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Application News

No. C233

LC-MS

Metabolome Analysis of Hydrophilic Metabolites in Saliva Using LCMS™-8060NX Triple Quadrupole Mass Spectrometer

The human mouth harbors as many as 9 billion bacteria of around 700 species that produce short-chain fatty acids similar to enteric bacteria. It is becoming clear that the short-chain fatty acids produced by oral bacteria have not only a harmful effect on periodontal tissue but also play a role in maintaining the oral environment. One example is anaerobic gram-negative bacteria in the mouth that produce large quantities of short-chain fatty acids (especially butyric acid) and are the main cause of periodontal disease.

Metabolome analysis is recently gaining popularity as a technique for the comprehensive study of metabolites in living organisms. Metabolomics, as a field of academic study, performs comprehensive analyses of low molecular weight metabolites such as amino acids and organic acids that arise from cellular activity to reveal differences between multiple samples. An exhaustive analysis is easier to perform by metabolome analysis compared with other types of omics analysis due to the relatively small number of analytes of interest.

This report describes an example metabolome analysis of saliva performed using a high-performance liquid chromatograph mass spectrometer (LC-MS-MS). Saliva is known to contain both oral bacteria and the metabolites produced by oral bacteria. The conditions under which saliva is stored prior to analysis can therefore affect the amounts of metabolites present in saliva due to the activity of these oral bacteria. We used metabolome analysis to investigate the effects of storage temperature on metabolites in saliva.

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Samples

Saliva obtained from one healthy adult male was centrifuged at 12000 rpm for 10 minutes. The resulting supernatant was divided between four microtubes and stored at -80, 4, 25, and 40 °C, respectively, for three days prior to analysis.

Pretreatment

As pretreatment for analysis of short-chain fatty acids and organic acids, samples were derivatized with 3-nitrophenylhydrazine (3-NPH) to improve ODS column retention and MS detection sensitivity. 3-NPH (derivatizing agent), pyridine (catalyst), carbodiimide (condensing agent), and 2-ethylbutyric acid (internal standard) were added to saliva and reacted at room temperature for 30 minutes. After this reaction, the saliva was diluted 5-fold with methanol solution containing formic acid.

As pretreatment for simultaneous analysis of hydrophilic metabolites, saliva was diluted 5-fold with ultra-pure water. During dilution, an internal standard (2-morpholinoethanesulfonic acid (MES)) was added to a concentration of 1 µmol/L.

Analytical Conditions

Short-chain fatty acids and organic acids were analyzed using LCMS-8060NX and an LC/MS/MS Method Package for Short Chain Fatty Acids. This method can analyze six short-chain fatty acids (acetic acid, propionic acid, butyric acid, etc.) and 16 organic acids related to central metabolic pathways. Table 1 shows the analytical conditions used for HPLC and MS.

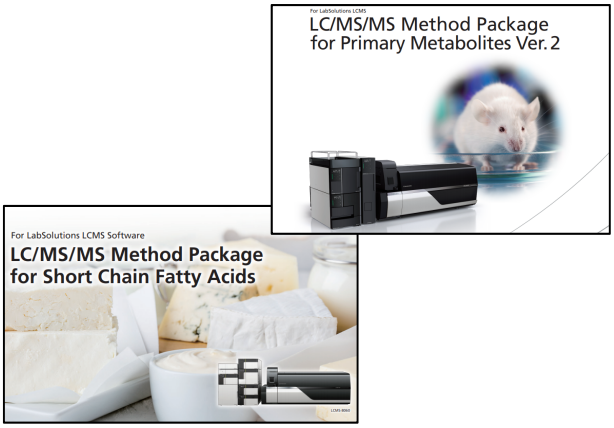
Table 1 Analytical Conditions
(Analysis of Short-Chain Fatty Acids and Organic Acids)

[HPLC conditions] (Nexera™ X3)		
Column	:	Reversed-phase column
Mobile phases	:	A) 0.1 % Formic acid in water
		B) Acetonitrile
Mode	:	Gradient elution
Flow rate	:	0.35 mL/min
Injection volume	:	3 µL
[MS conditions] (LCMS-8060NX)		
Ionization	:	ESI (Positive and negative mode)
Probe position	:	+3 mm
Mode	:	MRM
Nebulizing gas flow	:	2.0 L/min
Drying gas flow	:	10.0 L/min
Heating gas flow	:	10.0 L/min
DL temp.	:	250 °C
Block heater temp.	:	400 °C
Interface temp.	:	300 °C

Simultaneous analysis of hydrophilic metabolites was performed using LCMS-8060NX and the ion-pair free LC/MS/MS method included in the LC/MS/MS Method Package for Primary Metabolites Ver. 2. This method can simultaneously analyze 97 hydrophilic metabolites, including metabolites important for metabolome analysis in the life sciences such as amino acids, organic acids, nucleosides, and nucleotides. Table 2 shows the analytical conditions used for HPLC and MS.

