Non-Targeted Analysis of Metabolites in Sake by using Gas Chromatograph - Time-of-Flight Mass Spectrometer (GC-TOFMS)

Product used: Mass Spectrometer (MS)

Introduction

Sake is a Japanese alcoholic beverage made primarily from rice, kome-koji, and water. In addition to alcohol, sake contains amino acids, organic acids, and sugars, which produce a variety of flavors and tastes. Sake is also classified as 'Daiginjo-shu', 'Junmai-shu', and 'Honjozo-shu', depending on the rice polishing ratio and whether or not distilled alcohol is added and other types of sake are classified as 'futsu-shu'[1]. Amino acids, organic acids, and sugars in sake are thought to originate from the metabolism of microorganisms such as kome-koji and yeast. Therefore, it may be possible to classify these types of sake by metabolomic analysis.

Gas chromatograph-mass spectrometer (GC-MS) is widely used for metabolomics due to their rich database, easy operation, and high reproducibility of measurements. Measurement targets of GC-MS are volatile compounds. However, derivatization processes such as trimethylsilylation can be used to measure highly polar metabolites like sugars and amino acids. Additionally, in the field of metabolomics, "unknown compounds" that are not registered in the library database (DB) are sometimes detected. In this case, the molecular formula of even an unknown compounds can be determined by using a time-of-flight mass spectrometer (TOFMS) as a mass spectrometer and performing an "integrated analysis" that combines electron ionization and soft ionization[2]. Furthermore, by using automated structure analysis software named "msFineAnalysis AI" which uses artificial intelligence (AI) to predict EI mass spectra from chemical structures, it is possible to estimate the structural formula[3].

In this MSTips, we will report the results of non-targeted analysis of metabolites in sake using gas chromatograph time-of-flight mass spectrometry (GC-TOFMS).

Experimental

Derivatization

Four types of sake with different commercial classifications (Daiginjo-shu, Junmai-shu, Honjozo-shu, and Nigori-shu) were used as samples. About Nigori-shu it was centrifuged (4 °C, 10 min, 12,000 rpm), and only the supernatant was used as sample. We also prepared a Quality Check (QC) sample in which all samples were mixed in equal amounts. To 20 µL of each sample, 10 µL of 1 mg/mL sinapic acid methanol solution was added as an internal standard and mixed. Next, they were dried in an evaporator and left overnight in a desiccator. The next day, 100 µL of 20 mg/mL methoxyamine hydrochloride pyridine solution was added to each sample and shaken with a thermoshaker (30 °C, 90 min, 1200 rpm). Thereafter, 50 µL of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was added, and the mixture was heated and shaken with a thermoshaker again (37 °C, 30 min, 1200 rpm).

GC-MS measurement

A GC-TOFMS (JMS-T2000GC AccuTOF™ GC-Alpha, JEOL Ltd.) was used for the measurement. We used both EI and field ionization (FI) modes with a combination EI/FI/FD ion source. For each sample, n=3 measurements were performed using EI , and n=1 measurements were performed using FI. Other detailed conditions are shown in Table 1.

Data processing

EI data were converted to the GC-MS common format netCDF. These data were analyzed using the metabolomics analysis program MS-DIAL (Ver. 4.9, RIKEN) to detect peaks and identify compounds. For compound identification, msFineAnalysis AI (JEOL Ltd.) , which can integrate and analyze EI and FI data, was used to identify peaks with higher accuracy and prevent misidentification. After compound identification, we normalized each peaks by internal standard and LOWESS, and peak intensity of each samples was exported. Multivariate analysis was then performed in MetaboAnalyst 6.0 [4]. For multivariate analysis, principal component analysis and hierarchical cluster analysis were performed after normalization with AutoScaling.

