Application of 2-Picolylamine Derivatized Ultra-high Performance Liquid Chromatography Tandem Mass Spectrometry for Determination of Short-chain Fatty Acids in Feces Samples

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Abstract

We present a sensitive and selective method for simultaneous determination of short-chain fatty acids (SCFAs) such as acetic acid (AA), propionic acid, butyric acid (BA), isobutyric acid, valeric acid, isovaleric acid, hydroangelic acid, caproic acid, 4-methylvaleric acid and succinic acid (SA) in feces samples using a ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) with simple derivatization of 2-picolyamine. The main SFCAs were derivatized in same condition and showed the specific product ion ($m/z$ 109) in electrospray positive mode regarding to 2-picolyamine. The derivatized SA showed the different pattern of product ion ($m/z$ 191). The derivatized analytes showed the LOD < 75 nM, LOQ < 100 nM and $r^2$ in calibration curve > 0.991. The QuEChERS was used for sample preparation of feces samples. In the recovery test, the recovery values showed from 89.7 to 100.2% (RSD: 2.1 to 9.2%, n=6). This developed method was applied to evaluate the obese diabetes model mice. In the result, the branched-chain SCFAs levels in feces from model mice of spontaneous obese type II diabetes were on a declining trend compared with normal. The AA levels from model mice with high-calorie/fat diet were on a declining trend for 3 to 9 months. The BA levels showed that normal mice were increasing, and model mice were decreased tendency for breeding moths. High-calorie/fat diet showed that the SA levels were increased.

Keywords: short-chain fatty acids; 2-picolyamine; fluorescent derivatization
Introduction

Short-chain fatty acids (SCFAs) play an important role in the maintenance of health and the development of various diseases. Recent report showed that SCFAs are significant factor for the intestinal microbes system and the immune system of T-cell generation in host body. In addition, the association between microbial metabolism and psychiatric disorders in model animals has been discussed in the trend studies. Actually, we indicated that gut dysbiosis and consequent disruptions in plasma SCFA profiles occurred in metabolic syndrome-affected model mice. SCFAs are the main fermentation products of gut microbiota and a link between the gut microbiota and the host's physiology. Thus, it is very important to evaluate the concentration level and ratio pattern of SCFAs in experimental animals and/or clinical human samples for the confirmatory evidence between the intestinal flora and chronic diseases.

SCFAs are fatty acids with two to six carbon atoms that are produced based on the intestinal flora. Main SCFAs are acetic acid (AA), propionic acid (PA), butyric acid (BA), isobutyric acid (iso-BA), valeric acid (VA), isovaleric acid (iso-VA) and caproic acid (CA). Thus, it is needed to evaluate each analyte in biological samples that the use of separation technique is applied such as gas chromatography (GC) and liquid chromatography (LC). Primec et al. reviewed that analytical techniques of SCFAs in feces were introduced and discussed from 184 references. Although analytical techniques of SCFAs in biological samples have improved a lot in the past decades, the previous reports showed that useful method has not overcome GC technique, which still appears to be the commonly used quantification of SCFAs despite having some disadvantages. On the other hand, high
performance LC (HPLC) is another method for the analysis of SCFAs. The greatest advantage of the HPLC over the GC technique is the use of lower running temperatures and cost-effectiveness. Thus, the ion-exchanged HPLC columns were relatively used for the analysis of SCFAs based on the Primec’s review from 2004 to 2015 publications. If a mixture of weak acids is existed such as SCFAs in biological samples (included amino acids, peptides and other acids), the use of ion exclusion mode is not accessible to separate these biological chemicals. Among reversed phase (RP)-HPLC methods, a few of reports were presented for the analysis of SCFAs in feces. In fact, it is extremely difficult to retain these highly polar SCFAs from biological samples using common RP columns. Thus, the derivatization of SCFAs would be extremely useful as a way to use RP-HPLC methods.

