic solvents, B extract clearly offered the best overall performance when compared to
265 A.

3.2. Colon cancer cell metabolome identified by GC-MS

Metabolic information obtained from the four extraction protocols was then statistically investigated in order to find patterns and possible clustering in metabolic coverage obtained applying the four extraction solvents. Fig. 2 represents the unsupervised hierarchical clustering of the samples (i.e. metabolic extracts) according to the relative content of each metabolite in each sample (represented by the colour of the cell). As can be observed from the clustering information of Fig. 2, the four extracts were mainly differentiated according to the polarity of the solvent used in the extraction. Thus, as expected two clear clusters were observed corresponding to A with B (i.e. organic based solvents), and C with D (i.e. aqueous based solvents). This information was also observed and corroborated by means of PCA (see Fig. S1) and Venn diagram (see Fig. 3). In addition, PCA also showed that the metabolic information obtained from both organic solvents was more similar (presented less variation in their distribution in the plane defined by the main principal components from PC1 to PC5) than the one more heterogeneous obtained from both aqueous based extracts (more dispersion of aqueous samples in the plane defined by the PCs) (see Fig. S1).

Venn representation (Fig. 3) corroborated that the highest similarity of metabolic coverage was according to the extraction solvent polarity. Namely, organic based extracts (A and B) shared 72 metabolites, among which, 18 metabolites were exclusively detected in A and B. Similarly 80 metabolic species were common in both aqueous based extracts (C and D) from which 14 were only found in C and D. In addition, it could be observed that ACN and water extraction solvents (A and C, respectively) lead to the most different metabolic

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4 288 extracts with only 57 metabolites in common (from which 49 were found in all the four
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6 289 extracts) showing high complementarity.
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10 290 To deepen in the coverage of the different extracts according to the nature of the extracted
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12 291 metabolites and pathways involved, two web servers were used. Information about the
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14 292 biological role and nature of the identified metabolites was derived from MBRole web
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17 293 server analysis. Thus, the total set of metabolites with available KEGG identifiers in the
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19 294 four extracts (107 out of 150 identified metabolites) were submitted to analysis. Most
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22 295 important groups of metabolites identified were amino acids and peptides followed by fatty
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24 296 acids and lipids, nucleic acids and carbohydrates. Other groups of metabolites were
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27 297 represented to a lesser extent such as vitamins and cofactors (data not shown). Main
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29 298 metabolic pathways involving metabolites preferentially extracted (i.e. showing maximum
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32 299 levels) using the organic-based (A and B) and aqueous-based (C and D) protocols were
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34 300 analyzed using Metaboanalyst (Table S3 and S4 respectively). Fatty acid and nitrogenous
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37 301 bases (i.e. pyrimidine and purine) related pathways are well represented in organic based
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39 302 extracts. Among the two organic extracts, contribution of B extract is clearly superior to A
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41 303 whose metabolites are almost exclusively involved in nucleotide sugars metabolism. The
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44 304 main reason for this inequality could be explained for the lowest number of metabolites at
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46 305 highest intensity in A (see Table 1). On the other hand, pathways involving biosynthesis
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49 306 and metabolism of amino acids, glutathione, amino sugars and nitrogenous bases among
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51 307 others, are highly represented in aqueous extracts (C and D). Most of the metabolites
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54 308 involved in those pathways present higher performance of extraction using acidified water
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56 309 extraction (D) when compared with C (water extraction). Although in lower relative
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59 310 concentration, metabolic species in C showed the best reproducibility.
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311 Finally, identified metabolites were submitted to enrichment analysis (MSEA) by means of
312 overrepresentation analysis (ORA) to determine the more represented metabolic pathways
313 in the defined set of metabolites. As can be observed in Fig. S2, “protein biosynthesis” is
314 the metabolic pathway in which a higher number of the metabolites are involved. However,
315 more useful information can be obtained if solvents are subdivided regarding polarity.
316 When each protocol for metabolic extraction is investigated in detail, different trends are
317 observed. MSEA results of metabolites preferentially extracted (i.e. showing maximum
318 levels) in organic and aqueous based extracts are shown in Fig. 4 and Fig. 5 respectively.
319 As can be observed depending on the nature and polarity of the solvents used in the
320 extraction process diverse metabolic pathways are more suitable to study due to the very
321 different metabolic information. Some of the most relevant metabolic pathways that can be
322 studied with more efficiency in organic based solvents are “RNA transcription” and “beta
323 oxidation of very long fatty acids” (Fig. 4), while pathways most suitable to be evaluated in
324 aqueous based extracts include protein, sugars and amino acids biosynthesis and
325 metabolism (Fig. 5). Interestingly, pathways involving metabolism of glutathione and
326 nucleotides can be approached using both organic and/or aqueous extracts.

