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Title	Determination of total, free and esterified short-chain fatty acid in human serum by liquid chromatography-mass spectrometry	
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Instructions for use

1	Determination of Total, Free, and Esterified Short-Chain Fatty Acid					
2	in Human Serum by LC-MS/MS					
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16 Abstract

Background: Short-chain fatty acid (SCFA) are primarily absorbed through the portal vein during lipid digestion, which is utilized as the energy source, as well as prevents type 2 diabetes and some cancers. However, reports on the determination of these SCFA in human serum are limited.

21 **Methods:** Blood samples from human subjects (n = 547, male/female = 246/301, 22 age 58.85 ± 12.57) were collected. Saponification was applied to obtain total FA. After 23 derivatization by 2-nitrophenylhydrazine, FA 4:0 and FA 6:0 were measured by liquid 24 chromatography-mass spectrometry (LC-MS/MS).

**Results:** The developed method exhibited satisfied linearity ( $R^2 = 0.9996$  for both). 25 26 All the coefficient of variation of reproducibility and accuracy for FA 4:0 and FA 6:0 27 ranged in 3.0%-6.1%, with the average recoveries of 87.8%-102.4% and 28 92.2%-98.2%, respectively. In all the samples, the concentration of FA 4:0 29  $(162.4 \pm 76.4 \,\mu\text{mol/L})$  showed significantly higher than FA 6:0  $(2.0 \pm 2.5 \,\mu\text{mol/L})$ 30 P < 0.001). Furthermore, the esterified form showed predominant in both FA 4:0 and 31 FA 6:0 (98.2% and 82.4% of total FA, respectively). Besides, SCFA showed no 32 significant differences in concentration with sex or age differences.

33 Conclusion: This developed LC-MS/MS method is convenient and reliable, which
 34 might be useful for monitoring SCFA variation in blood.

35 Keywords: Short-chain fatty acid (SCFA), butanoic acid (FA 4:0), caproic acid
36 (FA 6:0), LC-MS/MS, serum

37 2 / 20

## 38 1 Introduction

39 Short-chain fatty acids (SCFAs), also referred to as volatile fatty acids (VFAs), are 40 widely known as the end products of bacterial metabolites generated via the 41 fermentation of dietary fibers (1). In all the SCFA in the human body, approximately 42 90% of the amount is absorbed in the colon and transported to the liver through the 43 hepatic vein, while the residual amount is secreted through the feces (2,3). In the recent 44 years, there is an increasing trend that recognizes SCFA as mediators of the local gut 45 and systemic health (4), along with the growing studies on their positive physiological 46 effects (5,6). Taking butyrate as an example, evidence has shown the uptake of butyrate 47 by the colonic epithelium, disclosing it preferentially utilized by colonocytes as the 48 primary energy source (7). After absorbed, butyrate is involved in various of 49 therapeutic effects, such as the improvement of type 2 diabetes (4,8), the reduction of 50 cholesterol production (2,9), the prevention of colon cancer (10), and the limit of 51 intestinal inflammation (1). Furthermore, among SCFA, butyrate serves as the most 52 potent promoter of intestinal regulatory T cells in vitro and the only one that showed 53 the niacin receptor 1 (NIACR1) ligand (11).

It should be noted that the esterified fatty acid (EFA) and free fatty acid (FFA) show different physiological characteristics. For example, tricaproin (TG 6:0/6:0/6:0) induces oxygen radical production in neutrophils, while the free caproic acid (FFA 6:0) exerts no clear such effect (12). Moreover, it is known that long chain FFA can affect gene expression of macrophages, adipocytes, or endothelial cells (13), and the elevation of its concentration is associated with most of the metabolic disorders, including impaired glucose utilization, impaired insulin secretion, dyslipidemia, pro-thrombotic, etc. (14,15). But there is a quite limited study on the effect of short-chain FFA. The bioactivity difference between EFA and FFA demands us for the attention on both of them. Thus, for SCFA determination, it is necessary to determine its not only the esterified but also the free form.

