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GC-MS based metabolomics of colon cancer cells using different extraction solvents

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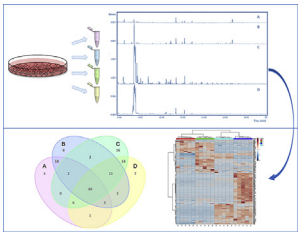
HIGHLIGHTS

- Metabolic information was highly influenced by the extraction solvent.
- Metabolic coverage was complementary among the extracts evaluated.
- This work uncovers the unexplored portion of HT29 cell line metabolome by GC-MS.
- Crucial information on solvent selection is given for target and profiling studies.

GRAPHICAL ABSTRACT



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ABSTRACT

The increasing incidence of colorectal cancer enforces the development of novel methodologies and protocols to deepen in the molecular mechanisms that govern disease pathophysiological events. The aim of this work is to deepen in the optimum metabolite extraction protocol from adherent mammalian cells of colon cancer for high throughput metabolomics using gas chromatography coupled to mass spectrometry (GC-MS). GC-MS results showed that metabolic information obtained from colon cancer cells was highly dependent on metabolite extraction selection, which at the same time is extremely influenced by the analytical platform. A further purpose of this investigation is to uncover an unexplored portion of HT-29 colon cancer cells metabolome, complementary to other already explored by CE-MS and LC-MS methods. At this respect, a total of 150 metabolites were identified in HT-29 colon cancer cells by GC-MS. The extraction protocol with acetonitrile-isopropanol-water was the most appropriate for fatty acids and related pathways analysis. Most of the metabolites involved in pathways of amino acids, glutathione, amino sugars and other polar metabolites were better extracted with acidified water, although water extraction showed the best overall reproducibility. Although pathways involving nitrogenous bases could be investigated using organic or aqueous extracts, a higher number of metabolites involved in these pathways were identified in the aqueous extracts. In addition, metabolite extraction protocol was observed to be crucial for the determination of potentially interesting clusters of metabolites.
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1. Introduction

In the past few years, cancer biology research has increasingly been focused on metabolism in cancer cells [1]. The complexity

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

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

Colorectal cancer is the second leading cause of cancer mortality in the USA [17] and Europe [18] with a continuous increasing incidence. Identifying soon the colon cancer apparition and/or progression will increase our knowledge on efficient drug discovery research and prevention. With this aim, in the last decade, Metabolomics has emerged to understand pathophysiological processes related to colon cancer. From the about 30 works related to colon cancer Metabolomics published so far, a minor part have been accomplished using NMR [19–23] with a clear trend toward the use of MS-based analytical platforms either alone [24–26] or in combination with separation techniques such as capillary electrophoresis (CE) [27–30], liquid chromatography (LC) [24,27–29,31–34] and gas chromatography (GC) [15,31–33,35–44].

Metabolomics investigations of colorectal cancer by GC-MS are summarized in Table S1. As can be seen in Table S1 a variety of samples including biofluids, tissues and cell culture models have been under scrutiny. The use of cell cultures is included in common reference model systems and is considered an invaluable biomedical research tool. However, the application of cell culture in metabolomics requires further development and standardization of study design steps, metabolism quenching method selection and optimization of metabolite extraction protocols, among others [45,46]. Namely, quenching step at the time of harvesting aims to prevent metabolic content to be altered before the analysis so that the metabolic state of the cell is preserved. There is a great controversy and continuous research in the determination of the optimum quenching method. Until date the most widely spread techniques to quench the metabolism in cells is a shock maintaining the cells below −20 °C [47,48], the addition of cold solvents such as cold methanol [49], use of cold isotonic PBS [49–51] or the combination in a single step of quenching and extraction procedures in mammalian adherent cells [45,52].

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

Considering that no single method is appropriate for the determination of all intracellular metabolites, metabolic information obtained from cell cultures will depend on multiple factors and metabolite extraction selection is highly influenced by the analytical platform [56]. Optimization and selection of the protocol for metabolite extraction from HT-29 cells [57] and evaluation of the subsequent cytosol sample treatment [30] for CE-MS metabolomics have been previously carried out. CE-MS is particularly suited for the rapid separation of ionic, weakly ionic, and/or highly polar metabolites and aqueous solvent was the preferred for metabolite extraction of HT-29 cells [57]. On the contrary, GC-MS is preferred for the analysis of less polar, volatile (or amenable to chemical derivatization) compounds and requires thermal stability of the analytes. Considering that no single analytical platform is able to determine all intracellular metabolites, in this work, a GC-MS method has been developed due to the high complementarity nature of GC-MS used in this work and the CE-MS [57] and LC-MS [28,29] methods already developed by our group. Further, taking into account all the above considerations, in this work GC-MS has been used to evaluate the coverage and reproducibility of four metabolic extraction solvents selected according to the physicochemical diversity of metabolites including the large variation on solubility (in aqueous or organic solvents) and based on previous published works [30,57–63]. To our knowledge this is the first time that HT-29 colon cancer cells are examined using GC-MS metabolomics which could be of special importance in future studies to detect biochemical alterations due to colon cancer or to explore new preventive interventions.

2. Materials and methods

2.1. Chemicals

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

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

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