Miwa & Yamamoto used 2-nitrophenylhydrazine hydrochloride for the analysis of SCFAs in serum and milk samples using HPLC with ultraviolet-visible (UV) detector. Yoshida et al. developed a preparation method using 9-anthryldiazomethane as a fluorescent labeling reagent for SCFAs. In addition, the benzofurazan derivatization reagents were developed for the analysis of SCFAs using HPLC coupled with electrospray tandem mass spectrometry (LC-ESI-MS/MS). In this derivatization, the mixed standard solution of AA, PA, BA, CA was applied. Recently, the stable isotope-labeled chemical derivatization was developed for the analysis of SCFAs by LC-ESI-MS/MS method. Han et al. reported that 3-nitrophenylhydrazine labeled with $^{12}$C/$^{13}$C was used for the analysis of AA, PA, BA, iso-BA, VA, iso-VA, CA and others (total 10 analytes) in feces. Moreover, Chan et al. reported that aniline labeled with $^{12}$C/$^{13}$C and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) was used for the
analysis of 12 SCFAs in infant feces. The stable isotope-labeled chemical derivatization can correct the observational effect of ionization on ESI-MS/MS. However, it is difficult to refer indirectly to calibrate the sample preparation and efficiency of derivatization. Thus, it is better use of stable isotope-labeled SCFAs than these derivatized regents. Current work has focused on separating isomeric SCFAs, streamlining derivatized reagent selection, and improving labeling conditions to enable parallel detection of SCFAs and related fatty acids using stable isotope-labeled analytes and, as a consequence, afford minimal interference and good reproducibility for early-chromatographic time of analytes in biological samples.

In 2018, fast quantification of SCFAs and ketone bodies by LC-ESI-MS/MS after facile derivatization (O-benzylhydroxylamine) coupled with liquid-liquid extraction. This drawback showed that reaction time is over 1 hour, and deficient liquid-liquid extraction with dichloromethane for recovery and clear-up of SCFAs from matrixes. Indeed, the convenient and accurate derivatized LC-ESI-MS/MS method may be not utilized to evaluate signature SCFAs from intestinal flora. Thus, in this study, we developed a convenient derivatization for the simultaneous determination of 10 SCFAs such as AA, PA, BA, iso-BA, VA, iso-VA, 2-methyl-butyric acid (2M-BA), CA, 4-methyl-valeric acid (4M-VA) and succinic acid (SA) using ultra-high performance LC with MS/MS (UHPLC-MS/MS). For these carboxyl group, we employ 2-picolyamine rapidly reacted with carboxylic acids in 2,2'-dipyridyl disulfide (DPDS) and triphenylphosphine (TPP) solution. Significantly, 2-picolyamine is commercially available and small reagents for increasing the sensitivity and specificity of carboxylic acids in the positive ESI-MS/MS. In addition, a simple, cost-effective and rapid sample preparation such QuEChERS (quick,
easy, cheap, effective, rugged, and safe) method is applied for SCFAs. This QuEChERS was used without dichloromethane and other hazardous solvents for sample preparation of biological samples\textsuperscript{18}. The 10 SCFAs and derivatized reaction was shown in Fig. 1, and examined for model mice feces in this study. The application for the determination of SCFAs in feces using the derivatization with 2-picolylamine and QuEChERS method by UHPLC-ESI-MS/MS is worthy to evaluate SCFAs biomarkers between the intestinal flora and chronic diseases. In addition, this is first report to analysis SCFAs in biological sample using the combination technique of 2-PA derivatization, QuEChERS and UHPLC-ESI-MS/MS.

**Experimental**

**Reagents and materials**

The standards of AA, PA, BA, iso-BA, VA, iso-VA, CA, 4M-VA and SA were obtained from Wako Pure Chemical Co. (Osaka, Japan). The 2-picolylamine, DPDS and TPP, 2M-BA were obtained from Tokyo Chemical Co. (Tokyo, Japan). PA-d\textsubscript{6}, BA-d\textsubscript{5}, VA-d\textsubscript{9} were obtained from Central Chemicals Co. (Tokyo, Japan). CA-d\textsubscript{11} was obtained from Sigma-Aldrich Co. (St. Louis, MO. USA). AA-d\textsubscript{4} was obtained from Cambridge Isotope Laboratories Inc. (Cambridge, MA. USA). Methanol and formic acid were obtained from Wako Pure Chemical Co. (Osaka, Japan). Purified water was obtained using a PURELAB flex 5 system from ELGA Co. (London, UK). Stock solutions were adjusted using methanol. Concentrated solutions of mixed standard solutions were diluted as required by adding methanol for derivatization.
UHPLC-MS/MS equipment and conditions.

