**Metabolomics of adherent mammalian cells by capillary electrophoresis-mass spectrometry:
HT-29 cells as case study.**

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1 ABSTRACT

2 In this work, the optimization of an effective protocol for cell metabolomics is described with especial
3 emphasis in the sample preparation and subsequent analysis of intracellular metabolites from adherent
4 mammalian cells by capillary electrophoresis-mass spectrometry. As case study, colon cancer HT-29
5 cells, a human cell model to investigate colon cancer, are employed. The feasibility of the whole method

for cell metabolomics is demonstrated via a fast and sensitive profiling of the intracellular metabolites of HT-29 cells by capillary electrophoresis-time-of-flight mass spectrometry (CE-TOF MS). The suitability of this methodology is further corroborated through the examination of the metabolic changes in the polyamines pathway produced in colon cancer HT-29 cells by difluoromethylornithine (DFMO), a known potent ornithine decarboxylase inhibitor. The selection of the optimum extraction conditions allowed a higher sample volume injection that led to an increase in CE-TOF MS sensitivity. Following a non-targeted metabolomics approach, 10 metabolites (namely, putrescine, ornithine, gamma-aminobutyric acid (GABA), oxidized and reduced glutathione, 5'-deoxy-5'-(methylthio)adenosine, N-acetylputrescine, cysteinyl-glycine, spermidine and an unknown compound) were found to be significantly altered by DFMO ($p < 0.05$) in HT-29 cells. In addition to the effect of DFMO on polyamine metabolism, minor modifications of other metabolic pathways (e.g., related to intracellular thiol redox state) were observed.

Keywords: Metabolomics, capillary electrophoresis-mass spectrometry, human colon adenocarcinoma HT-29 cells, adherent mammalian cells, polyamines, difluoromethylornithine

Abbreviations:

CHO, chinese hamster ovary; PBS, phosphate-buffered saline; CE-TOF MS, capillary electrophoresis-time-of-flight mass spectrometry; DFMO, difluoromethylornithine; GABA, γ -aminoisobutyric acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; QC, quality control; W, water; AW, acidified water; IAW, isopropyl alcohol-acetonitrile-water; PCA, principal component analysis; EIE, extracted ion electropherogram; ODC, ornithine decarboxylase; MTA, 5'-deoxy-5'-(methylthio)adenosine; SAM, S-adenosylmethionine; SAH, S-adenosyl-L-homocysteine; GSH, reduced glutathione; GSSG, oxidized glutathione.

1. INTRODUCTION

Metabolomics is a valuable tool for investigating cellular responses and to explore the underlying mechanisms of external actions on metabolic pathways. Cell culture metabolomics represents a major opportunity in many different areas (including e.g., discovery of drug targets [1], biotechnology [2], phenotype markers [3], biopharmaceutical production [4], metabolic flux analysis [5,6], foodomics [7,8], among others), however, the application of metabolomics in the area of cell cultures is somewhat undeveloped. Although not specific to cell metabolomics, the broad physico-chemical diversity of metabolites as well as the broad dynamic range of their concentration, are main challenges in metabolomics. Extraction of metabolites from any given biological sample is one of the most important steps for the generation of meaningful results. A suitable metabolite extraction protocol should remove interfering media components, and quantitatively extract as many metabolites as possible without causing chemical or physical degradation. Moreover, it is assumed that the subset of observed metabolites will depend on both sample processing and analytical platform used to analyze them [9-10]. The development of robust and standardized sampling procedures for intracellular metabolite analysis from cell cultures represents today an important challenge [11]. Among others, the type of cellular organism under study affects the overall sample preparation process, because the leakage of metabolites during sample processing is likely to be dependent on cell wall and membrane structure. Until now, different extraction methods have been presented for intracellular metabolic profiling of different microorganisms [12,13], including yeast and bacteria [14]. Several approaches have been reported for the extraction of intracellular metabolites from mammalian cells growing in suspension, such as Chinese hamster ovary (CHO). In this latter case, special attention has been given to the interest of CHO for producing recombinant proteins [15-20]. The growing mode (adherent or non-adherent) of the different mammalian cells will also be critical for the sample preparation step prior to metabolomics, especially in the first stages of the sample preparation process. In cell cultures all the components of culture media comprise a significant potential source of variability, and thus, to reduce variance in metabolic profile/fingerprint the use of the same

growth medium is recommended for all cell cultures of a certain experiment. In any case, the growth medium is certainly a source of contamination for intracellular metabolomics, and thus, cells must be efficiently washed to avoid contamination with exogenous compounds. Therefore, general sample preparation of intracellular cell metabolomics comprises two steps; the first one provides washed and quenched cells while in the second step intracellular metabolites are extracted.

Metabolic content must also be representative of the metabolic state of the cell culture, however, the effectiveness of the different methods described in literature for metabolism quenching is still under discussion and in certain cases it is even possible to see contradictory results regarding their adequacy and performance. Temperature shock maintaining the cells below -20 °C has been widely used for metabolic quenching [21,22]. When applying quenching procedures using cold solvents the time lap between sampling and the inhibition of metabolism is minimal, however, contact with organic solvents may produce cell wall/membrane permeabilization. Indeed, drastic loss of intracellular metabolites during the quenching step has been reported for cold solvents such as methanol when used prior to intracellular metabolite extraction in bacteria [12,13], and in CHO mammalian cells grown in suspension [17]. On the other hand, the use of washing/quenching solutions with too low ionic strength might result in a significant metabolite leakage, so this approach should also be handled with care. Reducing temperature, enzymatic reaction velocity will strongly decrease, and with that, the unwanted metabolites changes will be reduced. The use of cold isotonic PBS to remove residues surrounding the cells (culture medium, for example) minimizes the conversion of metabolites with a rapid turnover [17-19]. Some recent works have claimed the benefits of combining in a single step quenching and extraction procedures in mammalian adherent cells [23,24], although the latter procedures skip any reliable normalization strategy. The importance of normalization is too often underestimated in cell culture metabolomics, and it is particularly critical when the cells are exposed to certain conditions that induce or inhibit cell proliferation [25].

Capillary electrophoresis-mass spectrometry (CE-MS) is a thoughtful methodology to analyze polar ionic compounds, and is becoming more and more routine in metabolomics field [26-31]. In this work, we describe in detail an effective sample preparation procedure for intracellular metabolite extraction in a model of adherent mammalian cells, namely, human colon cancer HT-29 cells. The feasibility of the proposed sample treatment prior to metabolomics of the intracellular content of adherent HT-29 cells was demonstrated through the examination via capillary electrophoresis-time-of-flight mass spectrometry (CE-TOF MS) of the metabolic changes produced by difluoromethylornithine (DFMO), an ornithine decarboxylase inhibitor with reported anti-proliferative activity against several cancer cell lines.

