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Application News

No. L555

High Performance Liquid Chromatography

Improvement of Productivity in Research on Intestinal Microbiota by Shim-pack™ Fast-OA High-Speed Organic Acid Analysis Column

Recent research suggests that intestinal microbiota that grows in the intestinal tract contributes to maintaining and improving the health of the host. In the field of research on intestinal microbiota, mass spectrometry is used for comprehensive measurements of metabolites, and HPLC is utilized in cases where the target is clear (for example, quantitation of short-chain fatty acids formed by microbial metabolism).

This article introduces an example of measurement of short-chain fatty acids in feces of monkeys by using a high-speed organic acid analysis column.

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Analysis of Short-Chain Fatty Acids Standard Solution

Shim-pack Fast-OA is an ion exclusion mode column that separates acidic compounds such as short-chain fatty acids. Because retention time changes depending on the mobile phase concentration and temperature, conditions are optimized by adjusting the column temperature according to the target compound of the analysis. Fig. 1 shows the retention times of short-chain fatty acids at temperatures from 35 °C to 50 °C. Although there are no temperature-dependent changes in the elution order, retention becomes weaker as the temperature is increased. In this study, 50 °C was used, as this temperature enables rapid analysis with no loss of separation performance.

Table 1 shows the analysis conditions when analyzing 6 components of a mixed short-chain fatty acid standard solution using a Shim-pack Fast-OA and a Shim-pack SCR-102H, which is also an ion exclusion mode column, and Fig. 2 shows the chromatograms. When the Shim-pack SCR-102H was used, the analysis time for one cycle was about 30 min, but with Shim-pack Fast-OA, elution of valeric acid with a retention time within 10 min was confirmed.

The Shim-pack SCR-102H has the advantage that high separation performance is possible owing to its column length of 300 mm. However, when the target is short-chain fatty acids, analysis time becomes an issue. Although it is difficult to achieve a reduction in analysis time in ion exclusion chromatography by changing the analysis conditions, analysis time can be shortened effectively by using Shim-pack Fast-OA, which has a smaller particle size than conventional columns and allows selection of the number of columns depending on the analysis target.

Table 1 Analysis Conditions

Column	: Shim-pack Fast-OA (100 mm L. × 7.8 mm I.D., 5 µm) Shim-pack SCR-102H (300 mm L. × 8.0 mm I.D., 7 µm)
Guard column	: Shim-pack Fast-OA (G) Shim-pack SCR-102H (G)
Mobile phase	: 5 mmol/L p-toluenesulfonic acid (Mobile phase reagent set for organic acid analysis, mobile phase)
Flow rate	: 0.8 mL/min
pH buffering solution	: 5 mmol/L p-toluenesulfonic acid 20 mmol/L Bis-Tris 0.1 mmol/L EDTA (Mobile phase reagent set for organic acid analysis, pH buffering reagent)
Flow rate	: 0.8 mL/min
Column temperature	: 50 °C
Detection	: Conductivity detector (CDD-10Avp)
Injection volume	: 10 µL

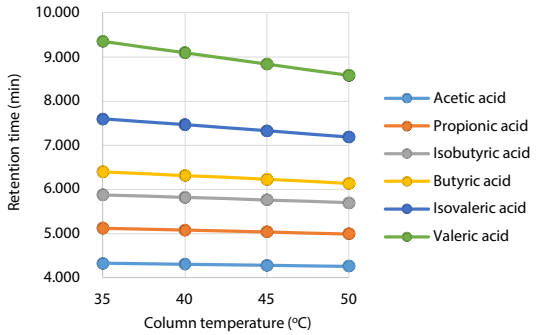

Fig. 1 Retention Behavior of Short-Chain Fatty Acids

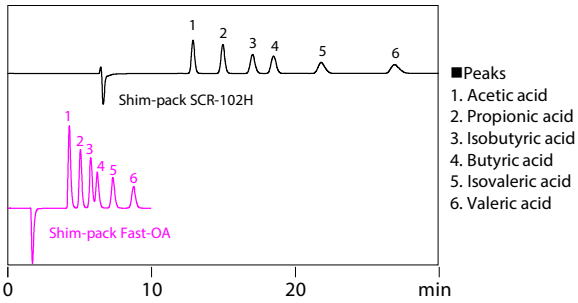

Fig. 2 Chromatograms of Standard Sample (500 mg/L each)

Calibration Curve

Calibration curves for each of the short-chain fatty acids were prepared in the concentration range of 5, 10, 50, 100, 500, and 1000 (mg/L). Table 2 shows the results of an evaluation of the linearity contribution ratio and area value reproducibility at 5 mg/L. Satisfactory results were obtained, with contribution ratio (r^2) values 0.999 or higher and area value reproducibility (%RSD) of 5% or less.