UHPLC system was a Waters Acquity H Class (Waters Co., Milford, MA). A RP analysis was performed via an Acquity BEH C$_{18}$ column (1.7 $\mu$m, 2.1×100 mm) at 40$^\circ$C. The injection volume was 5 $\mu$L. The mobile phase consisting of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol) was delivered at a flow rate of 0.3 mL/min. The gradient elution was as follows: 2% solvent B at 0 min, 2% solvent B at 2 min, 10% solvent B at 2.1 min, 10% solvent B at 2.5 min, 35% solvent B at 6 min, 98% solvent B at 6.1 min, 98% solvent B at 8 min, 2% solvent B at 8.1 min, 2% solvent B 10 min. A Waters Xevo TQD triple quadrupole mass spectrometer was operated with an ESI source in the positive mode. The ionization source conditions were as follows: capillary voltage; 2.00 kV, cone voltage; 30 V, collision energy; 15-20 eV, source temperature; 150$^\circ$C, and desolvation temperature; 400$^\circ$C. The cone and desolvation gas flows were 50 and 800 L/h, respectively, and were obtained using a nitrogen source (N$_2$ Supplier Model 24S; Anest Iwata Co., Yokohama, Japan). Detailed MS/MS conditions are indicated in Table 1.

Derivatization of SCFAs

Based on a previous report for carboxylic acids, SCFAs and IS solutions in methanol were derivatized$^{17}$. These solutions were reacted with 2-picolyamine in DPDS and TPP in acetonitrile at 60$^\circ$C for 10 min. The reaction mixtures were removed and re-dissolved in 100 $\mu$L of methanol/water (10:90, v/v). Finally, the derivatization solutions (5 $\mu$L) were analyzed by means of UPLC-ESI-MS/MS.
Sample preparation of SCFAs in mice feces

Mouse feces (about 2 mg) after thawing were added to IS (10 μL), mixed with 1 mL of methanol and QuEChERS (Supel QuE PSA (EN) 25 mg), vortexed vigorously, homogenized at 1,500 rpm for 10 min (Geno/Grinder, SPEXSamplePrep), and centrifuged at 10,000 rpm for 10 min at 4°C (himac CF15RN, HITACHI). The supernatant (100 μL) was then derivatized by using the process described above using 2-picolyamine. The sample was then analyzed by means of UPLC-ESI/MS/MS.

Calibration curves and limit of detection/quantification (LOD/LOQ)

Fixed concentrations of the standard solutions (0.1 nM – 1000 μM for AA, PA, BA, and SA; 0.01 μM – 100 μM for iso-BA, VA, iso-VA, 2M-BA, CA and 4M-VA) were prepared by sequential dilutions of the stock solutions. The working solutions were pretreated and processed in the same manner as the feces samples prior to UHPLC-MS/MS. The LOD and LOQ values were evaluated based on the signal-to-noise ratio (S/N) obtained while detecting the concentration of analytes and indicated S/N =3 and S/N >10. The calibration curves were constructed from different concentrations to evaluate linearity at each concentration level by plotting the peak area ratio of the standard solutions: internal standard (y) vs. each concentration of the adjusted standard solution (x, μM).

Recovery test

The standard solutions (10 μL) were spiked in pooled control samples (n = 6). The
spiked (at low and high levels) and unspiked solutions were pretreated and subjected to UHPLC-MS/MS as described above. The percentage recovery was defined as follows:

\[ \text{Percentage recovery} = \frac{F}{(F_0 + A)} \times 100\% \]

where \( F \) and \( F_0 \) are the concentrations of the analytes in the spiked and unspiked samples, respectively, and \( A \) is the spiked concentration. For repeatability, the same operator ran the six feces samples from mice over a short period of time at room temperature.

