

# GC/MS Detection of Short Chain Fatty Acids from Mammalian Feces Using Automated Sample Preparation in Aqueous Solution

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## Abstract

This Application Note presents a method for the profiling of short chain fatty acids (SCFAs) in mammalian feces using the Agilent 5977B GC/MSD equipped with an Agilent 7890B GC and an Agilent 7693A autosampler. Despite the importance of profiling and quantifying SCFAs in feces, technical difficulties in the analysis remain due to the volatility and hydrophilicity of SCFAs. We used isobutyl chloroformate/isobutanol derivatization in aqueous solution without drying the samples. Using this protocol, a method for C1 to C7 (formic to heptanoic) acids was developed, and more than seven SCFAs were successfully identified and quantified from mammalian feces samples by GC/MS.

## Introduction

Short chain fatty acid (SCFA) profiling has been a major topic in gut bacteria studies<sup>1,2</sup>. However, the high volatility and hydrophilic characteristics of SCFAs makes the detection of these analytes difficult. GC/MS can be a suitable technique, offering advantages for the detection of volatile compounds. These highly polar species need derivatization for proper GC/MS analysis.

Derivatization can involve silylation (that is, trimethylsilyl (TMS))<sup>3</sup>, alkylation<sup>4</sup>, and esterification<sup>5</sup>. Many derivatization protocols require nonaqueous conditions, calling for removing water from the biological samples before the derivatization reaction. SCFAs can be adversely affected during the drying process, resulting in inaccurate results. In previous studies, esterification using propyl chloroformate/propanol was used for derivatization under aqueous conditions<sup>6</sup>. Unfortunately, this derivatizing reagent could not be chromatographically separated from the formic acid, making identification and detection of formic acid difficult.

An improved GC/MS derivatization protocol was developed using isobutyl chloroformate/isobutanol, which allows proper separation, identification, and detection of 14 SCFAs, using sensitive GC/MS analysis. This Application Note introduces an SCFA profiling platform for mammalian feces samples using GC/MS.

## Experimental

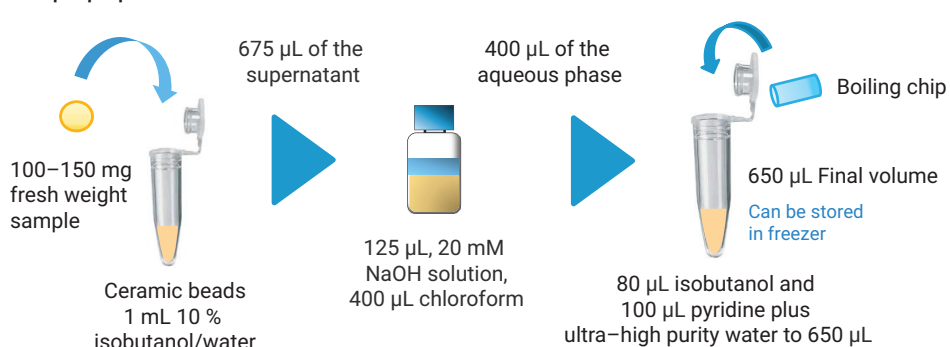
Sample pretreatment is important, especially in the case of lipid-rich biological samples such as feces, because lipids can influence the derivatization process. Therefore, remove the many lipophilic compounds in biological samples by phase separation before the derivatization process. This helps to avoid blocking the derivatization due to contaminants, and removes the contaminant peaks in the GC chromatogram.

### Sample preparation

The freshly collected feces samples (human and cat, 100–150 mg fresh weight) were placed into 2-mL screw cap tubes with ceramic beads (KT03961-1, Bertin Technologies, France). After the addition of 1 mL 10 % isobutanol, the samples were homogenized mechanically (Precellys Evolution;

Bertin Technologies, France) at 6,000 rpm for 20 seconds, twice, with a 30 second interval. Each sample was then centrifuged at 21,000 g for five minutes, and 675  $\mu$ L of the supernatant was transferred to a new tube. Next, to monitor recoveries, a 20  $\mu$ g aliquot of 3-methylpentanoic acid was added to the supernatant. Then, 125  $\mu$ L of 20 mM NaOH solution and 400  $\mu$ L of chloroform were added to the tube, and the sample was vortexed and centrifuged at 21,000 g for two minutes. A 400  $\mu$ L aliquot of the upper aqueous phase was transferred into a new tube, then 80  $\mu$ L isobutanol and 100  $\mu$ L pyridine were added to the tube along with ultrahigh quality water to adjust to 650  $\mu$ L total volume. To minimize foaming, one boiling chip was placed into the tube. The sample can be stored in a freezer after this step.

