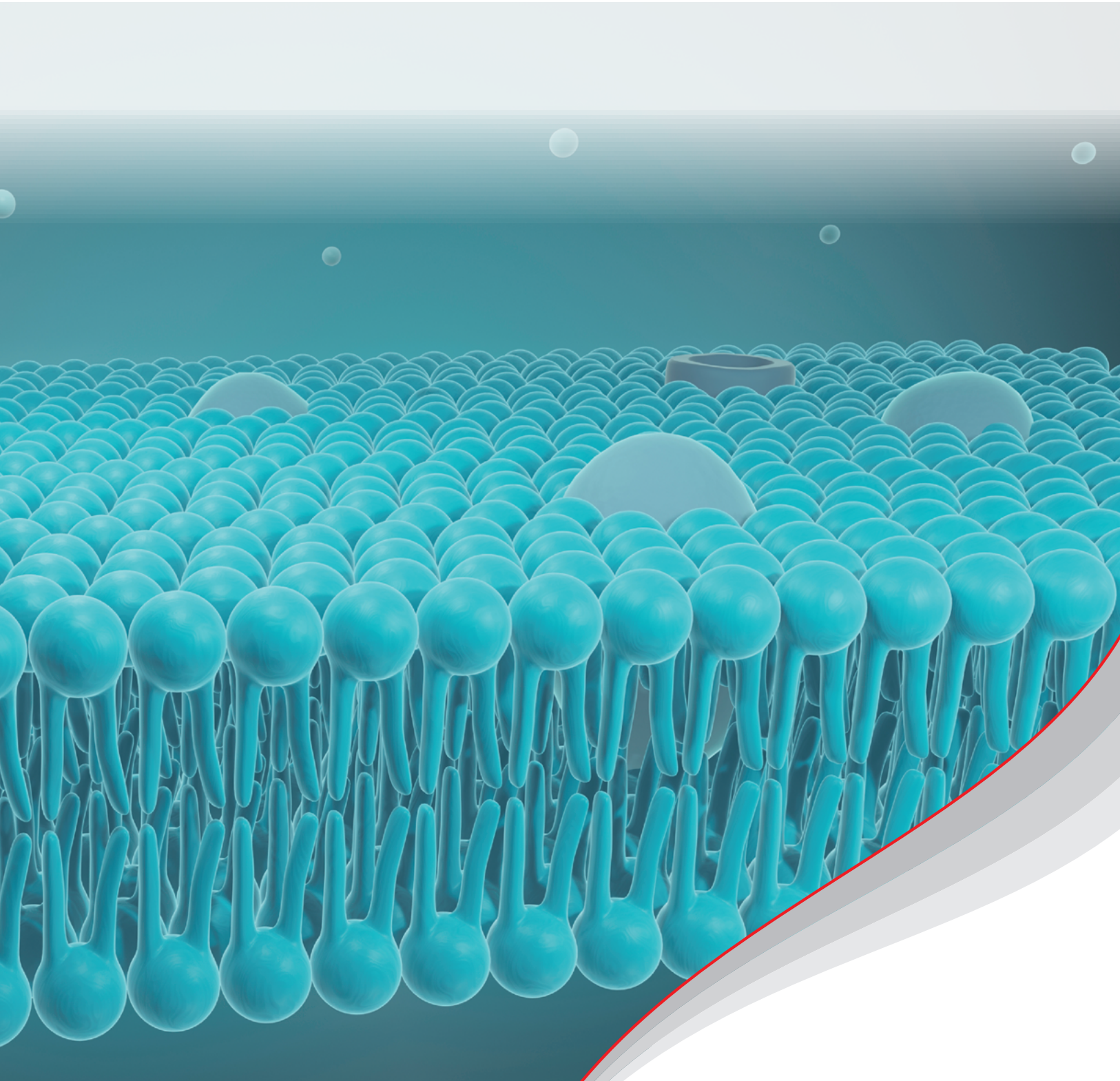
C10G-E101

SHIMADZU
Excellence in Science

Lipid and Fatty Acid Analysis Solutions



Lipids and Fatty Acids

Lipids are one of the four major biological substances, along with proteins, carbohydrates, and nucleic acids. Fatty acids are also important in living organisms and make up cell membranes (examples: phospholipids, sphingolipids, cholesterol esters). Both play essential roles in the physiology of living organisms, serving as structural components of biological membranes, mediators of energy storage, and signaling molecules within and between cells.

Lipids

Lipids are a general term for substances that are soluble in nonpolar solvents. Nonpolar solvents are usually hydrocarbons, and waxes, sterols, vitamins, acylglycerols, and phospholipids that are soluble in them are generally classified as lipids. Fig. 1 shows eight categories of lipids and Fig. 2 shows major lipids in plasma.