327 **CONCLUDING REMARKS**

328 To date it is not possible to study the whole metabolome of a biological system and this is
329 partially due to the selection of a certain sample treatment and analytical platform. In this
330 work, four reliable sample preparation protocols have been evaluated to extract metabolites
331 from HT-29 colon cancer cells and to uncover a portion of this cell line metabolome by
332 GC-MS.

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333 The unsupervised statistical analysis showed that the four extracts evaluated were mainly
334 differentiated according to the polarity of the solvent used in the extraction process and that
335 the overall GC-MS variability of aqueous extracts was higher compared to the organic
336 ones.

337 In summary, the protocol involving ACN-Isopropanol-water (3:3:1, v/v/v) was shown to be
338 the most appropriate extraction procedure for fatty acid and/or related pathways analysis
339 among the four metabolic extraction procedures. Metabolic extraction with ACN yielded
340 the lowest reproducibility of extraction together with the smallest metabolite coverage. On
341 the other hand, metabolic coverage was similar in both aqueous extracts. However, overall
342 performance of extraction of individual metabolites seemed to be higher using acidified
343 water (5% formic acid) compared to just water, which on the contrary showed better GC-
344 MS reproducibility. Most of the metabolites involved in pathways of amino acids,
345 glutathione, amino sugars and other polar metabolites present higher performance of
346 extraction in the acidified water extract, although metabolic species in water extract show
347 the best overall reproducibility. Although pathways involving nitrogenous bases could be
348 investigated using organic or aqueous extracts, more metabolites involved in those
349 pathways can be identified in the aqueous extracts.

350 In light of the results obtained, qualitative information about the metabolic coverage and
351 related pathways exhibited high complementarity among the extracts. This information is
352 useful for future hypothesis-driven studies for the evaluation of a certain metabolic pathway
353 or a group of metabolites (i.e. if they are thought to be altered in HT29 colorectal cells by
354 the action of a drug or a natural bioactive compound).

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355 **Conflict of interest statement**

356 The authors declare no conflict of interest.

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364 **References**

365 [1] R.A. Cairns, I.S. Harris, T.W. Mak, Regulation of cancer cell metabolism, *Nat. Rev. Cancer* 11
366 (2011) 85-95.

367 [2] O. Warburg, On the origin of cancer cells, *Science* 123 (1956) 309-314.

368 [3] M.D. Williams, R. Reeves, L.S. Resar, H.H. Hill Jr, Metabolomics of colorectal cancer: past and
369 current analytical platforms, *Anal. Bioanal. Chem.* 405 (2013) 5013-5030.

370 [4] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, *Cell* 144 (2011) 646-
371 674.

372 [5] M.G. Vander Heiden, J.W. Locasale, K.D. Swanson, H. Sharfi, G.J. Heffron, D. Amador-
373 Noguez, H.R. Christofk, G. Wagner, J.D. Rabinowitz, J.M. Asara, L.C. Cantley, Evidence for an
374 alternative glycolytic pathway in rapidly proliferating cells, *Science* 329 (2010) 1492-1499.

375 [6] H. Kondoh, M.E. Lleonart, J. Gil, J. Wang, P. Degan, G. Peters, D. Martinez, A. Carnero, D.
376 Beach, Glycolytic enzymes can modulate cellular life span, *Cancer Res.* 65 (2005) 177-185.

377 [7] N.J. Serkova, K. Glunde, Metabolomics of cancer, *Methods Mol. Biol.* 520 (2009) 273-295.