### Sample preparation



### Derivatization



GC/MS

Figure 1. Sample preparation and derivatization process.

### Calibration standards preparation

The desired concentration of the SCFAs standard with 125  $\mu\text{L}$  20 mM NaOH, 100  $\mu\text{L}$  pyridine, and 80  $\mu\text{L}$  isobutanol were combined in a tube. The final volume was adjusted to 650  $\mu\text{L}$  with water. To avoid foaming, one boiling chip was added into the tube.

### Derivatization process

Calibration standards and samples were subjected to the same derivatization procedure. A 50- $\mu\text{L}$  aliquot of isobutyl chloroformate was carefully added to the 650  $\mu\text{L}$  sample or standard solution. To release the gases generated by the reaction, the tube lid was kept open for 1 minute, then the lid was closed and the sample vortexed. Next, 150  $\mu\text{L}$  hexane was added, and the tubes were centrifuged at 21,000 g for 2 minutes. The upper hexane-isobutanol phase was transferred into an autosampler vial for GC/MSD analysis. Preslit screw caps (p/n 5185-5824) are recommended for the vials because  $\text{CO}_2$  gas is produced.

Figure 2 shows the reaction mechanism of the process.

### Instrumentation

For SCFA quantification, GC/MS measurements were carried out on an Agilent 5977B GC/MSD single quadrupole mass spectrometer equipped with an Agilent 7890B GC and an Agilent 7693A autosampler. Table 1 lists the GC/MS analytical conditions.

## Results and discussion

### Chromatographic separation

Table 2 lists the monitored 14 SCFAs with their retention times and quantification ions.

Figures 3 and 4 show that the modified derivatization process allowed the derivatization reagent to be completely separated from the early eluting SCFAs, formic acid, and acetic acid.