Fatty acyls	Long-chain fatty acids Fatty acid ester Fatty acid amide Sophorolipid
Glycerolipids	Acylglycerol Glyceryl ether Glyceroglycolipid Archaeol Crenarchaeol
Glycerophospholipids	Phosphatidylcholine Phosphatidylinositol Plasmalogen Ether-type phospholipids
Sphingolipids	Sphingosine Ceramide Sphingolin lipid Sphingoglycolipid
Sterol Lipids	Sterol Steroid hormone Bile acid
Prenol Lipids	Carotenoids Retinal Triterpenoids Diterpenoids Sesquiterpenoids
Saccharolipids	Lipid A
Polyketides	Macrolide Tetracycline

Fig. 1 Eight classes of lipids

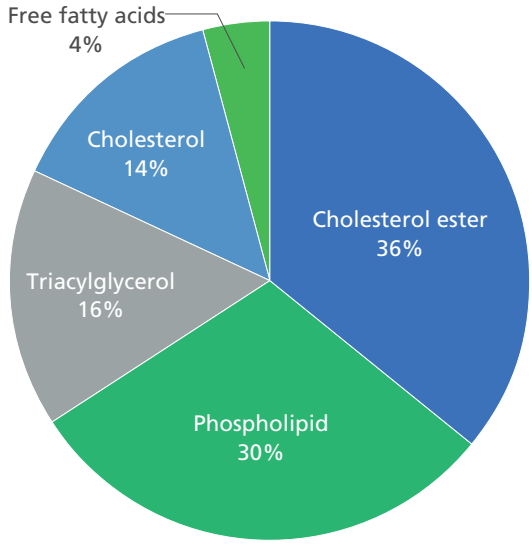


Fig. 2 Major lipids in plasma

LIPID MAPS® Structure Database (LMSD)
https://www.lipidmaps.org/databases/lmsd/browse

Fatty Acids

Fatty acid analysis is used in a variety of fields, including food, clinical, and chemical. LC-MS/MS or GC-FID (or GC-MS) is commonly used in fatty acid analysis, and instruments are selected based on analytical purposes and target species. A simple comparison is shown in Fig. 3 and 4.

		LC	GC
Profiling	Percentage in total fatty acids (%)	△	◎
	Profiling of specific fatty acids	○	◎
Targeted	Trace analysis at <1 ppm	○	△
	Analysis at > 1 ppm	○	◎

Fig. 3 instrument comparison by application

◎ : Well suited
○ : Doable
△ : Limited use
× : Not recommended

		LC	GC
Free fatty acids	Short	○	△
	Middle/Long	◎	○
Fatty acid ester	Short	○	△
	Middle/Long	○	◎
Unsaturated fatty acids	Double bond position	×	○
	Isomer	×	◎
Sensitivity		◎	○
Sample preparation		○	△

Fig. 4 Instrument comparison based on fatty acid types, sensitivity, and sample preparation

LC-MS advantage

- LC-MS is a highly sensitive instrument and suitable for analyzing trace amounts of free fatty acids existing in the body (e.g., cascade metabolites from arachidonic acid).

LC-MS shortcomings

- LC-MS analysis cannot distinguish isomers like *cis-trans*.
- Linear (n-) and branched fatty acids (iso-, anteiso-) cannot be separated.
- Ion suppression occurs, making it unsuitable for an analysis involving complex matrices.

Examples

1) If you want to measure short-chain fatty acids produced by gut bacteria in the life sciences field
   ➡ LC is preferred over GC.

2) When a phospholipid headgroup needs to be identified
   ➡ LC is preferred over GC.

GC-MS advantage

- GC-MS is suitable for analyzing the constituent fatty acids in fatty acid esters (e.g., wax, glycerides, and phospholipids), a form in which fatty acids are abundant in the body, as well as free fatty acids, which are found in small amounts in the body.

GC-MS shortcomings

- GC-MS analysis needs to ensure that the methylation is 100% complete (by thin layer chromatography, for example).
- Determining which lipids contained measured fatty acids in vivo can be difficult by GC/MS.

Examples

1) When considering consumer health in the food sector, such as with these three examples:
   - Unsaturated vs Saturated
   - cis isomer vs trans isomer
   - Omega -3 vs Omega -6

   ➡ GC is suitable because LC cannot separate isomers.

2) If you want to know the fatty acid composition of cells (e.g., ratios of C 16, C 17, and C 18)
   ➡ GC is preferred over LC.

Rapid Profiling of 39 Bile Acids in Plasma, Urine, and Fecal Samples

Application

The colanic acid structure constitutes the basic backbone of bile acids and is known to be difficult to fragment by MS/MS. As such, it is difficult to measure fragment ions based on differences in the bile acid structure. In order to accurately quantify various structurally similar bile acids in a simultaneous LC-MS/MS analysis, the isomers must be sufficiently separated by HPLC. In this application, we carefully optimized HPLC conditions for the separation of bile acids and were able to achieve both high throughput and high sensitivity.

benefits

This is a rapid and versatile quantitative method for bile acids. Combined with fully automated sample preparation of plasma, urine, and feces, the developed method enables a highly sensitive measurement while processing a large number of samples.

Measurement

In the case of mouse fecal samples, hydrolysis is performed first to liberate bile acids from the sulfate and glucuronide conjugates produced in the intestinal tract. Potassium hydroxide was added to 5 to 10 mg of feces, incubated at 80 C for 20 minutes, and the pH was lowered using a potassium phosphate buffer for an automatic extraction. For plasma, urine, and hydrolyzed fecal samples, 250 µL was used for extraction. An Evolute Express ABN 30 mg 96 well plate (Biotage®) was used as the extraction plate, and water and methanol, both containing formic acid, were used as the extraction solvents.

