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Analysis of short- and medium-chain fatty acids in human fecal, plasma, and serum samples

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1 Introduction

Short-chain fatty acids (SCFAs) and medium-chain fatty acids (MCFAs) are not only important energy-supplying metabolites, but they are also signaling molecules involved in the regulation of carbohydrate and lipid metabolism. Genetic defects in SCFA and MCFA metabolism may cause inborn errors of metabolism. Long-term aberrant levels of SCFAs and MCFAs can lead to inflammation and insulin resistance, which may result in a wide range of disorders including diabetes, neurodegenerative diseases, and cancer.

In humans, SCFAs are primarily generated by the gut microbiome through fermentation of dietary fibers. Dietary proteins and peptides can also be a source of SCFAs. The contained branched-chain amino acids valine, leucine, and isoleucine can be metabolized by intestinal bacteria to branched-chain fatty acids (BCFAs) like isobutyric acid and isovaleric acid. These BCFAs are crucial mediators of the gut-brain axis, modulating the release of the neurotransmitter serotonin by specialized cells in the gut epithelium. The most abundant SCFAs – acetic acid, propionic acid, and butyric acid – perform important tissue-specific regulatory functions and are central mediators of the gut-immune axis. In general, SCFAs use a variety of mechanisms to boost immunity and promote metabolic health.

MCFAs, such as capric acid and lauric acid, mainly derive from dietary medium-chain triglycerides, as found in milk or plant oils. MCFAs may have beneficial effects on glucose and lipid metabolism, and have been shown to have ameliorating effects on metabolic and neurological disorders in clinical trials. Overall, combining the analysis of SCFAs and MCFAs can provide crucial insights into the microbiome-host interaction, energy homeostasis, and health status.

Here, we demonstrate the Short-chain fatty acid PLUS (SCFA+) assay: a new liquid chromatography tandem mass spectrometry (LC-MS/MS) assay, covering the broadest panel of SCFAs and MCFAs currently on the market. Analytical validation was performed on a Waters® ACQUITY UPLC® I-Class system, coupled to a Xevo® TQ-S mass spectrometer. The assay enables the analysis of up to 19 metabolites (Table 1) covering not only SCFAs (up to 6 carbon atoms) but also MCFAs (7–12 carbon atoms). The calibration range was optimized for fecal samples. Analytical validation was performed according to guidelines set out by the European Medicines Agency (EMA) and the US Food and Drugs Administration (FDA).

2 Materials and method

The wet human fecal sample was weighted and homogenized with the threefold volume of isopropanol, using a Precellys® homogenizer with ceramic beads. Two sampling approaches were used to compare 3-spot sampling, e.g., 3 x 200 mg + 3 x 600 µL, with 1-spot sampling, e.g., 200 mg + 600 µL (see below for details). After centrifugation, 10 µL of supernatant were transferred to a 96-well deep well plate for derivatization. For human plasma and serum samples, the sample volume was 50 µL. The derivatization was based on 3-nitrophenylhydrazine (NPH) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC).

The Waters® Xevo® TQ-S was operated in negative electrospray ionization mode to detect the derivatized SCFAs and MCFAs. Using the biocrates ultra-high performance liquid chromatography (UHPLC) column, all 19 target compounds were separated within a 10-minute chromatographic run. The analysis of a full 96-well plate (up to 75 samples, apart from calibrators, quality control, zero and blank samples) was achieved in less than 19 hours (overnight).

Absolute quantification of the 19 analytes was carried out using a 7-point external calibration and 16 corresponding isotope labeled internal standards (ISTD). The accuracy of the measurement results was guaranteed through the inclusion of quality control (QC) samples at three concentration levels, always measured in replicates. For the two sample matrices, feces and plasma or serum, a separate set of QC samples was developed. This was necessary due to large differences in concentrations of fatty acids in these two sample matrices.

In case of fecal samples, the obtained analyte concentrations are usually normalized to wet weight. It also possible to normalize against dry weight, which is recommended when the samples show great differences in the water content.

