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An isotope-labeled chemical derivatization method for the quantitation of short-chain fatty acids in human feces by liquid chromatography–tandem mass spectrometry

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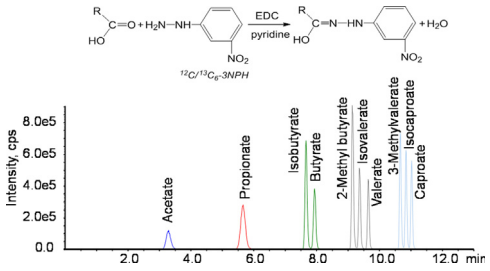
HIGHLIGHTS

- 3-Nitrophenylhydrazine was used to derivatize short-chain fatty acids (SCFAs) for LC-MS/MS.
- $^{13}C_6$ analogues were produced for use as isotope-labeled internal standards.
- Isotope-labeled standards compensate for ESI matrix effects in LC-MS/MS.
- Femtomolar sensitivities and 93–108% quantitation accuracy were achieved for human fecal SCFAs.

GRAPHICAL ABSTRACT



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ABSTRACT

Short-chain fatty acids (SCFAs) are produced by anaerobic gut microbiota in the large bowel. Qualitative and quantitative measurements of SCFAs in the intestinal tract and the fecal samples are important to understand the complex interplay between diet, gut microbiota and host metabolism homeostasis. To develop a new LC-MS/MS method for sensitive and reliable analysis of SCFAs in human fecal samples, 3-nitrophenylhydrazine (3NPH) was employed for pre-analytical derivatization to convert ten C_2–C_6 SCFAs to their 3-nitrophenylhydrazones under a single set of optimized reaction conditions and without the need of reaction quenching. The derivatives showed excellent in-solution chemical stability. They were separated on a reversed-phase C_{18} column and quantitated by negative-ion electrospray ionization – multiple-reaction monitoring (MRM)/MS. To achieve accurate quantitation, the stable isotope-labeled versions of the derivatives were synthesized in a single reaction vessel from $^{13}C_6$-3NPH, and were used as internal standard to compensate for the matrix effects in ESI. Method validation showed on-column limits of detection and quantitation over the range from low to high femtomoles for the ten SCFAs, and the intra-day and inter-day precision for determination of nine of the ten SCFAs in human fecal samples was $\leq$8.8% (n = 6). The quantitation accuracy ranged from 93.1% to 108.4% (CVs $\leq$ 4.6%, n = 6). This method was used to determine the SCFA concentrations and compositions in six human fecal samples. One of the six samples, which was collected from a clinically diagnosed type 2 diabetes patient showed a significantly high molar ratio of branch-chain SCFAs to straight-chain SCFAs than the others. In summary, this work provides a new LC-MS/MS method for precise and accurate quantitation of SCFAs in human feces.

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1. Introduction

Some dietary components that we intake each day pass through the upper gastrointestinal tract unaffected or are only partially digested. These indigestible or partially digested components are fermented in the cecum and colon by anaerobic gut microbiota. This results in the production of many classes of metabolites in the large bowel [1], and short-chain fatty acids (SCFAs) are the major group [2,3]. SCFAs are small fatty acids that contain one to six carbon atoms. In the colon, the straight-chain SCFAs are predominantly from bacterial fermentation of dietary fibers and resistant starch [4], while the branched-chain SCFAs are mostly derived from bacterial metabolism of the branched-chain amino acids (valine, leucine, and isoleucine) [5]. These SCFAs provide *ca.* 10% of the daily energy requirements in humans [6] and the rest are excreted through defecation [7].

The biological relevance of SCFAs to human health is more than the salvage of energy for the host, and is multifaceted [8,9]. SCFAs produced in the gut are absorbed in the colon in exchange for bicarbonate and are used to maintain redox balance in fermentation reactions. They are transported from the intestinal lumen into the blood compartment of the host, and can be found in hepatic, portal, and peripheral blood [10,11]. SCFAs are taken up by organs to act as substrates or signal molecules to regulate whole-body energy homeostasis [12], including modulation of glucose, lipid, and cholesterol metabolism in tissues *via* multiple mechanisms [13–16]. SCFAs act not only as important modulators of the epigenome by altering histone acetylation, but also as endogenous ligands for the G-protein-coupled free fatty acid receptors (FFAR), FFAR2 and FFAR3 [17]. For example, SCFAs were shown to trigger secretion of the incretin hormone glucagon-like peptide-1 from mixed colonic cultures through regulation of the SCFA receptors *ffar2* and *ffar3*, highlighting SCFAs as potential targets for the treatment of diabetes [18]. A recent study revealed that the SCFAs propionate and butyrate activate intestinal gluconeogenesis (IGN) *via* two complementary mechanisms [19]. Butyrate activates IGN gene expression through a cAMP-dependent mechanism, while propionate activates IGN gene expression *via* a gut–brain neural circuit involving FFAR3. In the colon, SCFAs (*e.g.*, butyrate) are the nutrients for colonic epithelium, regulate the colonic and intracellular environment, modulate cell proliferation and gene expression, and provide protection against gastrointestinal disorders and colon carcinogenesis [20]. Accumulating evidence has shown a direct link between SCFAs and several human health conditions, including inflammatory bowel disease [21], irritable bowel syndrome (IBS) [22,23], diarrhea [24], and cancer [25]. Therefore, qualitative and quantitative measurements of SCFAs in the intestinal tract and the fecal samples have the potential to increase our understanding of the complex interplay between diet, gut microbiota, and host metabolism homeostasis.