2. MATERIALS AND METHODS

2.1. Chemicals

Ammonium acetate, sodium hydroxide, sodium chloride, methanol, dimethyl sulfoxide (DMSO) and isopropyl alcohol were from Sigma-Aldrich (St. Louis, MO, USA) and purchased with the highest purity available. MS grade formic acid and isopropyl alcohol were from Riedel-de Haën (Seelze, Germany) and acetonitrile was from Labscan (Gliwice, Poland). Deionized water was obtained by using a Milli-Q system from Millipore (Bedford, MA, USA). The following commercial standards were from Sigma-Aldrich: glutathione, glutathione oxidized, spermine, spermidine, putrescine, cadaverine, N-acetylspermine, ornithine, lysine, arginine, S-adenosylmethionine, adenine, N-acetylputrescine, S-adenosylhomocysteine, cysteine, cytidine, methionine, adenosine, 5'-deoxy-5'-(methylthio)adenosine, γ -aminoisobutyric acid (GABA), tyramine and methionine sulfone.

2.2. Cell culture and DFMO treatment

Colon adenocarcinoma HT-29 cell line obtained from ATCC (American Type Culture Collection, LGC Promochem, UK) was grown in McCoy's 5A (from Lonza, Barcelona, Spain) supplemented with 10%

heat-inactivated fetal calf serum (from Biowest, Nuaille, France), 50 U/mL penicillin G and 50 U/mL streptomycin (Lonza), at 37 °C in humidified atmosphere with 5% CO₂. When cells reached ~50% confluence, they were trypsinized, neutralized with culture medium, plated in different culture plates and allowed to adhere overnight at 37 °C. To study the effect of DFMO on proliferation of HT-29 line, cells were seeded onto 96-well culture plates at 10,000 cells/cm², permitted to adhere overnight at 37 °C, and exposed to 4.6 mM DFMO for 0-72 h. After incubation, cell proliferation was estimated by the MTT assay as follows: 0.5 mg/mL of MTT reagent (Sigma-Aldrich) was added and incubated for 3 h at 37 °C in humidified atmosphere with 5% CO₂. After incubation, the media was aspirated and 100 µL of DMSO was added to each well to dissolve the formazan (the metabolic product from MTT). Then, the absorbance at 570 nm was measured in a microplate reader Multiskan™ FC Microplate Photometer from Thermo Fisher Scientific (Vantaa, Finland). Results are provided as the mean ± SEM (standard error of the mean) of five replicates. The results were analyzed by the analysis of variance (ANOVA) with Tukey post hoc test and differences were considered significant at p<0.05. To evaluate different protocols for metabolite extraction, 10,000 cells/cm² were seeded onto P150 tissue culture dishes, permitted to adhere overnight at 37 °C, and incubated with culture medium for a given time. Cells from different plates were pooled, and after counting, ten million cells were subjected to intracellular metabolite extraction with four different extraction solvents (five replicates each). To study the effect of DFMO on HT-29 line using a metabolomic approach, P150 tissue culture dishes were seeded with 10,000 cells/cm², permitted to adhere overnight at 37 °C, and incubated with 4.6 mM of DFMO in complete culture medium for 48 h. Three independent experiments were performed to compare metabolic profiles from DFMO-treated and non-treated cells. After incubation, cells were subjected to intracellular metabolite extraction as described below.

2.3. Intracellular metabolite extraction

After incubation, growth medium from culture plates was removed by aspiration, and cells were trypsinized and pelleted [31]. Cells were then washed with 1 mL of cold PBS and counted in a Neubauer counting chamber using a light microscope (ID3, Carl Zeiss, Jena, Germany). After counting, 10×10^6 cells were subjected to metabolite extraction. 300 μ L cold extraction solvent spiked with internal standards (tyramine and methionine sulfone at 125 μ M) and 300 mg abrasive 212-300 μ m beads glass balls (Sigma-Aldrich) were added to and immediately frozen in liquid nitrogen for 3 min. Thawing was carried out in a ultrasonic bath at 50 Hz (Ultrasons from JP Selecta, Barcelona, Spain). Then, cells were disrupted using a mixer mill MM 400 from Retsch GmbH (Haan, Germany) at a frequency of 30 Hz for 3 min. Three cycles of freezing/thawing and mechanical disruption were performed. Cell debris was separated from intracellular metabolic content by centrifugation at 24,000xg for 10 min at 4°C. When aqueous solutions were used for extractions, 200 μ L of the supernatant were ultrafiltrated by using 3 kDa Amicon Ultra 0.5 mL centrifugal devices from Millipore (Billerica, MA, USA) at 14,000xg for 40 min at 20 °C and then stored at -80 °C until CE-MS analysis. Supernatants obtained with organic solvents were directly stored at -80 °C until CE-MS analysis.

2.4. Metabolite analysis by CE-MS

Metabolic profiling was carried out using a P/ACE 5500 capillary electrophoresis (CE) system (Beckman Instruments, Fullerton, CA, USA) connected to a time-of-flight mass spectrometer (TOF MS) instrument (micrOTOF model) from Bruker Daltonics (Bremen, Germany) using an orthogonal electrospray ionization (ESI) interface G1607A from Agilent Technologies (Palo Alto, CA, USA). Metabolites were separated in a fused silica capillary (80 cm, 50 μ m i.d., 375 μ m o.d.) filled with 3 M formic acid as background electrolyte (BGE) applying a voltage of +27 kV at 25°C. Before first use, the fused-silica separation capillary was conditioned by rinsing with 1 M NaOH for 10 min, followed by 20 min with Milli-Q water. Before each CE-MS analysis the capillary was conditioned with the separation buffer during 4 min. Sample injections were made at the anodic end using N₂ pressure of 0.5 psi for 16 s (12 nL)

or 32 s (24 nL). Isopropyl alcohol-water (1:1, v/v) was delivered as the sheath liquid at 0.24 μ L/min by a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA). ESI-TOF MS was performed in the positive ion mode, and the capillary voltage was set at 4 kV. The flow rate of heated dry nitrogen gas (heater temperature, 200 °C) was maintained at 0.4 bar. External and internal calibration of the TOF-MS instrument was performed by introducing a 10 mM sodium formate solution through the separation capillary. The ions used for the calibration of the TOF-MS instrument were 90.9766, 158.9641, 226.9515, 294.9389, 362.9263, 430.9138, 498.9012 and 566.8886 m/z. Each sample was analyzed in duplicate by CE-MS