Sample

Biological sample (Human plasma, human urine, and mouse feces)

Instrument configuration

LC-MS System	: LCMS-8060NX
Column	: ACE Excel C18 Amide (ADVANCED CHROMATOGRAPHY TECHNOLOGIES LTD)

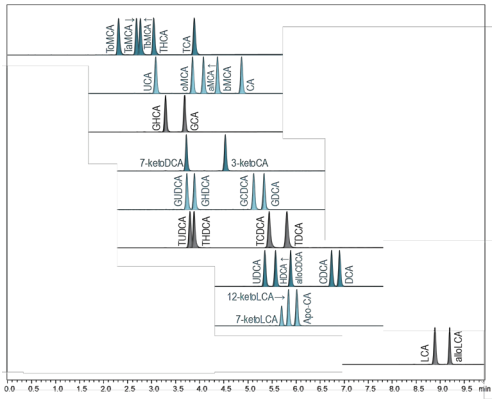


Fig. 5 MRM chromatograms of bile acid isomers (a standard solution of 10 ng/mL)

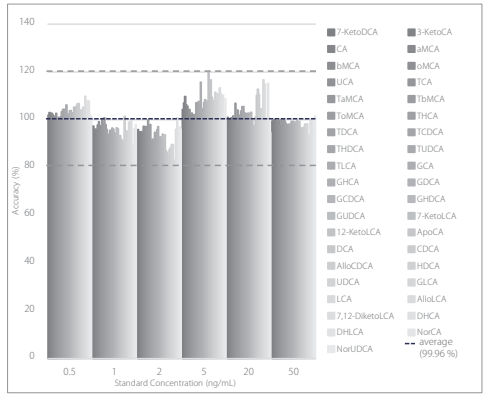


Fig. 6 Accuracy of the standard solution

Summary

39 bile acids in biological samples (e.g., human plasma, human urine, and mouse feces) were quantitatively analyzed using 10 internal standards. The LC/MS/MS Method Package for Bile Acids Ver. 2 was used for the analysis. This package includes optimized conditions and automated sample preparation protocols for LC-MS/MS analysis. Having rigorously optimized the HPLC conditions, we were able to achieve both high throughput and high sensitivity while realizing the separation of bile acids.

LC/MS/MS Method Package for Bile Acids Ver. 2

This method package contains optimized LC separation conditions and MS parameters for 49 bile acids. Examples of sample preparation for biological samples are also included. The method package enables a comprehensive analysis of bile acids in biological samples without any method development.

Product

Method Development for Triglycerides in Blood

Application

Routine blood tests estimate the total amount of triglycerides, but do not provide quantitative information about the various fatty acids that are bound to triglycerides. Therefore, Shimadzu developed an LC-MS/MS method for the analysis of blood triglycerides as the LC/MS/MS MRM Library for Triglycerides.

benefits

The developed method analyzes 47 types of triglycerides in blood in 11 minutes (equivalent to 130 analyses per day) and identifies fatty acid combinations in triglycerides. Therefore, it is useful for finding biomarkers in a high-throughput screening.

Measurement

960 µL of a methanol/butanol mixture solution was added to 20 µL of plasma or serum and shaken for 3 minutes. Centrifugation was performed for 15 minutes, and the supernatant was transferred into a new tube and was diluted 10 times. 3 µL of the diluted supernatant was injected into the LC-MS/MS. In the MRM of triglycerides, the ions detected by neutral loss (NL) of fatty acids were set as the product ions, while the precursor ions were set as $[M+NH_4]^+$.

Sample

Two types of human plasma (Plasma 1 and 2), and serum

Instrument configuration

LC-MS System	: LCMS-8060
Column	: Shim-pack Velox™, C18 (2.1 mm I.D.×50 mm, 2.7 µm)

