Application Note

Application Note No.10 (Lifescience)

Lifescience

Application of Metabolomics Techniques using LC/MS and GC/MS Profiling Analysis of Green Tea Leaves

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1. Introduction

Metabolomics, or metabolome analysis, refers to the in-depth analysis of metabolites in a biological organism, and in recent years has been applied across a wide range of fields, including diagnostic biomarker search and etiological analysis in the field of medical treatment, marker search for indications of drug efficacy and toxicity in the field of drug manufacture, and quality control and quality prediction in the field of food products, to name a few[1)].

A great number of metabolites spanning a wide range of chemical characteristics have become targets for metabolome analysis. For this reason, separation analytical techniques are often used for comprehensive analysis of these substance, with such techniques as gas chromatography-mass spectrometry (GC/MS), liquid chromatography-mass spectrometry (LC/MS), and capillary electrophoresis mass spectrometry being reported.

This Application Note presents the profiling of green tea leaves as an example of one application of the metabolome analysis technique using LC/MS and GC/MS. A search of the substances believed to play roles in product quality was conducted, and a quality prediction model was constructed based on the results.

2. A Typical Metabolomics Research Flow

Metabolome analysis starts with the design of experiment plan, then proceeds to sample procurement and data acquisition using analytical instrumentation, and finally analysis of the data using a variety of statistical techniques[1)].
Fig. 1 shows the typical scheme employed in metabolome analysis.

