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Sample preparation optimization in fecal metabolic profiling

Olga Deda[a], Anastasia Chrysovalantou Chatziioannou[a], Stella Fasoula[a], Dimitris Palachanis[a], Nicolaos Raikos[b], Georgios A. Theodoridis[a], Helen G. Gika[b,*]

[a] Department of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
[b] Laboratory of Forensic Medicine and Toxicology, School of Medicine, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

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ABSTRACT

Metabolomic analysis of feces can provide useful insight on the metabolic status, the health/disease state of the human/animal and the symbiosis with the gut microbiome. As a result, recently there is increased interest on the application of holistic analysis of feces for biomarker discovery. For metabolomics applications, the sample preparation process used prior to the analysis of fecal samples is of high importance, as it greatly affects the obtained metabolic profile, especially since feces, as matrix are diversifying in their physicochemical characteristics and molecular content. However there is still little information in the literature and lack of a universal approach on sample treatment for fecal metabolic profiling.

The scope of the present work was to study the conditions for sample preparation of rat feces with the ultimate goal of the acquisition of comprehensive metabolic profiles either untargeted by NMR spectroscopy and GC–MS or targeted by HILIC-MS/MS. A fecal sample pooled from male and female Wistar rats was extracted under various conditions by modifying the pH value, the nature of the organic solvent and the sample weight to solvent volume ratio. It was found that the 1/2 (w_f/v_s) ratio provided the highest number of metabolites under neutral and basic conditions in both untargeted profiling techniques. Concerning LC–MS profiles, neutral acetonitrile and propanol provided higher signals and wide metabolite coverage, though extraction efficiency is metabolite dependent.

1. Introduction

Recent evidence shows that gut microflora plays a key role on the health state of the host organism. Human gut contains a trillion of microbes, a number that far exceeds the number of somatic and germ cells in the human body [1]. Gut microbiota has a total biomass of 1.5 kg and has been characterized as a hidden metabolic organ [2] that reinforces the epithelial barrier against pathogenic bacteria, strengthens the immune system and manages the acquisition of nutrients [3]. In cases where the balance of intestinal flora is disrupted and the number of gut microbiota grows excessively, certain diseases may appear and pro-colitogenic perturbations of the symbiotic relation may be caused [4]. Investigation of the relation between microbial community and its host may aid in the treatment of severe, chronic diseases that appear to be directly or indirectly involved with the intestinal flora such as obesity [5–7] and type 2 diabetes [8,9]. Chronic inflammatory diseases, inflammatory bowel disease, ulcerative colitis, Crohn's disease in humans and rodents [4,10–16] and colorectal cancer [17] have also been connected with the role of gut microbiome. Culture-based techniques used to determine the composition of the intestinal bacteria can identify only 10–50% of the existing 500–1000 species [2]. Additional research in this area has led to the development of microbial culturomics [2,18] that enable the identification of non-cultivated gut bacteria, and DNA sequencing mostly targeting 16S rRNA [19–22].

Omics technologies, namely metatranscriptomics, metaproteomics and metabolomics provide a powerful tool for the investigation of the role of intestinal microbes. Co-metabolism resulting from symbiosis of gut bacteria and their host can be monitored by metabolomics. Both untargeted and targeted metabolomics approaches have been used to identify potential disease biomarkers [23]. Metabolomic analysis of fecal samples has revealed calprotectin or lactoferrin as valuable biomarkers for early detection of inflammatory bowel diseases [24,25]. NMR spectroscopy [26–29], GC–MS [30–32] and LC–MS [33–35] have been used to find biomarkers of intestinal diseases in stool material from human and rodents. The use of metabolomics in the study of bowel diseases was recently reviewed [23,36].

* Corresponding author.
E-mail address: gkikae@auth.gr (H.G. Gika).

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Regarding to the analytical procedures used for fecal analysis in the published studies, there are only a few papers dealing with sample treatment method development, hence there is a lack of a well-defined and universal protocol applied for sample preparation, as we have recently reported [37]. Stool material presents critical and limiting factors since feces represent a specimen of diversifying and complex mixture [38]. In metabolomics an optimal sample preparation method should be robust and capable of extracting the majority of metabolites effectively and reproducibly [39]. The method used for sample preparation can have major impact on the obtained metabolic profile. A metabolomics research "dogma" was that the best sample preparation is no sample preparation. Obviously this is not valid in feces, as recently reported by our group sample preparation can improve the information quality even for conventional specimens such as urine [40]. Till now only few studies have dealt with the optimisation of the method used for the sample preparation of feces. In these studies metabolomics analysis was performed by NMR spectroscopy [41–43] and GC–MS [44–48] and the investigated parameters included freeze-drying, sonication, filtration, homogenization, sample weight to buffer/solvent ratio, extraction solvent, pH value, extraction duration and repetitions numbers. Up to date there is no study of the optimum treatment conditions of fecal samples for metabolic fingerprinting by LC–MS.

In the present work we aimed to optimize sample preparation of rat feces for subsequent analysis by NMR and GC–MS (untargeted mode) and targeted HILIC-MS/MS the three commonly applied analytical techniques in metabolomics studies.