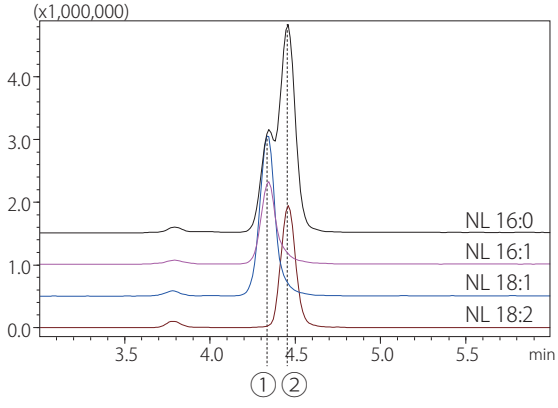


Fig. 7 MRM chromatogram of TG 50: 2

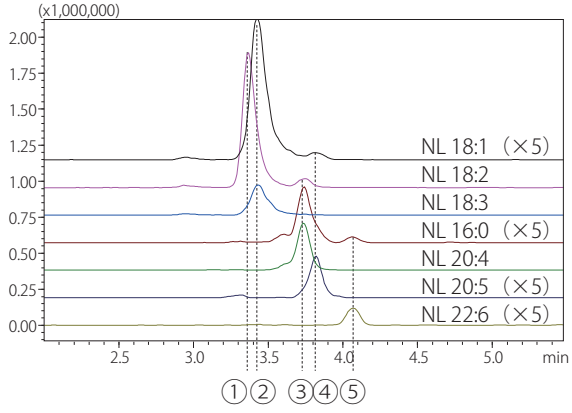


Fig. 8 MRM chromatogram of TG 54: 6

Summary

The developed method can measure 47 blood triglycerides with different molecular weights in 11 minutes per analysis (or 130 analyses per day). In addition, with the acquisition of 195 MRM data, co-eluting triglyceride isomers can be differentiated and identified by the fatty acid peak combinations. Using this method, we analyzed commercially available human plasma and serum samples and were able to detect minute inter-sample differences in triglycerides by a multivariate analysis.

LC/MS/MS MRM Library for Triglycerides

195 MRM transitions were prepared for 47 triglycerides in this library, reflecting the estimated fatty acid combinations. Various triglycerides can be compared between samples.

Product

Omics Approach with Metabolomics and Lipidomics

Application

In the study of living organisms, it is necessary to monitor the target metabolites as well as their precursors and intermediates. In this application, an omics approach was employed by evaluating metabolic changes (i.e., metabolomics) and combining the evaluation results with lipidomics on phospholipids.

benefits

LC/MS/MS MRM Library for Phospholipid Profiling realizes a trouble-free data analysis.

Measurement

We cultured E. coli in a jar fermenter using a media supplemented with 50 mM thiosulfate or 100 mM sulfate as a sulfur source. In order to assess metabolic variation depending on the culture media, some of the cells were recovered from the culture suspension after 0, 24, 48, 72, 96, 120, 168 and 216 hours. After measuring the OD value of the recovered E. coli, the media was adjusted to OD = 2, equivalent to 1 mL, and then rinsed with ultrapure water. Next, we used the Bligh-Dyer method to extract hydrophilic metabolites and phospholipids from the cells. The water and chloroform layers were collected, dried by a concentration centrifuge, and then dissolved in ultrapure water and methanol. The extract was diluted as required and subjected to simultaneous analysis using the LCMS-8060.

For the metabolite analysis, the non-ion pair method of the LC/MS/MS Method Package for Primary Metabolites Ver. 2 was used, and for the phospholipid analysis, a simultaneous analysis was performed with the LC/MS/MS MRM Library for Phospholipid Profiling.

Sample

An E. coli extract

Instrument configuration

LC-MS System	: LCMS-8060
Column	: C8 column (2.1 mm I.D.×150 mm, 2.6 µm)

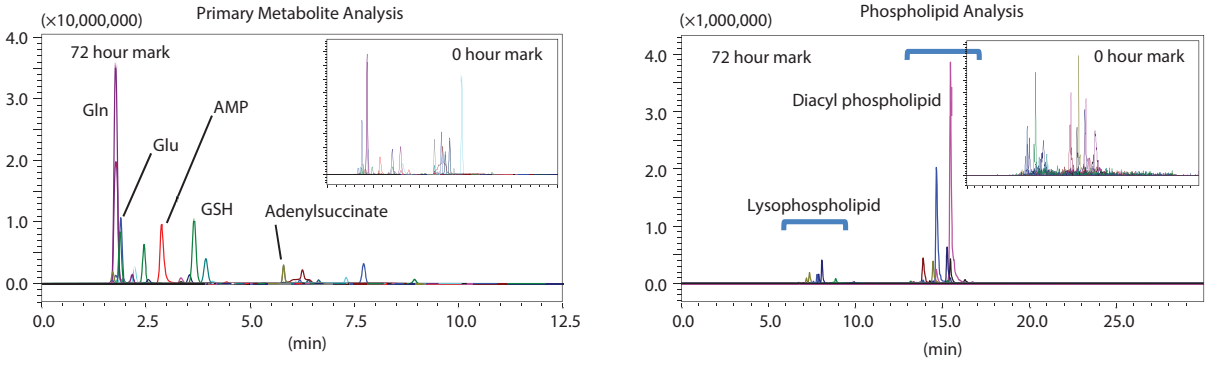


Fig. 9 MRM chromatograms (primary metabolites and phospholipids) of E. coli extracts cultured in thiosulfuric acid-supplemented medium.

Summary

This application captured metabolic changes in an E. coli extract, showing its applicability for food and biotechnology companies and researchers studying microorganisms. An analytical approach based on two omics (i.e., metabolomics and lipidomics) was used and proven effective in evaluating metabolic fluctuations.

LC/MS/MS MRM Library for Phospholipid Profiling

This method package is designed to analyze phospholipids containing fatty acids from C 14 to C 22 and includes a phospholipid class determination method for major phospholipids in living organisms. MRM transitions of up to 867 components are registered in the library.