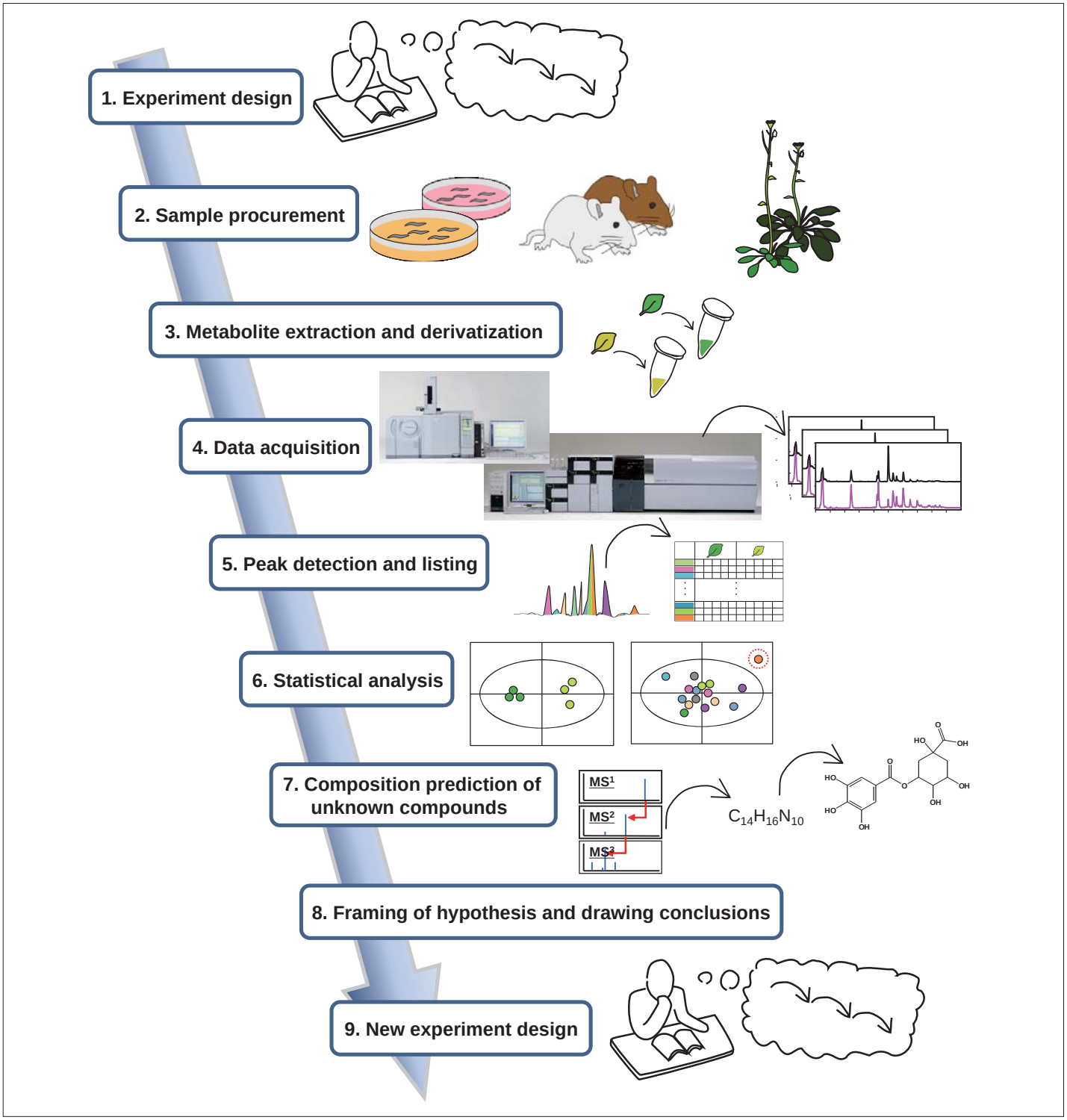


Fig. 1 Example of Metabolome Analysis Scheme

3. Metabolome Analysis and Analytical Method

A great number of metabolites spanning a wide range of chemical characteristics have become targets for metabolome analysis. Therefore, separation by gas chromatography or liquid chromatography and detection using mass analysis of these metabolites is typically conducted using combination technologies such as gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-mass spectrometry (LC/MS), etc. as powerful means of analysis. The profiling and identification of such a complex metabolites can be efficiently performed through the effective use of GC/MS and LC/MS.

3-1 Metabolome Analysis by GC/MS

GC, compared to other types of chromatography, provides excellent separation, and allows efficient and precise separation of structurally similar volatile metabolites and metabolites that can be converted into volatile derivatives through derivatization. GC/MS offers both high resolution by GC and high-sensitivity MS detection.
In addition, identification of compounds is possible using the mass spectrum specific to each compound obtained using the EI (Electron Ionization) method in MS analysis.

Metabolite identification is greatly facilitated by creating a database of mass spectra and standardized retention indices of retention times. Use of Shimadzu's GC/MS metabolite database and mass spectral libraries allows identification of many metabolites.
GC/MS can be used for analysis of low-polarity volatile metabolites of fats and esters, and high-polarity metabolites of amino acids and organic acids converted into volatile derivatives.

Metabolome Analysis by GC/MS

- ✓ Analytical targets include low-polarity volatile metabolites of fats and esters, and high-polarity metabolites of amino acids and organic acids converted into volatile derivatives
- ✓ Detection and identification of many peaks using high-resolution and high-speed scanning
- ✓ Compound search is available using Shimadzu GC/MS metabolite database and NIST mass spectral libraries

Gas Chromatograph Mass Spectrometer
GCMS-QP2010 Ultra
<Features>
High-sensitivity, high-speed scan measurement
Shortened wait time with Easy-sTop function
Energy conservation using Ecology Mode

Shimadzu GC/MS Metabolite Database
(Amino acids, fatty acids, and organic acids)
<Features>
Includes information on about 300 metabolites
Database with retention indices

3.2 Metabolome Analysis by LC/MS

LC has features making it applicable for analysis of a wide range of low-to high-polarity metabolites, including non-volatile compounds that cannot be analyzed by GC and metabolites that are difficult to derivatize. Though LC does not achieve the excellent resolution possible with GC, ultra-high speed, high-resolution analysis has become possible with recent advances in technology allowing production of micro-particle column packing material and instrument performance. This LC/MS consisting of an ultra-high-speed LC combined with a mass spectrometer greatly contributes to achieving high sample throughput in metabolome analysis. Moreover, this high-throughput analysis can be expected to provide the secondary benefit of maintaining the stability of measurement samples and mass spectrometric results, in addition to shortening measurement time.

