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GC-MS ANALYSIS OF SHORT-CHAIN FATTY ACIDS IN FECES, CECUM CONTENT, AND BLOOD SAMPLES

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ABSTRACT

Short-chain fatty acids, the end products of fermentation of dietary fibers by the gut microbiota, have been shown to exert multiple effects on mammalian metabolism. For the analysis of short-chain fatty acids, gas chromatography–mass spectrometry is a very powerful and reliable method. Here, we describe a fast, reliable, and reproducible method for the separation and quantification of short-chain fatty acids in mouse feces, cecum content, and blood samples (i.e., plasma or serum) using gas chromatography–mass spectrometry. The short-chain fatty acids analysed include acetic acid, propionic acid, butyric acid, valeric acid, hexanoic acid, and heptanoic acid.
INTRODUCTION

Lipids and fatty acids (FAs) are essential molecules in the regulation and control of various biological functions and play a role in the onset and progression of disease [1]. FAs with less than 8 carbon atoms are considered short-chain fatty acids (SCFAs) [2]. SCFAs (predominantly acetic acid, propionic acid, and butyric acid with respectively 2, 3, and 4 carbon atoms) are mainly produced in the colonic lumen after anaerobic fermentation of indigestible carbohydrates by saccharolytic gut bacteria [3]. The link between diet, the gut microbiota, the production of SCFAs and their role in human health and disease is an active area of research [4]. This requires suitable analytical techniques for sensitive and accurate quantification of SCFAs. One technique traditionally used for the analysis of small, volatile molecules is gas chromatography–mass spectrometry (GC-MS). Here, were describe step by step the quantitative analysis of the SCFAs acetic acid, propionic acid, butyric acid, valeric acid, hexanoic acid, and heptanoic acid using GC-MS in feces, cecum content, as well as in blood samples (i.e., plasma or serum).

GC-MS is an analytical technique, well suited for the analysis of SCFAs and other (longer) FAs [5]. However, one critical step in the GC-MS analysis of FAs is their conversion into suitable volatile derivatives by derivatization (e.g., by alkylation or silylation) [6]. Traditionally, FAs are being transformed into their methyl ester, or trimethylsilyl ester derivatives in GC-MS analysis [7]. While both approaches work well for longer chain FAs, the intrinsically low boiling point of the SCFA methyl ester or trimethylsilyl ester derivatives results in some issues with their GC-MS based analysis. For example, the trimethylsilyl ester of acetic acid roughly presents the same boiling point as the commonly used derivatization reagents, leading to severe signal overlap.

Alternatively, for SCFA analysis FAs can be derivatized by the alkylation reagent pentafluorobenzyl bromide (PFBBr) [8,9]. The benzyl bromide group reacts with the carboxylic acid group to form an ester, allowing analysis as pentafluorobenzyl ester. Additionally, this so-formed ester presents ideal properties for electron-capture negative ionisation (ECNI), which is a highly selective and sensitive ionisation technique. ECNI allows analysis of the
negatively charged molecular ions, usually detected in the single ion monitoring mode (SIM) on quadrupole based mass spectrometers.

For targeted analysis of SCFAs, ideally isotopically labelled internal standards (IS) should be used. The use of IS enables quantitative analysis of biological samples and greatly improves specificity [7,9].

Apart from GC-MS, Nuclear magnetic resonance (NMR) spectroscopy can be used to analyse SCFAs in feces or cecum content. An interplatform comparison performed in our lab, comparing GC-MS and NMR spectroscopy, showed good correlations for the measurements of SCFA concentrations. However, the advantage of GC-MS over NMR is its higher sensitivity, which is essential for the analysis of SCFAs at low concentrations such as SCFAs present in blood.

MATERIALS

Use only high purity solvents (preferably LC-MS grade) in order to prevent elevated background signals (see Note 1). An overview of the amount of materials and chemicals is provided in Table 1. If vendors of different materials are specifically mentioned in this section, the use of these materials are recommended based on our previous experiences.