Product

Determination of Glucosylceramides in Supplements

Application

Glucosylceramides cannot be analyzed by an ultraviolet-visible (UV-VIS) detector as they have little light absorption. Also, in terms of LC, It is common to use a normal-phase mode (e.g., chloroform as a mobile phase) and determine the total content because glucosylceramide species vary widely. In such an analysis, the molecular species are eluted together without separation. The Evaporative Light Scattering Detector (ELSD) is a versatile detector that measures the scattered light of a target component after atomizing and vaporizing the mobile phase, and it can also detect substances without UV absorption (e.g., glucosylceramides). Supercritical chromatography (SFC) uses carbon dioxide, which is less polar, as the mobile phase, making it possible to perform analyses without using large amounts of highly hazardous organic solvents.

benefits

- Since chloroform is not used in the mobile phase, glucosylceramides can be analyzed safely compared to the normal phase mode.
- A high-speed analysis of glucosylceramides can be performed with the same level of reproducibility and sensitivity as achieved in normal phase mode.
- Since carbon dioxide is less expensive than the organic solvents used in HPLC, running costs will be reduced.

Measurement

A commercial supplement was added with 9 mL of a chloroform/methanol mixture solution and sonicated for 5 minutes. The supernatant was then centrifuged for 10 minutes, filtered, and diluted 5 times with the chloroform/methanol mixture solution. 5 µL of the diluted supernatant was injected into the SFC.

Sample

Over-the-counter supplements

Instrument configuration

SFC System	: Nexera UC
Column	: Shim-pack™ UC Sil (4.6 mm I.D.×150 mm, 5.0 µm)

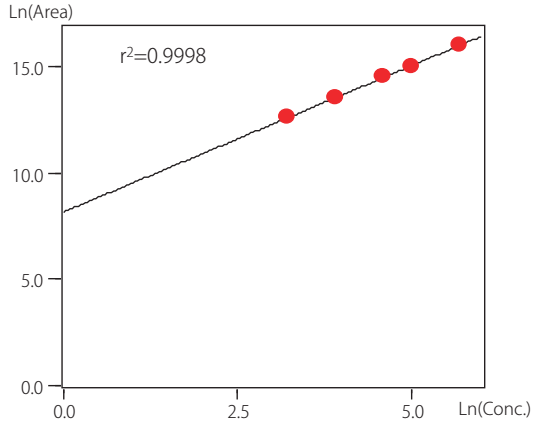


Fig. 10 Calibration curve

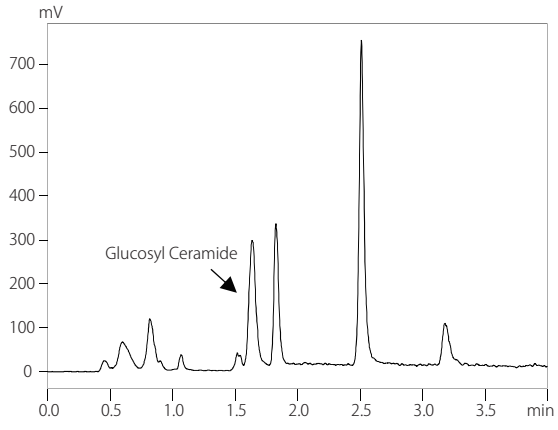


Fig. 11 Chromatogram of Supplement

Summary

Quantification of glucosylceramides in rice-derived supplements was performed using SFC and ELSD. In SFC, the use of carbon dioxide instead of the highly hazardous chloroform as the mobile phase not only improved safety but also allowed glucosylceramides to elute within two minutes. In addition, with ELSD, we were able to perform a highly sensitive and reproducible analysis.

Supercritical Fluid Extraction/Chromatograph System

Nexera™ UC / ELSD-LT III

The Nexera UC eliminates the need for complicated sample preparation, enabling more efficient lipid analysis.

Product

Analysis of Short-Chain Fatty Acids in Plasma

Application

Short-chain fatty acids easily evaporate due to their low boiling points, making them difficult to quantitate. GC/MS requires derivatization of the hydroxyl group, but many derivatization steps require water present in the sample to be dried before the derivatization. During this process, much of the short-chain fatty acid is lost due to evaporation. In this application, short-chain fatty acids were derivatized with amines to enable GC/MS analysis.

benefits

A simple derivatization method makes it possible to analyze short-chain fatty acids, which are often difficult to quantitatively analyze.

Measurement

Short-chain fatty acids were derivatized with amines using 4- (4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), a condensing agent that can promote the condensation reaction of carboxylic acids with amines, even in water and methanol. The derivatized compounds were analyzed by GC-MS. Short-chain fatty acids (e.g., formic acid, acetic acid, propionic acid, butyric acid, valeric acid, etc.), for which traditional analytical methods are limited, were derivatized with DMT-MM and n-octylamine for analysis.