Due to the extremely narrow peaks generated in ultra-high-speed LC, high-speed data handling performance is required in the MS segment of the analysis. The Shimadzu LCMS-2020 and LCMS-IT-TOF are suitable MS instruments for ultra-high-speed LC, as they offer analysis with high-speed scanning and high-speed polarity switching. The Shimadzu ultra-high-speed LC "Prominence UFLC" combined with these either of these MS instruments results in a system that is suitable for metabolome analysis. Furthermore, highly accurate composition prediction and the acquisition of partial structural information are possible with LCMS-IT-TOF due to the accurate mass measurement obtained in MS^n analysis. It is an instrument that offers effective unknown compound search in LC/MS even without the use of a general-purpose spectral library.

Metabolome Analysis by LC/MS

- ✓ Analytical targets include high-polarity metabolites such as amino acids, organic acids, sugars, etc., and secondary metabolites such as catechins, etc.
- ✓ Using ultra-high-speed LC, much higher speed and higher resolution are possible compared to conventional systems
- ✓ Using the LCMS-2020, profiling and marker search are possible.
- ✓ With the LCMS-IT-TOF, in addition to profiling and marker search, unknown metabolite compound information can be obtained.

Ultra-High-Speed Liquid Chromatograph "Prominence UFLC"
Quadrupole Mass Spectrometer "LCMS-2020"

<Features>
High-throughput analysis with high speed and high resolution
Excellent ease of maintenance
High cost performance

Ultra-High-Speed Liquid Chromatograph "Prominence UFLC"
Ion Trap Time-of-Flight Mass Spectrometer "LCMS-IT-TOF"

<Features>
High-throughput analysis with high speed and high resolution
High scanning sensitivity
Unknown metabolite compound information obtained due to high-accuracy MS^n analysis

4. Application of Metabolomics Techniques using LC/MS and GC/MS (Green Tea Leaves)

4.1 Overview

After preparing analysis samples from 9 types of high-grade tea leaves which were highly ranked at a green tea quality selection contest, analysis was conducted using the Prominence UFLC/LCMS-IT-TOF and the GCMS-2010 Plus.
Principal component analysis, a type of multivariate analyses, was conducted using the measurement results to elucidate which compounds contributed highly in determination of the ranking order. Among these, MS^n analysis was performed for typical unknown compounds, and prediction of the candidate compounds was conducted.

4.2 Experiment

4.2.1 Equipment

For LC/MS, the Prominence UFLC/LCMS-IT-TOF was used, and LCMSsolution was used for data processing. For GC/MS, the GCMS-QP2010 Plus was used, and GCMSsolution was used for data processing. Also, for the mass spectral libraries, the NIST 2008 Mass Spectral Library and the Shimadzu Metabolite Database were used, and Umetrics SIMCA-P was used as the multivariate analysis software.

4.2.2 Samples and Preparation

Of the green tea leaves ranked at a green tea quality selection contest held at Prefecture A, 109 leaves ranked 5, 10, 15th …, 45th place were used. The green tea leaves were processed according to a previously reported preparation procedure[2) 3)]. (Fig. 2, Fig. 3)

4.2.3 Analysis

Examination of the analytical conditions (Tables 1-2) was conducted by verifying the separation of standard solution compounds that are known to exist in green tea.

4.2.4 Data Analysis

In the case of LC/MS, the peaks were detected from the results of MS1 analysis, and a peak list was generated using the respective peak area values.
In GC/MS, the peaks were detected from the TIC and mass chromatograms, and identification was conducted using the GC/MS metabolite database and the NIST mass spectrum library. The peak list was generated based on the ratios of the areas of the identified peaks areas and that of the internal standard (ribitol).
Multivariate analysis was conducted in conjunction with these peak lists.