Application of mouse feces

Tsumura-Suzuki obese diabetes (TSOD) mice were used as a model of spontaneous obese type II diabetes mellitus, and Tsumura-Suzuki non obesity (TSNO) mice were used as controls. Five-week-old male TSOD and TSNO mice were given a normal chow and high-fat and high-cholesterol (HFHC) diet ad libitum under specific pathogen-free condition at the Institute for Animal Reproduction (Kasumigaura, Ibaraki, Japan). After 3, 6, 9 months, feces were collected for a day and stored at -80°C. The feces that were collected from 10-week-old female Balb/c mice fed a normal chow were used as an application sample. All the animal experiments were performed in compliance with the rules for animal experimentation established by the Institute for Animal Reproduction and Tokushima University Committee on Animal Care and Use.

Results and Discussion

Derivatization of SCFAs

Recent study indicated that the UHPLC with Orbitrap MS was applied to measure
AA, PA, BA, iso-BA, VA, iso-VA, SA and others in fasces, and discussed the main SFCAs in fasces is carbon chain with C$_1$ to C$_5$ and similar fatty acid such as SA and lactic acid (LA)$^{19}$. In preliminary study, we applied that the derivatization of SFCAs, SA and LA was examined with 2-picolyamine in previous condition$^{17}$. In this result, the SFCAs were derivatized in same condition and showed the specific product ion ($m/z$ 109) regarding to 2-picolyamine added proton ion (Fig. 2-(A)). On the other hand, the derivatized SA showed the different pattern of product ion that is formed by the dehydration of unreacted carbonyl group (Fig. 2-(B)). The derivatized LA was not detected for the specific product ions in this MS/MS condition. Possibility, this derivatization reaction and/or ionization would be not done in this reaction, regent, instrument and MS condition. Thus, in this study, we apply that the nine SFCAs and SA ions were used for the UHPLC-MS/MS analysis (Table 1). In addition, the LOD and LOQ indicated from 10 to 50 nM and 25 to 100 nM (Table 1). The calibration curves showed $r^2 > 0.991$ with each stable isotope internal standard.

Sample preparation of SCFAs in feces samples

In this study, we applied the QuEChERS for simple and useful sample preparation of feces samples. Originally, QuEChERS was a particular "method" for pesticide residue analysis, but it is very flexible and has evolved into an "approach," which has been used in many methods, and not just for pesticide residues$^{20}$. In addition, it is much decreased opportunities to keep researcher’s hand off direct sample solution. Commonly, the solid phase extraction presents the transferred sample to cartridge and/or tube by pipette-mediated process as a matter of routine. Thus, the QuEChERS is applied for the
sample preparation of SFCAs in feces samples. Various QuEChERS substances were
investigated and decided to Supel QuE PSA (EN). This result of recovery test was shown in
Table 2. The UHPLC-MS/MS chromatograms were shown in Fig. 3. These recovery and
reparability were satisfactory values for the simultaneous determination of SFCAs in feces
samples.

Application of 2-picolylamine derivatized UHPLC-MS/MS for SCFAs in feces samples

In experiment of intestinal microorganism and metabolome, mice as a model of
spontaneous obese type II diabetes mellitus has been used for the examination of SCFAs
effects\(^2\). This background information was referenced by our previous report\(^2\). In the result,
the 4M-VA were not detected or trace levels. On the other hands, the branched-chain
SCFAs (iso-BA, iso-VA and 2M-BA) showed that the concentration levels of these SCFAs
in feces samples from TSOD model with normal diet were on a declining trend compared
with TSNO (Fig. 4-(A)). Based on the result of straight-chain SCFAs, the AA levels from
TSOD with HFHC diet were on a declining trend for 3 to 9 moths (Fig. 4-(B)). The PA
levels could be not observed of characteristic tendency (Fig. 4-(C)). Relatively, the BA
levels showed that TSNO was increasing, and TSOD was decreased tendency for breeding
moths (Fig. 4-(D)). Moreover, HFHC diet showed that the SA levels were increased for
both mice models (Fig. 4-(E)). However, these SCFAs levels with either pattern would be
reevaluated by large-scale and detailed background (individuality, gauge environment,
amount of intake, relationship for pathology, and alteration of the intestinal flora). Equally,
our preliminary analysis of SCFAs levels in feces samples from mice as a model of
spontaneous obese type II diabetes mellitus elucidated a link between characteristic
tendency of SCFAs in feces and disease-associated alteration.