Sample

Human standard plasma (Kohjin Bio Co., Ltd.: human plasma and pooled EDTA-2Na (12271450))

Instrument configuration

GC-MS System	: GCMS-TQ™8040 NX
Column	: BPX-5 (0.25 mm I.D.×30 m, 0.25 µm)
Carrier Gas	: Helium
Carrier Gas Control	: Linear Velocity

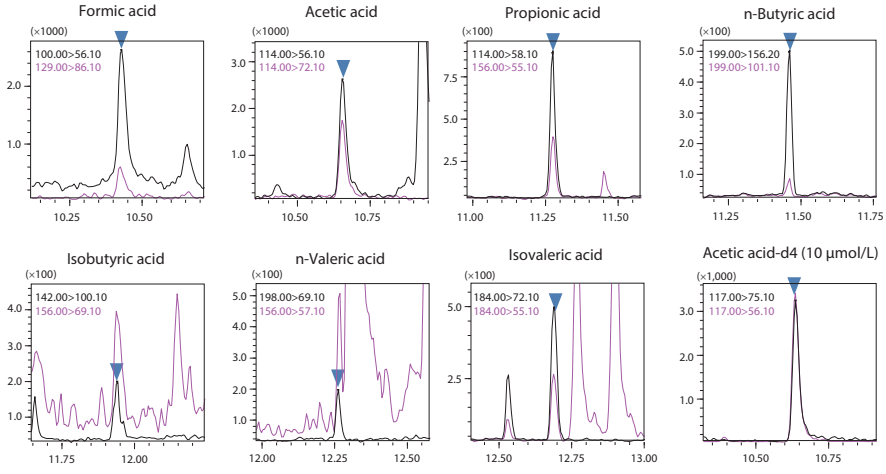


Fig. 12 MRM chromatograms of a spiked sample at 1 µmol/L

Summary

Short-chain fatty acids (e.g., formic acid, acetic acid, propionic acid, butyric acid, valeric acid, etc.) were derivatized with DMT-MM and n-octylamine and analyzed by GC-MS. This method can be applied to analysis of the intestinal environment and other metabolites. Compounds such as formic acid, acetic acid, propionic acid, butyric acid and valeric acid, which were previously difficult to analyze by GC-MS, can now be analyzed using a special derivatization method.

Triple Quadrupole GC-MS/MS

In addition to its ultra-high sensitivity, this instrument reduces maintenance frequency and running cost in long-term use.

Products

Quantitation of Fatty Acid Methyl Esters (FAMEs)

Application

FAMEs are prone to fragmentation during ionization and many similar low mass ions are detected. In this application, CI-MRM was used to combat this problem. CI-MRM by GC-MS/MS fragments ionized protonated molecules by CID, improving mass separation between impurities and FAMEs.

benefits

- PCI-MRM is more sensitive than EI-MRM, especially for unsaturated fatty acids, and is ideal for fatty acid analysis.
- The Smart EI/CI ion source can switch between EI and PCI methods without breaking vacuum and stopping the instrument.
- The Smart Metabolites Database and a fatty acid methylation kit make it easy to analyze fatty acids in foods.

Measurement

Standard solutions were prepared by serially diluting the FAME Reference Standard (AccuStandard Inc., cat.: FAMQ005), which contains 37 FAMEs. Food samples were homogenized, freeze-dried, and weighed to 50 mg. 2 mL of acetone was added, and the tube was shaken and centrifuged. 2 mL of hexanes was added to the tube, and the extract (i.e., acetone and hexanes) was recovered after shaking and centrifugation. 2 mL of deionized water was added, and the tube was shaken and centrifuged. The upper layer (i.e, organic solvents) was collected and dried. The dried samples were further processed using a Nakalai tesque fatty acid methylation kit (Nakalai tesque INC.). The extracted sample was diluted 100 times with hexanes before the measurement.

Sample

Commercially available beef and mackerel

Instrument configuration

GC-MS System	: GCMS-TQ8050 NX
Column	: DB-5MS (0.25 mm I.D.×30 m, 0.25 µm)
Carrier Gas	: Helium
Carrier Gas Control	: Linear Velocity

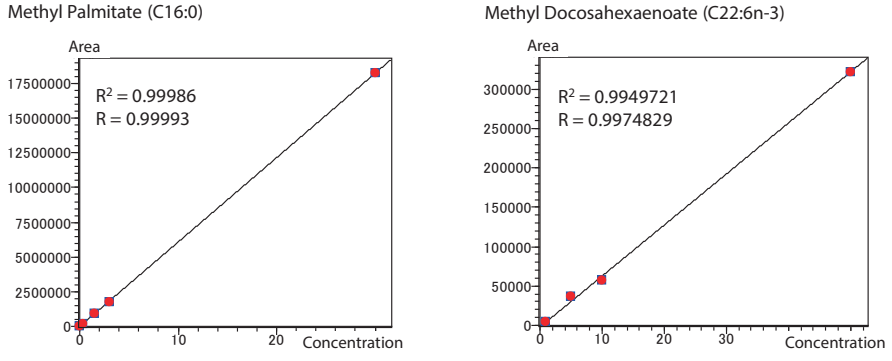


Fig. 13 Calibration curves of methyl palmitate and methyl docosahexaenoate acquired with the PCI mode

Summary

For quantitative analysis of fatty acids in foods, PCI-MRM is more sensitive than EI-MRM, and its accuracy is comparable to that of EI-MRM. The major disadvantage of PCI has been the need to replace the EI ion source with the PCI ion source, but by using the Smart EI/CI ion source, you can switch between the EI and PCI modes without breaking vacuum and stopping the instrument.