MATERIALS FOR SAMPLE PREPARATION

1. Glass autosampler vials, inserts, and caps. It is important to use the highest quality glass ware. We recommend to use the following items: Agilent certified 2 mL vials with screw top; Agilent certified 250 μL inserts with polymer feet; Agilent screw caps with PTFE/red silicone septum.

2. 1 μg/mL IS solution in ethanol (EtOH) (see Note 2): mix acetic acid-d4, propionic acid-d6 and butyric acid-d8 and dissolve in EtOH to a final concentration of 1 μg/mL. Store at −80°C. Apart from butyric acid, butyric acid-d8 is also used as the IS for valeric acid, hexanoic acid, and heptanoic acid.
Table 1. Chemicals and materials required per sample for the quantification of SCFAs. For every type of biological matrix, take along three blank samples. Blank samples should be processed in exactly the same way as biological samples.

<table>
<thead>
<tr>
<th>Chemical/material</th>
<th>Calibration series sample</th>
<th>Biological sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calibration series sample</td>
<td>Biological sample</td>
</tr>
<tr>
<td></td>
<td>Feces</td>
<td>Cecum content</td>
</tr>
<tr>
<td>Biological sample</td>
<td>± 50 mg</td>
<td>± 10 mg</td>
</tr>
<tr>
<td>Water</td>
<td>250 µL</td>
<td>550 µL</td>
</tr>
<tr>
<td>Acetone</td>
<td>250 µL</td>
<td>250 µL</td>
</tr>
<tr>
<td>1 µg/mL IS solution</td>
<td>10 µL</td>
<td>10 µL</td>
</tr>
<tr>
<td>Standards in EtOH</td>
<td>10 µL²</td>
<td>-</td>
</tr>
<tr>
<td>EtOH</td>
<td>-</td>
<td>10 µL</td>
</tr>
<tr>
<td>172 mM PFBBr</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>n-hexane</td>
<td>500 µL</td>
<td>500 µL</td>
</tr>
<tr>
<td>1.5 mL plastic tubes</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Clean steel beads</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Glass autosampler vials</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Glass autosampler inserts</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Glass autosampler caps</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

*For every individual sample of the calibration series, a specific concentration of standards in EtOH is used.

3. Concentration series of SCFA standards in EtOH: use the SCFA standard mixture (Sigma-Aldrich) and dilute with EtOH. Prepare concentrations ranging from the lower limit of quantification (LLOQ) (see Table 2) to 1000 µM. Store at −80°C.

4. 172 mM PFBBr in acetone: add 26.8 µL PFBBr to 1 mL acetone. Prepare fresh daily.

5. Clean steel beads (only for fibrous biological matrices): rinse 3.2 mm stainless steel beads with methanol and dry at room temperature.

MATERIALS FOR GC-MS

1. GC with split/splitless injector, coupled to a quadrupole mass spectrometer with chemical ionisation source.

2. Injection: autosampler (recommended).
3. GC column: use an Agilent VF-5 ms column (5% phenylmethyl; 25 m × 0.25 mm internal diameter; 0.25 μm film thickness).

4. Pure helium (99.9990%) and methane (99.9995%) should be used as carrier and as chemical ionisation gas, respectively.

Table 2. Overview of FAs. For each SCFA, an indication of the retention time (RT), the m/z-value, an indication of the LLOQ, and the IS to be used are shown.