378 [8] R.D. Beger, A review of applications of metabolomics in cancer, *Metabolites* 3 (2013) 552-574.

379 [9] K. Dettmer, P.A. Aronov, B.D. Hammock, Mass spectrometry-based metabolomics, *Mass*
380 *Spectrom. Rev.* 26 (2007) 51-78.

381 [10] A. Smolinska, L. Blanchet, L.M. Buydens, S.S. Wijmenga, NMR and pattern recognition
382 methods in metabolomics: from data acquisition to biomarker discovery: a review, *Anal. Chim.*
383 *Acta* 750 (2012) 82-97.

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62
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64
65

384 [11] W.B. Dunn, D.I. Ellis, *Metabolomics: current analytical platforms and methodologies*, *TrAC-*
385 *Trend. Anal. Chem.* 24 (2005) 285-294.

386 [12] C. Denkert, E. Bucher, M. Hilvo, R. Salek, M. Orešič, J. Griffin, S. Brockmöller, F. Klauschen,
387 S. Loibl, D.K. Barupal, *Metabolomics of human breast cancer: new approaches for tumor typing*
388 *and biomarker discovery*, *Genome Med.* 4 (2012) 37.

389 [13] Q. Huang, Y. Tan, P. Yin, G. Ye, P. Gao, X. Lu, H. Wang, G. Xu, *Metabolic characterization*
390 *of hepatocellular carcinoma using nontargeted tissue metabolomics*, *Cancer Res.* 73 (2013) 4992-
391 5002.

392 [14] T. Zhang, D.G. Watson, L. Wang, M. Abbas, L. Murdoch, L. Bashford, I. Ahmad, N. Lam,
393 A.C. Ng, H.Y. Leung, *Application of holistic liquid chromatography-high resolution mass*
394 *spectrometry based urinary metabolomics for prostate cancer detection and biomarker discovery*,
395 *PloS one* 8 (2013) e65880.

396 [15] S. Nishiumi, T. Kobayashi, A. Ikeda, T. Yoshie, M. Kibi, Y. Izumi, T. Okuno, N. Hayashi, S.
397 Kawano, T. Takenawa, *A novel serum metabolomics-based diagnostic approach for colorectal*
398 *cancer*, *PloS one* 7 (2012) e40459.

399 [16] S. Hori, S. Nishiumi, K. Kobayashi, M. Shinohara, Y. Hatakeyama, Y. Kotani, N. Hatano, Y.
400 Maniwa, W. Nishio, T. Bamba, *A metabolomic approach to lung cancer*, *Lung Cancer* 74 (2011)
401 284-292.

402 [17] American Cancer Society, *Colorectal Cancer Facts & Figures 2011-2013*, Atlanta: American
403 Cancer Society (2011).

404 [18] F. Bray, *The burden of cancer in Europe, Responding to the challenge of cancer in Europe.. in:*
405 *M.P. Coleman, D.M. Alexe, T. Albrecht, M. McKee (Eds.) Responding to the challenge of cancer in*
406 *Europe. Institute of Public Health of the Republic of Slovenia, Ljubljana, 2008, pp. 7-40.*

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58
59
60
61
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407 [19] D. Monleon, J.M. Morales, A. Barrasa, J.A. Lopez, C. Vazquez, B. Celda, Metabolite profiling
408 of fecal water extracts from human colorectal cancer, *NMR Biomed.* 22 (2009) 342-348.

409 [20] M. Tessem, K.M. Selnaes, W. Sjursen, G. Tranø, G.F. Giskeødegård, T.F. Bathen, I.S.
410 Gribbestad, E. Hofslis, Discrimination of patients with microsatellite instability colon cancer using
411 ¹H HR MAS MR spectroscopy and chemometric analysis, *J. Proteome Res.* 9 (2010) 3664-3670.

412 [21] H. Wang, D.E. Schiller, V. Tso, C. Slupsky, C.K. Wong, R.N. Fedorak, A novel highly
413 sensitive test for detecting colon cancer using spot urine metabolomics, *Gastroenterology* 140
414 (2011) S-40.

