Simultaneous Determination of 7 Short-Chain Fatty Acids in Human Saliva by High-Sensitivity Gas Chromatography-Mass Spectrometry

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Abstract
Upon absorption in the intestine of the host animal, the main function of short-chain fatty acids (SCFAs), mainly acetate, propionate and n-butyrate, is as metabolic energy. SCFAs, n-butyrate in particular, can also be found in the mouth. An excess of oral SCFAs may cause not only periodontal diseases but also systemic abnormalities in humans. Previously, we reported a method for simultaneous detection by gas chromatography-mass spectrometry (GC-MS) of acetate, propionate and n-butyrate in serum, urine and saliva. In the present study we used a modified version of this method to detect not only acetate, propionate and n-butyrate, but also iso-butyrate, n-valerate, iso-valerate and caproate, because the latter are suggested to be associated with periodontal diseases. Detection ranges of SCFAs were as follows; 6.25-400 µmol/L (acetate), 0.781-100 µmol/L (propionate and n-butyrate), 0.391-50 µmol/L (iso-butyrate), 0.781-50 µmol/L (n-valerate and iso-valerate) and 1.56-50 µmol/L (caproate). Furthermore, we validated the modified detection method with triple freeze-thaw-cycle recovery tests and intra- and inter-day repeatability. Freezing and thawing did not influence the concentrations of SCFAs in saliva. Upon analysis of five clinical saliva samples, it was observed that, except for n-valerate, which was detected only in two samples, all SCFAs were detected in saliva samples. To conclude, we were able to use a modified method to analyze successfully by GC-MS the salivary concentrations of SCFAs. In addition, we simultaneously detected the salivary concentrations of iso-butyrate, iso-valerate, n-valerate and caproate. This improved method was proved to be reliable to measure the concentrations of SCFAs in saliva.

Keywords: Short-chain fatty acid; GC-MS; Saliva; Simultaneous determination

1. Introduction
Microbiota living in the alimentary tract produces many types of metabolites via anaerobic fermentation including hydrogen, methane, hydrogen sulfide, ammonia, amines, phenols and short-chain fatty acids (SCFAs) [1]. SCFAs are chiefly used as metabolic energy upon absorption in the intestine of the host animal [2]. SCFAs, in particular n-butyrate, can also be detected in the mouth, which may induce not only periodontal diseases, but also systemic abnormalities in humans [3]. Therefore, salivary detection of SCFAs may be reasonable to diagnose the periodontal disease with noninvasive manners. Previously, we reported a method to detect trace concentrations of SCFAs such as acetate, propionate, and n-butyrate, in serum, urine and saliva, using gas chromatography-mass spectrometry (GC-MS) [4]. Bacteria residing in the oral cavity can produce propionate, iso-butyrate, n-valerate, iso-valerate and caproate, some of them being considered as biomarkers for periodontal diseases.
which can compromise the viability of human gingival fibroblasts [5]. Separately, Zhao et al. [6] reported a method for the simultaneous detection by GC-MS of acetate, propionate, n-butyrate, iso-butyrate, n-valerate, iso-valerate, caproate and enanthate, in human urine. However, the method by Zhao et al. was used to analyze only urine samples. Moreover, the extraction method (packed-fiber solid-phase extraction) reported by Zhao et al. made the subsequent analysis of SCFAs difficult.

In light of the above, in the present study, we modified our previously reported simultaneous GC-MS method for the analysis of SCFAs [4], using human saliva as samples. In addition, the newly improved method was analytically validated.

2. Materials and methods

2.1. Reagents

Sodium acetate, sodium propionate, sodium n-butyrate, sodium iso-butyrate, sodium n-valerate and sodium iso-valerate were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). Sodium caproate was purchased from Sigma-Aldrich Japan (Tokyo, Japan). These compounds were used as standard samples.

Sodium acetate-d3 was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA) and propionic acid-d3, butyric-d7 acid, isobutyric acid-1-13C, valeric acid-1-13C and caproic acid-2,2-d2 were purchased from Sigma-Aldrich Japan. These compounds were used as internal standards. Except for valeric acid-1-13C, which was used as internal standard for both n-valerate and iso-valerate, other internal standards were used to analyze the respective SCFAs.