<table>
<thead>
<tr>
<th>FA</th>
<th>Name</th>
<th>RT (min)</th>
<th>Monitored m/z</th>
<th>LLOQ (μM)*</th>
<th>IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA 2:0-d4</td>
<td>acetic acid-d4</td>
<td>7.19</td>
<td>62</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>FA 2:0</td>
<td>acetic acid</td>
<td>7.22</td>
<td>59</td>
<td>20</td>
<td>FA 2:0-d4</td>
</tr>
<tr>
<td>FA 3:0-d6</td>
<td>propionic acid-d6</td>
<td>7.86</td>
<td>78</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>FA 3:0</td>
<td>propionic acid</td>
<td>7.89</td>
<td>73</td>
<td>5</td>
<td>FA 3:0-d6</td>
</tr>
<tr>
<td>FA 4:0-d8</td>
<td>butyric acid-d8</td>
<td>8.41</td>
<td>94</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>FA 4:0</td>
<td>butyric acid</td>
<td>8.44</td>
<td>87</td>
<td>2</td>
<td>FA 4:0-d8</td>
</tr>
<tr>
<td>FA 5:0</td>
<td>valeric acid</td>
<td>8.99</td>
<td>101</td>
<td>1</td>
<td>FA 4:0-d8</td>
</tr>
<tr>
<td>FA 6:0</td>
<td>hexanoic acid</td>
<td>9.51</td>
<td>115</td>
<td>5</td>
<td>FA 4:0-d8</td>
</tr>
<tr>
<td>FA 7:0</td>
<td>heptanoic acid</td>
<td>9.99</td>
<td>129</td>
<td>1</td>
<td>FA 4:0-d8</td>
</tr>
</tbody>
</table>

* An indication of the lowest concentration to be included in the calibration series. This LLOQ is determined for every individual experiment. The calibration series samples are measured twice. A specific concentration is included if signal/noise >10 and if the accuracy based on the calibration obtained ≥80 and ≤120% for both measurements.

Methods

SCFAs are ubiquitous. Hence, extra care has to be taken to prevent sample contamination. Possible sources of contamination include environmental air, pipettes, pipette tips, low quality plastics, and (low purity) solvents (see Note 1). Interday and intraday repeatability of the method, validated in fetal calf serum (FCS), is provided in Table 3.
Table 3. Interday and intraday repeatability data in FCS. FCS was spiked with 5 μM and 100 μM SCFA. Acetic acid, propionic acid and butyric acid in these samples were quantified in triplicate on three different days using the described method.

<table>
<thead>
<tr>
<th>FA</th>
<th>Day</th>
<th>FCS</th>
<th>FCS + 5 μM SCFA</th>
<th>FCS + 100 μM SCFA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean (µM)</td>
<td>RSD</td>
<td>Mean (µM)</td>
</tr>
<tr>
<td>Acetic</td>
<td>intra1</td>
<td>108.5</td>
<td>0,12</td>
<td>113.4</td>
</tr>
<tr>
<td></td>
<td>intra2</td>
<td>126.5</td>
<td>0,05</td>
<td>120.8</td>
</tr>
<tr>
<td></td>
<td>intra3</td>
<td>117.8</td>
<td>0,11</td>
<td>121.4</td>
</tr>
<tr>
<td></td>
<td>inter</td>
<td>117.6</td>
<td>0,08</td>
<td>118.5</td>
</tr>
<tr>
<td>Propionic</td>
<td>intra1</td>
<td>&lt;LLOQ</td>
<td>N/A</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>intra2</td>
<td>&lt;LLOQ</td>
<td>N/A</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>intra3</td>
<td>8.1</td>
<td>0,24</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>inter</td>
<td>N/A</td>
<td>N/A</td>
<td>11.1</td>
</tr>
<tr>
<td>Butyric</td>
<td>intra1</td>
<td>&lt;LLOQ</td>
<td>N/A</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>intra2</td>
<td>&lt;LLOQ</td>
<td>N/A</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>intra3</td>
<td>&lt;LLOQ</td>
<td>N/A</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>inter</td>
<td>&lt;LLOQ</td>
<td>N/A</td>
<td>6.6</td>
</tr>
</tbody>
</table>

RE, based on the difference in the determined concentrations. For acetic acid, the RE is determined based on the difference between the FCS and FCS + 100 μM SCFA sample. For propionic acid and butyric acid, the RE is determined based on the difference between the FCS + 5 μM SCFA and FCS + 100 μM SCFA sample.

N/A not applicable, RE relative error, RSD relative standard deviation

SAMPLE PREPARATION OF FECES, CECUM CONTENT, AND BLOOD

1. Facilitate rapid sampling. Store the samples at −80 °C upon collection if the samples are not prepared immediately (see Note 3).