Table 1: Analyte panel of SCFA+ assay, the quantitation range and concentration range measured in human fecal (n = 15), serum (n = 20), and plasma samples (n = 20). Abbreviations: LLOQ, lower limit of quantification; ULOQ, upper limit of quantification.

No.	Analyte	Short name	Fecal samples [nmol/g wet weight]		Plasma and serum samples [µM]		
			Quantitation range (LLOQ-ULOQ)	Median concentration (min-max)	Quantitation range (LLOQ-ULOQ)	Median concentration (min-max)	
						Plasma	Serum
1	Acetic acid	FA 2:0	180-150,000	25,261 (7,378-56,012)	12-10,000	39 (23.2-70)	31 (12.8-68.6)
2	Propionic acid	FA 3:0	72-60,000	10,295 (3,726-21,347)	4.8–4,000	1.5 (0.69-4.9)	2.4 (1.3-4.5)
3	Isobutyric acid	FA 3:0-2M	7.2-6,000	1,239 (703-1,948)	0.48-400	0.31 (0.19-0.63)	0.60 (0.40-1.0)
4	Butyric acid	FA 4:0	720-120,000	8,653 (2,313-21,101)	9.6–8,000	0.22 (0-7.3)	0.54 (0.05-3.1)
5	2-Methylbutyric acid	FA 4:0-2M	7.2–6,000	840 (356-1,225)	0.48-400	<LOD	<LOD
6	Isovaleric acid	FA 4:0-3M	7.2-6,000	991 (412-1,428)	0.48-400	0.29 (0.07-0.53)	0.26 (0.11-0.58)
7	Valeric acid	FA 5:0	14.4-12,000	1,662 (505-3,289)	0.96-800	0.17 (0.14-0.66)	<LOD
8	2-Methylvaleric acid	FA 5:0-2M	0.36-300	0.54 (0.37-1.1)	0.024-20	0.024 (0.012-0.059)	0.028 (0.011-0.059)
9	3-Methylvaleric acid	FA 5:0-3M	0.36-300	0.11 (0.068-0.8)	0.024-20	<LOD	<LOD
10	Isocaproic acid	FA 5:0-4M	0.36-300	11.7 (2.5-56.3)	0.024-20	0.045 (0.016-0.16)	0.059 (0.01-0.2)
11	Caproic acid	FA 6:0	10.8-9,000	351 (12.7-3,766)	0.72-600	0.55 (0.42-0.83)	0.37 (0.21-0.65)
12	4-Methylhexanoic acid	FA 6:0-4M	0.36-300	0.82 (0.45-2.8)	0.024-20	0.039 (0.024-0.082)	0.046 (0.025-0.087)
13	5-Methylhexanoic acid	FA 6:0-5M	0.36-300	1.3 (0.34-5.4)	0.024-20	<LOD	<LOD
14	Heptanoic acid	FA 7:0	1.8-1,500	99 (1.6-456)	0.12-100	0.093 (0.058-0.15)	0.044 (0.015-0.11)
15	Caprylic acid	FA 8:0	0.9-750	19 (0.43-322)	0.06-50	0.93 (0.26-2.6)	1.1 (0.29-2.9)
16	Nonanoic acid	FA 9:0	1.8-300	1.7 (0.43-7.2)	0.12-20	0.58 (0.45-0.82)	0.32 (0.12-0.47)
17	Capric acid	FA 10:0	1.8-1,500	10.7 (3.5-255)	0.12-100	1.4 (0.39-4.2)	1.8 (0.5-4.8)
18	Undecanoic acid	FA 11:0	0.36-300	1.3 (0.65-3.3)	0.024-20	0.026 (0.005-0.053)	0.032 (0.003-0.07)
19	Lauric acid	FA 12:0	7.2-6,000	64.2 (9.5-2,354)	0.48-400	2.4 (0.38-4.5)	3.1 (0.53-7.2)