65 Most of the studies on SCFA determination relied on gas chromatography (GC), 66 combined with either flame ionization detector (FID) or mass spectrometry (MS) (16-67 19). High-performance liquid chromatography (HPLC) is a good alternative method 68 for SCFA analysis, of which the greatest advantage over GC is the use of lower running 69 temperatures (17,20). In terms of HPLC, approaches with derivatization could improve 70 the separation and sensitivity. One of the derivatization-based methods, using acidic 2-71 nitrophenylhydrazine hydrochloride (2-NPH·HCl) with or without saponification, has 72 been described for the determinations of both FFA and EFA in biological materials (21). 73 The author demonstrated that the developed method was simple, rapid, and reliable. 74 And this method has been applied in our laboratory for determining medium-chain FA 75 (MCFA) and long-chain FA (LCFA) of their total and free forms in various of samples, 76 ranging from the clinical blood sample to the commercial milk (22–24). However, the 77 strategy of HPLC with NPH-derivatization was not applicable for SCFA in our 78 laboratory, due to the poor resolution and the interference in chromatography.

79 LC/MS is considered as an ideal approach for analyzing targets in the complex

80 matrix like biological samples, as it provides chromatographic separation along with 81 highly sensitive and selective detection. Derivatization for LC/MS can help enhancing 82 the ionization efficiency, as well as increasing the retention time and m/z, which avoid 83 potential suppression effects. Also, an ingenious derivatization will be a strategy for 84 controlling fragmentation in MS (25). But to the best of our knowledge, there has not 85 been reported on SCFA determination by LC-MS/MS and NPH-derivatization yet. 86 Therefore, the aims of the present study are to develop a practical NPH-derived LC-87 MS/MS method for the determination of SCFA in the blood sample and to apply the 88 developed method to clinical samples. The butanoic acid (FA 4:0) and caproic acid 89 (FA 6:0) were treated as our targets, and their contents of both free and esterified forms 90 were investigated.

91

# 92 2 Materials and methods

93 2.1 Chemicals

LC/MS grade methanol, n-hexane, and water were purchased from Wako Pure
Chemical (Osaka, Japan). Ammonium acetate was purchased from Sigma-Aldrich
(St. Louis, MO). Other chemicals and reagents were of analytical grade and purchased
from Kanto Chemical Industry (Tokyo, Japan) unless specified.

100 previously synthesized in our laboratory (22). The FA NPH-derivatization kit, which

<sup>98</sup> FA 4:0, FA 6:0, and the internal standard (ISTD) undecanoic acid (FA 11:0) were

<sup>99</sup> purchased from Sigma-Aldrich, while 2-nitrophenyl hydrazide (NPH) of these FA were

101 contains 2-NPH·HCl, *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide
102 hydrochloride (EDC·HCl), potassium phosphate buffer (pH 4.6), was provided by
103 YMC Co., Ltd. (Kyoto, Japan).

104

## 2.2 Specimens and serum collection

Ethical approvals for this study were obtained, and informed consent was obtained from all individuals. Fasting blood sample was collected from 547 Japanese volunteers (246 males, mean age  $\pm$  SD: 58.4  $\pm$  12.6 years; 301 females, mean age  $\pm$  SD: 59.1  $\pm$  12.4 years; shown in **Figure S1 of Supplemental Material**). All of the volunteers were living in the country of Suttsu District, Hokkaido, Japan (42°47′N, 140°14′E). All of the serum was separated by centrifugation within 30 min of collection, and then stored at -80°C until tested.

112 **2.3** Sample preparation

113 The serum sample preparation procedure was in according to Shrestha et al as 114 previously reported (23). In brief, a portion of serum (25  $\mu$ L for total FA, 100  $\mu$ L for 115 free FA) was spiked with the ISTD FA 11:0 (2 nmol). For total FA determination, the 116 saponification was performed as mixing serum with  $100 \,\mu\text{L}$  of 0.3 M KOH-EtOH, and 117 heating at 80°C for 45 min. Then, derivatization was performed by adding 2-NPH·HCl 118 and EDC·HCl, and incubating at 60°C for 20 min. FA-NPH derivatives were obtained 119 by potassium phosphate buffer and n-hexane extraction, followed by vacuum dryness 120 of the n-hexane layer. Finally, the residue was dissolved in 200 mL of methanol and 121 stored at -80 °C before injection.