MS-graded purified water was purchased from FUJIFILM Wako Pure Chemical. Hydrochloric acid (Junsei Chemical, Tokyo, Japan), diethyl ether and sulfosalicylic acid dihydrate were purchased from Sigma-Aldrich Japan. tert-Butyldimethylchlorosilane was purchased from Tokyo Chemical Industry (Tokyo, Japan).

2.2. Preparation of stock solutions and working solution

Mixed solutions of standard and internal standards were separately prepared, using the above-mentioned MS-graded purified water. Solutions of acetate, propionate and n-butyrate were prepared in a final concentration of 20 mmol/L and those of iso-butyrate, n-valerate, iso-valerate and caproate in a final concentration of 10 mmol/L. Internal standards acetate-d3, propionate-d3 and n-butyrate-d7 solutions were prepared in a final concentration of 5 mmol/L. Lastly, iso-butyrate-1-13C, n-valerate-1-13C, and caproate-2,2-d2 solutions were prepared in a final concentration of 10 mmol/L. These solutions were kept at -80 °C until further use.

2.3. Samples of saliva

Saliva samples of five patients suffering from severe periodontal disease were received from Okayama University (Okayama, Japan). These saliva samples were stored at -80 °C until further use.

The experimental protocol of the present study was approved by the Ethics Committee of Okayama University (Approval number; Ken 1807-025), and in agreement with the code of ethics stated in the Declaration of Helsinki.

2.4. Sample preparation

The method of sample preparation for detecting SCFAs was previously described [4]. In the present study, a version of the method with slight modifications was used. Briefly, thawed saliva samples (450 µL) were transferred to 1.5 mL micro-tubes. Ten µL of each internal standard solution was added, and the mixture was vortexed for 10 s. The newly mixed samples were deproteinized with 10 µL of a 5-sulfosalicylic acid solution [1 g/mL of ultra-pure water (w/v)] and centrifuged at 2,000× g for 10 min at 4 °C. The supernatant was collected and transferred to a new test tube. Ten µL of hydrochloric acid (35%) and 3 mL of diethyl ether were added, vortexing the mixture (30 min) after each addition. Then, the final mixture was centrifuged at 1,200× g for 10 min at room temperature. The ether phase was retrieved and transferred to a new test tube. Next, tert-butyldimethylchlorosilane (8 µL) was added and the new solution heated at 60 °C for 20 min. After heating, the solution was cooled down and transferred to a glass vial for GC-MS analysis.

2.5. Instruments

Analytical conditions for the GC-MS analysis were similar to those reported elsewhere, with some modifications [4]. Briefly, a gas chromatography-mass spectrometry apparatus (GCMS-QP2010 Ultra; Shimadzu, Kyoto, Japan), equipped with an autosampler (AOC-5000; Shimadzu, Kyoto, Japan), was used. The GC-MS equipment was run in chemical ionization mode, with ammonia as the reagent gas. Chromatographic separation was carried out using a DB-5 MS column (30 m×0.25 mm I.D.×0.25 μm; Agilent Technologies, Santa Clara, CA, USA). Helium (1.5 mL/min) was used as the carrier gas. The stepwise thermal conditions of the column oven were as follows: the temperature was raised to and maintained at 40 °C for 1 min; then the temperature was raised to 60 °C at a rate of 70 °C/min. Afterwards, the temperature was raised again to 110 °C at a rate of 10 °C/min. Finally, the temperature was elevated to 250 °C at a rate of 70 °C/min, and maintained for 2 min. The total run time was 10.3 min. The mass spectrometer was set in selected ion monitoring mode. The m/z of acetate, acetate-d3, propionate, propionate-d3, n-butyrate and iso-butyrate, iso-butyrate-1-13C, n-butyrate-d7, n-valerate and iso-valerate, n-valerate-1-13C, caproate and caproate-2,2-d2 were 192.0, 195.0, 206.0, 209.0, 220.0, 222.0, 227.0, 234.0, 236.0, 248.0
and 251.0, respectively. iso-Butyrate-1-13C, n-valerate-1-13C and caproate-2,2-d2 were not monitored at their original m/z. Instead, the target ion was monitored after adding +1 to their original m/z. The sample injection was used in split-less mode, with an injection volume of 1 μL and an injector temperature of 250 °C. The ion source and interface temperatures for the chemical ionization were 200 and 250 °C, respectively. Moreover, for the electron impact mode ionization, voltage was 1.8 kV.