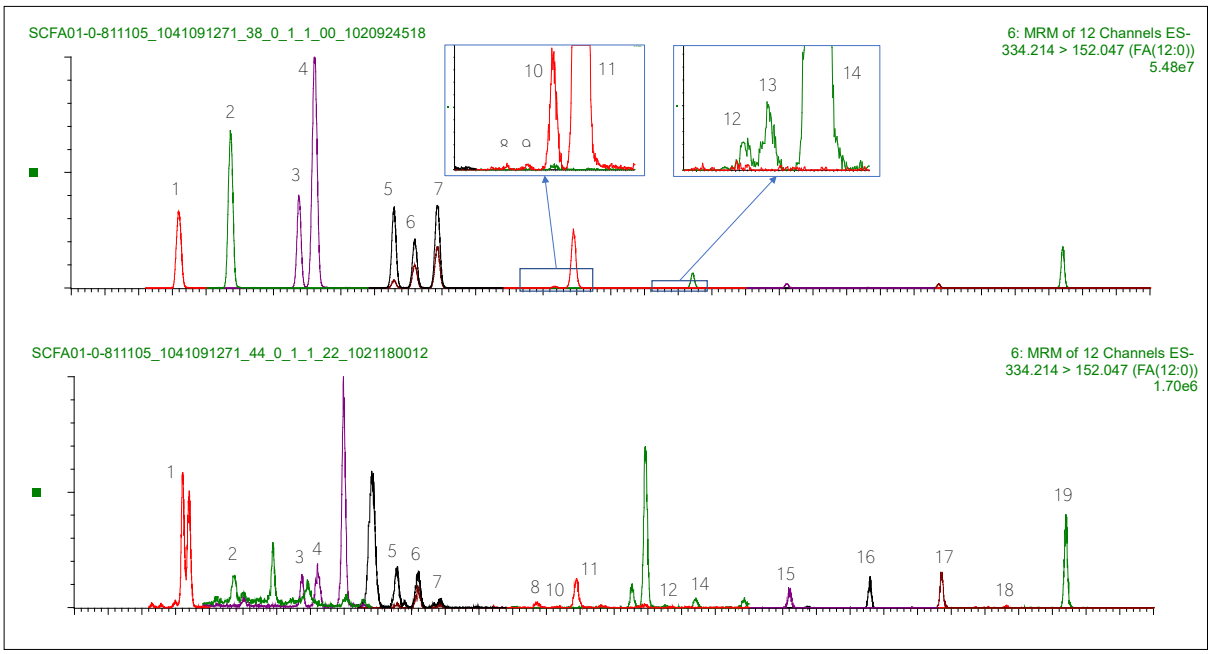

Figure 1: LC ion chromatograms derived from a human pooled fecal extract sample (upper panel) and a human pooled plasma sample (lower panel). Detected peaks are numbered according to Table 1.

3 Results and discussion

Figure 1 shows typical chromatograms of SCFAs and MCFAs in human fecal (upper panel) and plasma (lower panel) samples. In fecal samples, the signal intensity of target analytes was much higher than in plasma samples, which indicated much larger amounts of SCFAs and MCFAs in feces than in plasma. The range of concentrations measured in individual samples (15 for fecal and 20 for either plasma or serum samples) is given in Table 1. At least 17 out of 19 fatty acids could be detected consistently in each analyzed fecal sample, with the majority showing concentrations well above the lower limit of quantification (LLOQ). In the plasma and serum sample sets, 13–15 analytes could be detected, though at very low concentrations, and many in the range between the limit of detection (LOD) and LLOQ. This is to be expected given the naturally low levels of circulating SCFAs and MCFAs.

Several pre-analytical factors might strongly influence the outcome of metabolome analyses in general, and analyses of SCFAs and MCFAs in particular. Two worth noting are the level of homogeneity of a sample, especially of a fecal sample, and the stability of metabolites during sample storage. Since higher amounts of SCFAs and MCFAs could result from degradation of other metabolites like long-chain fatty acids during sample storage, the effect of storage time and temperature was assessed.