#### 122 2.4 LC-MS/MS analysis

123 LC-MS/MS was performed using a Surveyor HPLC system and a TSQ Quantum 124 Access MAX mass spectrometer with a heated electrospray ionization (H-ESI) probe 125 (Thermo Fisher Scientific Inc., Waltham, MA). LC was carried out on an Ascentis® Express Phenyl-Hexyl column (5 cm  $\times$  2.1 mm I.D., 2.7  $\mu$ m, Supelco, Inc., Bellefonte, 126 127 PA) at 45 °C. The injection volume was set at 1.0  $\mu$ L. The mobile phase consisted of 128 5 mM aqueous ammonium acetate (A), isopropanol (B), and methanol (C) at a flow 129 rate of 200  $\mu$ L/min. The following gradient elution was applied: 0.0–0.5 min 65% A 130 and 35% C; 0.5-1.0 min 30% A, 20% B, 50% C; 1.0-4.5 min 5% A, 30% B, 65% C; 4.5-5.0 min 33% B, 67% C; this ratio was kept to 8.0 min; 8.0-10.0 min returned to 131 132 initial gradient for re-equilibration. 133 The selected reaction monitoring (SRM) under negative mode was utilized for MS

detection, and the main parameters were optimized. Spray voltage was set at 3000 V.
Nitrogen was used as the sheath gas and the auxiliary gas (set at 50 psi and 10 psi,
respectively). The vaporizer temperature and the capillary temperature was set at
350 °C and 200 °C, respectively. Collision gas (argon) pressure was set at 1.8 mTorr.
Quantification was conducted by the workstation Thermo Xcalibur 2.1 software
(Thermo Fisher Scientific, Inc., Waltham, MA).

140 **2.5 Method validation** 

141 Stock solutions (200  $\mu$ mol/L) of FA 4:0, FA 6:0, and FA 11:0 were prepared in 142 methanol by measuring their dry weight on an ultrasensitive electro-balance (Cubis®

143	ultra micro balance, Sartorius Inc., Göttingen, Germany), and stored at -40 °C. To
144	determine linearity, the FA 4:0 and FA 6:0 standard solutions with amounts of 0.25, 0.5,
145	1.5, 2.5, 5, 10 nmol and 3.125, 6.25, 10, 25, 50, 200 pmol were prepared, respectively.
146	Each standard solution contained 2 nmol of the ISTD FA 11:0. The integration of the
147	peak area and the plotting of each calibration curve were performed by Xcalibur. The
148	limit of quantification (LOQ) was defined as the level of signal-to-noise ratio $\geq 10$ .
149	Both reproducibility and recovery assays were tested in 6 replicated analyses for the
150	independently prepared serum samples. Recovery was calculated with the following
151	formula:
152	Recovery (%) = [(amount found – amount original)/amount spiked] $\times$ 100%.
153	2.6 Statistics
154	Statistical analysis was conducted using the unpaired two-tailed <i>t</i> -test, one-way and
155	two-way ANOVA (using the Tukey post-hoc test), which was performed by Prism 6.0
156	(GraphPad Software, Inc., La Jolla, CA). $P < 0.05$ was considered to be statistically
157	significant. All of the shown data are presented as the means $\pm$ standard deviation (SD).
158	Pearson's correlation coefficient was performed with JMP 10.0 (SAS Institute Inc.,
159	Cary. NC).
160	
161	3 Results and discussion
162	3.1 Optimization of LC-MS/MS

163 The optimized SRM conditions of these FA-NPH together with their acquired

164 chromatograms were shown in **Figure 1**. All of the three analytes showed identical 165 sharp peaks of similar retention times in both standards mixture solution and serum 166 sample, respectively, indicating the acceptable interfere by matrix effect. Besides, all 167 of the analytes (ISTD included) could be eluted in less than 5 minutes, and the total run 168 time for one injection could be controlled within 10 minutes by this method, suggesting 169 it convenient, commercial, and capable of testing a large number of samples.

170 **3.2 Method validation** 

The calibration curve was constructed by plotting the FA 4:0-NPH or FA 6:0-NPH to FA 11:0-NPH peak area ratio (y) against the amount of corresponding FA (x, nmol). Both of the calibration curves showed good linearity and acceptable ranges (for FA 4:0, y = 0.0058x - 0.0006,  $R^2 = 0.9996$ ; for FA 6:0, y = 0.1715x - 0.0004,  $R^2 = 0.9996$ ; shown in **Figure S2 of Supplemental Material**). The LOQ were 5 pmol and 0.12 pmol for FA 4:0 and FA 6:0, respectively.