2.5. Data analysis

Metabolomic data signals obtained from CE-TOF MS were exported to the MS exchange format *.mzXML using MSConvert tool from Proteowizard program (available at <http://proteowizard.sourceforge.net/downloads.shtml>). CE-MS data was then processed with MZmine program (version 2.7.2) [33]. Parameters applied for mass detection, peak deconvolution and sample alignment are described elsewhere [7]. Peaks showing high variability (with a value of median/average > 1.5) and peaks not found in at least 80% of samples within the same data set (i.e. same culture conditions and extraction solvent) were removed from the subsequent data processing steps. The resulting output *.csv data tables of high quality time-aligned detected peaks with their corresponding migration time, m/z and peak area obtained for each sample were merged into one file to perform statistical analysis using STATISTICA program (v.7, Statsoft, Tulsa, OK, USA, www.statsoft.com). In order to compare and to evaluate the different metabolite coverage from the four extraction protocols a principal component analysis (PCA) was performed. An analysis of variance (ANOVA) with p-value set at 0.05 was applied to determine the differences between control and DFMO treated HT-29 cells after the application of the optimum metabolite extraction protocol. Metabolites showing significant differences ($p < 0.05$) after DFMO treatment were subjected to tentative identification process by matching the experimental accurate

m/z values and those contained in different free available databases, namely, Human Metabolome Database [34], METLIN [35], and KEGG [36], with a mass accuracy window of 10 ppm. When available, commercial standards were co-injected to support tentative metabolite identifications.

3. RESULTS AND DISCUSSION

3.1. CE-TOF-MS analysis

As mentioned above, metabolites were separated in a fused silica capillary (80 cm, 50 μ m i.d.) filled with 3 M formic acid as background electrolyte applying a voltage of +27 kV at 25°C. For the metabolomic study, a quality control (QCs) mixture containing 19 metabolite standards (spermine, spermidine, putrescine, cadaverine, N-acetylspermine, ornithine, lysine, arginine, S-adenosylmethionine, adenine, N-acetylputrescine, S-adenosyl-L-homocysteine, N-acetylornithine, cytidine, methionine, adenosine, 5'-deoxy-5'-(methylthio)adenosine, oxidized glutathione and reduced glutathione) at 0.1 mM each, was used to monitor the reliability and repeatability of the results. An example of the separations obtained is shown in **Fig. 1**. As can be seen, under these conditions an efficient and fast separation of the 19 metabolites could be obtained in less than 12 minutes by CE-TOF-MS.

3.2. Intracellular metabolite extraction from HT-29 cells

The issue of normalization is still unresolved in cell metabolomics. The changes in metabolite concentrations can greatly vary because of the effect of the treatment on the cell proliferation and hence, in the number of cells. To overcome this limitation, in the present work, cell normalization was accomplished counting cells. To undertake this, a distinction has to be made between cells grown in suspension and adherent cells. Adherent mammalian cells, as the ones used in this work, entail additional methodological difficulties, compared to those in suspension, since cell detachment has to be carried out

as a prerequisite prior to cell counting. Although some sample preparation procedures focused on metabolomics have been described for mammalian adherent cells [37], comparative studies are rare, and thus, currently there is not a validated and standardized procedure for this purpose [15]. Therefore, for all metabolite extractions carried out in this work, HT-29 cells were first harvested, subsequently detached, and finally counted. We selected 10 million cells for metabolite extraction as a good compromise between sensitivity, size and ease of handling of cell culture. Namely, after a washing step with cold isotonic PBS, detached cells were counted and sample volumes containing 10 million cells were subjected to metabolite extraction. After obtaining the selected number of cells, a mechanical procedure to disrupt cells and release metabolites was applied. Three cycles of freezing/thawing and homogenization, were used for this purpose [19,38]. A scheme of the experimental procedure for intracellular metabolite extraction from adherent HT-29 cells can be seen in **Fig. 2**. Given the variety of extraction solvents available, the choice of the most suitable one is not an easy task, as it is expected that different solvents will bring the extraction of different classes of metabolites based on their different polarity. It is expected that aqueous solutions will result in poor extraction of hydrophobic compounds, whereas organic solvents provide higher efficiency extraction for non-polar metabolites. Based on our own experience [39] and literature [40-44], four different extraction solvents were tested in this work, namely, water (W), water-formic acid (95:5, v/v) (AW), acetonitrile (ACN) and isopropyl alcohol-acetonitrile-water (3:3:1, v/v/v) (IAW). Thus, the efficiency of each solvent for extracting intracellular metabolites from HT-29 was firstly studied on cultured HT-29 cells incubated with complete medium for 48 h using a non-targeted metabolomic approach. Special focus was next put on the study of metabolites related to the polyamines pathway, since polyamine metabolism has been described as an attractive target for cancer chemoprevention and chemotherapy [45]. The compatibility of the extraction solvent with the subsequent CE-TOF MS analysis was also taken into account, as well as the additional advantage provided by organic solvents regarding protein precipitation. For comparison purposes, injected sample volume was 12 nL.. When organic extracts were directly injected unstable current at the beginning of the CE-TOF MS analysis or even current failure, was observed. Therefore, prior to their injection organic extracts were diluted (1:1, v/v) in

CE separation buffer (24 nL were injected in those cases). More specifically, the choice of the extraction solvent was made according to the best performance in terms of metabolite number and signal intensity, as well as the reliability and repeatability of the extraction method. Thus, CE-TOF MS metabolic profiles obtained for each extraction solvent were evaluated using non-targeted approaches by determining features (unique metabolite signals at a given migration time value). Different algorithms for automatic low molecular weight compounds detection (m/z signals detection, peak building, peak deconvolution, etc.) were applied for non-targeted metabolomics. Alignment of metabolic signals among all samples was then carried out [7]. Only those features with lower variability (median/average values <1.5) and detected in 80% of extracts obtained with each extraction solvent were considered. MZmine data processing and the subsequent peak filtering and adduct grouping processes allowed us to detect 105, 87, 61 and 44 metabolites for W, AW, IAW, and ACN, respectively. In **Fig. 3** the metabolite coverage overlap is represented by means of a Venn diagram. As can be deduced from this figure, only 27 out of 145 total detected metabolites are common to the four extracts indicating a high complementarity in metabolic information provided by the four solvents. The overall metabolic differences obtained for each extraction solvent was also studied by PCA (supplementary information, **Fig. S1**). PC1 clearly separates water extraction from the other three solvents suggesting metabolic information in water is very different. On the other hand, PC2 is the responsible of the separation between aqueous from organic extracts. As can be observed in the PC1 vs. PC2 plot, the two organic-based extracts are the more similar and their differentiation is explained by PC3 (data not shown).