Table 2 Analytical Conditions
(Simultaneous Analysis of Hydrophilic Metabolites)

[HPLC conditions] (Nexera X3)		
Column	:	Reversed-phase column
Mobile phases	:	A) 0.1 % Formic acid in water
		B) 0.1 % Formic acid in acetonitrile
Mode	:	Gradient elution
Flow rate	:	0.25 mL/min
Injection volume	:	3 µL
[MS conditions] (LCMS-8060NX)		
Ionization	:	ESI (Positive and negative mode)
Probe position	:	+3 mm
Mode	:	MRM
IonFocus voltage	:	±2 kV
Nebulizing gas flow	:	3.0 L/min
Drying gas flow	:	10.0 L/min
Heating gas flow	:	10.0 L/min
DL temp.	:	250 °C
Block heater temp.	:	400 °C
Interface temp.	:	300 °C



Sensitivity Improvement by IonFocus™ Unit

When analyzing a sample that contains a large amount of sample matrix, such as a biological or food sample, matrix effects can be reduced by moving the ion source away from the MS inlet. However, this also reduces the amount of ions that enter the MS and hence reduces sensitivity. The IonFocus unit (Fig. 1) in LCMS-8060NX uses focus electrodes to more efficiently transport ions only into the mass spectrometer while removing unwanted neutral particles. This allows the ion source to be moved away from the MS inlet without reducing sensitivity, and ensures both high-sensitivity analysis and excellent instrument robustness. We checked the effect of the IonFocus unit on hydrophilic metabolites detected in saliva and confirmed no loss of sensitivity and even an approx. 1.4-fold increase in sensitivity on average, even when the ion source was moved away from the MS inlet. Fig. 2 shows an example of this sensitivity improvement.

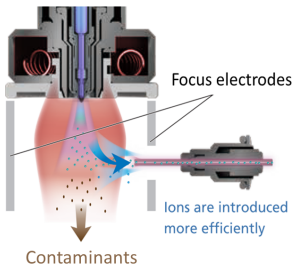


Fig. 1 IonFocus Unit Concept

Fig. 2 Sensitivity Improvement by IonFocus Unit

Peak Integration by Peakintelligence™

Peak integration was performed on the detected peaks using Peakintelligence, optional software for LabSolutions Insight™. Peakintelligence incorporates an AI system that is taught how peak integration is performed by experienced analysts, which allows a user to perform peak analysis at an expert level. Fig. 3 shows how Peakintelligence detects and processes a peak correctly even when close to another component peak. Peakintelligence reduces the number of peaks that are detected incorrectly or go undetected, and reduces the time it takes to verify and correct results obtained from peak integration.

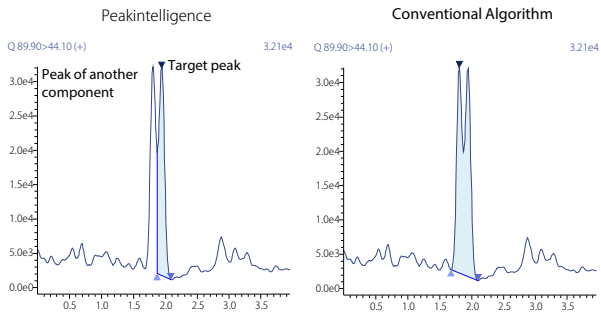


Fig. 3 Peak Integration by Peakintelligence

Metabolome Analysis

Analyzing the saliva sample for short-chain fatty acids and organic acids detected 6 short-chain fatty acids and 14 organic acids. Simultaneous analysis for hydrophilic metabolites detected 50 components that were mainly amino acids, organic acids, and nucleoside metabolites. Principal component analysis was performed by SIMCA® 16 software using the peak area ratio of each component relative to an internal standard. Fig. 4 shows a score plot and loading plot for analysis of short-chain fatty acids and organic acids, and Fig. 5 shows a score plot and loading plot for simultaneous analysis of hydrophilic metabolites.