Variance of concentrations with position in fecal sample

Fecal samples from 15 individuals were pre-processed in two different ways (Figure 2): 1-spot (unpooled) and 3-spot sampling (pooled). With 1-spot sampling, a portion of approx. 200 mg fecal matter was transferred from one position on the sample heap to a Precellys® vial, weighted, and homogenized after the addition of the threefold volume of isopropanol. Three such replicates were processed for each sample. In 3-spot sampling, the fecal matter was taken from

three different positions on the sample heap, amounting to a total of approx. 1 g added to a test tube. After weighting, the threefold volume of isopropanol was added and then vortexed to form a homogenous suspension. Approximately 1 mL of the suspension was transferred to a Precellys® vial for homogenization.

Figure 2 shows the precision of the triplicate measurements as overall coefficient of variance (CV) in % from all analytes for each individual. For 1-spot sampling, the repeatability of the results is strongly dependent on the homogeneity of the sample. It is difficult to obtain a representative sample when it is taken from only one position in the sample heap. Therefore, 3-spots sampling is recommended.

Stability depends on storage time of plasma and serum samples

To investigate the effect of storage time on the stability of SCFAs and MCFAs, a batch of freshly acquired plasma and serum samples was compared to a batch of samples already stored for four and half years. The first batch contains samples from 20 individuals, who gave matching plasma and serum samples. These samples were stored for one month at -80 °C. The second batch contains matching plasma and serum samples from eight individuals. These samples were acquired four and half years earlier and stored at -80 °C. The donors of the two sample batches come from the same demographic region.

In plasma samples, a significant increase in concentrations with storage time was observed for several analytes (Figure 3), such as acetic acid (FA 2:0), valeric acid (FA 5:0), 2-methylvaleric acid (FA 5:0-2M), caproic acid (FA 6:0), 4-methylhexanoic acid (FA 6:0-4M), and heptanoic acid (FA 7:0). The concentrations in matching serum samples were stable and unaffected during sample storage. These findings indicate that using samples with the same sample type and similar storage time is essential for correct data analysis and interpretation in any study involving SCFAs or MCFAs. Additionally, caution needs to be taken when comparing studies with different sample sets.

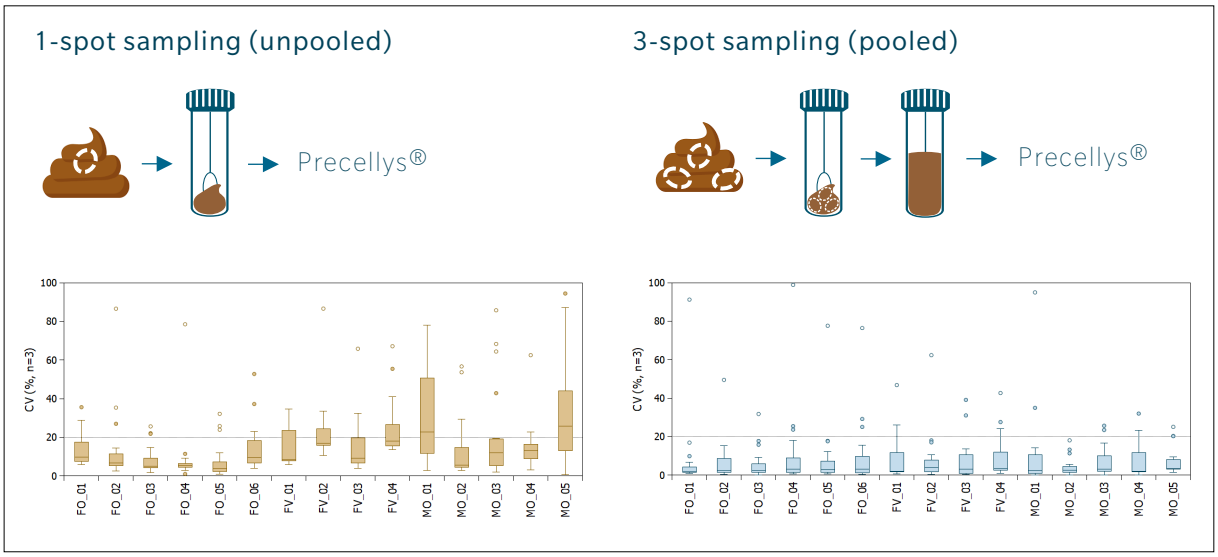

Figure 2: Precision (CV, %) of fecal sample triplicates with different sampling protocols: 1-spot sampling (left panel) and 3-spot sampling (right panel) with additional pre-homogenization step. Samples were taken from 15 individuals with different genders and diets (FO, female omnivore; FV, female vegetarian; MO, male omnivore).