2.6. Validation method

Validation was conformed to the guidelines for analytical procedures and methods validation by Food and Drug Administration (FDA) [7].

2.6.1. Linearity of the calibration curve

The ranges of the standards solutions were as follows: acetate: 3.13-400 μM; propionate and n-butyrate: 0.391-100 μM; iso-butyrate: n-valerate and iso-valerate: 0.391-50 μM; and caproate: 0.781-50 μM. To evaluate the linearity of the calibration curve, three replicates of the standard solutions were prepared and loaded into the GC-MS on a same day. The peak area value of each calibration standard was calculated by dividing it by the peak area of its respective internal standard. After that, the data were plotted. Calibration curves were constructed with the plotted data using linear regression, after which the coefficient of determination was calculated. Linearity of the calibration curve was considered acceptable when the mean coefficient of determination (r²) for the three replicates was greater than 0.995.

2.6.2. Intra- and inter-day repeatability

To calculate accuracy and precision, intra-day repeatability was defined by the total value of three replicates of the standard solutions in a day. Accuracy was defined by the following expression: (the measured mean values of three replicates) / (the theoretical value) ×100. Precision was defined by the following expression: (the standard deviation of three replicates) / (the measured mean values) ×100. The accuracy and precision percentages were considered acceptable when they were 85-115% and <15%, respectively.

Inter-day repeatability for accuracy was defined by the total value of the three replicates of the working solutions in consecutive 3 days. The procedure to validate the inter-day repeatability for accuracy was as same as for the intra-day repeatability.

2.6.3. Carryover test

A carryover test was conducted using an injection of the working standard solution at the highest concentrations, followed by a blank solution (ultrapure water). This procedure was conducted three times. The appearance of the peaks of the standard samples and internal standards were evaluated during the blank solution loading.

2.6.4. Validation of dilution

When the periodontal disease are deteriorated, SCFA concentrations in the oral cavity are expected to increase [8]. Therefore, we further evaluated the validation of dilution in saliva.

To validate dilution, we mixed the five saliva samples and diluted them 2-, 4-, 8-, 16-, 32-, 64-fold with ultrapure water. All samples were prepared in triplicate and as described in Chapter 2.4. Both diluted and undiluted saliva samples were added internal standards and afterwards, their peak area was measured. Next, the relative peak area percentages of the diluents and precision within diluents were calculated. In addition, the concentration ratios of the diluents vs undiluted saliva were calculated. A relative peak percentage was considered acceptable when its value was 85-115%, and the precision values and those of the concentration percentages were considered acceptable when they were 85-115% or <15%, respectively.

2.6.5. Recovery test

Three (low, middle and high) different concentrations of the standard substances were prepared by diluting with ultrapure water (acetate: 100, 200 and 1,000 μmol/L; other fatty acids: 50, 100 and 500 μmol/L). Saliva samples were mixed and diluted 10- or 50-fold with a phosphate-buffered saline solution. Fifty-fold diluents were used to determine the recovery percentage of acetate, and 10-fold diluents were used to determine the recovery percentage of propionate. Undiluted saliva was used to determine the recovery percentages of the other SCFAs. Next, each of the abovementioned concentrations of the standard substrates were added to individual mixed saliva samples (400 μL) and the SCFAs extracted as per the protocol mentioned in Chapter 2.4. The SCFA concentrations in standard-free saliva were also measured. The recovery percentage of each standard sample was defined by the following expression: Recovery percentage = (standard-added saliva – standard-sample) / (theoretical value) ×100. The recovery test was considered successful with a percentage of 70-110%. Recovery tests were conducted in triplicate.

2.6.6. Freeze-thaw stability

Freeze-thaw stability was evaluated with undiluted saliva. Undiluted saliva was divided in six parts: three parts were frozen at -80 °C for 12 hours; the remaining three parts were used as unfrozen control. On the next day, frozen saliva was thawed out to room temperature and re-froze at -80 °C for further 12 hours. This freeze-thaw procedure was conducted three times in total. Saliva samples were analyzed by GC-MS after freezing and thawing, and the stability percentage of the
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freeze-thaw procedure was defined by the following expression: Freeze-thaw stability percentage = (frozen-thawed sample/unfrozen control) × 100.