2. Materials and methods

2.1. Samples

A pooled sample was obtained by mixing fecal samples collected at the same time of the day over a period of 3 days from 1 male and 1 female Wistar rat (age of 8 months). Individual samples were stored directly at −80 °C upon their collection and were pooled after were thawed, the day of their analysis. The rats were acclimated in regulated light/dark cycle of 12 h, controlled temperature and humidity. They were fed ad libitum with standard chow and were allowed to have free access to water. The study was in accordance to the Helsinki Declaration; ethical approval was obtained from the bio-ethics committee of the Aristotle University.

2.2. Sample preparation for untargeted metabolic profiling

The pooled sample was mechanically homogenized and divided in two representative portions. One portion was used to obtain extract for global metabolic profiling by NMR and GC–MS and the other portion was used for targeted LC–MS/MS profiling.

For NMR and GC–MS three ratios of sample weight to buffer volume ratios namely 1/10, 1/5 and 1/2 (w_f/v_s) and three pH values were tested. More specifically either 100, 200 or 500 mg of pooled fecal sample were weighted and mixed with 1 mL of phosphate buffer (PBS) of either acidic, neutral or basic pH (pH values of 3.0, 7.4 and 9.1).

In the PBS solution sodium chloride was also added (25 mg NaCl/10 mL buffer solution) in order to facilitate extraction. Each extraction was performed in two replicates. Thus, in total 9×2 fecal extracts were prepared according to the procedure illustrated in Scheme 1. Homogenization was achieved by ultrasonic homogenizer for 15 min and then the fecal slurry was centrifuged for 20 min in 18000*g*. From the clear supernatant 400 mL were transferred and diluted with 150 μL of D_2O.

For NMR analyses 50 μL of 0.1% 3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP) in D_2O was added. After a second centrifugation the clear extracts were placed in 5 mm NMR tubes for analysis.

From the aforementioned clear supernatant another aliquot of 300 μL was taken for GC–MS metabolic profiling (aqueous fecal extract, Scheme 1). Finally the remaining precipitate was treated further to obtain the organic extract for GC–MS analysis. The precipitate was rinsed by PBS and vortex mixed for 1 min with 1 mL of MeOH-$CHCl_3$ 1:1 (v/v). After centrifugation for 20 min (18000*g*), 200 μL of the supernatant was used for analysis. Both aqueous and organic extracts were subjected to trimethylsilyl-derivatization, (after drying under N_2 stream) according to the procedure described elsewhere [49], before GC–MS analysis. As a measure to assist in Quality Control (QC), equal volume aliquots from all extracts were mixed to produce a pooled sample; the latter was split in three portions which were derivatised and analysed in the beginning, the middle and the end of the chromatographic run. An aliquot of 10 μL of 1 mM solution of 2-fluorobiphenyl was added in all samples as internal standard.

2.3. Sample preparation for targeted metabolic profiling

For LC–MS/MS analysis, the examined extraction parameters were: the sample weight to solvent volume ratio (w_f/v_s), the pH and the extraction solvent mixture. An amount of 250 mg from the pooled stool material was weighted every time and mixed with the extraction solvent in two w_f/v_s ratios, either of 1/2 or of 1/5. Three extraction solvents namely methanol, 1-propanol and acetonitrile were examined in a 1:1 (v/v) mixture with aqueous solutions of 3 different pH values: acidic, neutral and basic. Aqueous solution of pH 3.1 was prepared by addition of concentrated acetic acid, of pH 10.7 with concentrated ammonia and of pH 6.5 with ammonium acetate salt. Every mixture was vortex-mixed for 2 min followed by sonication for 10 min and then was centrifuged for 30 min at 4 °C (20.000*g*). Supernatants were filtered through syringe filters PTFE 0.22 μm before subjected to LC–MS analysis. In total 18 extracts were prepared as illustrated in Scheme 1. As described above a QC pooled sample was generated mixing equal volume aliquots from all extracts. The QC sample was analysed in the beginning (several times to condition the system), the middle and the end of the chromatographic runs, to assist in system stability and data quality assessment. Prior to the analysis of extracts a series of reference standards were injected as described in [50] to allow for analyte quantitation.

2.4. NMR conditions

A final volume of 550 μL water fecal extract (including D_2O and TSP used for chemical shift reference) was analysed by NMR spectroscopy. ^1H NMR spectra of aqueous fecal extracts samples were acquired using an Agilent 500 MHz spectrometer equipped with a 5 mm triple resonance probe at 300 K. The water signal was suppressed by applying presat power of 10 dB during a recycle delay of 2 s. The 90° pulse length was adjusted to 7.67 μs during a relaxation delay of 4 s. All spectra were acquired with the same receiver gain (0). The Carr-Purcell-Meiboom-Gill (CPMG) sequence was recorded for the samples to suppress the signal from proteins and lipids in order to raise signal of small metabolites. The spectral width covered the range from −1.0 to 10.0 ppm acquiring a total of 256 scans in 16384 data points for each spectrum. The total acquisition time for each spectrum was 30.37 min.