Stability depends on storage temperature of plasma and serum samples

The effect of storage temperature is shown in Figure 4. In this experiment, matching plasma and serum samples from one individual, stored for approximately two years at different temperatures (-80 °C and -20 °C) were compared. Again, concentrations of SCFAs and MCFAs differed between plasma and serum samples. In addition, the storage temperature had a clear effect on the stability of the analytes. In most cases, concentrations of SCFAs and MCFAs were increased at -20 °C compared to -80 °C, independent of the sample matrix. This suggests that the continuous generation of SCFAs and MCFAs during storage might accelerate at higher temperatures. Therefore, if samples are not measured soon after collection, they need to be stored at -80 °C to minimize changes in SCFA and MCFA concentrations. Otherwise, there is a risk of incorrect analysis and interpretation of study results.

4 Conclusions

The new standardized and quality controlled SCFA+ assay provides absolute quantitative results for a broad panel of 11 SCFAs and 8 MCFAs in human fecal, plasma and serum samples. For fecal samples in general, pre-analytical aspects play a very important role in providing correct results for data analysis and interpretation due to the inhomogeneous nature of this sample matrix. A multi-spot, pooled sampling approach is highly recommended to minimize within-sample variance. Whichever sample matrix is used, samples need to be collected within a short period of time and stored as soon as possible at -80 °C to guarantee their stability before analysis.