2.7. Measurement of clinical saliva sample

Clinical validation was carried out for the saliva samples as described in Chapter 2.3. When SCFA concentration was not within the calibration curve range, the sample was diluted for 10- or 50-fold with a phosphate-buffered saline solution. Samples were analyzed in duplicate.

3. Results and discussion

3.1. Peak specificity

Representative chromatograms of the standard samples, internal standards, ultrapure water and mixed saliva are shown in Figs. 1 and 2. Peaks of the standard samples and internal standards were detected (Fig. 1). The peak times of SCFAs were as follows: 5.04 min (acetate), 5.01 min (acetate-d3), 6.28 min (propionate), 6.25 min (propionate-d3), 7.19 min (n-butyrate), 7.15 min (n-butyrate-d7), 6.75 min (iso-butyrate), 6.75 min (iso-butyrate-1-13C), 7.94 min (n-valerate), 7.94 min (n-valerate-1-13C), 7.60 min (iso-valerate), 8.53 min (caproate) and 8.53 min (caproate-2,2-d2). In contrast, no peaks of the standard samples or the internal standards were observed in ultrapure water at the same retention times (data not shown).

Except for iso-valerate, individual peaks were also clearly detected in mixed saliva (Fig. 2). Indeed, an adjacent peak (at 7.57 min) was found near the peak of iso-valerate (7.63 min). Nonetheless, the adjacent peak resulting from the detection of an unknown matter did not overlap with the iso-valerate. In addition, when the saliva was diluted, this adjacent peak area was decreased. Based on the current results, it was determined that the present method successfully detected SCFAs in saliva by means of column separation and molecular calculation that differentiated them from other endogenous components.
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3.2. Linearity of calibration curve

The acceptable ranges of the calibration curves with the mean determination coefficient were as follows (Table 3): acetate, 3.13-400 µmol/L, \( r^2 = 0.9995 \); propionate, 0.391-100 µmol/L, \( r^2 = 0.9998 \); n-butyrate, 0.391-100 µmol/L, \( r^2 = 0.9998 \); iso-butyrate, 0.391-50 µmol/L, \( r^2 = 0.9999 \); n-valerate, 0.391-50 µmol/L, \( r^2 = 0.9998 \); iso-valerate, 0.391-50 µmol/L, \( r^2 = 0.9999 \); and caproate, 1.56-50 µmol/L, \( r^2 = 0.9998 \). Other lower or higher concentrations were rejected.

In light of the above results, we estimated that the linearity of the calibration curves for SCFAs fitted within the respective ranges.

3.3. Intra- and Inter-day repeatability

The calculated intra-day repeatability of SCFAs is shown in Table 1. For intra-day repeatability, accuracy was acceptable when it fitted within ranges as defined in Chapter 2.6.2. The calculated inter-day repeatability of SCFAs is shown in Table 2. For inter-day repeatability, accuracy was acceptable when it fitted within ranges as defined in Chapter 2.6.2.

Based on the results of the intra- and inter-day validations and to draw the calibration curve, we corrected the acceptable ranges as follows: acetate, 6.25-400 µmol/L; propionate and n-butyrate, 0.781-100 µmol/L; iso-butyrate, 0.391-50 µmol/L; n-valerate and iso-valerate, 0.781-50 µmol/L; caproate, 1.56-50 µmol/L (Table 3).

3.4. Carryover test

Peaks of standard samples and internal standards did not appear in the first replicate of blank samples (data not shown) and thus, it was established that no carryover took place. Due to this, the analysis of the remaining replicates was not conducted.

3.5. Validation of dilution

The precision and concentration percentages of diluents (2- to 64-fold) are shown in Table 4. At 0.7-2.1%, 0.3-3.0% and 1.1-13.5% precision rages of acetate, propionate and n-butyrate were acceptable in all diluents. While within 2- to 32-fold, the precision range (4.0-13.5%) of iso-butyrate was acceptable, at 64-fold (concentration=0.391 µmol/L), the precision of iso-butyrate was rejected. A 2-fold or greater dilution of saliva caused the detection of n-valerate to fail.
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Similar to iso-butyrate, while within 2- to 16-fold, the precision range (0.2-9.0%) of iso-valerate was acceptable, at 32- and 64-fold (concentration<0.781 µmol/L), the precision of iso-valerate was rejected. Likewise, while at 2-fold the precision percentage (5.0%) of caproate was acceptable, within 4- and 64-fold (concentration<1.56 µmol/L), the precision of caproate was rejected. According to these results, it can be recommended that for a successful detection of SCFAs by GC-MS, saliva should be first analyzed undiluted, and only when the concentration of a given SCFA exceeds the quantitation limit, should saliva be diluted for that specific SCFA quantitation.

