Journal of Pharmaceutical and Biomedical Analysis 124 (2016) 337–340

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Procedure optimization for extracting short-chain fatty acids from human faeces

Justyna Dobrowolska–Iwanek[a], Paweł Zagrodzki[a,b], Michał Woźniakiewicz[c], Aneta Woźniakiewicz[c], Małgorzata Zwolińska –Wcisło[d], Diana Winnicka[a], Paweł Paśko[a,e,*]

[a] Department of Food Chemistry and Nutrition, Medical College Jagiellonian University, 30-688 Kraków, Medyczna 9, Poland
[b] Department of Nuclear Physical Chemistry, Institute of Nuclear Physics, Polish Academy of Scienece, 31-142 Kraków, Radzikowskiego 152, Poland
[c] Laboratory for Forensic Chemistry, Faculty of Chemistry, Jagiellonian University in Kraków, 30-060, Ingardena 3, Poland
[d] Department of Gastroenterology, Hepatology and Infectious Diseases, Medical College Jagiellonian University, 31-501 Kraków, Śniadeckich 5, Poland
[e] Faculty of Health and Medical Science, Andrzej Frycz Modrzewski Kraków University, 30-705, Herlinga–Grudzińskiego 1, Poland

ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form 24 February 2016
Accepted 25 February 2016
Available online 3 March 2016

Keywords:
Short-chain fatty acids
Extraction
Optimization
Stool

ABSTRACT

Short-chain fatty acids play an important role in the physiology and metabolism of the colon. Disturbed balance of such compounds in human gut can significantly contribute to etiological factors of various gastrointestinal disorders and it may also increase the risk of developing cancer or cardiovascular diseases. The aim of the study was to select the optimal parameters for acetic, propionic and butyric acids extraction from stool samples. The experimental conditions were optimized with respect to the solvent sample shacking time, sample ultrasounds (Ultrasound-Assisted Extraction, UAE) exposure time and the number of extractions from the particulate stool samples. The screening of experimental parameters was conducted with fractional factorial design of experiments, namely 3^{3-1}. The optimal conditions for UAE were found, namely ultrasound digestion time of 40 min (at 35 °C), shaking time of 4 min, and the three subsequent extractions.

1. Introduction

Short-chain fatty acids (SCFA) such as acetic acid, propionic acid, and butyric acid are the end-products of bacteria fermentation processes of polysaccharides such as cellulose, pectin, hemicelluloses and also oligosaccharides [1]. These acids form the essential part of intestinal environment as they contribute to normal bowel function, colonic vasculature and musculature and they also protect against pathology in the colon lumen [2]. They also have a stimulatory effect on the epithelial growth, in particular the butyric acid, while the stimulated cell turnover may also provide a source of energy for colonic mucosa [3,4]. Butyric acid is particularly important as it promotes apoptosis in tumor cells, and due to its ability of regulating the gene expression and promoting the genetic stability by differentiation of precancerous and transformed cells; the latter is done most likely via inhibition of histone deacetylase [5,6]. SCFAs by lowering pH in gut prevent pathogenic bacteria therein from replicating, hence they also inhibit development of infectious diseases [7]. As SCFA are poorly absorbed dietary fibre breakdown products, they correlate positively with faecal weigh and may cause fluid retention in the colon leading to a laxative effect [8]. Due to such functions of SCFAs, their disturbed balance in human gut can be a significant factor in aetiology of various gastrointestinal disorders, and it may also rise the risk of developing cancer or cardiovascular diseases.

Scarce investigation to determine organic acids in stool samples [9–11] can be found in the literature. Extraction procedures seems particularly limited. Hoshi et al. [10] used the method that comprised ultrasonication of 300 mg faecal contents in 2 mL of hydroxide aqueous solution (10 mmol L^{-1}) with addition of crotonic acid (0.5 g L^{-1}), centrifugation of the sample, and subsequent removal of fat-soluble substances by extraction with chloroform. Other methods, such as proposed by Kikuhi et al. [9] and Usami et al. [12], included homogenisation of the sample (0.3–0.5 g) with water (15 mL or 1 mL, respectively), centrifugation of the homogenate, and addiction of perchloric acid ca. 10% (0.1 mL) to the supernatant

Abbreviations: CE, capillary electrophoresis; MCE, mixed cellulose esters; SCFA, short-chain fatty acid; UAE, ultrasound-assisted extraction.

* Corresponding author at: Department of Food Chemistry and Nutrition, Medical College, Jagiellonian University, Medyczna 9, 30-688, Krakow, Poland.
E-mail address: paskopaw@poczta.fm (P. Paśko).

http://dx.doi.org/10.1016/j.jpba.2016.02.042
0731-7085/

(0.9 mL), which is then left in a cool place. The particular steps of the procedures differ in details. Ohigashi at el. [11] applied a sample preparation procedure where homogenization of the sample (circa 1 g), was followed by addition of 0.15 mol L^{-1} perchloric acid into a fourfold volume, the mixture was left over at +4 °C for 12 h and then centrifuged at the last stage.

Still, the analytical procedures for short-chain organic acid extraction presented therein raise doubts with regard to the effectiveness of analyte isolation from the matrix. The procedures proposed in previous publications have not been validated, therefore the efficiency of the extraction process with known parameters is unknown. Moreover, for the isolation processes of the tested organic acids the completeness of carrying the analytes into the solution has not been verified. As the procedures were applied only once, the extraction of the entire amount of analytes from the sample remains doubtful.

The available literature sources indicate that the problem of influence of various biochemical and analytical factors on the process of organic acids isolation from human stool has not been scrutinized closely yet. To clear the objections, and in order to obtain reliable and accurate results we have developed a methodology and procedure for effective organic acid extraction from stool samples.