415 [22] I. Bertini, S. Cacciatore, B.V. Jensen, J.V. Schou, J.S. Johansen, M. Kruhoffer, C. Luchinat,
416 D.L. Nielsen, P. Turano, Metabolomic NMR fingerprinting to identify and predict survival of
417 patients with metastatic colorectal cancer, *Cancer Res.* 72 (2012) 356-364.

418 [23] B. Jiménez, R. Mirnezami, J. Kinross, O. Cloarec, H.C. Keun, E. Holmes, R.D. Goldin, P.
419 Ziprin, A. Darzi, J.K. Nicholson, ¹H HR-MAS NMR spectroscopy of tumor-induced local
420 metabolic “field-effects” enables colorectal cancer staging and prognostication, *J. Proteome Res.* 12
421 (2013) 959-968.

422 [24] S.A. Ritchie, P.W. Ahiachonu, D. Jayasinghe, D. Heath, J. Liu, Y. Lu, W. Jin, A. Kavianpour,
423 Y. Yamazaki, A.M. Khan, Reduced levels of hydroxylated, polyunsaturated ultra long-chain fatty
424 acids in the serum of colorectal cancer patients: implications for early screening and detection,
425 *BMC Med.* 8 (2010) 1.

426 [25] F. Li, X. Qin, H. Chen, L. Qiu, Y. Guo, H. Liu, G. Chen, G. Song, X. Wang, F. Li, Lipid
427 profiling for early diagnosis and progression of colorectal cancer using direct-infusion electrospray
428 ionization Fourier transform ion cyclotron resonance mass spectrometry, *Rapid Commun. Mass Sp.*
429 27 (2013) 24-34.

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430 [26] E.G. Armitage, H.L. Kotze, J.S. Fletcher, A. Henderson, K.J. Williams, N.P. Lockyer, J.C.
431 Vickerman, Time-of-flight SIMS as a novel approach to unlocking the hypoxic properties of cancer,
432 Surf. Interface Anal. 45 (2013) 282-285.

433 [27] A. Hirayama, K. Kami, M. Sugimoto, M. Sugawara, N. Toki, H. Onozuka, T. Kinoshita, N.
434 Saito, A. Ochiai, M. Tomita, H. Esumi, T. Soga, Quantitative metabolome profiling of colon and
435 stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry,
436 Cancer Res. 69 (2009) 4918-4925.

437 [28] C. Ibáñez, C. Simó, V. García-Cañas, Á Gómez-Martínez, J.A. Ferragut, A. Cifuentes,
438 CE/LC-MS multiplatform for broad metabolomic analysis of dietary polyphenols effect on colon
439 cancer cells proliferation, Electrophoresis 33 (2012) 2328-2336.

440 [29] C. Ibáñez, A. Valdés, V. García-Cañas, C. Simó, M. Celebier, L. Rocamora-Reverte, Á
441 Gómez-Martínez, M. Herrero, M. Castro-Puyana, A. Segura-Carretero, Global Foodomics strategy
442 to investigate the health benefits of dietary constituents, J. Chromatogr A 1248 (2012) 139-153.

443 [30] C. Simó, C. Ibáñez, Á Gómez-Martínez, J.A. Ferragut, A. Cifuentes, Is metabolomics
444 reachable? Different purification strategies of human colon cancer cells provide different CE-MS
445 metabolite profiles, Electrophoresis 32 (2011) 1765-1777.

446 [31] B. Tan, Y. Qiu, X. Zou, T. Chen, G. Xie, Y. Cheng, T. Dong, L. Zhao, B. Feng, X. Hu,
447 Metabonomics identifies serum metabolite markers of colorectal cancer, J. Proteome Res. 12 (2013)
448 3000-3009.

449 [32] D.C. Montrose, X.K. Zhou, L. Kopelovich, R.K. Yantiss, E.D. Karoly, K. Subbaramaiah, A.J.
450 Dannenberg, Metabolic profiling, a noninvasive approach for the detection of experimental
451 colorectal neoplasia, Cancer. Prev. Res. (Phila) 5 (2012) 1358-1367.