### Table 2. Calculated inter-day repeatability of accuracy and precision for short-chain fatty acids.

| SCFA   | Theoretical value (µmol/L) | Mean value (µmol/L) | Standard deviation | Accuracy | Precision |
|--------|-----------------------------|---------------------|--------------------|----------|-----------|
| Acetate | 0.391 0.781 1.56 3.13 6.25 12.5 25 50 100 200 400 | - - - - 6.02 11.8 24.3 50.7 103 198 400 | - - - - 0.11 1.6 2.3 0.5 4 5 4 | - - - - 96.4% 94.0% 97.0% 101.3% 103.0% 98.9% 100.1% | - - - - 18.3% 13.4% 9.4% 1.0% 4.3% 2.6% 0.9% |
| Propionate | 0.458 0.824 1.71 3.15 6.56 12.2 24.4 50 99 198 400 | 0.141 0.83 0.06 0.29 0.43 0.2 0.6 0.6 1.1 | 117.2% 105.5% 109.4% 100.9% 105.0% 97.6% 97.5% 100.0% 99.9% | 30.9% 10.1% 3.8% 9.1% 6.6% 1.3% 2.3% 1.3% 1.1% |
| n-Butyrate | 0.391 0.781 1.56 3.13 6.25 12.5 25 50 100 200 400 | 0.467 0.835 1.65 3.29 6.54 12.3 24.7 49.3 100.2 | - - - - 0.11 1.6 2.3 0.5 4 5 4 | - - - - 96.4% 94.0% 97.0% 101.3% 103.0% 98.9% 100.1% | - - - - 18.3% 13.4% 9.4% 1.0% 4.3% 2.6% 0.9% |
| iso-Butyrate | 0.391 0.781 1.56 3.13 6.25 12.5 25 50 100 200 400 | 0.368 0.844 1.54 3.07 6.23 12.4 25.4 50 99 198 400 | - - - - 0.11 1.6 2.3 0.5 4 5 4 | - - - - 96.4% 94.0% 97.0% 101.3% 103.0% 98.9% 100.1% | - - - - 18.3% 13.4% 9.4% 1.0% 4.3% 2.6% 0.9% |
| n-Valerate | 0.391 0.781 1.56 3.13 6.25 12.5 25 50 100 200 400 | 0.467 0.835 1.65 3.29 6.54 12.3 24.7 49.3 100.2 | - - - - 0.11 1.6 2.3 0.5 4 5 4 | - - - - 96.4% 94.0% 97.0% 101.3% 103.0% 98.9% 100.1% | - - - - 18.3% 13.4% 9.4% 1.0% 4.3% 2.6% 0.9% |
| iso-Valerate | 0.391 0.781 1.56 3.13 6.25 12.5 25 50 100 200 400 | 0.368 0.844 1.54 3.07 6.23 12.4 25.4 50 99 198 400 | - - - - 0.11 1.6 2.3 0.5 4 5 4 | - - - - 96.4% 94.0% 97.0% 101.3% 103.0% 98.9% 100.1% | - - - - 18.3% 13.4% 9.4% 1.0% 4.3% 2.6% 0.9% |
| Caproate | 0.391 0.781 1.56 3.13 6.25 12.5 25 50 100 200 400 | 0.467 0.835 1.65 3.29 6.54 12.3 24.7 49.3 100.2 | - - - - 0.11 1.6 2.3 0.5 4 5 4 | - - - - 96.4% 94.0% 97.0% 101.3% 103.0% 98.9% 100.1% | - - - - 18.3% 13.4% 9.4% 1.0% 4.3% 2.6% 0.9% |

### Table 3. Calibration ranges, linear regression equations, and coefficients of determination of the calibration curves for short-chain fatty acids.