Several analytical techniques have been used to analyze the SCFAs in biological samples, with gas chromatography (GC) in combination with various detectors often being used [14]. For example, acidified water extraction – direct injection GC was used for the determination of eight SCFAs in the human and rat colonic and fecal samples [26]. Capillary electrophoresis with indirect UV detection was used to determine SCFAs in the culture media of anaerobic strains [27] and in urine [28]. Liquid chromatography (LC) with electrochemical detection has also been used to determine human fecal SCFAs [29], and the determination of some SCFAs in equine cecal liquor [30] and in human and rat sera [31] was performed by ion-exchange LC–UV. Schiffels et al. used 4-nitrophenol as a derivatizing reagent for HPLC–UV detection of SCFAs in biogas fermentation media, though separation of some SCFAs from other organic acids was not achieved [32]. 2-Nitrophenylhydrazine (2NPH) has previously been utilized to derivatize fatty acids for LC–UV analysis [33,34], including serum SCFAs [35]. However, due to the difficulties encountered when LC–UV was used for the separation of the 2NPH derivatives of SCFAs from other interfering compounds in the sample, the endogenous concentrations of SCFAs in human serum could not actually be measured [35].

A deeper understanding of the multilevel molecular network in which SCFAs exert the beneficial effects on human health is currently hampered by the lack of quantitative metabolic flux data for the SCFAs [36]. Metabolic flux analysis using isotope (*e.g.*, ^{13}C) labeling is a powerful model-based technique for determining intracellular metabolic fluxes in living systems and represents a next-level metabolomics technique [37]. Mass spectrometry (MS) plays an important role in this regard [38,39]. The use of mass spectrometry for isotope-labeled metabolic flux analysis requires sensitive and reliable analytical techniques to produce accurate qualitative and quantitative measurements of various endogenous compounds. Due to the volatility of the SCFAs, most chemical derivatization procedures for GC/MS of organic acids are not suitable for the analysis of SCFAs in biological samples because losses that occur during sample preparation and phase transition. To address this issue, a chemical derivatization procedure using chloroformates was developed for GC/MS of SCFAs in human blood, urine, and fecal samples [40,41].

LC/electrospray ionization (ESI)-MS is now the most widely used analytical technique in metabolomics. Ion-exclusion LC–MS [42], and reversed-phase LC–MS with post-column neutralization [43], were used for the determination of SCFAs in the pig colon and blood. The special and complex instrument setup for these two methods, however, makes them unsuitable for routine analysis in most laboratories. On the other hand, LC/ESI-MS quantitation without the use of an isotopically-labeled internal standard (IS) for an analyte often makes the analysis questionable because of the notorious matrix effects in ESI [44,45]. While the use of an isotopically-labeled analog of an analyte is a commonly used practice in LC/MS bioanalysis, an alternative strategy is to employ isotopically-labeled chemical derivatization for enhanced metabolomic analysis of endogenous compounds [46]. Over the past few years, several isotopically-labeled pairs of derivatizing reagents have been reported for the identification and relative quantitation of fatty acids by LC-ESI/MS, although these reagents were not developed specifically for the targeted analysis of SCFAs. These reagents include D_0/D_9-cholamine [47], D_0/D_3-3-acyloxymethyl-1-methylpyridinium [48], and $^{12}C/^{13}C_2$-*para*-dimethylaminophenacyl bromide [49], among others. The aim of this current work was to develop a new isotopically-labeled chemical derivatization-LC/ESI-MS method that is capable of precise and accurate quantitation of SCFAs in human fecal samples. This method uses $^{12}C/^{13}C_6$-3-nitrophenylhydrazine (3NPH) for pre-analytical chemical derivatization under a mild reaction condition to quantitatively convert SCFAs to their 3-nitrophenylhydrazones for subsequent analysis by ultrahigh performance liquid chromatography (UPLC)/multiple-reaction monitoring (MRM)-MS. $^{13}C_6$-3NPH was used to generate a mixture of the stable isotope-labeled SCFA derivatives in a single reaction vessel for internal standard calibration.

2. Material and methods

2.1. Chemicals

Authentic compounds of ten straight-chain and branched-chain SCFAs, including acetic acid (C_2), propionic acid (C_3), butyric acid (C_4), isobutyric acid (C_4; 2-methylpropanoic acid), 2-methylbutyric acid (C_5), isovaleric acid (C_5; 3-methylbutyric acid), valeric acid (C_5; pentanoic acid), caproic acid (C_6; hexanoic acid), 3-methylvaleric acid (C_6; 3-methylpentanoic acid), and isocaproic acid