The first principal component score confirms that storage temperature had an effect, with saliva stored at 40 °C distinguished by the presence of succinic acid and glutamic acid and saliva stored at 25 °C distinguished by the presence of pyruvic acid, histidine, and tyrosine. Saliva that was stored at 4 °C and -80 °C tended to contain the same short-chain fatty acids, organic acids, amino acids, and nucleoside metabolites and showed no substantial quantitative difference between samples for almost any component.

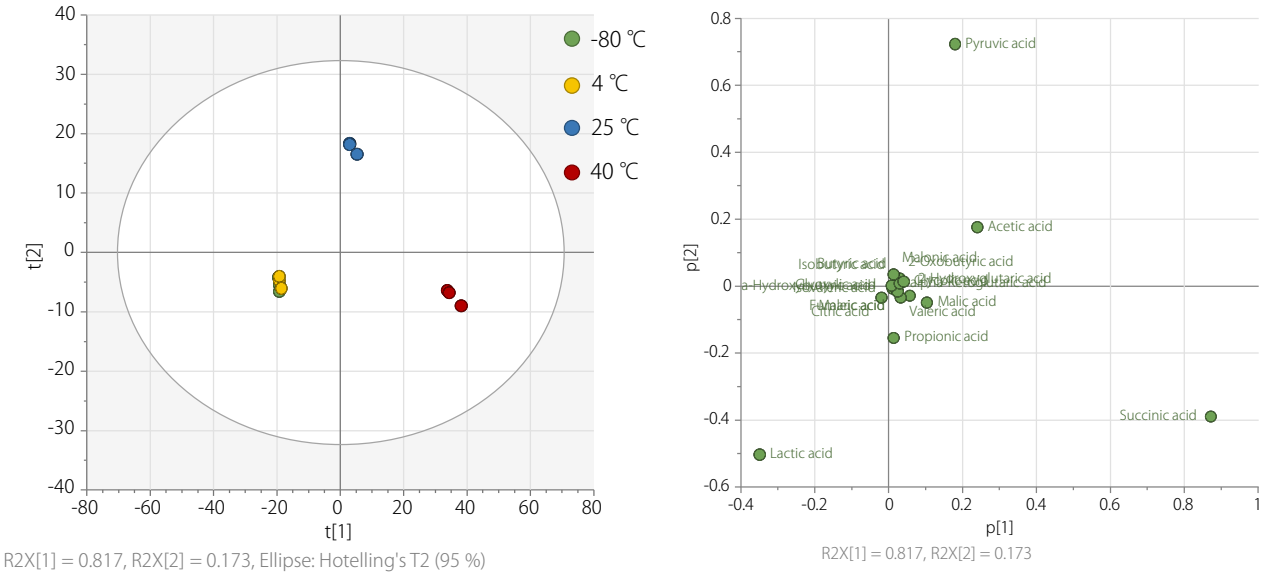


Fig. 4 Principal Component Analysis Results of Short-Chain Fatty Acids and Organic Acids