| Tested standards | Calibration range (µmol/L) | Plot | Linear regression | Coefficient of determination |
|------------------|---------------------------|------|-------------------|-------------------------------|
| Acetate          | 6.25-400                   | 7    | y = 0.0044x + 0.0055 | 0.9995±0.0002                |
| Propionate       | 0.781-100                  | 8    | y = 0.0044x + 0.0013 | 0.9998±0.0002                |
| n-Butyrate       | 0.781-100                  | 8    | y = 0.0050x + 0.0004 | 0.9998±0.0003                |
| iso-Butyrate     | 0.391-50                   | 8    | y = 0.0080x + 0.0011 | 0.9999±0.0001                |
| n-Valerate       | 0.781-50                   | 7    | y = 0.0121x + 0.0030 | 0.9997±0.0003                |
| iso-Valerate     | 0.781-50                   | 7    | y = 0.0113x + 0.0016 | 0.9998±0.0001                |
| Caproate         | 1.56-50                    | 6    | y = 0.0123x + 0.0002 | 0.9999±0.0001                |

The coefficient of determination was calculated from the analysis of three replicates. Parameter “x” in the linear regression indicated the concentration (µmol/L), and parameter “y” indicated peak area / internal standard area.
3.6. Recovery test

The recovery percentages of the three different concentrations of SCFAs are shown in Table 5. Recovery percentage ranges of acetate, propionate, n-butyrate, iso-butyrate, n-valerate, iso-valerate and caproate were 87.3-100.5%, 97.8-100.4%, 90.6-98.3%, 98.7-101.7%, 103.9-106.3%, 101.0-106.9 and 90.3-106.0%, respectively. It was estimated that since all data fitted within the range of our criteria (70-110%), the results of the SCFAs recovery tests were acceptable.

3.7. Stability of SCFA concentration of the freeze-thaw procedure

The recovery percentages for acetate, propionate, n-butyrate, iso-butyrate, n-valerate, iso-valerate and caproate were 98.5%, 101.9%, 106.3%, 103.1%, 93.5%, 100.9% and 96.4%, respectively. The percentages were within our criterium rage (85-115%). Thus, based on these results, it was demonstrated that the triple freeze-thaw cycle procedure did not affect the concentrations of SCFAs in saliva.

3.8. SCFA detection in clinical saliva samples

The results of the analysis of the SCFAs concentrations in saliva from five clinical samples of patients with severe periodontal disease are shown in Table 6.

Acetate had the highest concentrations among SCFAs. Indeed, the concentration range of acetate was 1,812-4,117 µmol/L, and its mean concentration was 2,819 µmol/L. Propionate had the second highest concentration, but relatively low compared with that of acetate. Indeed, the concentration range of propionate was 185-836 µmol/L, and its mean concentration was 473 µmol/L. n-Butyrate and iso-butyrate were detected in all saliva samples. While the concentration range and the mean concentration of n-butyrate were 11.9-161.7 µmol/L, and 83.7 µmol/L, respectively, those of iso-butyrate were 2.73-49.55 µmol/L and 20.04 µmol/L, respectively. n-Valerate was detected in two saliva samples, at concentrations levels of 1.76 and 2.56 µmol/L. iso-Valerate was detected in all saliva samples. The concentration range and the mean concentration of caproate were 1.73-4.47 µmol/L, and 2.94 µmol/L, respectively.