As mentioned above, special focus was put on metabolites related to the polyamines pathway, because of their role in cell proliferation and carcinogenesis [46]. Representative extracted ion electropherograms (EIEs) of 13 polyamine-related metabolites are given in **Fig. 4**. As can be seen, the peaks were separated without interferences from other endogenous substances in the samples. Peaks corresponding to polyamine-related compounds were eluted from 4 to 10 min. These compounds (namely, spermine, spermidine, putrescine, acetylspermine, ornithine, arginine, S-adenosylmethionine, adenine,

acetylputrescine, S-adenosyl-L-homocysteine, methionine, adenosine, and 5'-deoxy-5'-(methylthio)adenosine) belong to the polyamine pathway (*vide infra*). As indicated above, five injections were carried out for each extraction solvent, and thus, % RSD values of peak areas for the 13 polyamine-related metabolites were calculated for each solvent, the results are given in **Table 1**. Marked differences in terms of the selectivity of metabolite extraction were observed among the four extraction solvents. AW (**Fig. 4A**) and W (**Fig. 4B**) solvents gave the best overall performance in terms of intensity, number of extracted metabolites and repeatability. Only three polyamines-related metabolites were found in higher concentration in the organic-based extracts: acetylspermine and adenosine in ACN extract (**Fig. 4C**), and 5'-Deoxy-5'-(methylthio)adenosine in the IAW extract (**Fig. 4D**), showing in both cases (ACN and IAW) much higher relative standard deviation values (RSD): 9-88%. When W and AW were compared important differences were also found. Spermine and spermidine, as well as adenosine and S-adenosylmethionine were found in higher quantity in the AW extract. Spermine, spermidine, adenosine and S-adenosylmethionine were also found in the W extract, although peak intensities were lower. On the contrary, arginine, adenine, S-adenosyl-L-homocysteine, methionine, and 5'-deoxy-5'-(methylthio)adenosine, were found in higher quantity in the W extract. Unfortunately, putrescine and ornithine, were not detected using any of the four selected extraction solvents and analysis conditions. Moreover, it was also observed that the best repeatability values for metabolites extraction were obtained using W with %RSD ranging from 6 to 9 % (see **Table 1**).

In order to evaluate the extraction protocol in more detail, additional evaluation of the data using principal component analysis (PCA) was performed (supplementary information, **Fig. S2**). PC1, which explains the most of the data total variance (45%), revealed that W was the most different solvent among the four extraction solvents. Arginine, adenine, S-adenosyl-L-homocysteine, methionine are directly correlated with PC1, and as mentioned before, they were found at higher levels in W extract. PC2 explains 31% of the total variance of the data and segregates AW extract from the other three solvents. PC2 is directly correlated with adenosine indicating higher levels of that metabolite in IAW, ACN and W solvents.

However, PC2 inversely correlates with spermine, spermidine and S-adenosylmethionine that are found at higher concentrations in the AW extract. Overall, extraction with W provided better repeatability and higher number of small-size compounds were detected when a non-targeted analysis was carried out and, therefore, water was selected as extraction solvent for the following experiments.

3.3. Evaluation of intracellular metabolic extraction: Effect of DFMO on HT-29 proliferation

In order to evaluate both the antiproliferative effect of DFMO on colon cancer cells and the usefulness of the proposed methodology, HT-29 cells were incubated with 4.6 mM of DFMO for 24, 48 and 72 h and cell proliferation compared to the non-treated HT-29 cells (control) was analyzed by the MTT assay.

After 24 h, no statistically significant differences were observed between the viability of control and treated cells. However, a reduction of ~20% of the cell proliferation was observed at 48 h (p-value = 0.002), while DFMO exhibited its maximum antiproliferative effect at 72 h, decreasing the cell viability in ~40% (p-value = 0.0002). DFMO is an inhibitor that blocks the action of ornithine decarboxylase (ODC) [47], an enzyme that produces the decarboxylation of ornithine into putrescine, which is the first step of polyamine biosynthesis (see **Fig. 5**). Polyamine biosynthesis has been shown to be necessary for cell proliferation, and plays a crucial role in rapidly dividing cells such as cancerous cells [48,49].

Using the extraction protocol described above (see **Fig. 2**) and water as extraction solvent, larger sample volumes could be injected in the CE capillary without compromising the CE current stability. Therefore, up to 60 nL could be injected in the CE-MS system. This brought an increase of sensitivity, and 13 polyamine-related metabolites could be detected, namely, spermine, spermidine, putrescine, acetylspermine, ornithine, arginine, S-adenosylmethionine (SAM), adenine, acetylputrescine, S-adenosyl-L-homocysteine (SAH), methionine, adenosine, and 5'-deoxy-5'-(methylthio)adenosine (MTA).

Compounds related to the polyamine pathway mapping were first studied comparing the HT-29 cells treated for 48h vs. control cells. Significant depletion of spermidine and spermine as well as putrescine, acetylspermine and 5'-deoxy-5'-(methylthio)adenosine was observed after DFMO cell treatment.

Spermidine is derived from putrescine by addition of an aminopropyl group via spermidine synthase, and spermine is derived from spermidine by addition of another aminopropyl group catalyzed by spermine synthase. According to that, the observed putrescine and spermidine depletion was in good agreement with the expected activity of DFMO. Moreover, ornithine, which could not be detected under the analytical conditions described in the previous section “*Intracellular metabolite extraction from HT-29 cells*”, was detected in a greater amount in DFMO-treated cells when higher amount of sample was injected. These results are also consistent with the selective blockade of the ODC enzyme by DFMO. Interestingly, MTA levels decreased significantly in DFMO-treated cells relative to control cells. Recent evidences suggest a key role for methionine cycle intermediates, including SAM, SAH and MTA, in the cytostatic activity of DFMO [50]. More specifically, DFMO treatment reduces methionine cycle metabolism in normal human colon epithelial cells and colorectal cancer cells as well, which has been associated with decreases in one-carbon-dependent thymidine metabolite pools. Other polyamine-related metabolites indicated in **Fig. 5** were detected but not significantly altered with the DFMO treatment.