Table 2 Linearity Contribution Ratio and Area Value Reproducibility

	Linearity (r^2)	Reproducibility (N=6, %RSD)
Acetic acid	0.9997	1.36
Propionic acid	0.9997	3.51
Isobutyric acid	0.9997	2.48
Butyric acid	0.9998	2.24
Isovaleric acid	0.9999	3.40
Valeric acid	0.9997	1.95

Samples and Pretreatment

The monkey feces used as samples were frozen on site and stored in a frozen condition at -80 °C. Fig. 3 shows the pretreatment protocol. First, 700 µL of phosphate buffered saline (PBS) was added to 100 mg of the sample. The sample for use in the analysis was then prepared by mixing the solution, followed by centrifugal separation and ultrafiltration of the supernatant.

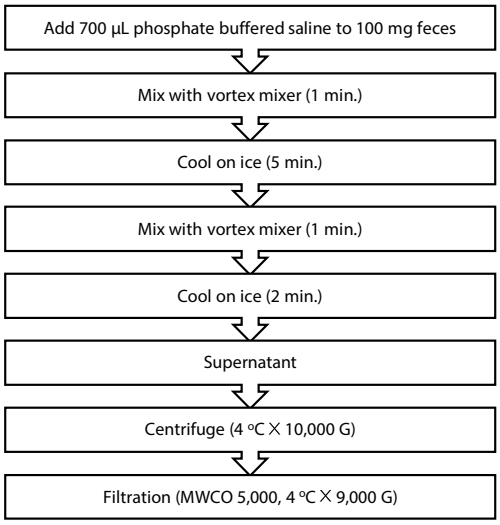

Fig. 3 Pretreatment Protocol for Monkey Feces Samples

Analysis of Monkey Feces

Feces samples from 5 monkeys were taken from points A, B, and C. Table 3 shows the details of the samples, and Fig. 4 shows a schematic illustration of the sampling locations.

Table 3 Details of Samples

Sample No.	Sampling location	Monkey	Time until freezing
1	A	Unknown	Half day to full day
2	B	Male, age 4 years	Immediately after excretion
3	B	Male, age 2 years	Immediately after excretion
4	C	Female, adult	Immediately after excretion
5	C	Female, adult	Immediately after excretion

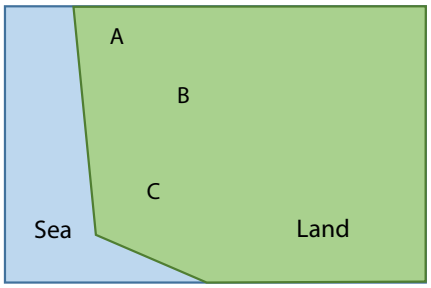

Fig. 4 Schematic Illustration of Sampling Locations

After pretreatment by the protocol in Fig. 3, the above-mentioned 5 samples were analyzed by HPLC under the conditions in Table 1 using Shim-pack Fast-OA columns. Fig. 5 shows the chromatogram of Sample 2. A large peak originating from the buffer solution used in the pretreatment, which contained phosphoric acid, was observed around t0 (around 2.5 min). However, peaks of the short-chain fatty acids eluted after acetic acid (Fig. 5, after 4 min), which are particularly important in research on intestinal bacterial flora, could be quantified with no problems.

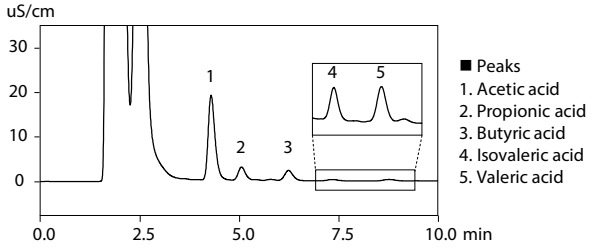

Fig. 5 Chromatogram of Sample 2

Table 4 shows the amounts of each of the short-chain fatty acids contained in Samples 1 to 5. In the case of Sample 1, half day to full day passed from excretion until sampling and freezing, and virtually no short-chain fatty acids were detected. This is thought to be due to volatilization of the short-chain fatty acids during the period from excretion to sampling, or metabolism by microorganisms in feces. Based on this, for accurate measurement, collection of samples as soon as possible after excretion and storage under conditions that prevent microbial metabolism and volatilization are critical for correct measurement of the effect of intestinal microbiota from feces samples.

Table 4 Amount of Short-Chain Fatty Acids Contained in Samples

	Amount of compounds ($\times 10^{-2}$ mol/kg)					
	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid
Sample 1	0.042	-----	-----	-----	-----	-----
Sample 2	5.6	0.99	-----	1.0	0.16	0.23
Sample 3	5.5	0.96	-----	0.90	0.19	0.34
Sample 4	5.8	1.3	-----	1.2	-----	0.25
Sample 5	6.3	0.88	-----	0.84	-----	0.23

Conclusion

Short-chain fatty acids, which are an object of research in the field of intestinal microbiota, were analyzed by using a Shim-pack Fast-OA column. Quantitation of the target compounds was possible within a time of 10 min and was unaffected by contamination. As a result of measurements of the amounts of short-chain fatty acids contained in feces samples from 5 monkeys, virtually no short-chain fatty acids were found in one sample that was collected and frozen more than half a day after excretion, but it was possible to measure the existence of short-chain fatty acids with satisfactory sensitivity in the samples that were frozen immediately after excretion.

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