### Table 4. Results of the repeatability of dilutions.

| Parameters     | Undiluted | 2-fold | 4-fold | 8-fold | 16-fold | 32-fold | 64-fold |
|----------------|-----------|--------|--------|--------|---------|---------|---------|
| **Acetate**    | 2893      | 1490   | 758    | 377    | 204     | 101     | 48.5    |
| Mean value (µmol/L) | 2893      | 1490   | 758    | 377    | 204     | 101     | 48.5    |
| Standard deviation | 87.4      | 22.0   | 7.33   | 2.68   | 4.21    | 1.38    | 0.541   |
| Accuracy       | 103.00%   | 104.8% | 104.3% | 112.7% | 111.4%  | 107.3%  |
| Precision      | 1.0%      | 0.7%   | 2.1%   | 1.4%   | 1.1%    |         |
| **Propionate** | 453       | 227    | 114    | 56.4   | 29.8    | 15.0    | 7.36    |
| Mean value (µmol/L) | 453       | 227    | 114    | 56.4   | 29.8    | 15.0    | 7.36    |
| Standard deviation | 7.92      | 1.77   | 1.48   | 0.192  | 0.562   | 0.443   | 0.035   |
| Accuracy       | 100.4%    | 100.8% | 99.5%  | 105.2% | 105.9%  | 103.9%  |
| Precision      | 1.7%      | 0.8%   | 1.3%   | 0.3%   | 1.9%    | 3.0%    | 0.5%    |
| **n-Butyrate** | 61.6      | 31.4   | 15.5   | 7.80   | 4.06    | 2.03    | 1.09    |
| Mean value (µmol/L) | 61.6      | 31.4   | 15.5   | 7.80   | 4.06    | 2.03    | 1.09    |
| Standard deviation | 0.852     | 0.607  | 0.178  | 0.346  | 0.160   | 0.247   | 0.142   |
| Accuracy       | 101.9%    | 100.5% | 101.3% | 105.6% | 105.3%  | 112.8%  |
| Precision      | 1.4%      | 1.9%   | 1.1%   | 4.4%   | 8.0%    | 12.2%   | 13.5%   |
| **iso-Butyrate** | 19.5      | 9.87   | 5.09   | 2.42   | 1.35    | 0.650   | <0.391  |
| Mean value (µmol/L) | 19.5      | 9.87   | 5.09   | 2.42   | 1.35    | 0.650   | <0.391  |
| Standard deviation | 0.245     | 0.396  | 0.441  | 0.223  | 0.142   | 0.0878  | N.C.    |
| Accuracy       | 101.0%    | 104.2% | 99.2%  | 110.3% | 106.4%  | N.C.    |
| Precision      | 1.3%      | 4.0%   | 8.7%   | 9.2%   | 10.6%   | 13.5%   | N.C.    |
| **n-Valerate** | 1.09      | 0.781  | 0.781  | 0.781  | 0.781   | 0.781   | 0.781   |
| Mean value (µmol/L) | 1.09      | 0.781  | 0.781  | 0.781  | 0.781   | 0.781   | 0.781   |
| Standard deviation | 0.106     | N.C.   | N.C.   | N.C.   | N.C.    | N.C.    | N.C.    |
| Accuracy       | 106.4%    | N.C.   | N.C.   | N.C.   | N.C.    | N.C.    |
| Precision      | 1.6%      | N.C.   | N.C.   | N.C.   | N.C.    | N.C.    |
| **iso-Valerate** | 11.9      | 6.16   | 3.24   | 1.54   | 0.808   | <0.781  | <0.781  |
| Mean value (µmol/L) | 11.9      | 6.16   | 3.24   | 1.54   | 0.808   | <0.781  | <0.781  |
| Standard deviation | 0.682     | 0.129  | 0.00766| 0.0234 | 0.0725  | N.C.    | N.C.    |
| Accuracy       | 108.8%    | N.C.   | N.C.   | N.C.   | N.C.    | N.C.    |
| Precision      | 3.0%      | N.C.   | N.C.   | N.C.   | N.C.    | N.C.    |
| **Caproate**   | 4.80      | 2.51   | <1.56  | <1.56  | <1.56   | <1.56   | <1.56   |
| Mean value (µmol/L) | 4.80      | 2.51   | <1.56  | <1.56  | <1.56   | <1.56   | <1.56   |
| Standard deviation | 0.169     | 0.125  | N.C.   | N.C.   | N.C.    | N.C.    | N.C.    |
| Accuracy       | 104.7%    | N.C.   | N.C.   | N.C.   | N.C.    | N.C.    |
| Precision      | 3.5%      | 5.0%   | N.C.   | N.C.   | N.C.    | N.C.    |

N.C.: Not calculated.
These SCFA concentrations was relatively high (as much as 10-times) rather than those in the saliva of healthy pigs [4]. Therefore, severe periodontitis might induce the stimulation of SCFA production in oral cavity. Nonetheless, we have no data about the salivary SCFA concentrations in healthy human, this points needs further elucidations.

4. Conclusion

As more than 80% of adults suffer from periodontal disease [9], it can be considered a fairly common chronic disease. Moreover, the concentrations of SCFAs in saliva of patients suffering from periodontal disease are relatively higher than those in healthy subjects [10], as SCFAs are produced in excess by gingival pathogens such as Porphyromonas gingivalis [11].