A non-targeted metabolomic approach was also carried out with the aim to gain insight into other metabolic pathways that could be altered upon DFMO treatment. In order to extract the most significant differences between treated HT-29 cells with respect to the control cells, 70 detected metabolite compounds were aligned and subjected to statistical analysis. Among the 10 significantly different metabolites detected applying an ANOVA (p-value set at 0.05), cysteinyl-glycine was assigned by matching the experimental mass spectra with information contained in HMDB, METLIN and KEGG databases. Eight out of ten metabolites significantly altered were first tentatively identified by using metabolite databases and they were next confirmed by standard co-injection, while only one metabolite (170.067 m/z migrating at 7.1 min) remained unknown. **Fig. 6** summarizes the 10 compounds that changed significantly (p-value <0.05) after the DFMO treatment detected by a non-target metabolomics approach. As can be seen in **Fig. 6**, the levels of three metabolites increased whereas seven metabolites decreased their concentration in DFMO-treated HT-29 cells. Interestingly, following a non-targeted

metabolomic approach, the polyamines pathway also came out as the main route altered by DFMO as can be deduced from the 5 metabolites related to the mentioned polyamines synthesis (namely, putrescine, ornithine, MTA, N-acetylputrescine, and spermidine) corroborating the usefulness of this approach. The non-targeted metabolomic analysis also suggested that DFMO treatment induces changes in several compounds related with glutathione metabolism, including GSH, GSSG and cysteinyl-glycine. More precisely, increases in intracellular GSH and cysteinyl-glycine contents were concomitant with a decrease in GSSG levels in treated cells. Glutathione-related metabolites participate in antioxidant defense and many other metabolic processes [51,52]. The elevation of intracellular GSH in DFMO-treated cells was in good agreement with reported data in literature [53-56]. Hunter et al. observed that elevation of GSH concentration induced by 1 mM DFMO in rat brain tumor cells was the result of increased GSH synthesis [53]. Polyamines have a recognized reactive oxygen species scavenger function [54,55]. Thus, the observed changes in GSH levels can be explained as the cellular response to altered ROS levels occurring upon intracellular polyamine depletion induced by DFMO-treatment. As mentioned before, the dipeptide cysteinyl-glycine, the GSH catabolite generated by gamma-glutamyltranspeptidase, significantly increased in DFMO-treated cells. Cysteinyl-glycine has been suggested to participate in GSH homeostasis, controlling GSH synthesis, and therefore, affecting the antioxidant defense of the cell [56]. In addition, decreased levels of gamma-aminobutyric acid (GABA) in DFMO-treated cells compared to control cells, were observed. GABA accumulation in several tumor processes [57-59] including colorectal cancer [60] has been well documented. Specifically, diamine oxidase is responsible for GABA generation from putrescine, and increased levels of this enzyme have been associated with higher malignancy probability in cancer progress [58]. Furthermore, there is certain feedback between GABA levels and polyamine biosynthesis since the former metabolite stimulates ODC activity [61,62]. Thus, increased GABA levels induce ODC activity resulting in higher putrescine levels, which is in turn a precursor of GABA.

4. CONCLUSIONS

An effective protocol for metabolomics of adherent mammalian cells has been developed in this work. The protocol allows intracellular metabolite extraction and normalization prior to metabolomic analysis, making possible a quantitative comparison of cell metabolomic profiles. Our method comprises a reliable sample preparation step to extract metabolites with especial emphasis on polyamine related metabolites from HT-29 cell line. Although acidified water gave best extraction yields for spermine, spermidine, adenosine and S-adenosylmethionine, extraction with water gave better yields for arginine, adenine, S-adenosyl-L-homocysteine, methionine, and 5'-deoxy-5'-(methylthio)adenosine. Furthermore, this last polyamine compound was not detected in acidified water extract. The selection of water as the optimum extraction solvent for polyamines profiling led to an increase in CE-TOF MS sensitivity by means of a higher sample volume injection, by which putrescine and ornithine could be detected. These two compounds together with 8 more metabolites, namely gamma-aminobutyric acid (GABA), oxidized and reduced glutathione, 5'-deoxy-5'-(methylthio)adenosine, N-acetylputrescine, cysteinyl-glycine, spermidine and an unknown compound were found to be significantly altered ($p < 0.05$) by DFMO following a non-targeted metabolomics approach. This finding indicates that DFMO treatment especially affects the polyamine metabolism, and other relevant metabolic pathways (e.g., related to intracellular thiol redox state) as well. The results from DFMO treated and control cells corroborate the usefulness of the whole method for intracellular cell metabolomics. Besides, the protocol showed to be robust and reliable for intracellular metabolic profiling of HT-29 cells. CE-TOF MS is particularly suited for the rapid separation of ionic, weakly ionic, and/or highly polar metabolites with very high resolution using minimal volumes of reagents and sample. Taking into account this last point, further improvements on sample preparation using less quantity of cells and less extraction solvents are therefore affordable without analytical limitations. However, when using one single analytical technique for a particular metabolomic study it has to be assumed that a significant bias is introduced into the final results. It would be therefore important to point out that in order to provide more global metabolomic information, a

375 combination of NMR, GC/MS, LC/MS and CE/MS for metabolomics analysis should be ideally
376 employed.

377

378 **Conflict of interest statement**

379 The authors declare no conflict of interest.

380

381 **ACKNOWLEDGEMENTS**

382 This work was supported by an AGL2011-29857-C03-01 project (Ministerio de Educacion y Ciencia,
383 Spain).

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REFERENCES

- [1] C. Russell, A. Rahman, A.R. Mohammed, Application of genomics, proteomics and metabolomics in drug discovery, development and clinic, *Ther. Deliv.* 4 (2013) 395-413.
- [2] H.F. Kildegaard, D. Baycin-Hizal, N.E. Lewis, M.J. Betenbaugh, The emerging CHO systems biology era: harnessing the 'omics revolution for biotechnology, *Curr. Op. Biotech.* 24 (2013) 1102-1107.
- [3] W.N.P. Lee, Characterizing phenotype with tracer based metabolomics, *Metabolomics* 2 (2006) 31-39.
- [4] Z. Yang, F. Marotta, Pharmacometabolomics in drug discovery & development: applications and challenges, *Metabolomics* 2 (2012) 1000e122.
- [5] J.K. Sims, S. Manteiga, K. Lee, Towards high resolution analysis of metabolic flux in cells and tissues, *Curr. Opin. Biotechnol.* 24 (2013) 933-939.
- [6] D. Mueller, E. Heinzle, Stable isotope-assisted metabolomics to detect metabolic flux changes in mammalian cell cultures, *Curr. Opin. Biotechnol.* 24 (2013) 54-59.
- [7] C. Ibáñez, A. Valdés, V. García-Cañas, C. Simó, M. Celebier, L. Rocamora-Reverte, Á. Gómez-Martínez, M. Herrero, M. Castro-Puyana, A. Segura-Carretero, E. Ibáñez, J.A. Ferragut, A. Cifuentes, Global Foodomics strategy to investigate the health benefits of dietary constituents, *J. Chromatogr. A* 1248 (2012) 139-153.
- [8] A. Valdés, C. Simó, C. Ibáñez, L. Rocamora-Reverte, J.A. Ferragut, V. García-Cañas, A. Cifuentes, Effect of dietary polyphenols on K562 leukemia cells: a Foodomics approach, *Electrophoresis* 33 (2012) 2314-2327.
- [9] C. Ibáñez, C. Simó, V. García-Cañas, J.A. Ferragut, A. Cifuentes, CE/LC-MS multiplatform for broad metabolomic analysis of dietary polyphenols effect on colon cancer cells proliferation, *Electrophoresis* 33 (2012) 2328-2336.