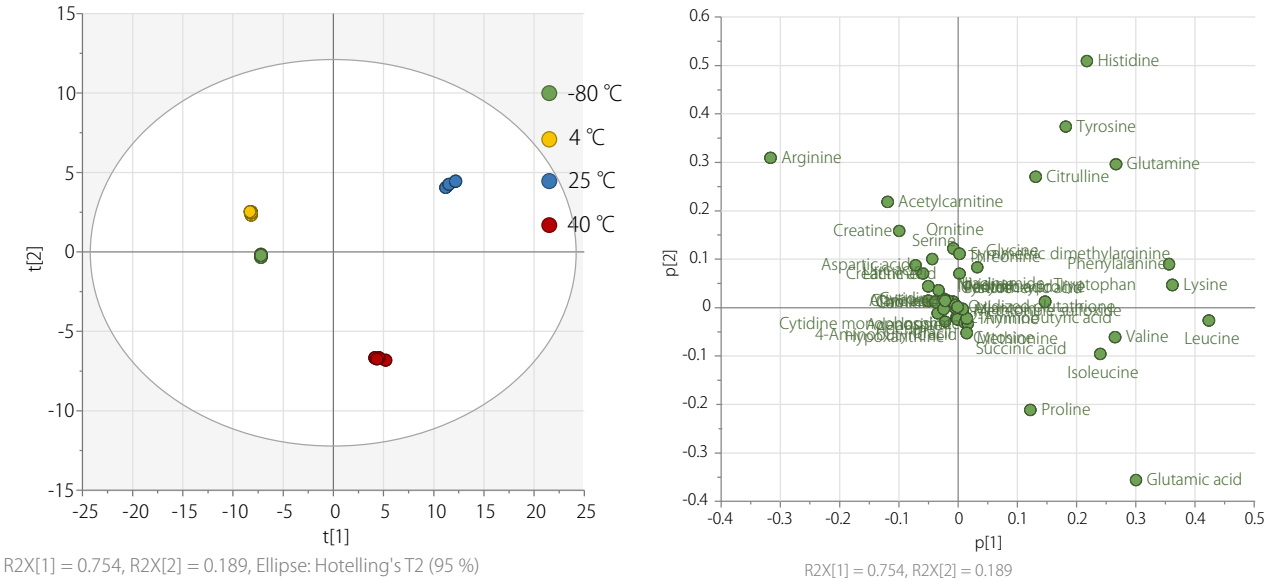


Fig. 5 Principal Component Analysis Results of Hydrophilic Metabolites

Fig. 6 shows the variation in peak area ratio after storage at different temperatures for metabolites with large quantitative differences between samples. The charts show amounts of amino acids such as arginine, glutamic acid, and histidine, and organic acids such as lactic acid, pyruvic acid, and succinic acid vary substantially depending on the storage temperature of the saliva. A possible cause of this variation is that oral bacteria in saliva consumed and/or produced these components during storage.

Summary

This report shows that metabolome analysis of saliva by LC/MS/MS can be used for an exhaustive analysis of hydrophilic metabolites produced by oral bacteria. This analytical method could be extremely effective for metabolome analysis in the study of oral flora.

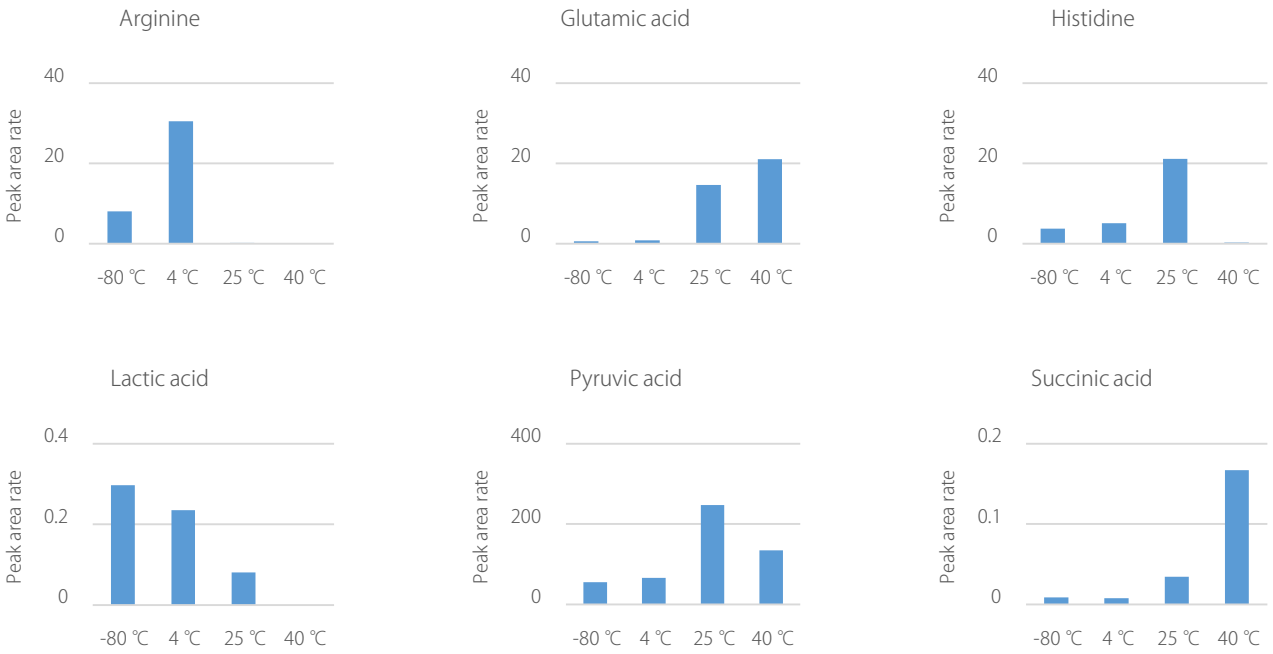


Fig. 6 Profiles of Short-Chain Fatty Acids and Organic Acids in Saliva

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