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59
60
61
62
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64
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452 [33] Y. Cheng, G. Xie, T. Chen, Y. Qiu, X. Zou, M. Zheng, B. Tan, B. Feng, T. Dong, P. He,
453 Distinct urinary metabolic profile of human colorectal cancer, *J. Proteome Res.* 11 (2011) 1354-
454 1363.

455 [34] Y. Ma, H. Qin, W. Liu, J. Peng, L. Huang, X. Zhao, Y. Cheng, Ultra-high performance liquid
456 chromatography–mass spectrometry for the metabolomic analysis of urine in colorectal cancer, *Dig.*
457 *Dis. Sci.* 54 (2009) 2655-2662.

458 [35] D. Zimmermann, M. Hartmann, M.P. Moyer, J. Nolte, J.I. Baumbach, Determination of
459 volatile products of human colon cell line metabolism by GC/MS analysis, *Metabolomics* 3 (2007)
460 13-17.

461 [36] E.C.Y. Chan, P.K. Koh, M. Mal, P.Y. Cheah, K.W. Eu, A. Backshall, R. Cavill, J.K.
462 Nicholson, H.C. Keun, Metabolic profiling of human colorectal cancer using high-resolution magic
463 angle spinning nuclear magnetic resonance (HR-MAS NMR) spectroscopy and gas chromatography
464 mass spectrometry (GC/MS), *J. Proteome Res.* 8 (2008) 352-361.

465 [37] C. Denkert, J. Budczies, W. Weichert, G. Wohlgemuth, M. Scholz, T. Kind, S. Niesporek, A.
466 Noske, A. Buckendahl, M. Dietel, Metabolite profiling of human colon carcinoma–deregulation of
467 TCA cycle and amino acid turnover, *Mol. Cancer* 7 (2008) 72.

468 [38] Y.L. Ma, W.J. Liu, J.Y. Peng, P. Zhang, H.Q. Chen, H.L. Qin, Study on specific metabonomic
469 profiling of serum from colorectal cancer patients by gas chromatography-mass spectrometry,
470 *Zhonghua Wei Chang Wai Ke Za Zhi* 12 (2009) 386-390.

471 [39] Y. Ma, W. Liu, J. Peng, L. Huang, P. Zhang, X. Zhao, Y. Cheng, H. Qin, A pilot study of gas
472 chromatograph/mass spectrometry-based serum metabolic profiling of colorectal cancer after
473 operation, *Mol. Biol. Rep.* 37 (2010) 1403-1411.

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474 [40] Y. Qiu, G. Cai, M. Su, T. Chen, Y. Liu, Y. Xu, Y. Ni, A. Zhao, S. Cai, L.X. Xu, Urinary
475 metabonomic study on colorectal cancer, *J. Proteome Res.* 9 (2010) 1627-1634.

476 [41] Y. Kondo, S. Nishiumi, M. Shinohara, N. Hatano, A. Ikeda, T. Yoshie, T. Kobayashi, Y.
477 Shiomi, Y. Irino, T. Takenawa, Serum fatty acid profiling of colorectal cancer by gas
478 chromatography/mass spectrometry, *Biomark. Med.* 5 (2011) 451-460.

479 [42] M. Mal, P.K. Koh, P.Y. Cheah, E.C.Y. Chan, Metabotyping of human colorectal cancer using
480 two-dimensional gas chromatography mass spectrometry, *Anal. Bioanal. Chem.* 403 (2012) 483-
481 493.

482 [43] T. Yoshie, S. Nishiumi, Y. Izumi, A. Sakai, J. Inoue, T. Azuma, M. Yoshida, Regulation of the
483 metabolite profile by an APC gene mutation in colorectal cancer, *Cancer Sci.* 103 (2012) 1010-
484 1021.

485 [44] L.C. Phua, M. Mal, P.K. Koh, P.Y. Cheah, E.C.Y. Chan, H.K. Ho, Investigating the role of
486 nucleoside transporters in the resistance of colorectal cancer to 5-fluorouracil therapy, *Cancer*
487 *Chemother. Pharmacol.* 71 (2013) 817-823.