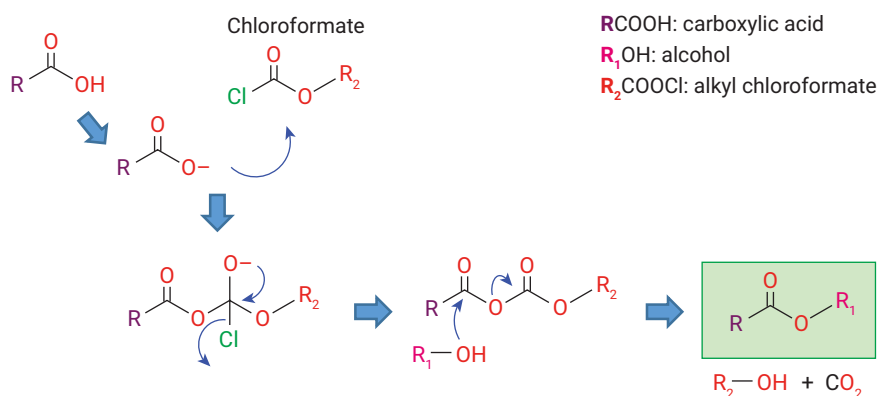


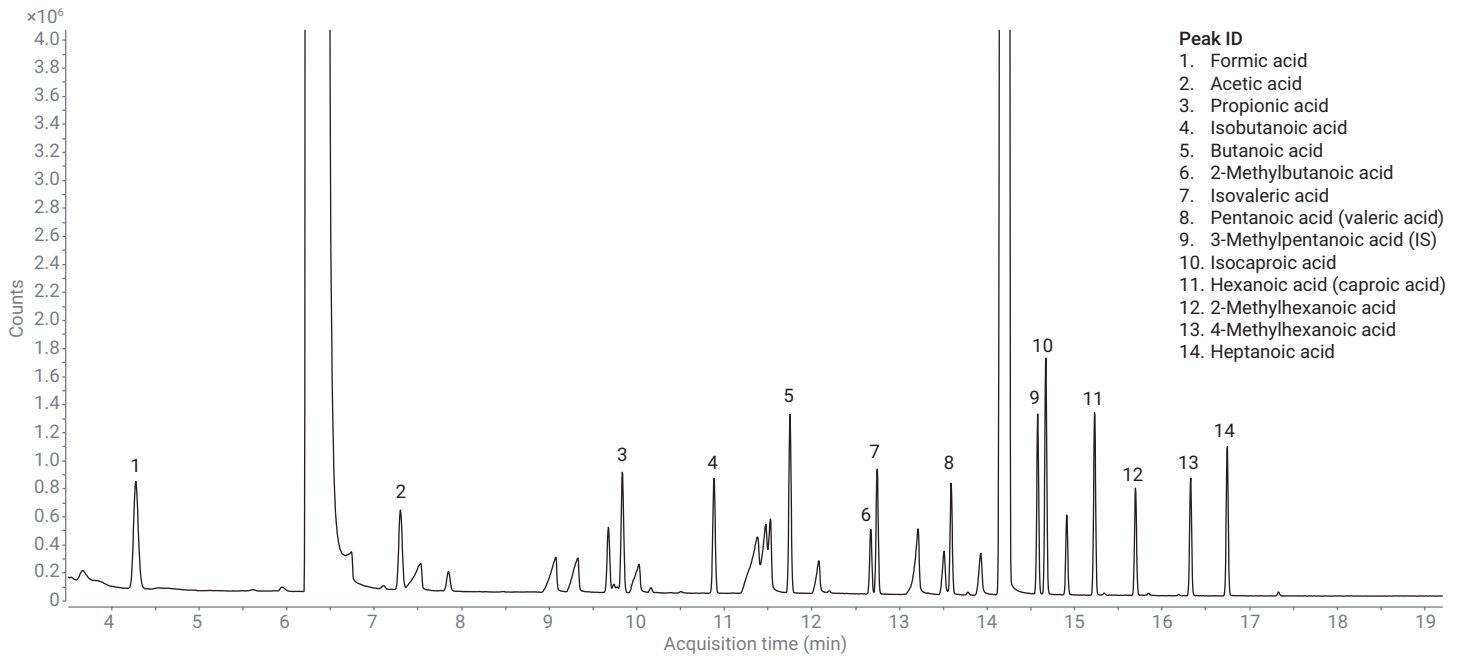
Figure 2. Derivatization reaction mechanism.

Table 1. GC/MS Analytical method.