In conclusion, we developed the UHPLC-MS/MS with simple derivatization of
2-picolyamine for the determination of 10 SCFAs in feces. The overall results indicate that
this approach of derivatized UHPLC-MS/MS and QuEChERS preparation is a useful
process specification for the evaluation of SCFAs from an imbalance in gut bacteria and
host-expressed effects. Future work will investigate the host-bacteria relationship in
intestines based on various SCFAs levels in biological samples.

References

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Figure Captions

Fig. 1 Derivatization of targeted SCFAs.

Fig. 2 MS/MS spectra and fragment patterns of specific SCFA standards.

(A) MS/MS spectrum of BA (m/z 165.0 →)

(B) MS/MS spectrum of SA (m/z 209.0 →)

Fig. 3 UHPLC-MS/MS chromatograms of SCFA in feces samples for recovery test.

(A) Chromatograms of AA and AA-d₄

(B) Chromatograms of PA and PA-d₆

(C) Chromatograms of BA, iso-BA and BA-d₅

(D) Chromatograms of VA, iso-VA, 2M-VA and VA-d₆

(E) Chromatograms of CA, 4M-VA and CA-d₁₁

(F) Chromatograms of SA and SA-d₄

Fig. 4 The concentration levels and trends of SCFAs in mice feces samples from model of spontaneous obese type II diabetes mellitus.

(A) The concentration levels and trends of branched-chain SCFAs (iso-BA, iso-VA and 2M-BA) were used of feces samples (3, 6, 9 moths with normal diet, n = 9 for one group).

(B) The concentration levels of AA were used of feces samples (3, 6, 9 moths with normal and HFCF diet, n=3 for one group).

(C) The concentration levels of PA were used of feces samples (3, 6, 9 moths with normal
and HFCF diet, n=3 for one group).

(D) The concentration levels of BA were used of feces samples (3, 6, 9 moths with normal and HFCF diet, n=3 for one group).

(E) The concentration levels of SA were used of feces samples (3, 6, 9 moths with normal and HFCF diet, n=9 for one group).
Fig. 1

[10 SCFAs analytes]

- **R = CH$_3$**
  - SCFAs: AA, PA
- **R = CH$_2$CH$_3$**
  - SCFAs: VA, iso-VA, 4M-VA
- **R = CH$_2$CH$_2$CH$_3$**
  - SCFAs: BA, 2M-BA, CA, SA

SCFAs: Acetic Acid (AA), Propionic Acid (PA), Butyric Acid (BA), Valeric Acid (VA), Isovaleric Acid (iso-VA), 4-Methylvaleric Acid (4M-VA), 2-Methylbutyric Acid (2M-BA), Caproic Acid (CA), Caproic Acid (SA)

DPDS and TPP in acetonitrile at 60°C for 10 min

2-picolylamine

2-picolylamine derivative
Fig. 2
Fig. 3
Fig. 4
### Table 1 SCFAs information, MS/MS conditions and internal standards

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<th>Analytes</th>
<th>Abbreviated name</th>
<th>M.W.</th>
<th>Precursor ions</th>
<th>Cone voltage (V)</th>
<th>Product ions</th>
<th>Collision energy (eV)</th>
<th>LOD (nM)</th>
<th>LOQ (nM)</th>
<th>Internal standard</th>
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<td>60</td>
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<td>50</td>
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<td>PA</td>
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<td>165.0</td>
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<td>15</td>
<td>25</td>
<td>75</td>
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<td>25</td>
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<td>15</td>
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<td>193.0</td>
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Table 2 Recovery test of SCFAs in pooled mice feces samples

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<th>Analytes</th>
<th>Added concentration levels (μM)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
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(n=6)