2. Matrix dependent pre-processing of feces:
   Prepare an aqueous extract of feces. Weigh feces (approximately 50 mg mouse feces) (see Note 4) in a 1.5 mL plastic tube with 0.1 mg accuracy and add 300 μL water. Homogenise the sample using a bullet blender; add two clean 3.2 mm steel beads and blend the sample for 5 min. Centrifuge at 1400 × g for 10 min. Transfer the supernatant to a fresh 1.5 mL plastic tube.
3. Matrix dependent pre-processing of cecum content:

Weigh cecum content (approximately 10 mg mouse cecum content) (see Note 4) in a 1.5 mL plastic tube with 0.1 mg accuracy and add 400 μL water. Homogenise by vortexing. Use a bullet blender if the material is fibrous: add two clean 3.2 mm steel beads and blend the sample for 5 min. Centrifuge at 1400 × g for 10 min. Transfer the supernatant to a fresh 1.5 mL plastic tube. Dilute the supernatant 1:5 with water in a total volume of 50 μL using a fresh 1.5 mL plastic tube.

4. Matrix dependent preprocessing of blood:

Obtain plasma and/or serum. No further pre-processing is needed.

5. Prepare a glass autosampler vial for every sample:

6. For calibration samples, add 250 μL acetone, 10 μL 1 μg/mL IS solution, and 10 μL of the calibration series SCFA standards at the desired concentration. In case of feces or cecum content analysis, add 10 μL water which is pre-processed exactly the same as the biological samples. This includes bullet blending if necessary.

7. For biological samples, add 250 μL acetone (see Note 5), 10 μL 1 μg/mL IS solution in EtOH (see Note 2), 10 μL EtOH (see Note 6), and 10 μL aqueous feces, 10 μL cecum content extract, or 10 μL plasma/serum into a glass autosampler vial.

8. For blank samples, add 250 μL acetone, 10 μL 1 μg/mL IS solution, and 10 μL EtOH into a glass autosampler vial. In case of feces or cecum content analysis, 10 μL water should be added which is pre-processed in exact the same way as the biological samples. This includes bullet blending if necessary. For every type of biological matrix used in an experiment, three blank samples should be included.

9. Vortex all samples.

10. Add 100 μL 172mM PFBBr in acetone (see Note 7). Vortex all samples.

11. Heat the samples at 60°C for 30 min in a laboratory stove. Let the samples cool down to room temperature (approximately 15 min) (see Note 8).

12. Add 500 μL n-hexane and 250 μL water to the samples. Shake the vial in vertical direction for approximately 10 sec. Let the samples rest for 1 min at room temperature.
13. Prepare a new empty glass autosampler vial with a glass insert for every sample. Transfer 250 μL of the n-hexane (upper layer) into the glass insert.

**GC-MS Analysis**

1. Inject 1 μL in the GC-MS, splitless at 280°C.
2. Use helium as carrier gas at a constant flow rate of 1.20 mL/min.
3. Use the following temperature gradient: 1 min at 40°C, linear increase at 40°C/min to 60°C, held for 3 min at 60°C, linear increase at 25°C/min to 210°C, linear increase at 40°C/min to 315°C, and held for 3 min at 315°C.
4. Set the transfer line temperature at 280°C.
5. Keep the ionisation source temperature at 280°C.
6. Use methane as chemical ionisation gas at approximately 15 psi.
7. Detect ions obtained in the negative mode using SIM (see Notes 9 and 10). Table 2 provides the m/z-values to be monitored and an indication of retention times (RT). As a consequence of small chromatographic differences (e.g., GC column length), the exact RT varies between various GC systems. Hence, calibration using external standards is mandatory.