Table 1 GC-MS measurement condition

GC	
Column	HP-5MS UI (Agilent) 30 m×0.25 mm I.D., df=0.25 µm
Inlet	250 °C, For multivariate analysis:Split 50:1, For compound identification: Split 5:1
Oven	80 °C (2 min) →15 °C/min→325 °C/min (7 min)
Carrier flow	He, 1.0 mL/min (Constant Flow)

MS	
Ion Source	EI/FI/FD combination ion source
Ionization	EI+:70 eV, 300 µA, FI+:-10 kV, 6 mA
m/z Range	For multivariate analysis: *m/z* 33 - 800 For compound identification: m/z 33 - 1600

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Results and Discussion

TICC comparison results

Figure 1 shows TICC of EI data. Many compounds specific to sake were detected in each sample, including sugar alcohols such as glycerol and glyceryl glucoside, sugars such as glucose, amino acids, organic acids. Since the peak intensity of each component differed from sample to sample, multivariate analysis was performed.

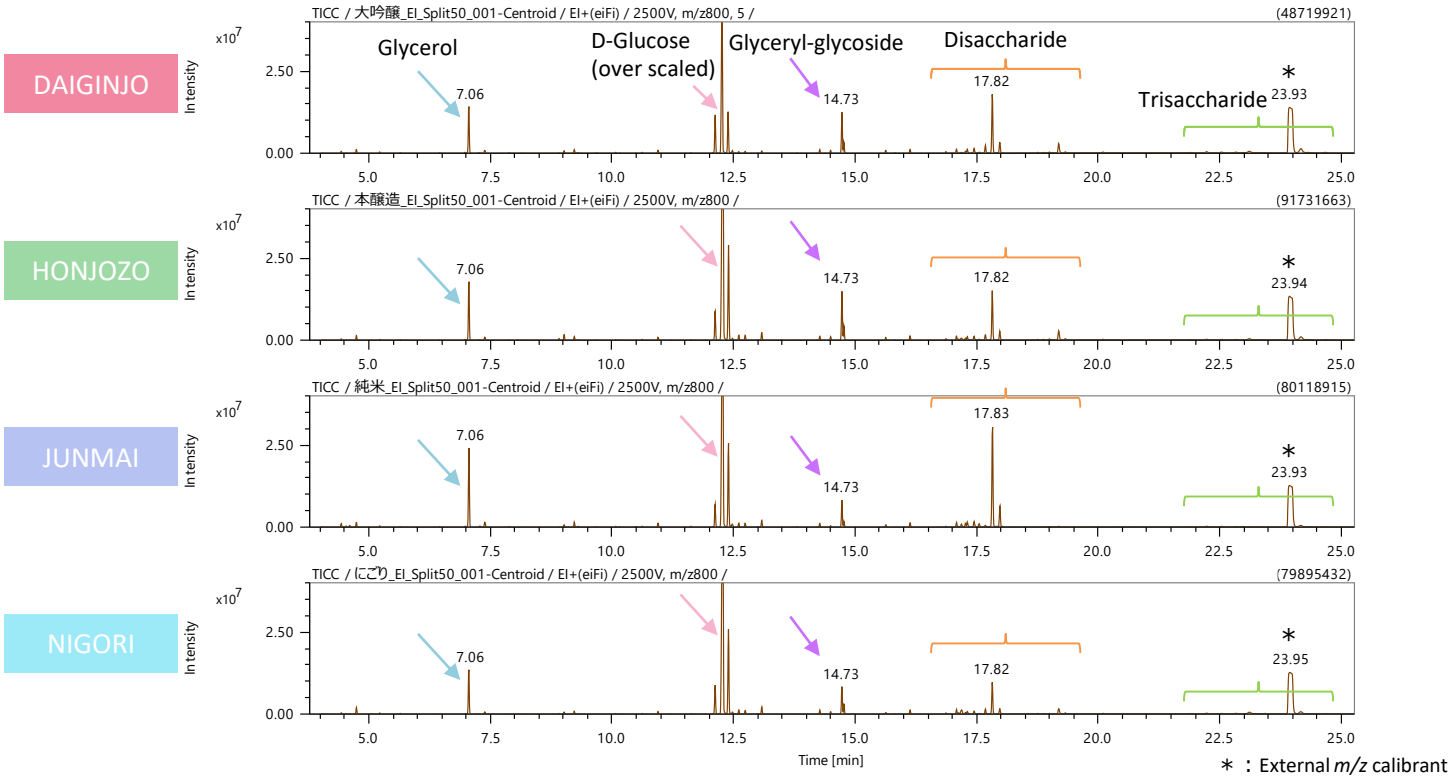

Figure 1 TICC of metabolites in sake

Principal component analysis

Figure 2 shows the results of Principal Component Analysis (PCA) performed by MetaboAnalyst on the data normalized by LOWESS and internal standard. The score plot in Figure 2-A shows that the positive and negative scores of the first principal component (PC1) can classify Junmai-shu and others. This may indicate differences due to the whether or not distilled alcohol added during