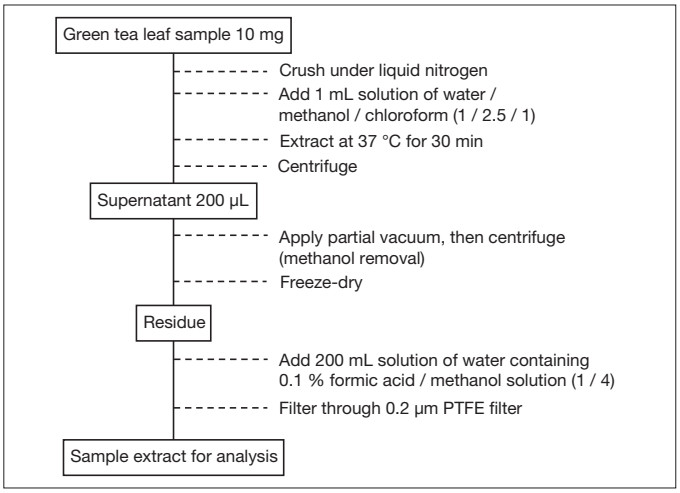


Fig. 2 Sample Preparation Procedure for LC/MS Analysis

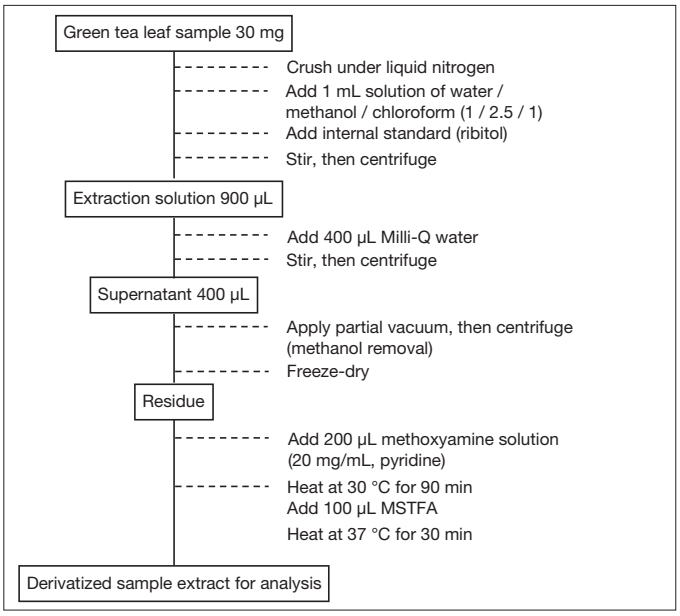


Fig. 3 Sample Preparation Procedure for GC/MS Analysis

Table 1 LC/MS Analytical Conditions

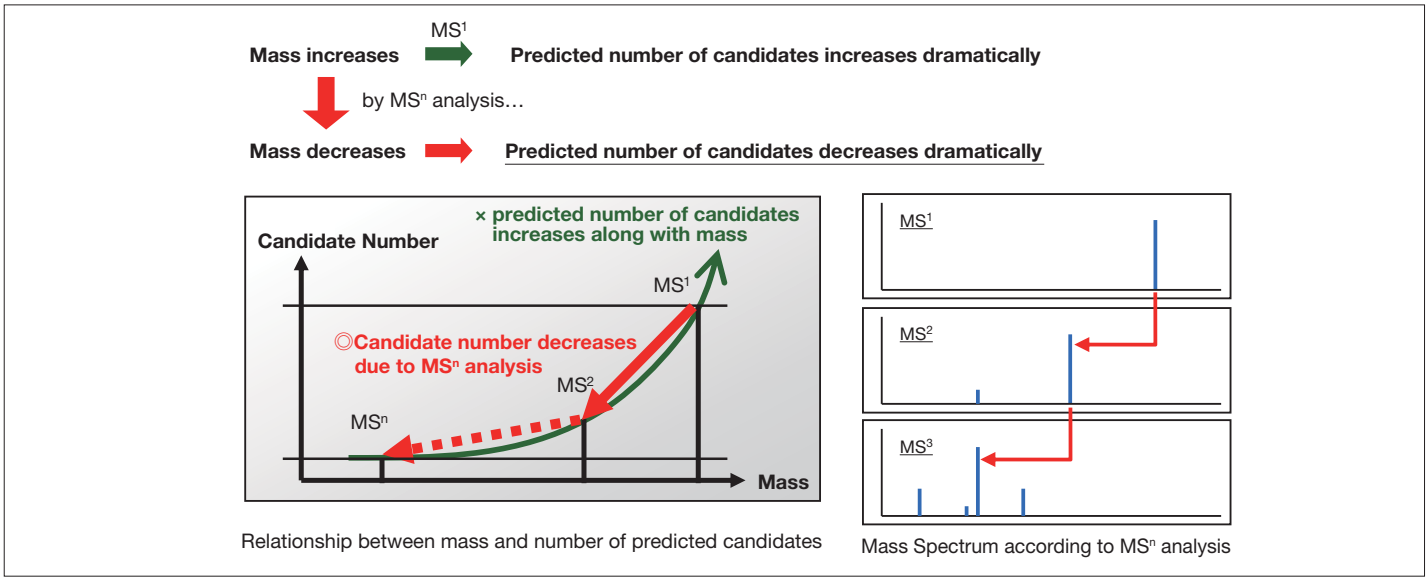
Instrument	Prominence UFLC series, LCMS-IT-TOF
[LC Conditions]	
Column	Shim-pack XR-ODS (50 mm L. × 2.0 mm I.D., 2.2 µm)
Mobile phase A	0.1 % formic acid aqueous solution
Mobile phase B	Methanol
Gradient program	B concentration 2 % (0 min) - B 60 % (10 min) - B 98 % (10.01 – 14 min) - B 2 % (14.01 min) - (STOP) (19 min)
Flow rate	4 mL/min
Column temperature	40 °C
[MS Conditions]	
Ionization mode	ESI (+ / - switching)
Measurement range	*m/z* 100 - 1000
CDL temperature	200 °C
BH temperature	200 °C

Table 2 GC/MS Analytical Conditions

Instrument	GCMS-QP2010 Plus, AOC-20i + s (auto injector)
[GC Conditions]	
Column	Rtx®-5MS (30 m L. × 0.25 mm I.D. df = 0.25 mm, Restek Corp.)
Injection temperature	250 °C
Column temperature	80 °C (2 min) - (15 °C/min) - 320 °C (20 min)
Injection mode	Split
Carrier gas	He (Constant Linear Velocity)
Linear velocity	36.8 cm/sec
Split ratio	25 : 1
Injection volume	1 µL
[MS Conditions]	
Ion source temperature	200 °C
Interface temperature	250 °C
Measurement range	*m/z* 50 - 1000
Event time	0.2 sec
Scan speed	5000 µ/sec

4.2.5 Unknown Compound Analysis by LC/MS

Accurate mass measurement and composition prediction by MS^3 analysis were conducted based on the *m/z* values of unknown compounds indicated as being important according to multivariate analysis. Fig. 4 shows how candidates are narrowed due to MS^n analysis.



Relationship between mass and number of predicted candidates

Mass Spectrum according to MS^n analysis

Fig. 4 Narrowing of Candidates due to MS^n Analysis

4.3 Results
4.3.1 LC/MS Analytical Results

A standard sample consisting of typical compounds contained in green tea was added to a commercially available green tea beverage, and the separation was evaluated. Fig. 5 shows the results. Separation of the 10 constituents of interest was completed in a 10-minute gradient analysis.
In green tea leaf sample analysis, the first sample was repeatedly analyzed interrupting the series of analyses, and the repeatability of retention times and peak areas were calculated to determine the degree of variation of the data with respect to the elapsed time during analysis. Fig. 6 shows the results. High retention time repeatability is evident with regard to the 5 constituents with the greatest intensity.