488 [45] M.A. Lorenz, C.F. Burant, R.T. Kennedy, Reducing time and increasing sensitivity in sample
489 preparation for adherent mammalian cell metabolomics, *Anal. Chem.* 83 (2011) 3406-3414.

490 [46] C.A. Sellick, D. Knight, A.S. Croxford, A.R. Maqsood, G.M. Stephens, R. Goodacre, A.J.
491 Dickson, Evaluation of extraction processes for intracellular metabolite profiling of mammalian
492 cells: matching extraction approaches to cell type and metabolite targets, *Metabolomics* 6 (2010)
493 427-438.

494 [47] B. Álvarez-Sánchez, F. Priego-Capote, M.L. de Castro, Metabolomics analysis II. Preparation
495 of biological samples prior to detection, *TrAC-Trend. Anal. Chem.* 29 (2010) 120-127.

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496 [48] B. Gonzalez, J. François, M. Renaud, A rapid and reliable method for metabolite extraction in
497 yeast using boiling buffered ethanol, *Yeast* 13 (1997) 1347-1355.

498 [49] S. Dietmair, N.E. Timmins, P.P. Gray, L.K. Nielsen, J.O. Krömer, Towards quantitative
499 metabolomics of mammalian cells: Development of a metabolite extraction protocol, *Anal.*
500 *Biochem.* 404 (2010) 155-164.

501 [50] S. Dietmair, M.P. Hodson, L. Quek, N.E. Timmins, P. Chrysanthopoulos, S.S. Jacob, P. Gray,
502 L.K. Nielsen, Metabolite profiling of CHO cells with different growth characteristics, *Biotechnol.*
503 *Bioeng.* 109 (2012) 1404-1414.

504 [51] J. Kronthaler, G. Gstraunthaler, C. Heel, Optimizing high-throughput metabolomic biomarker
505 screening: a study of quenching solutions to freeze intracellular metabolism in CHO cells, *Omics* 16
506 (2012) 90-97.

507 [52] Q. Teng, W. Huang, T.W. Collette, D.R. Ekman, C. Tan, A direct cell quenching method for
508 cell-culture based metabolomics, *Metabolomics* 5 (2009) 199-208.

509 [53] I. Chantret, A. Barbat, E. Dussaulx, M.G. Brattain, A. Zweibaum, Epithelial polarity, villin
510 expression, and enterocytic differentiation of cultured human colon carcinoma cells: a survey of
511 twenty cell lines, *Cancer Res.* 48 (1988) 1936-1942.

512 [54] D. Ahmed, P. Eide, I. Eilertsen, S. Danielsen, M. Eknæs, M. Hektoen, G. Lind, R. Lothe,
513 Epigenetic and genetic features of 24 colon cancer cell lines, *Oncogenesis* 2 (2013) e71.

514 [55] S. Fernández-Arroyo, A. Gómez-Martínez, L. Rocamora-Reverte, R. Quirantes-Piné, A.
515 Segura-Carretero, A. Fernández-Gutiérrez, J. Ferragut, Application of nanoLC-ESI-TOF-MS for the
516 metabolomic analysis of phenolic compounds from extra-virgin olive oil in treated colon-cancer
517 cells, *J. Pharm. Biomed. Anal.* 63 (2012) 128-134.

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518 [56] M. Čuperlović-Culf, D.A. Barnett, A.S. Culf, I. Chute, Cell culture metabolomics: applications
519 and future directions, *Drug Discov. Today* 15 (2010) 610-621.

520 [57] C. Ibáñez, C. Simó, A. Valdés, L. Campone, A.L. Piccinelli, V. García-Cañas, A. Cifuentes,
521 Metabolomics of adherent mammalian cells by capillary electrophoresis-mass spectrometry: HT-29
522 cells as case study, *J. Pharm. Biomed. Anal.* 110 (2015) 83-92.

523 [58] H. Meyer, H. Weidmann, M. Lalk, Methodological approaches to help unravel the intracellular
524 metabolome of *Bacillus subtilis*, *Microb. Cell. Fact.* 12 (2013) 69.