The reproducibility and recovery of the present method were determined by 177 178 analyzing the serum of volunteers added to the certain concentration of FA 4:0 and 179 FA 6:0 in 6 replicates, respectively (shown in Table 1). For FA 4:0, the coefficient of 180 variation (CV) of reproducibility and recovery were 3.1% and 5.3%, respectively, while 181 for FA 6:0 the values were 6.1% and 3.0%, respectively, suggesting the favorable precision. The average recoveries of them were  $94.7 \pm 5.0\%$  and  $96.1 \pm 2.9\%$ , 182 183 respectively. From these results, the developed method was confirmed to show sufficient performance to determine the two SCFA species in clinical specimens. 184

#### 185 **3.3** Comparison of the total, free, and esterified SCFA in human serum

186 Serum samples collected from 547 subjects were measured for FA 4:0 and FA 6:0 of 187 both total and free forms by the developed LC-MS/MS method. And the esterified FA 188 content was calculated as the difference total FA minus free FA. The concentrations 189 among FA 4:0 and FA 6:0 of their total, free, and esterified form exhibited significant 190 difference (P < 0.05, shown in Figure 2). The amount of FA 4:0 was significantly 191 higher than that of FA 6:0 (162.39  $\pm$  76.36 and 1.97  $\pm$  2.51  $\mu$ mol/L, respectively, 192 P < 0.001), suggesting FA 4:0 the major SCFA in this study. In terms of FA forms, the average concentration of free and esterified FA 4:0 were  $2.63 \pm 0.83$  and 193 194  $159.39 \pm 76.79 \,\mu$ mol/L, respectively, while for FA 6:0, the concentrations were 195  $0.35 \pm 0.16$  and  $1.62 \pm 2.49 \,\mu$ mol/L, respectively. The results showed that the esterified 196 form was predominant form in both FA 4:0 and FA 6:0, accounted for 98.2% and 82.4% 197 of its corresponded total form, respectively (Figure 2).

198 Most of the previous studies on FA 4:0 and FA 6:0 quantitation were performed by GC, with the sample preparation method including distillation, organic solvent 199 200 extraction, or hollow fiber supported liquid membrane extraction (16,26-28), while 201 van Eijk et al measured SCFA via LC/MS with the pretreatment of methanol extraction 202 (29). All of the above studies seemed targeting on free SCFA due to their pretreatment 203 procedures. In our experiment, the levels of free FA 4:0 and free FA 6:0 were similar 204 to their results (FA 4:0 varied from  $0.9 \pm 0.2 \,\mu$ mol/L to 2.5–29.6  $\mu$ mol/L, and FA 6:0 205 ranged from 1.4–9.7  $\mu$ mol/L to 4.1 ± 0.8  $\mu$ mol/L).

206 Furthermore, in the present work, the total and esterified forms of FA 4:0 and FA 6:0 207 were also investigated. Based on the concentration of total, free, and esterified forms 208 of FA 4:0 and FA 6:0, respectively, the Pearson's correlation coefficients were 209 calculated, and the scatter plots were generated in Figure 3. The very strong correlation 210 between the amount of total and its esterified form was observed (for the total and 211 esterified FA 4:0, r = 0.9999, P < 0.0001, Figure 3A; for the total and esterified FA 6:0, 212 r = 0.9984, P < 0.0001, Figure 3B). However, there was no correlation between free 213 and esterified form, or total and free form (r < 0.15 for all in Figure 3C-F). 214 Studies showed that the elevation of plasma FFA leads to a shift from unsaturated to 215 saturated fatty-acyl chains in membrane phospholipids, which promotes the physical 216 attractive van der Waals interactions between phospholipid acyl chains, increasing the 217 stiffness of both erythrocyte and endothelial membranes, and finally resulting in

as free SCFA does not directly construct or reconstruct the cell membrane, its content
variation might affect the body health through other ways, such as cell signaling or
enzymatic reaction.

vascular and neurological lesions, and a various of metabolic disease (30). However,

## 222 **3.4** SCFA variation with sex and ages

The variation of SCFA between male and female of different ages (grouped as 30-39, 40-49, 50-59, 60-69, and 70-79 years old) was compared (shown in **Figure 4**). For total FA 4:0 in male, the concentration ranged from  $184.47 \pm 73.99 \,\mu$ mol/L (50-59 group) to  $138.93 \pm 67.12 \,\mu$ mol/L (70-79 group), while in female the concentration