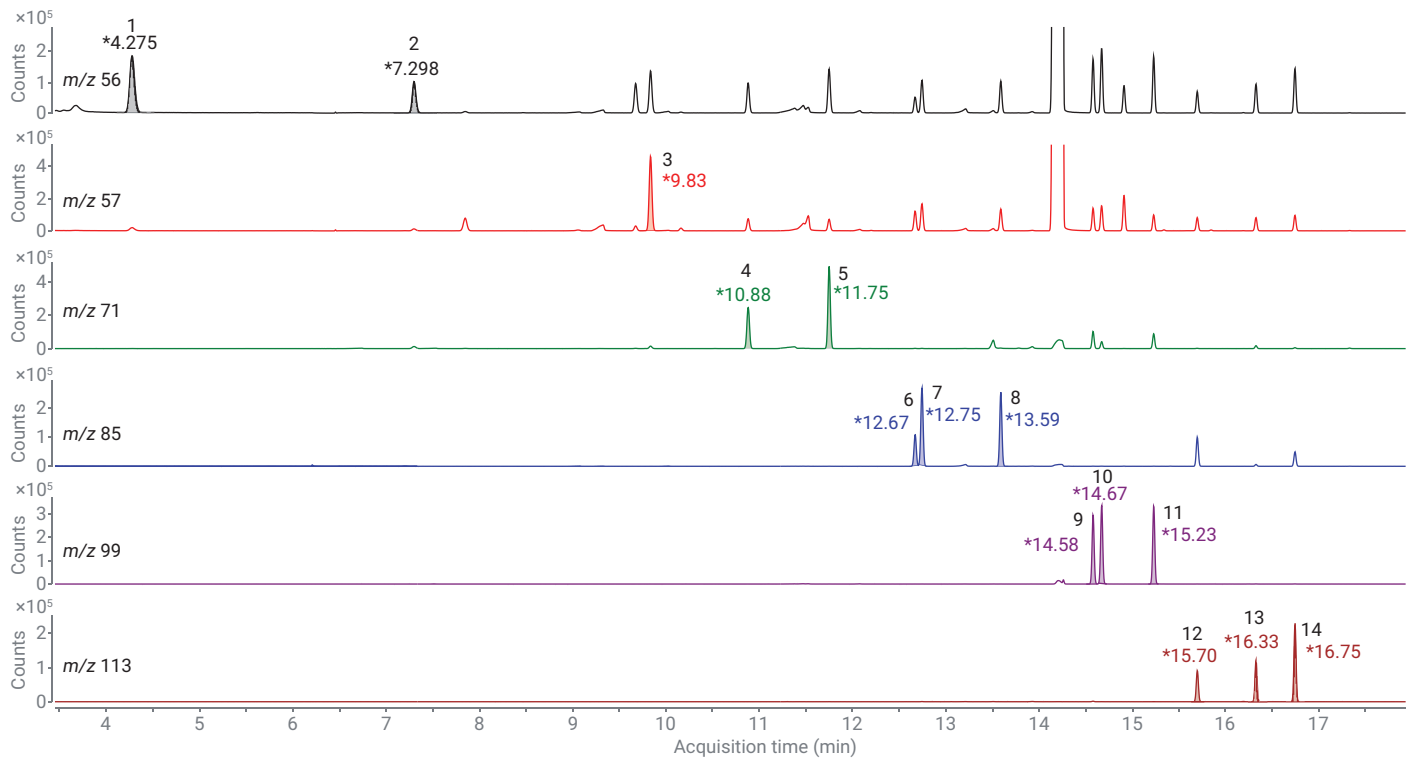
Parameter	Value
GC/MS System	Agilent 7890B GC/5977 MSD
Column	VF-5ms 30 m $\times$ 0.25 mm, 0.5 $\mu\text{m}$ (p/n CP8945)
Column flow	1.0 mL/min
Liner	Ultra Inert liner, Universal, Low PSI drop, Wool (p/n 5190-2295)
Injection mode	Split (50:1)
Injection temperature	260 $^{\circ}\text{C}$
Oven temperature	40 $^{\circ}\text{C}$ for 5 minutes, 10 $^{\circ}\text{C}/\text{min}$ to 310 $^{\circ}\text{C}$
Transfer line temperature	280 $^{\circ}\text{C}$
MS mode	Scan
Scan range	$m/z$ 30–350
Ion source temperature	250 $^{\circ}\text{C}$
Quad. temperature	150 $^{\circ}\text{C}$

Table 2. List of analytes, their retention times, and quantification ions.

No.	Compound	RT (min)	$m/z$
1	Formic acid	4.28	56
2	Acetic acid	7.30	56
3	Propionic acid	9.83	57
4	Isobutanoic acid	10.88	71
5	Butanoic acid	11.75	71
6	2-Methylbutanoic acid	12.67	85
7	Isovaleric acid	12.75	85
8	Pentanoic acid (valeric acid)	13.59	85
9	3-Methylpentanoic acid (IS)	14.26	99
10	Isocaproic acid	14.58	99
11	Hexanoic acid (caproic acid)	14.67	99
12	2-Methylhexanoic acid	15.23	113
13	4-Methylhexanoic acid	15.70	113
14	Heptanoic acid	16.33	113



**Figure 3.** Total ion current chromatogram (TICC) of the SCFAs.



**Figure 4.** Extracted ion chromatogram (EIC) of the quantitation ions.

The large peaks around 6.5 minutes and 14.3 minutes are from the derivatizing reagent. However, they do not interfere with the proper identification and quantitation of the SCFAs.

### Quantitative performance

Calibration was carried out to accommodate the naturally occurring concentrations in mammalian feces. Since C1 to C4 SCFAs are detected in biological samples at much higher concentrations than higher carbon SCFAs, calibration started at higher concentrations for the early eluting analytes than the later eluting compounds. In all ranges excellent linearity was observed.

### Sample results

We tested the feces samples of vertebrates using this technique. We commonly detected acetic acid (C2), propionic acid (C3), and butanoic acid (C4) in all tested samples. Acetic acid was the dominant SCFA among all feces.