In the present work, we successfully modified a previously reported method [4] to analyze acetate, propionate and n-butyrate concentrations in saliva, by GC-MS. We also used this newly modified method to simultaneously analyze the concentrations of other fatty acids such as iso-butyrate, iso-valerate, n-valerate and caproate. To prove its accuracy, the newly modified method was screened for robustness using a number of validation tests.

The detection ranges of SCFAs were as follows: acetate, 6.25-400 µmol/L; propionate and n-butyrate, 0.781-100 µmol/L; iso-butyrate, 0.391-50 µmol/L; n-valerate and iso-valerate, 0.781-50 µmol/L; and caproate, 1.56-50 µmol/L. Admittedly, our detection range did not always better that of Zhao et al. [6]. However, unlike that of Zhao et al., our method could detect almost all SCFAs in saliva (Table 6). Thus, as far as the analysis of SCFAs in saliva is concerned, our method is more suitable.

It is worth commenting that in separate analyses, we were able to detect SCFAs also in plasma and urine samples, using the newly modified method. We plan to report these data in the future. We envision that the method reported in the present work be used more widely in the future to analyze salivary samples of gingival disease-affected patients, in a more noninvasive manner.

Acknowledgement

The authors thank Mr. Kai Ohashi of the Kyoto Institute of Nutrition & Pathology for his excellent technical assistance. We also thank Bioscience Proofreaders (https://bioscience-proofreaders.com) for editing the English language of this manuscript.

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Table 5. Results of the recovery test.

| Parameters         | Test range | Low    | Middle | High   |
|--------------------|------------|--------|--------|--------|
| Acetate            | Recovery ratio | 100.5% | 95.7%  | 87.3%  |
|                    | Standard deviation | 7.8    | 9.4    | 3.2    |
|                    | Precision     | 7.7%   | 9.8%   | 3.6%   |
| Propionate         | Recovery ratio | 97.8%  | 100.4% | 99.8%  |
|                    | Standard deviation | 4.9    | 3.7    | 4.7    |
|                    | Precision     | 5.0%   | 3.7%   | 4.7%   |
| n-Butyrate         | Recovery ratio | 98.3%  | 97.0%  | 90.6%  |
|                    | Standard deviation | 12.3   | 7.5    | 3.1    |
|                    | Precision     | 12.5%  | 7.7%   | 3.4%   |
| iso-Butyrate       | Recovery ratio | 98.7%  | 99.5%  | 101.7% |
|                    | Standard deviation | 13.2   | 3.3    | 3.6    |
|                    | Precision     | 13.4%  | 3.3%   | 3.5%   |
| n-Valerate         | Recovery ratio | 105.8% | 103.9% | 106.3% |
|                    | Standard deviation | 5.3    | 4.7    | 3.2    |
|                    | Precision     | 5.0%   | 4.5%   | 3.0%   |
| iso-Valerate       | Recovery ratio | 101.0% | 103.5% | 106.9% |
|                    | Standard deviation | 14.4   | 9.1    | 2.1    |
|                    | Precision     | 14.2%  | 8.8%   | 1.9%   |
| Caproate           | Recovery ratio | 104.7% | 106.0% | 90.3%  |
|                    | Standard deviation | 7.9    | 4.3    | 2.6    |
|                    | Precision     | 7.5%   | 4.0%   | 2.9%   |

Table 6. Concentrations of short-chain fatty acids in clinical saliva samples.

| Gender | Age | Acetate (µmol/L) | Propionate | n-Butyrate | iso-Butyrate | n-Valerate | iso-Valerate | Caproate |
|--------|-----|-----------------|------------|------------|-------------|------------|--------------|----------|
| Male   | 62  | 1,812           | 185        | 11.9       | 2.73        | <0.781     | 1.57         | 2.91     |
| Female | 49  | 2,690           | 409        | 108.3      | 16.04       | <0.781     | 14.32        | 2.51     |
| Female | 70  | 2,255           | 476        | 67.3       | 12.35       | 1.76       | 6.35         | 1.73     |
| Female | 76  | 3,223           | 460        | 69.4       | 19.54       | 2.56       | 12.36        | 4.47     |
| Female | 77  | 4,117           | 836        | 161.7      | 49.55       | <0.781     | 26.47        | 3.08     |
Chromatography

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