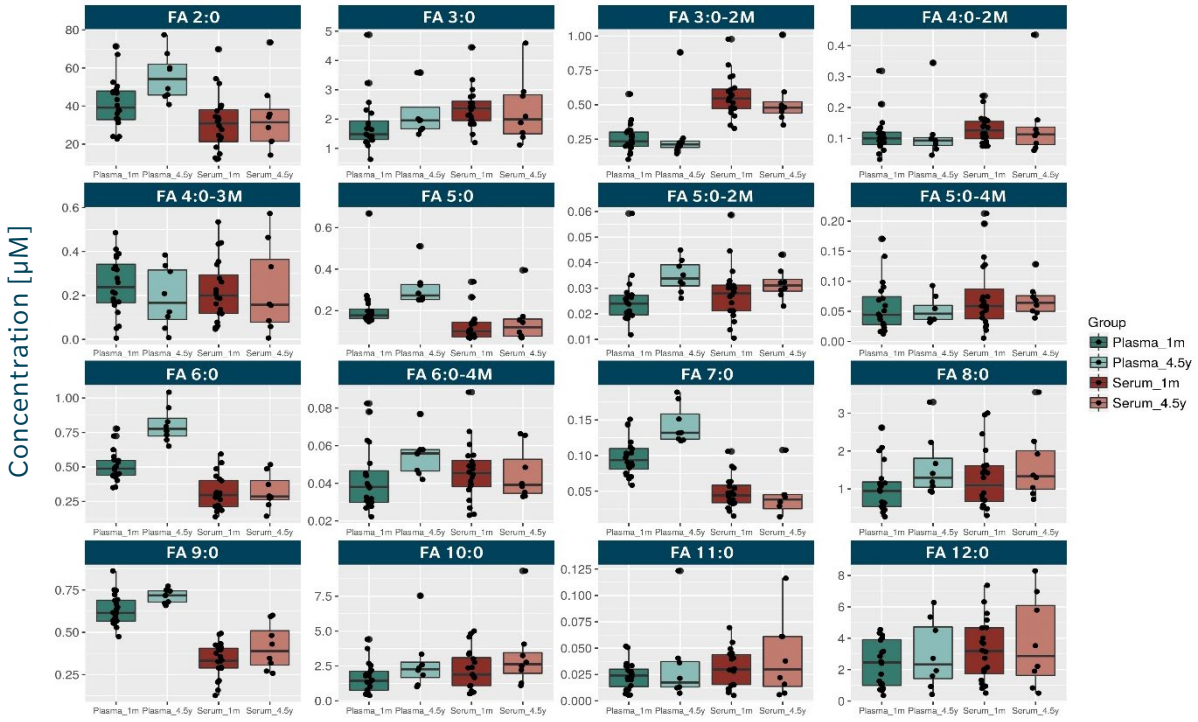

Figure 3: Concentrations of detected analytes measured with SCFA+ assay in individual plasma (green) and serum (red) samples with different storage times. The darker boxes represent matching plasma and serum samples from 20 individuals, stored for one month at -80 °C. The lighter boxes show matching plasma and serum samples from 8 other individuals, stored for four and a half years at -80 °C. All samples were analyzed on the same day.

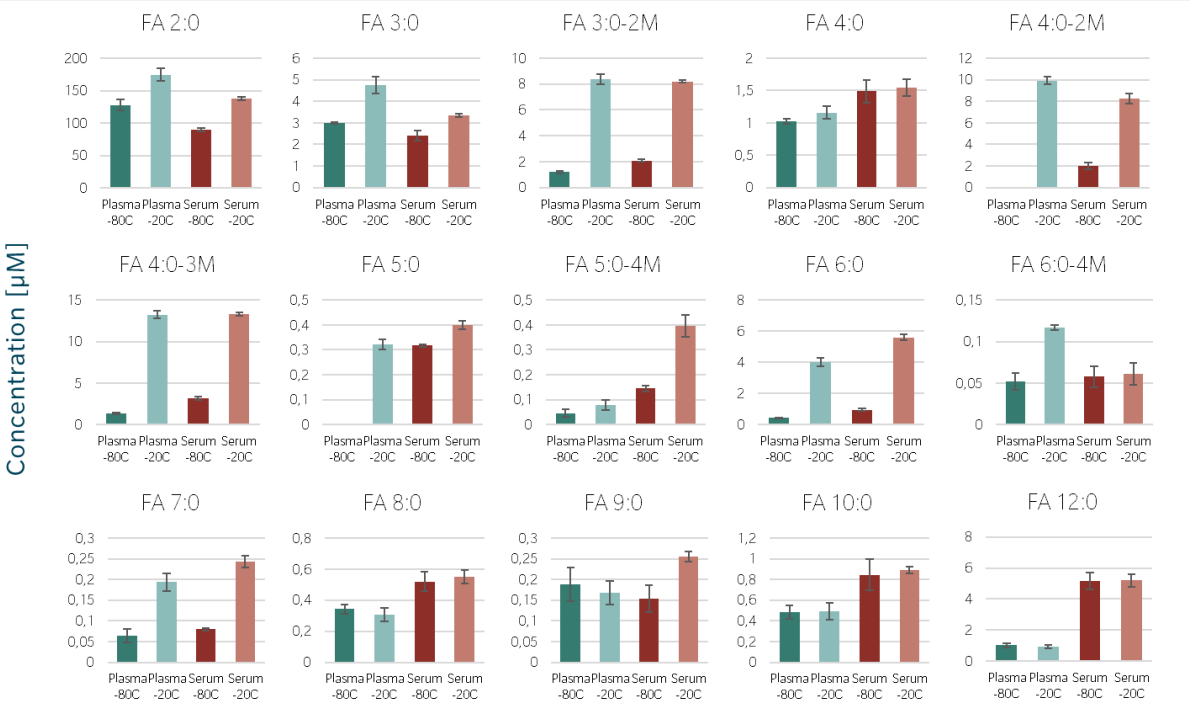

Figure 4: Concentrations of detected analytes measured with SCFA+ assay in matching plasma (green) and serum (red) samples from one individual, stored for two years at -80 ° (darker bars) and -20 °C (lighter bars). All samples were analyzed on the same day in triplicates.