sake production. In addition, it was possible to classify Daiginjo-shu and others based on the positive and negative scores of the second principal component (PC2). This is presumed to be due to the difference in the rice polishing rate. Note that the classification of Nigori-shu was not written on the bottle. However, Nigori-shu was plotted near Honjozo-shu and may have similar characteristics.

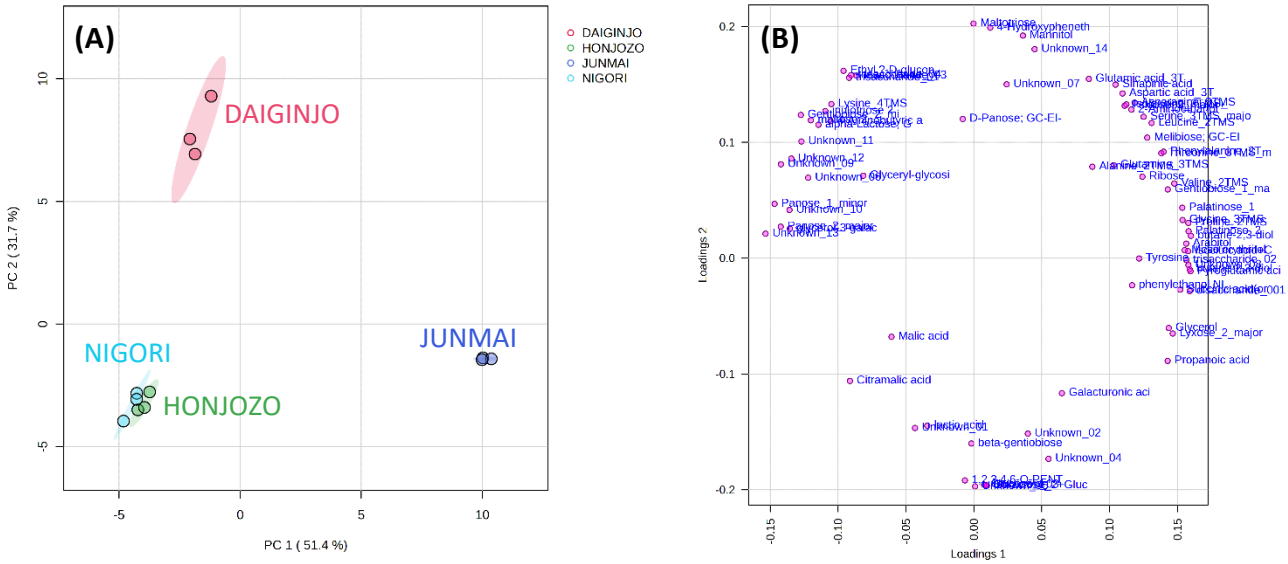

Figure 2 Principal component analysis results (A) Score plot, (B) Loading plot

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Hierarchical Cluster Analysis (HCA) results

A heat map of the Hierarchical Cluster Analysis (HCA) result is shown in the figure 3. The heat map shows the abundance of each component by color (red: high, blue: low), making it possible to visualize the compounds characteristic of each sample. For example, peak intensity comparison result of proline and valine compounds characteristic in Junmai-shu was shown in the Figure 4. The amino acids proline and valine are know bitterness component of sake[5]. Junmai-shu is made only with rice, kome-koji, and water, and does not contain distilled alcohol. Therefore, it is generally said to have a taste that allows you to feel the flavor and richness of rice. It is possible that valine and proline are related to the unique taste of Junmai-shu measured this time.

