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

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15

16 **Abstract**

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

21 **Methods:** Blood samples from human subjects (n = 547, male/female = 246/301,  
22 age  $58.85 \pm 12.57$ ) 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).

25 **Results:** The developed method exhibited satisfied linearity ( $R^2 = 0.9996$  for both).  
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

## 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).

54 It should be noted that the esterified fatty acid (EFA) and free fatty acid (FFA) show  
55 different physiological characteristics. For example, tricaproin (TG 6:0/6:0/6:0)  
56 induces oxygen radical production in neutrophils, while the free caproic acid (FFA 6:0)  
57 exerts no clear such effect (12). Moreover, it is known that long chain FFA can affect  
58 gene expression of macrophages, adipocytes, or endothelial cells (13), and the elevation

59 of its concentration is associated with most of the metabolic disorders, including  
60 impaired glucose utilization, impaired insulin secretion, dyslipidemia, pro-thrombotic,  
61 etc. (14,15). But there is a quite limited study on the effect of short-chain FFA. The  
62 bioactivity difference between EFA and FFA demands us for the attention on both of  
63 them. Thus, for SCFA determination, it is necessary to determine its not only the  
64 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**

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

98 FA 4:0, FA 6:0, and the internal standard (ISTD) undecanoic acid (FA 11:0) were  
99 purchased from Sigma-Aldrich, while 2-nitrophenyl hydrazide (NPH) of these FA were  
100 previously synthesized in our laboratory (22). The FA NPH-derivatization kit, which

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**

105 Ethical approvals for this study were obtained, and informed consent was obtained from  
106 all individuals. Fasting blood sample was collected from 547 Japanese volunteers (246  
107 males, mean age  $\pm$  SD: 58.4  $\pm$  12.6 years; 301 females, mean age  $\pm$  SD: 59.1  $\pm$  12.4  
108 years; shown in **Figure S1 of Supplemental Material**). All of the volunteers were  
109 living in the country of Suttu District, Hokkaido, Japan (42°47'N, 140°14'E). All of  
110 the serum was separated by centrifugation within 30 min of collection, and then stored  
111 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  $\mu$ 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®  
126 Express Phenyl-Hexyl column (5 cm × 2.1 mm I.D., 2.7 μm, Supelco, Inc., Bellefonte,  
127 PA) at 45 °C. The injection volume was set at 1.0 μL. The mobile phase consisted of  
128 5 mM aqueous ammonium acetate (A), isopropanol (B), and methanol (C) at a flow  
129 rate of 200 μL/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;  
131 4.5–5.0 min 33% B, 67% C; this ratio was kept to 8.0 min; 8.0–10.0 min returned to  
132 initial gradient for re-equilibration.

133 The selected reaction monitoring (SRM) under negative mode was utilized for MS  
134 detection, and the main parameters were optimized. Spray voltage was set at 3000 V.  
135 Nitrogen was used as the sheath gas and the auxiliary gas (set at 50 psi and 10 psi,  
136 respectively). The vaporizer temperature and the capillary temperature was set at  
137 350 °C and 200 °C, respectively. Collision gas (argon) pressure was set at 1.8 mTorr.  
138 Quantification was conducted by the workstation Thermo Xcalibur 2.1 software  
139 (Thermo Fisher Scientific, Inc., Waltham, MA).

## 140 **2.5 Method validation**

141 Stock solutions (200 μmol/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  $-40\text{ }^{\circ}\text{C}$ . 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:

$$152 \quad \text{Recovery (\%)} = [(\text{amount found} - \text{amount original})/\text{amount spiked}] \times 100\%.$$

## 153 **2.6 Statistics**

154 Statistical analysis was conducted using the unpaired two-tailed *t*-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**

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

177 The reproducibility and recovery of the present method were determined by  
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  
182 precision. The average recoveries of them were  $94.7 \pm 5.0\%$  and  $96.1 \pm 2.9\%$ ,  
183 respectively. From these results, the developed method was confirmed to show  
184 sufficient performance to determine the two SCFA species in clinical specimens.

### 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  $1.97 \pm 2.51 \mu\text{mol/L}$ , respectively,  
192  $P < 0.001$ ), suggesting FA 4:0 the major SCFA in this study. In terms of FA forms, the  
193 average concentration of free and esterified FA 4:0 were  $2.63 \pm 0.83$  and  
194  $159.39 \pm 76.79 \mu\text{mol/L}$ , respectively, while for FA 6:0, the concentrations were  
195  $0.35 \pm 0.16$  and  $1.62 \pm 2.49 \mu\text{mol/L}$ , 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  
199 GC, with the sample preparation method including distillation, organic solvent  
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  $0.9 \pm 0.2 \mu\text{mol/L}$  to  $2.5\text{--}29.6 \mu\text{mol/L}$ , and FA 6:0  
205 ranged from  $1.4\text{--}9.7 \mu\text{mol/L}$  to  $4.1 \pm 0.8 \mu\text{mol/L}$ ).

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  
218 vascular and neurological lesions, and a various of metabolic disease (30). However,  
219 as free SCFA does not directly construct or reconstruct the cell membrane, its content  
220 variation might affect the body health through other ways, such as cell signaling or  
221 enzymatic reaction.

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

223 The variation of SCFA between male and female of different ages (grouped as 30–39,  
224 40–49, 50–59, 60–69, and 70–79 years old) was compared (shown in **Figure 4**). For  
225 total FA 4:0 in male, the concentration ranged from  $184.47 \pm 73.99 \mu\text{mol/L}$  (50–59  
226 group) to  $138.93 \pm 67.12 \mu\text{mol/L}$  (70–79 group), while in female the concentration

227 ranged from  $183.76 \pm 81.08 \mu\text{mol/L}$  (40–49 group) to  $142.73 \pm 75.02 \mu\text{mol/L}$  (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 ( $2.05 \pm 2.42 \mu\text{mol/L}$ ) 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 Ascitti-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**

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

255

## 256 **Abbreviations**

257 CV, coefficient of variation; EDC·HCl, *N*-(3-Dimethylaminopropyl)-*N'*-  
258 ethylcarbodiimide hydrochloride; EFA, esterified fatty acid; FA 4:0, butanoic acid;  
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  
261 acid; LOD, limit of detection; LOQ, limit of quantification; MCFA, medium-chain fatty  
262 acid; NPH, 2-nitrophenyl hydrazide; SCFA, short-chain fatty acid; SD, standard  
263 deviation; SRM, selected reaction monitoring.

264

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268

269 **Declaration of conflicting interests**

270 The authors declare no conflict of interests.

271

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277

278 **Ethical approval**

279 Ethics review boards of Faculty of Health Sciences, Hokkaido University (No. 16–10)  
280 and of Graduate School of Medicine, Hokkaido University (No. 16–007) approved this  
281 study protocol.

282

283 **Guarantor**

284 S-PH

285

286 **Contributorship**

287 S-PH, and HC conducted the study design and discussion. AT, S-PH, and HC were  
288 involved in sample collection. ZC, YW, and RS performed the sample preparation. ZC,  
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  
292 the manuscript, and approved the final version of the manuscript.

293

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395 **Figure caption**

396

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

399

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).

410