409 [10] N.L. Kuehnbaum, P. Britz-McKibbin, New advances in separation science for metabolomics:
 410 resolving chemical diversity in a post-genomic era, *Chem. Rev.* 113 (2013) 2437-2468.

411 [11] M. Cuperlovic-Culf, D.A. Barnett, A.S. Culf, I. Chute, Cell culture metabolomics: applications and
 412 future directions, *Drug Discov. Today* 15 (2010) 610-621.

413 [12] C.J. Bolten, P. Kiefer, F. Letisse, J.C. Portais, C. Wittmann, Sampling for metabolome analysis of
 414 microorganisms, *Anal. Chem.* 79 (2007) 3843-3849.

415 [13] C. Wittmann, J.O. Krömer, P. Kiefer, T. Binz, E. Heinzle, Impact of the cold shock phenomenon on
 416 quantification of intracellular metabolites in bacteria, *Anal. Biochem.* 327 (2004) 135-139.

417 [14] C.L. Winder, W.B. Dunn, S. Schuler, D. Broadhurst, R. Jarvis, G.M. Stephens, R. Goodacre, Global
 418 metabolic profiling of *Escherichia coli* cultures: an evaluation of methods for quenching and extraction of
 419 intracellular metabolites, *Anal. Chem.* 80 (2008) 2939-2948.

420 [15] C.A. Sellick, R. Hansen, G.M. Stephens, R. Goodacre, A.J. Dickson, Metabolite extraction from
 421 suspension-cultured mammalian cells for global metabolite profiling, *Nat. Protoc.* 6 (2011) 1241-1249.

422 [16] C.A. Sellick, R. Hansen, A.R. Maqsood, W.B. Dunn, G.M. Stephens, R. Goodacre, A.J. Dickson,
 423 Effective quenching processes for physiologically valid metabolite profiling of suspension cultured
 424 Mammalian cells, *Anal. Chem.* 81 (2009) 174-183.

425 [17] S. Dietmair, N.E. Timmins, P.P. Gray, L.K. Nielsen, J.O. Krömer, Towards quantitative
 426 metabolomics of mammalian cells: Development of a metabolite extraction protocol, *Anal. Biochem.* 404
 427 (2010) 155-164.

428 [18] S. Dietmair, M.P. Hodson, L.E. Quek, N.E. Timmins, P. Chrysanthopoulos, S.S. Jacob, P. Gray, L.K.
 429 Nielsen, Metabolite profiling of CHO cells with different growth characteristics, *Biotechnol. Bioeng.* 109
 430 (2012) 1404-1414.

431 [19] J. Kronthaler, G. Gstraunthaler, C. Heel, Optimizing high-throughput metabolomic biomarker
 432 screening: a study of quenching solutions to freeze intracellular metabolism in CHO cells, OMICS 16
 433 (2012) 90-97.

434 [20] N. Sengupta, S.T. Rose, J.A. Morgan, Metabolic flux analysis of CHO cell metabolism in the late
 435 non-growth phase, Biotechnol. Bioeng. 108 (2011) 82-92.

436 [21] B. Álvarez-Sánchez, F. Priego-Capote, M.D. Luque de Castro, Metabolomics analysis II. Preparation
 437 of biological samples prior to detection, TrAC-Trends Anal. Chem. 29 (2010) 1210-1217.

438 [22] B. Gonzalez, J. François, M. Renaud, A rapid and reliable method for metabolite extraction in yeast
 439 using boiling buffered ethanol, Yeast 13 (1997) 1347-1355.

440 [23] M.A. Lorenz, C.F. Burant, R.T. Kennedy, Reducing time and increasing sensitivity in sample
 441 preparation for adherent mammalian cell metabolomics, Anal. Chem. 83 (2011) 3406-3414.

442 [24] Q. Teng, W. Huang, T.W. Collette, D.R. Ekman, C. Tan, A direct cell quenching method for cell-
 443 culture based metabolomics, Metabolomics 5 (2009) 199-208.

444 [25] B. Cao, J. Aa, G. Wang, X. Wu, L. Liu, M. Li, J. Shi, X. Wang, C. Zhao, T. Zheng, S. Guo, J. Duan,
 445 GC-TOFMS analysis of metabolites in adherent MDCK cells and a novel strategy for identifying
 446 intracellular metabolic markers for use as cell amount indicators in data normalization, Anal. Bioanal.
 447 Chem. 400 (2011) 2983-2993.

448 [26] C. Ibañez, C. Simo, V. Garcia-Canas, A. Cifuentes, and M. Castro-Puyana. Metabolomics,
 449 peptidomics and proteomics applications of capillary electrophoresis-mass spectrometry in Foodomics: A
 450 review. Anal.Chim.Acta 802 (2013) 1-13.

451 [27] A. Hirayama, M. Wakayama, and T. Soga. Metabolome analysis based on capillary electrophoresis-
 452 mass spectrometry. Trends Anal.Chem. 61 (2014) 215-222.

453 [28] A. Z. Buzatto, A. C. de Sousa, S. F. Guedes, Z. Cieslarova, and A. V. C. Simionato. Metabolomic
454 investigation of human diseases biomarkers by CE and LC coupled to MS. *Electrophoresis* 35 (2014)
455 1285-1307.

456 [29] V. Poinso, V. Ong-Meang, P. Gavard, and F. Couderc. Recent advances in amino acid analysis by
457 capillary electromigration methods, 2011-2013. *Electrophoresis* 35 (2014) 50-68.

458 [30] V. Garcia-Cañas, C. Simo, M. Castro-Puyana, A. Cifuentes, Recent advances in the application of
459 capillary electromigration methods for food analysis and Foodomics, *Electrophoresis* 35 (2014) 147-169.

460 [31] R. Ramautar, G.W.Somsen, G.J.de Jong, CE-MS for metabolomics: Developments and applications
461 in the period 2012-2014, *Electrophoresis* 36 (2015) 212-224.

462 [32] A. Valdés, V. García-Cañas, L. Rocamora-Reverte, A. Gómez-Martínez, J.A. Ferragut, A. Cifuentes.
463 Effect of rosemary polyphenols on human colon cancer cells: transcriptomic profiling and functional
464 enrichment analysis. *Genes Nutr.* 8 (2013) 43-60.