**Data Analysis**

1. Integrate the obtained signal (see Note 11).
2. Calculate the relative retention time (RRT) and area ratios using the respective IS (see Table 2) (see Notes 12 and 13).
3. Determine the slope and LLOQ for every SCFA by performing linear regression. It is recommended to use a weighing factor of 1/x^2 [10].
4. Calculate the SCFA concentrations by using the area ratios obtained from the biological samples, average signal of the blank samples as intercept (see Note 14), and the slopes obtained from the analysis of the calibration series samples. Take into account the sample dilution for feces and cecum content.
1. SCFAs (especially acetic acid and propionic acid) usually show high background signals, resulting in a relatively high LLOQ (see Fig. 1). SCFA background signals can be diminished by using high purity solvents (preferably LC-MS grade). Additionally, use glass vials for organic solvents. For plastic tubes, we strongly recommend to use Eppendorf polypropylene tubes.

![Fig. 1 Background signal of SCFAs.](image)

Fig. 1 Background signal of SCFAs. Several SCFAs usually show a high background signal. As a consequence, the LLOQ for these SCFAs is dependent on the background obtained. In our experience, the background signal of acetic acid is higher than that of propionic acid, which in turn is higher than the background signal of butyric acid. The dashed lines in the graph show the extent of the background signals.

2. The IS signal should be present in every sample. The IS is used to correct for differences in sample preparation between the samples. Use exactly the same batch of 1 μg/mL IS solution in EtOH for the entire experiment, as minor differences in IS composition potentially translate into systematic under- or overestimation of SCFAs in samples.

3. Collect the biological samples as quick as possible and store the samples at −80°C. Levels of SCFAs within biological samples are vulnerable for change, especially when the collection is performed slowly or when samples are improperly stored. SCFAs can evaporate from the samples or SCFAs from the air can contaminate the samples.
4. The amount of sample that is required for the analysis of SCFAs might vary between biological samples from different species.

5. Acetone facilitates the precipitation of proteins.

6. The addition of 10 μL EtOH to the samples ensures that the solvents of the biological samples are matched to the solvents in the calibration samples.

7. Within this protocol no base is added to catalyse the derivatization reaction, since the addition of base can severely increase SCFA background signal [8].

8. n-Hexane is added after the samples have been cooled down in order to prevent evaporation and spilling.

9. Sensitivity is higher when the MS is operated in SIM mode as compared to the full scan mode. However, the full scan mode can be useful to detect FAs that are not incorporated in the SIM method, or to determine the RT of a specific SCFA. If one decides to operate in full scan mode, an m/z-range of 50–150 is recommended to be used.

10. For isotopolog analysis, either m/z-values corresponding to isotopologs can be added to the SIM method (e.g., M0, M1, M2, etc. for every SCFA) [8], or the MS can be operated in scheduled scan mode (e.g., scan window including m/z-values corresponding to M0, M1, M2, etc., for every SCFA).

11. Pyruvic acid has the same mass and almost the same RT as butyric acid and both acids are derivatized by PFBBr. Consecutively, care has to be taken when both analytes are simultaneously present in the sample (see Fig 2). Particularly in plasma and serum samples, pyruvic acid and butyric acid are simultaneously present. In feces and cecum content, pyruvic acid is usually not detected.
Fig. 2 Signal interference between butyric acid (RT = 8.22 min) and pyruvic acid (RT = 8.25 min). Butyric acid and pyruvic acid are eluting closely while being monitored in the same SIM trace. Particularly in plasma (a) and serum samples, pyruvic acid and butyric acid are simultaneously present. In feces and cecum content (b), pyruvic acid is usually not detected.

12. \[ \text{RRT} = \frac{\text{retention time analyte}}{\text{retention time IS}} \]

13. \[ \text{Area ratio} = \frac{\text{area analyte}}{\text{area IS}} \]

14. The blank samples reflect the background signal of the biological samples more accurately than the intercept obtained from the linear regression of the calibration series samples. Therefore, use the average area ratio of the blank samples as background signal/intercept to calculate the concentrations of the biological samples. Use the following formula:

\[
\text{Concentration} = \frac{\text{area ratio} - \text{average area ratio blank samples}}{\text{slope}}
\]
REFERENCES


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