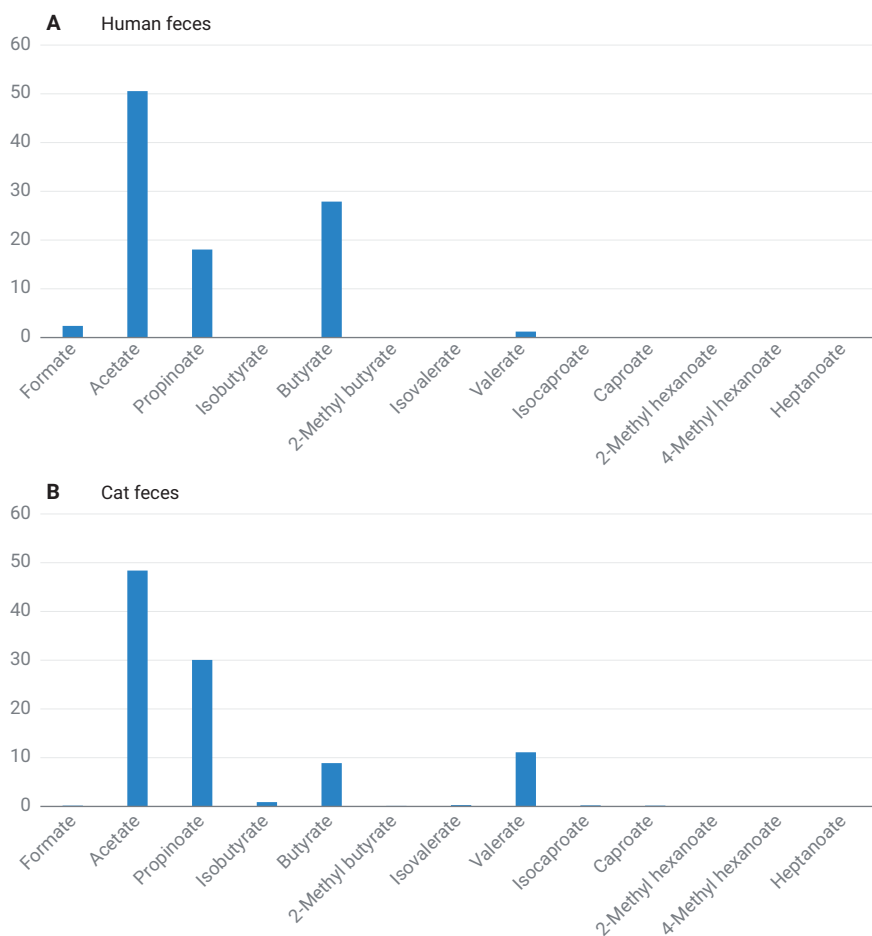
This isobutylation protocol by chloroformate enables rapid SCFA profiling for various biological samples, such as feces. This improved protocol facilitates the investigation of gut bacteria fermentation processes.

## Conclusions

Esterification by chloroformate can be conducted in aqueous solutions. Compared with esterification in nonaqueous solutions (for example,  $\text{BF}_3$  or  $\text{H}_2\text{SO}_4$  in methanol)<sup>5</sup>, the protocol is easy and practical. Our approach can be performed with an autosampler, allowing automation of the derivatization step. This protocol has the potential to be applied in routine measurements.

**Table 3.** Excellent calibration results were obtained both for the naturally occurring high and trace concentration ranges.

No.	Compound	Range (on-column)	R <sup>2</sup>
1	Formic acid	40 pg–1 ng	0.9963
2	Acetic acid	20 pg–1 ng	0.9979
3	Propionic acid	20 pg–1 ng	0.9992
4	Isobutanoic acid	10 pg–1 ng	0.9993
5	Butanoic acid	10 pg–1 ng	0.9993
6	2-Methylbutanoic acid	10 pg–1 ng	0.9987
7	Isovaleric acid	10 pg–1 ng	0.9985
8	Pentanoic acid (valeric acid)	10 pg–1 ng	0.9995
9	3-Methylpentanoic acid (IS)	–	–
10	Isocaproic acid	4 pg–1 ng	0.9995
11	Hexanoic acid (caproic acid)	4 pg–1 ng	0.9996
12	2-Methylhexanoic acid	4 pg–1 ng	0.9987
13	4-Methylhexanoic acid	4 pg–1 ng	0.9990
14	Heptanoic acid	4 pg–1 ng	0.9996



**Figure 5.** Mammalian sample results.

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