Analysis of Short-Chain Fatty and Organic Acids in Fecal Samples from Mice Treated with SPF and Antibiotics

Application

In general, short-chain fatty acids are highly volatile and hydrophilic, making an LC/MS analysis difficult in a commonly used reversed-phase system. Furthermore, derivatization methods (e.g., trimethylsilylation) widely used for GC/MS require the sample to be dried out, which can result in the loss of volatile components such as short-chain fatty acids. In this application, carboxylic acids were derivatized with 3-nitrophenylhydrazine (3-NPH) in an aqueous solution to enable an LC/MS analysis.

benefits

Because 3-NPH also reacts with ketone functional groups, pyruvate and oxaloacetic acid, which have ketones, are also derivatized.

Measurement

The weighed sample was suspended in ethanol and then the supernatant was collected by centrifugation. The supernatant was subjected to derivatization with 3-NPH. For the 3-NPH derivatization, pyridine was used as the catalyst and carbodiimide as the condensing agent, and the reaction was carried out at room temperature for 30 minutes. After the reaction, the solution was diluted with a methanol solution containing formic acid and subjected to analysis by LCMS-8060. We used MRM transitions and analysis methods registered in the LC/MS/MS Method Package for Short-Chain Fatty Acids.

Sample

Fecal samples from SPF and antibiotic-treated mice

Instrument configuration

LC-MS System	: LCMS-8060
Column	: Mastro C18 (2.1 mm I.D.×150 mm, 3 µm)
Injection Volume	: 3 µL

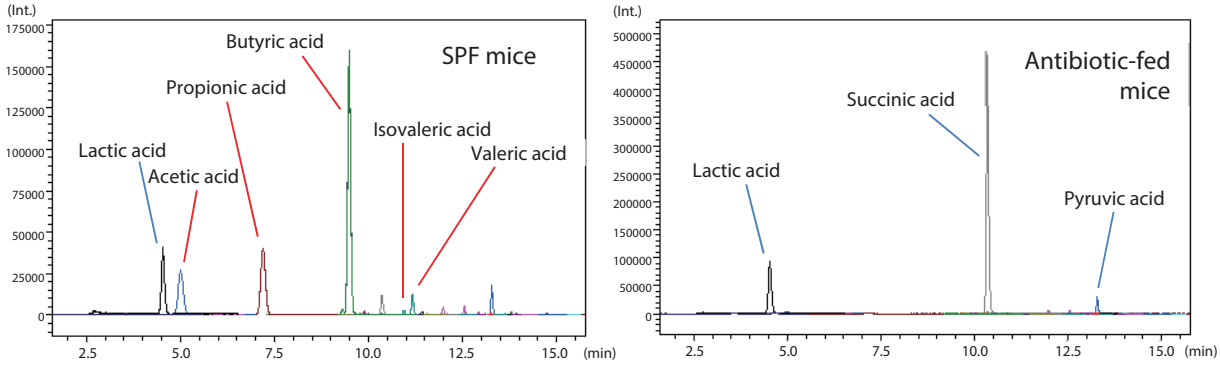


Fig. 14 MRM chromatograms of short-chain fatty acids and organic acids (3-NPH) in fecal samples from SPF and antibiotics-fed mice.

Summary

Using conventionally housed and antibiotic-treated mice, we evaluated the effects of altered gut microbiota on short-chain fatty acids and organic acids in feces. Short-chain fatty acids have been linked to lifestyle-related diseases such as obesity and diabetes and are also associated with improved immune function. This application will be useful for medical researchers researching gut microbiota.

LC/MS/MS Method Package for Short-Chain Fatty Acids

The method package includes a sample preparation protocol in the instruction manual. The complete workflow from sample preparation to measurement and data analysis can be easily implemented in your laboratory.

Product

Analysis of Hydrophilic Metabolites in Saliva

Application

Because short-chain fatty acids are not retained on an ODS column, they are prone to co-elute with sample matrix and are less sensitive to detection by a mass spectrometer. In this application, a derivatization with 3-nitrophenylhydrazine (3-NPH) enhanced retention on an ODS column and improved sensitivity with a mass spectrometer.

benefits

Short-chain fatty acids can be easily analyzed using the LC/MS/MS Method Package for Primary Metabolites Ver. 3 and LCMS-8060NX.

Measurement

For the analysis of short-chain fatty acids, saliva was mixed with 3-NPH (derivatization reagent), pyridine (catalyst), carbodiimide (condensing agent) and 2-ethylbutyric acid (internal standard) and allowed to react for 30 minutes at room temperature. After the reaction, the mixture was diluted 5-fold with a methanol solution containing formic acid. For the analysis of primary metabolites, saliva was diluted five times with ultrapure water and 2-Morpholinoethanesulfonic acid (MES) was added as an internal standard.