227	ranged from 183.76 ± 81.08 $\mu$ mol/L (40–49 group) to 142.73 ± 75.02 $\mu$ mol/L (30–39
228	group) (Figure 4A). For total FA 6:0, the highest and lowest concentrations in male
229	were presented in 60–69 group ( $2.05 \pm 2.42 \mu$ mol/L) and 40–49 group
230	$(1.58 \pm 2.15 \mu \text{mol/L})$ , respectively, while in female they were presented in 40–49
231	group $(2.27 \pm 3.37 \mu\text{mol/L})$ and $30-39$ group $(1.42 \pm 0.74 \mu\text{mol/L})$ , respectively
232	(Figure 4B). There was no significant difference between male and female, or among
233	different ages ( $P > 0.05$ for all the comparison), suggesting the limited influence from
234	sex and age factors to these two SCFA in the human serum. Besides, as the quite large
235	deviations shown, it might be indicated that serum SCFA content especially for FA 6:0,
236	varies along with individual differences, which might include multiple factors,
237	including hormone, dietary, sports, etc. Moreover, the similar trend could be observed
238	in free FA 4:0, free FA 6:0, esterified FA 4:0, and esterified FA 6:0 (Figure 4C-F).
239	According to the review by Lohner et al, the long-chain FA in plasma total lipid,
240	such as FA 20:4 n-6 and FA 20:5 n-3, showed significantly lower contribution in men
241	than in women (31). And Asciutti-Moura et al demonstrated that the older people have
242	less circulating essential fatty acids (such as FA 18:2 n-6 and FA 20:4 n-6) due to diet
243	and metabolism (32). These polyunsaturated FAs exert the biological actions, including
244	maintaining cell-membrane fluidity, inhibiting inflammatory processes, decreasing
245	secretion of proinflammatory cytokines by monocytes/macrophages, etc. (33), which
246	are different from SCFA. That might be likely the explanation why the trend of SCFA
247	content is not obvious in relation to sex or ages.

### 248 **4** Conclusion

In the present work, a simple, precise, and accurate method for determining SCFA by LC-MS/MS was developed and applied to a large scale of human serum. The developed method might be useful for monitoring both free and esterified SCFA variation in clinical samples. For the future study, SCFA profile will be combined with biological information of subjects, to reveal the relationship between SCFA changes and lifestylerelated diseases.

255

# 256 Abbreviations

257 coefficient variation; EDC·HCl, N-(3-Dimethylaminopropyl)-N'-CV, of ethylcarbodiimide hydrochloride; EFA, esterified fatty acid; FA 4:0, butanoic acid; 258 259 FA 6:0, caproic acid; FA 11:0, undecanoic acid; FFA, free fatty acid; HPLC, high-260 performance liquid chromatography; ISTD, internal standard; LCFA, long-chain fatty acid; LOD, limit of detection; LOQ, limit of quantification; MCFA, medium-chain fatty 261 acid; NPH, 2-nitrophenyl hydrazide; SCFA, short-chain fatty acid; SD, standard 262 263 deviation; SRM, selected reaction monitoring.

264

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# **Declaration of conflicting interests**

- 270 The authors declare no conflict of interests.

n Strategy
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roved this
HC were
ation. ZC,
at

289 YW and ZG operated the LC-MS/MS and data process. YZ and YM advised statistics.

- 290 S-PH and HC reviewed the relevant literature. ZC wrote the first draft of the manuscript.
- 291 YZ, ZG, and WY helped correcting the mistakes. All the authors reviewed and edited
- the manuscript, and approved the final version of the manuscript.
- 293

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## **Figure caption**

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397	Figure 1.	The representative	SRM chromatogram	s of FA-NPH in	standards mixture
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398 solution (left) and in the serum sample (right).

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400 Figure 2. The average concentrations of FA 4:0 and FA 6:0 with their total, free, and
401 esterified forms. Bars without a common letter represented significantly different at the
402 0.001 probability level. Values above bars were presented as percentages of the
403 corresponded total forms.

404

405 Figure 3. Pearson's correlation coefficient among the total, esterified, and free forms
406 of FA 4:0 (A, C, E) and FA 6:0 (B, D, F).

407

- 408 Figure 4. Variations of FA 4:0 and FA 6:0 with total, free, and esterified forms between
- 409 male and female of different ages. Sample numbers are presented as M/F (male/female).