525 [59] A.D. Patterson, H. Li, G.S. Eichler, K.W. Krausz, J.N. Weinstein, A.J. Fornace, F.J. Gonzalez,
526 J.R. Idle, UPLC-ESI-TOFMS-based metabolomics and gene expression dynamics inspector self-
527 organizing metabolomic maps as tools for understanding the cellular response to ionizing radiation,
528 *Anal. Chem.* 80 (2008) 665-674.

529 [60] D.Y. Lee, O. Fiehn, High quality metabolomic data for *Chlamydomonas reinhardtii*, *Plant*
530 *Methods* 4 (2008) 7.

531 [61] J.L. Au, M.H. Su, M.G. Wientjes, Extraction of intracellular nucleosides and nucleotides with
532 acetonitrile, *Clin. Chem.* 35 (1989) 48-51.

533 [62] L. von Stechow, A. Ruiz-Aracama, B. van de Water, A. Peijnenburg, E. Danen, A. Lommen,
534 Identification of cisplatin-regulated metabolic pathways in pluripotent stem cells, *PloS one* 8 (2013)
535 e76476.

536 [63] O. Fiehn, T. Kind, Metabolite profiling in blood plasma, *Method. Mol. Biol.* 358 (2007) 3-17.

537 [64] O. Fiehn, G. Wohlgenuth, M. Scholz, Setup and Annotation of Metabolomic Experiments by
538 Integrating Biological and Mass Spectrometric Metadata. In: Ludäscher B., Raschid L. (eds) *Data*
539 *Integration in the Life Sciences. DILS 2005. Lecture Notes in Computer Science*, vol 3615.
540 Springer, Berlin, Heidelberg.

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51
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61
62
63
64
65

541 [65] M. Scholz, O. Fiehn, SetupX--a public study design database for metabolomic projects, Pac.
542 Symp. Biocomput. (2007) 169-180.

543 [66] M. Chagoyen, F. Pazos, Tools for the functional interpretation of metabolomic experiments,
544 Brief Bioinform. 14 (2013) 737-744.

545 [67] J. Xia, N. Psychogios, N. Young, D.S. Wishart, MetaboAnalyst: a web server for metabolomic
546 data analysis and interpretation, Nucleic Acids Res. 37 (2009) W652-60.

547 [68] M. Chagoyen, F. Pazos, MBRole: enrichment analysis of metabolomic data, Bioinformatics 27
548 (2011) 730-731.

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549 **FIGURE CAPTIONS**

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551 **Figure 1.** GC-MS total ion chromatograms (TIC) of metabolite extracts obtained using
552 ACN (A), ACN-IsopOH-water (3:3:1, v/v/v) (B), water (C), and 5% formic acid in water
553 (D). GC-MS conditions are described in the text.

554 **Figure 2.** Hierarchical clustering and heat map of total identified metabolites (rows) in all
555 samples (columns). A (ACN), B (ACN-IsopOH-water (3:3:1, v/v/v)), C (water) and D (5%
556 formic acid) extracts are represented in red, green, dark blue and light blue respectively.
557 Cell color represents metabolite relative content.

558 **Figure 3.** Venn diagram representation of number of HT-29 metabolites identified in A, B,
559 C and D extracts.

560 **Figure 4.** Summary plot for Metabolite Set Enrichment Analysis (MSEA) of metabolites
561 identified in the organic extracts (A and B) from HT-29 cells.

562 **Figure 5.** Summary plot for Metabolite Set Enrichment Analysis (MSEA) of metabolites
563 identified in the aqueous extracts (C and D) from HT-29 cells.

Table 1. Summary of metabolite extraction performance of the four extraction solvents used in this work.

Metabolic extract	Extraction solvent	Detected metabolites	Identified metabolites	Metabolites at maximum intensity ^a	Metabolites RSD<15% ^a
A	ACN	141	82	13	17
B	ACN-IsopOH-H ₂ O (3:3:1, v/v/v)	182	96	36	51
C	H ₂ O	172	100	25	62
D	5% HCOOH	168	94	76	20

^aMetabolites at maximum intensity (5th column) and with RSD <15% (6th column) out of the total identified metabolites.