465 [33] M. Katajamaa, M. Oresic, Processing methods for differential analysis of LC/MS profile data, *BMC*
466 *Bioinformatics* 6 (2005) 179.

467 [34] D.S. Wishart, C. Knox, A.C. Guo, R. Eisner, N. Young, B. Gautam, D.D. Hau, N. Psychogios, E.
468 Dong, S. Bouatra, R. Mandal, I. Sinelnikov, J. Xia, L. Jia, J.A. Cruz, E. Lim, C.A. Sobsey, S. Shrivastava,
469 P. Huang, P. Liu, L. Fang, J. Peng, R. Fradette, D. Cheng, D. Tzur, M. Clements, A. Lewis, A. De Souza,
470 A. Zuniga, M. Dawe, Y. Xiong, D. Clive, R. Greiner, A. Nazyrova, R. Shaykhtudinov, L. Li, H.J. Vogel,
471 I. Forsythe, HMDB: a knowledgebase for the human metabolome, *Nucleic Acids Res.* 37 (2009) D603-
472 D610.

473 [35] C.A. Smith, G. O'Maille, E.J. Want, C. Qin, S.A. Trauger, T.R. Brandon, D.E. Custodio, R.
474 Abagyan, G. Siuzdak, METLIN: a metabolite mass spectral database, *Ther. Drug. Monit.* 27 (2005) 747-
475 751.

476 [36] M. Kanehisa, M. Araki, S. Goto, M. Hattori, M. Hirakawa, M. Itoh, T. Katayama, S. Kawashima, S.
477 Okuda, T. Tokimatsu, Y. Yamanishi, KEGG for linking genomes to life and the environment, *Nucleic*
478 *Acids Res.* 36 (2008) D480-D484.

479 [37] Z. Leon, J.C. García-Cañaveras, M.T. Donato, A. Lahoz, Mammalian cell metabolomics:
480 experimental design and sample preparation, *Electrophoresis* 34 (2013) 2762-2775.

481 [38] A.P.H. Danielsson, T. Moritz, H. Mulder, P. Spéigel, Development and optimization of a
482 metabolomic method for analysis of adherent cell cultures, *Anal. Biochem.* 404 (2010) 30-39.

483 [39] C. Simo, C. Ibañez, A. Gomez-Martinez, J.A. Ferragut, A. Cifuentes, Is metabolomics reachable?
484 Different purification strategies of human colon cancer cells provide different CE-MS metabolite profiles,
485 *Electrophoresis* 32 (2011) 1765-1777.

486 [40] A.D. Patterson, H. Li, G.S. Eichler, K.W. Krausz, J.N. Weinstein, A.J. Fornace Jr., F.J. Gonzalez,
487 J.R. Idle, UPLC-ESI-TOFMS-based metabolomics and gene expression dynamics inspector self-
488 organizing metabolomic maps as tools for understanding the cellular response to ionizing radiation, *Anal.*
489 *Chem.* 80 (2008) 665-674.

490 [41] D.Y. Lee, O. Fiehn, High quality metabolomic data for *Chlamydomonas reinhardtii*, *Plant Methods* 4
491 (2008) 7.

492 [42] J.L. Au, M.H. Su, M.G. Wientjes, Extraction of intracellular nucleosides and nucleotides with
493 acetonitrile, *Clin. Chem.* 35 (1989) 48-51.

494 [43] L. Von Stechow, A. Ruiz-Aracama, B. van de Water, A. Peijnenburg, E. Danen, A. Lommen,
495 Identification of cisplatin-regulated metabolic pathways in pluripotent stem cells, *PLoS One* 8 (2013)
496 e76476.

497 [44] H. Meyer, H. Weidmann, M. Lalk, Methodological approaches to help unravel the intracellular
498 metabolome of *Bacillus subtilis*, *Microb. Cell Fact.* 12 (2013) 69.

499 [45] E.W. Gerner, F.L. Meyskens Jr., Polyamines and cancer: old molecules, new understanding, Nat.
 500 Rev. Cancer 4 (2004) 781-792.

501 [46] D. Ramani, J.P. de Bandt, L. Cynober, Aliphatic polyamines in physiology and diseases, Clin. Nutr.
 502 33 (2014) 14-22.

503 [47] B.W. Metcalf, P. Bey, C. Danzin, M.J. Jung, J. Casara, J.P. Vever, Catalytic irreversible inhibition
 504 of mammalian ornithine decarboxylase (EC 4.1.1.17) by substrate and product analogues, J. Am. Chem.
 505 Soc. 100 (1978) 2551-2553.

506 [48] D.H. Russell, Clinical relevance of polyamines, Crit. Rev. Clin. Lab. Sci. 18 (1983) 261-311.

507 [49] D. Russell, S.H. Snyder, Amine synthesis in rapidly growing tissues: ornithine decarboxylase
 508 activity in regenerating rat liver, chick embryo, and various tumors, Proc. Natl. Acad. Sci. USA 60 (1968)
 509 1420-1427.

510 [50] M. Witherspoon, Q. Chen, L. Kopelovich, S.S. Gross, S.M. Lipkin, Unbiased metabolite profiling
 511 indicates that a diminished thymidine pool is the underlying mechanism of colon cancer chemoprevention
 512 by alpha-difluoromethylornithine, Cancer Discovery 3 (2013) 1072-1081.

513 [51] G. Wu, Y.Z. Fang, S. Yang, J.R. Lupton, N.D. Turner, Glutathione metabolism and its implications
 514 for health, J. Nutr. 134 (2004) 489-492.

515 ~~[49]~~ [52] F. Carlucci, A. Tabucchi, Capillary electrophoresis in the evaluation of aminothiols in body
 516 fluids, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877 (2009) 3347-3357.

517 [53] K.J. Hunter, D.F. Deen, L.J. Marton, Changes in the glutathione content of rat 9L cells induced by
 518 treatment with the ornithine decarboxylase inhibitor α -difluoromethylornithine, Cancer Res. 47 (1987)
 519 5270-5273.

520 [54] G.Y. Liu, Y.C. Hung, P.C. Hsu, Y.F. Liao, W.H. Chang, G.J. Tsay, H.C. Hung, Ornithine
521 decarboxylase prevents tumor necrosis factor alpha-induced apoptosis by decreasing intracellular reactive
522 oxygen species, *Apoptosis* 10 (2005) 569-581.

523 [55] H.C. Ha, N.S. Sirisoma, P. Kuppusamy, J. Zweier, P.M. Woster, R. A. Casero, The natural
524 polyamine spermine functions directly as a free radical scavenger, *PNAS* 95 (1998) 11140-11145.