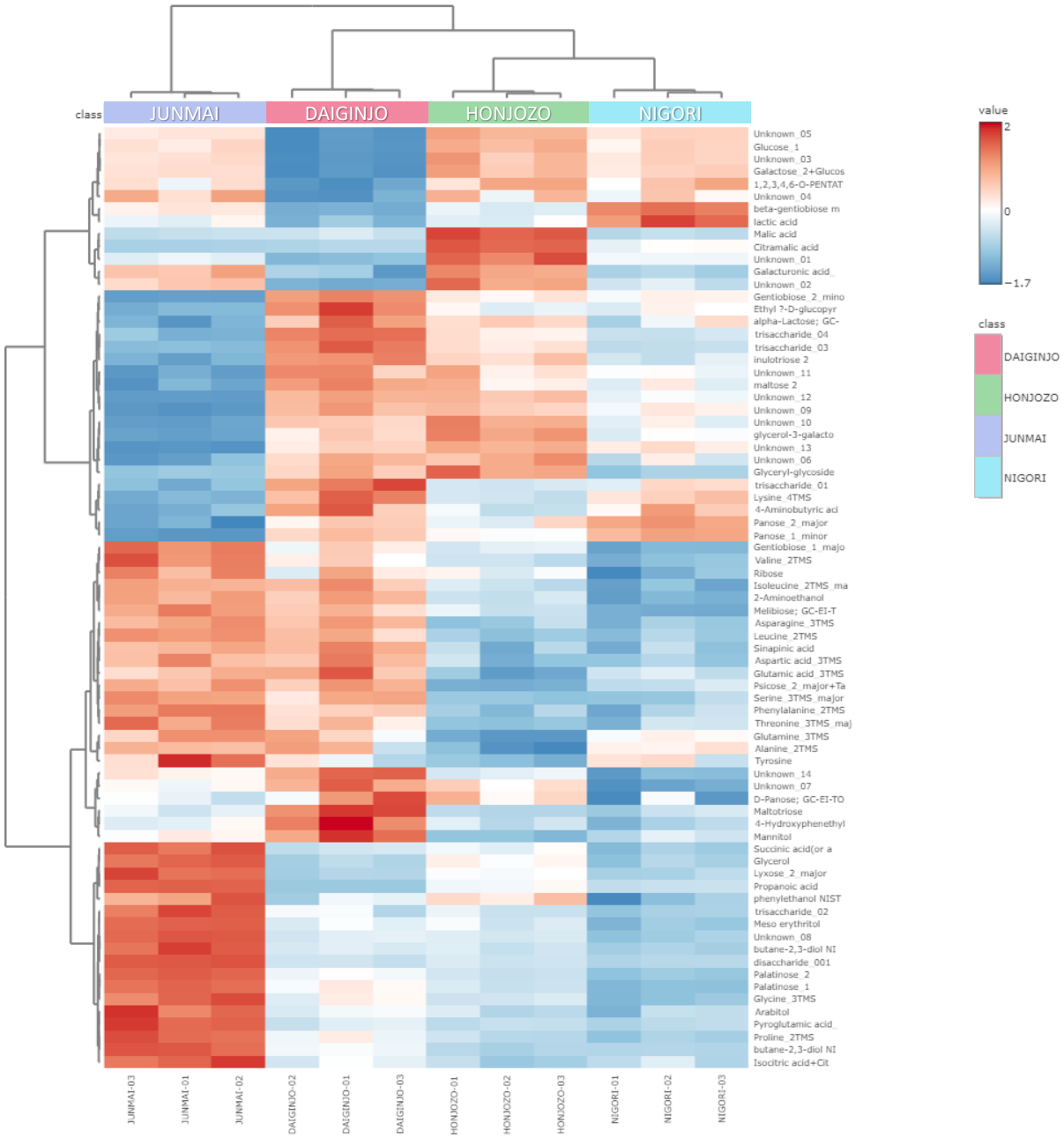

Figure 3 Hierarchical Cluster Analysis (HCA) results

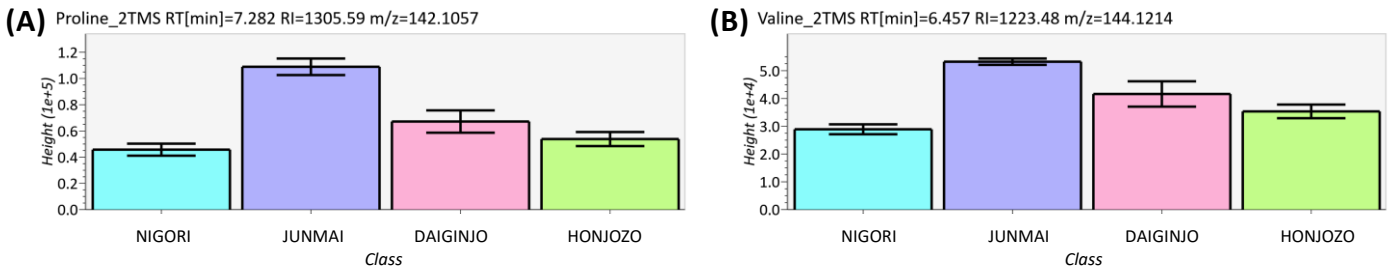

Figure 4 Peak intensity comparison results
(A) Proline, (B) Valine

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AI structure analysis result of unknown compound

In this work, several compounds that were not registered in the library DB were detected. For example, we will explain the integrated analysis and structure analysis result for peak RT 17.82 min in Figure 2. Mass spectra of this compound in Honjozo-shu were shown Figure 5. Molecular ion m/z 947 was detected only by soft ionization FI and not by EI. The molecular formula was estimated to be $C_{37}H_{89}NO_{11}Si_8$ from the elemental composition estimation result of the molecular ion in FI. This molecular formula is presumed to be derived from oxime and TMS derivatization of a reducing disaccharide. However, the RI value of the compound with a matching molecular formula and the No. 1 hit in the library DB (β-gentiobiose, octakis(trimethylsilyl) ether, methyloxime (isomer 1)) had a large difference in RI value of Δ169 (Table 2). Therefore, this component was presumed to be an unregistered component in the Library DB, although its molecular formula is $C_{37}H_{89}NO_{11}Si_8$. Figure 6 indicates AI structure analysis result of this compound. The first candidate was an isomaltulose derivative of a disaccharide. Since isomaltulose derivatives have not been registered in the NIST library, it is possible that this component is isomaltulose.

In this report, FI was able to clearly detect molecular ion even for high-mass components that are difficult to analyze by metabolomics analysis using EI. Furthermore, it was possible to estimate the structure formula even when it was not yet registered in the library DB.