This suitable and time-saving method is highly required to be implemented, as it facilitates the evaluation of such compounds in human stool, which proves both essential and helpful in diagnosing and treating such human gastrointestinal tract disorders as diarrhoea, lactose intolerance, or colitis ulcerosa. The knowledge about the methodology of SCFA extraction and further investigation may be also useful for further studies on their influence on the energy balance, anticancer process, antibacterial effects and many other biological processes, where clinical implications are attributable to SCFAs. The current studies are focused on SCFA evaluation and thus they contribute to bowel disease prevention.

2. Materials and methods

2.1. Chemicals

Deionised water of 18 MΩ cm was obtained from Milli Ro & Q water purification system (Merck-Millipore, Billerica, MA, USA). Organic acids were of analytical standard grade. Caproic acid (99.5%) was obtained from Fluka (Steinheim, Germany), acetic acid (99%), propionic acid (99.5%), butyric acid (99.5%), and perchloric acid (70%) were purchased from Sigma-Aldrich (Steinheim, Germany), whereas 36–38% hydrochloric acid was from Baker Analyzed, methylhydroxyethylcellulose (M-HEC) from HERCULES (Prague, Czech Republic), and L-histidine was from Serva (Heidelberg, Germany). CEofix™ Anion test "5" kit designed for inorganic and organic anion analysis by the capillary electrophoresis (CE) technique was obtained from Analis (Namur, Belgium).

2.2. Subject

The subject, a man, 35 years of age, underwent a physical examination, and laboratory examinations including a complete blood count, blood chemical analyses, serum high sensitive C-reactive protein and faecal haemoglobin. The subject was apparently healthy and had not received any antibiotics or probiotics over a year preceding the study. Informed consent was obtained from the participant included in the study.

The subject, over a week prior to the sample collection, had kept a low fat (55.1 ± 23.7 g/24 h) and low energy diet (1888 ± 357 kcal). The diet (g/24 h) was composed of 69.7 ± 9.6 of total proteins, 308.1 ± 48.1 of total carbohydrates and 32.8 ± 13.2 of dietary fibre.

The study was approved by the Local Ethics Committee (KBET/206/B/2012).

2.3. Faeces samples preparation

The stool sample of approximately 150 g, was collected after defecation with disinfected plastic equipment. The sample was immediately dried in a forced-air draying oven at 40 °C for 48 h. Next, the sample was ground in the automatic mill (PREDOM Mesko, Poland) and thoroughly mixed prior to be aliquoted.

Faeces aliquots of 1 g of dry weight each, were transferred into 25 mL plastic vials. Then, with the tubes tightly twisted, the content of each vessel was immersed in 10 mL of perchloric acid (0.15 M).

The prepared samples were ready for the further extraction procedure. All experiments were performed in triplicate.

Following the extraction, the extracts were placed in 1.5 mL new plastic tubes and centrifuged for 10 min at 10000 rpm at +4 °C. The suspension was then filtered with mixed cellulose esters (MCE) membrane filters of the pore size of 0.45 μm (Nantong FilterBio Membrane Co., China). The samples were stored at −20 °C until analysis. The samples were analysed for organic acids by an isotachophoresis method. In order to confirm the obtained results the extracts were also analysed with the capillary electrophoresis method (the reference method).

2.4. Instrumentation

To determine the organic acids in stool samples, the isotachophoretic analyses were performed by means of the Electrophoretic Analyser EA 202 M (Villa Labeco, Spisska Nova Ves, Slovakia) with a conductivity detector. The system was equipped with a sample valve of 30 μL fixed volume and two capillaries, namely 90 mm × 0.9 mm I.D. pre-separation capillary and a 160 mm × 0.3 mm I.D. analytical capillary. The pre-separation involved the current of 250 μA, while the actual separation in the analytical capillary column was performed at 60 μA and 50 μA during the during the initialization and detection phase, respectively [13]. The leading electrolyte of pH 4.5 was 10 mM hydrochloric acid with 0.2% M-HEC, and 22 mM ε-aminocaproic acid. The terminating electrolyte contained 5 mM of caproic acid and 5 mM of L-histidine. The exemplary isotachophoreogram was shown on Fig. 1. For acetic, propionic and butyric acids, LOD ranged from 0.27 to 0.33 mg L^{-1} and LOQ values ranged between 0.80 and 1.00 mg L^{-1}.

Ultrasonic cleaner Sonic-6 by POLSONIC, Poland, was used for the ultrasound-assisted extraction from the biological material. A laboratory shaker type 358S by ELPAN Poland, was used for shaking samples.

PA 800 plus capillary electrophoresis (CE) system by Beckman Coulter (Brea, CA, USA) with the diode array detector (DAD) was used to identify the organic anions in human stool samples. The separation was carried out in the fused capillary I.D. 75 μm, of the total length of 60.2 cm/50 cm to the detector. The capillary was thermostated at 25 °C and the spectrophotometric detection was performed at 233 nm. The sample was injected hydrodynamically at 0.8 psi for 8 s, and the separation potential of −30 kV. Initially, the capillary was rinsed with 0.1 M NaOH and then with water. The separation was performed using chemicals from CEofix Anions "5" kit, according to its procedure. Octanoic anion was applied as the internal standard (IS).

2.5. Experimental design and statistical approach

The combination of three parameters, namely the time of shacking the sample with the solvent (at room temperature), the time of sample exposure to ultrasounds (at 35 °C), and the number of extractions from a single sample, was optimized to achieve the best