Sample

Saliva collected from a healthy adult male

Instrument configuration

LC-MS System	: LCMS-8060NX
Column	: Reversed-phase column

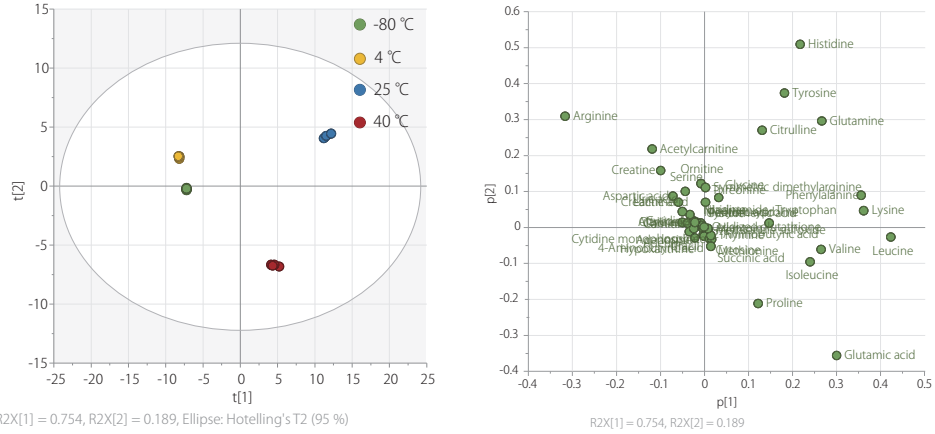


Fig. 15 Principal component analysis of hydrophilic metabolites

Summary

The LCMS-8060NX features enhanced sensitivity with IonFocus. The LC/MS/MS Method Packages for Primary Metabolites and Short-Chain Fatty Acids, together with the AI-learned Peakintelligence peak-picking algorithm, allowed a comprehensive analysis of hydrophilic metabolites in saliva.

LC/MS/MS Method Package for Primary Metabolites Ver. 3

LC and MS parameters have been optimized for 200 compounds. Two methods with different columns and reagents are available, and you can choose the one that best suits your needs.

Product

Metabolic Map Analysis of 196 Eicosanoid Metabolites

Application

A wide variety of eicosanoid metabolites are known to exist, making method development a complicated process. For this reason, Shimadzu offers the LC/MS/MS Method Package for Lipid Mediators Ver. 3 for simultaneous analysis of 196 eicosanoid metabolites.

benefits

A metabolic map for the LC/MS/MS Method Package for Lipid Mediators Ver. 3 is available. With quantitative values displayed on a metabolic map, the reactivity of involved metabolic enzymes can be studied.

Measurement

300 µL of a methanol solution containing 0.1% formic acid and 10 µL of an 18-component internal standard solution were added to 30 µL of the sample and shaken for about 3 minutes. After centrifugation, the supernatant was diluted three-fold with 0.1% formic acid water and loaded on to a solid-phase extraction cartridge. The collected eluate was dried and reconstituted in 30 µL of methanol, and 5 µL was injected for LC-MS analysis. Each sample was analyzed three times.

Sample

Human plasma and serum

Instrument configuration

LC-MS System	: LCMS-8060NX
Column	: Kinetex® C8 (2.1 mm I.D.×150 mm, 2.6 µm)

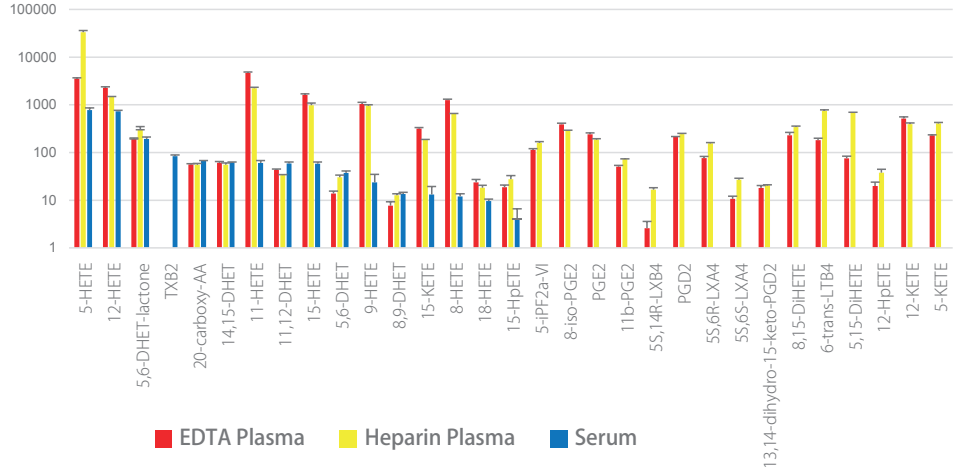


Fig. 16 Quantitative profiling of 31 arachidonic acid metabolites in human plasma and serum
(The vertical axis shows the area ratio to the internal standard multiplied by 1000.)

Summary

A metabolic map of 196 eicosanoid metabolites was developed and applied in a comparative analysis of metabolites in human plasma and serum. A total of 68 metabolites were detected in plasma and serum. Using this analytical tool, the enzymes involved in the detected metabolites were easily identified and can be further studied for reactivity.

LC/MS/MS Method Package for Lipid Mediators Ver. 3

LC and MS conditions are optimized and ready-to-use for simultaneous analysis of 214 components, including 196 metabolites in the arachidonic acid cascade and 18 internal standards. All the compounds can be measured in a mere 20 minutes.

Product

Shimadzu Corporation
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