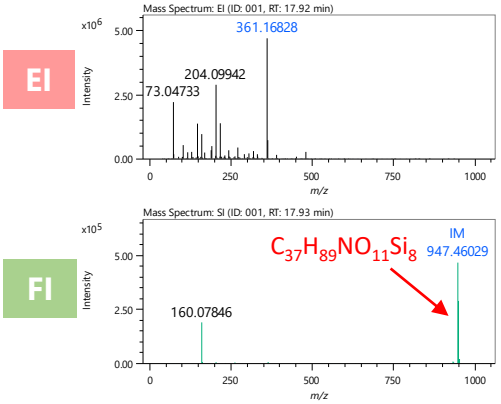

Figure 5 Mass spectra of unknown compound

Table 2 Integrated qualitative analysis result of unknown compound

Elemental Composition of IM (m/z: 947.46029)							Integration	Library Search											
#	Formula	DBE	Calculated m/z	Mass Error [mDa]	Isotope Matching	Coverage	Adduct/Loss	#	Library Name	CAS #	Lib.	Similarity	Reverse Similarity	Lib. RI [iu]	ΔRI [iu]	Formula	DBE	MW	EI Base Peak (Lib.)	Coverage
A01	C36 H93 N O8 Si10	1.0	947.45884	1.45	0.63	100														
								L24	Dehydrologanin, 4TMS derivative	-	main lib	774	794	2937	32	C29 H56 O10 Si4	6.0	676	361	94
								L27	Ketologanic acid, 5TMS derivative	-	main lib	770	792	2976	7	C31 H62 O10 Si5	6.0	734	361	94
								L29	Sweroside, 4TMS derivative	-	main lib	765	789	2991	22	C28 H54 O9 Si4	6.0	646	73	94
								L37	Methyl (1S)-7-hydroxy-7-methyl-1-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-4a,5,6,7a-tetrahydro-1H-cyclopenta[c]pyran-4-carboxylate, 5TMS derivative	-	main lib	758	781	2944	25	C32 H66 O10 Si5	5.0	750	73	100
A02	C37 H89 N O11 Si8	2.0	947.45843	1.86	0.86	100	none	L01	β-Gentiobiose, octakis(trimethylsilyl) ether, methyloxime (isomer 1)	-	main lib	924	926	2800	169	C37 H89 N O11 Si8	2.0	947	204	100

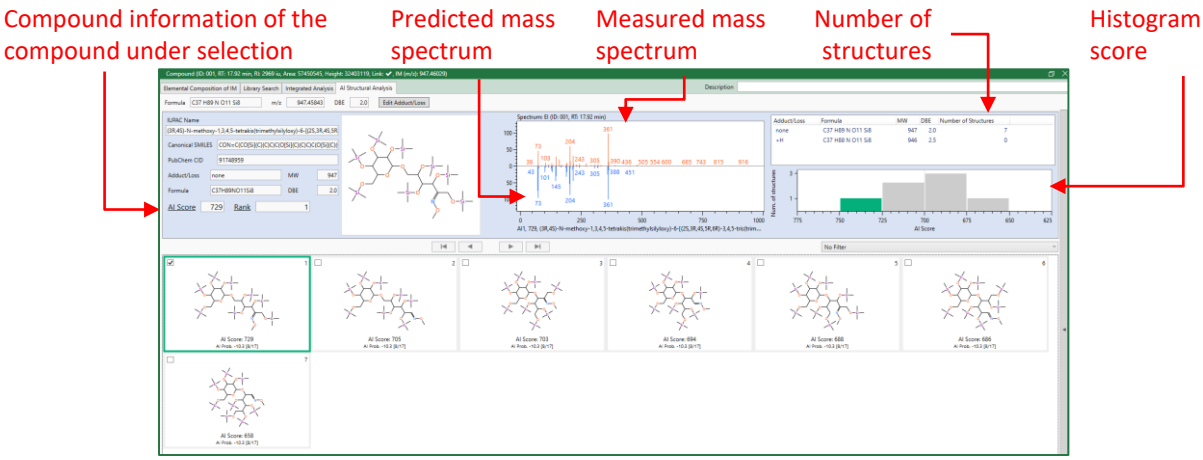

Figure 6 AI structure analysis result of unknown compound

Conclusions

In this MSTips, we introduced the results of non-targeted analysis of metabolites in sake using gas chromatograph time-of-flight mass spectrometry (GC-TOFMS). Even highly polar metabolites that are difficult to measure using conventional GC-MS measurements could be easily measured by TMS derivatization. Additionally, multivariate analysis has made it possible to classify sake. Even for TMS derivatives of disaccharides, which are difficult to measure by GC-QMS due to their large molecular weights, we were able to measure molecular ions using GC-TOFMS and FI . For compound that have not been registered in the library DB, we were able to estimate their structural formula using the unknown compound structure analysis software msFineAnalysis AI.

From the above results, it was confirmed that GC-TOFMS and msFineAnalysis AI are effective in the field of metabolomics using GC-MS.

Reference

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