525 [56] G.M. De Donatis, R. Moschini, M. Cappiello, A. Del Corso, U. Mura, Cysteinyl-glycine in the
526 control of glutathione homeostasis in bovine lenses, *Mol. Vision* 16 (2010) 1025-1033.

527 [57] R. Chanda, A.K. Ganguly, Diamine oxidase activity and tissue di and polyamine contents of human
528 ovarian, cervical and endometrial carcinoma, *Cancer Lett.*, 89 (1995), 23-28.

529 [58] W.A. Fogel, GABA and polyamine metabolism in peripheral tissues, in: S.L. Erdö, N.G. Bowery
530 (Eds.), *GABAergic Mechanisms in the Mammalian Periphery*, Raven Press, New York, 1986, pp. 35-56.

531 [59] T. Nishiyama, I. Hayashi, I. Inoue, C.W. Lin, G. Akagi, T. Sano, Distribution of histaminase in
532 human tumor tissues. An immunohistochemical study, *Oncodev. Biol. Med.* 4 (1983) 197-207.

533 [60] Z. Kleinrok, M. Matuszek, J. Jesipowicz, B. Matuszek, A. Opolski, C. Radzikowski, GABA content
534 and GAD activity in colon tumors taken from patients with coloncancer or from xenografted human colon
535 cancer cells growing as s.c. tumors in athymic nu/nu mice, *J. Physiol. Pharmacol.* 49 (1998) 303-310.

536 [61] N. Seiler, G. Bink, J. Grove, Regulatory Interrelations between GABA levels and polyamine
537 metabolism, *Neurochem. Res.* 4 (1979) 425-435.

538 [62] N. Seiler, On the role of GABA in vertebrate polyamine metabolism, *Physiol. Chem. Phys.* 12
539 (1980) 411-429.

FIGURE CAPTIONS

Figure 1. CE-TOF MS base peak electropherogram (BPE) and extracted ion electropherograms (EIE) of the QC mixture containing 19 polyamine related compounds: 1: spermine; 2: spermidine; 3: putrescine; 4: cadaverine; 5: N-acetylspermine; 6: ornithine; 7: lysine; 8: arginine; 9: S-adenosylmethionine; 10: adenine; 11: N-acetylputrescine; 12: S-adenosyl-L-homocysteine, 13: N-Acetylornithine; 14: cytidine; 15: methionine; 16: adenosine; 17: 5'-deoxy-5'-(methylthio)adenosine; 18: oxidized glutathione; 19: reduced glutathione (0.1 mM each). Metabolites were separated in a bare fused silica capillary with 80 cm total (and effective) length and 50 μm i.d., filled with 3 M formic acid as BGE; running voltage was +27 kV and temperature 25°C. Injection was made at the anodic end using N₂ pressure of 0.5 psi for 16 s (12 nL).

Figure 2. Experimental flowchart for intracellular metabolite extraction from HT-29 colon cancer cells.

Figure 3. Venn diagram representation of number of HT-29 intracellular metabolites extracted with acidized water (AW), water (W), acetonitrile (ACN) and isopropyl alcohol-acetonitrile-water (IAW) following a non-targeted metabolomic approach.

Figure 4. Representative extracted ion electropherograms of the 13 polyamine-related metabolites from extracts obtained with (A) acidized water, (B) water, (C) acetonitrile, and (D) isopropyl alcohol-acetonitrile-water. From top to bottom: Spermine, spermidine, putrescine, acetylspermine, ornithine, arginine, S-adenosylmethionine, adenine, acetylputrescine, S-adenosyl-L-homocysteine, methionine, adenosine, 5'-deoxy-5'-(methylthio)adenosine. Injections were made at the anodic end using N₂ pressure of 0.5 psi for 16 s (12 nL) for acidized water and water extracts and for 32 s (24 nL) for acetonitrile and isopropyl alcohol-acetonitrile-water extracts (previously diluted 1:1 (v/v) with BGE). Rest separation conditions as in **Fig. 1**.

Figure 5. Polyamine pathway mapping. Oval shape nodes represent genes and rectangular nodes represent metabolites.

Figure 6. Whisker plot representing the mean values ($\pm$ SD) of the metabolites showing significant differences ($p < 0.05$) after DFMO treatment of the HT-29 colon cancer cells.

567 **Table 1.** Variability of metabolite extraction (five extractions for each condition): Water (W), acidified water
568 (AW), acetonitrile (ACN) and isopropyl alcoholacetonitrile-water (IAW).

Compound	Molecular formula	Metabolite database			m/z [M+H] ⁺	% RSD peak area (n=5)			
		METLIN	KEGG	HMDB		W	AW	ACN	IAW
Spermine	C ₁₀ H ₂₆ N ₄	255	C00750	HMDB01256	203.2230	6	6	ND	54
Spermidine	C ₇ H ₁₉ N ₃	254	C00315	HMDB01257	146.1652	9	6	29	41
Putrescine	C ₄ H ₁₂ N ₂	3226	C00134	HMDB01414	89.1073	ND	ND	ND	ND
Acetylspermine	C ₁₂ H ₂₈ N ₄ O	3369	C02567	HMDB01186	245.2336	ND	ND	88	ND
Ornithine	C ₅ H ₁₂ N ₂ O ₂	27	C01602	HMDB00214	133.0972	ND	ND	ND	ND
Arginine	C ₆ H ₁₄ N ₄ O ₂	13	C00062	HMDB00517	175.119	6	14	27	10
S-Adenosylmethionine	C ₁₅ H ₂₃ N ₆ O ₅ S	3289	C00019	HMDB01185	399.1445	6	6	32	9
Adenine	C ₅ H ₅ N ₅	85	C00147	HMDB00034	136.0618	9	10	23	23
Acetylputrescine	C ₆ H ₁₄ N ₂ O	3252	C02714	HMDB02064	131.1179	ND	ND	ND	ND
S-Adenosyl-L-homocysteine	C ₁₄ H ₂₀ N ₆ O ₅ S	296	C00021	HMDB00939	223.0747	9	11	51	19
Methionine	C ₅ H ₁₁ NO ₂ S	26	C00073	HMDB00696	150.0583	8	12	15	9
Adenosine	C ₁₀ H ₁₃ N ₅ O ₄	86	C00212	HMDB00050	268.104	7	13	52	29
5'-Deoxy-5'-(methylthio)adenosine	C ₁₁ H ₁₅ N ₅ O ₃ S	3425	C00170	HMDB01173	298.0968	9	ND	ND	15

ND: Not detected

569