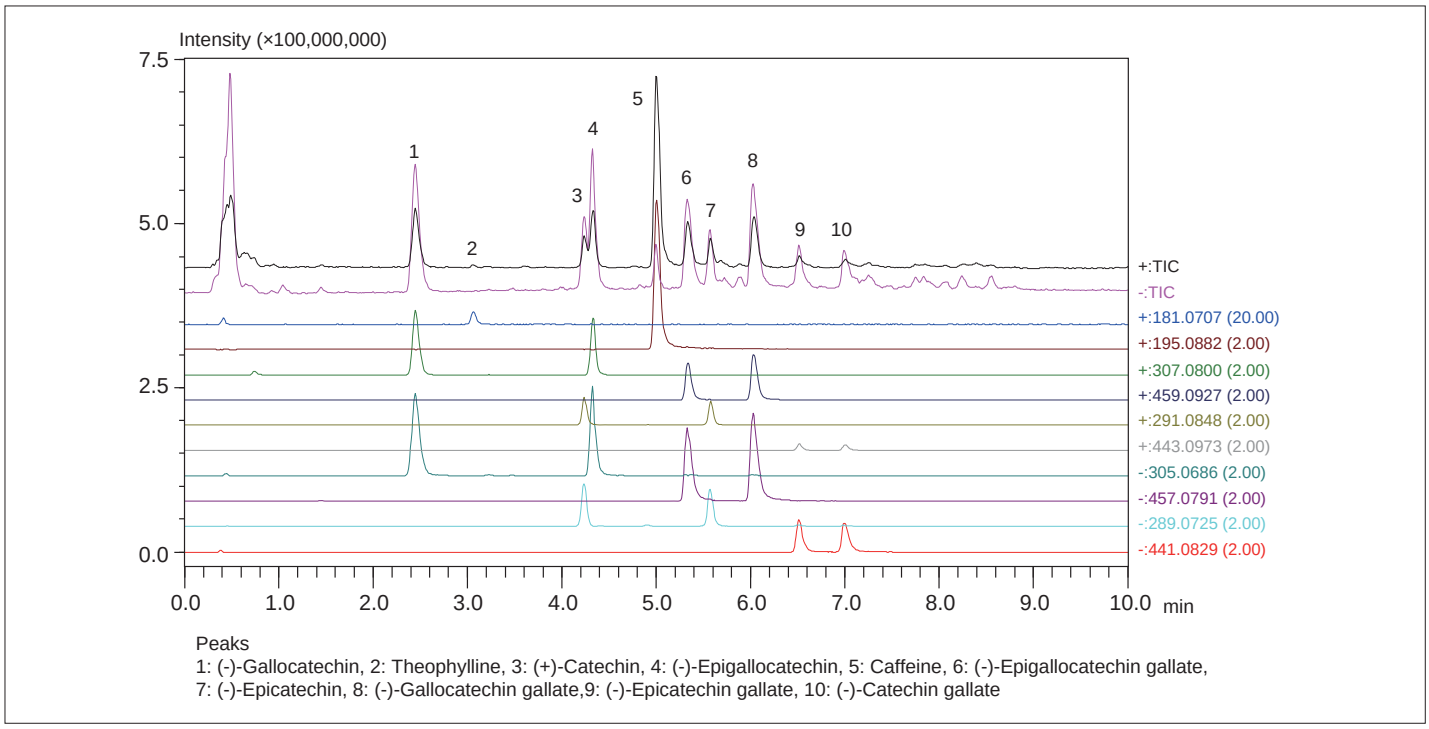
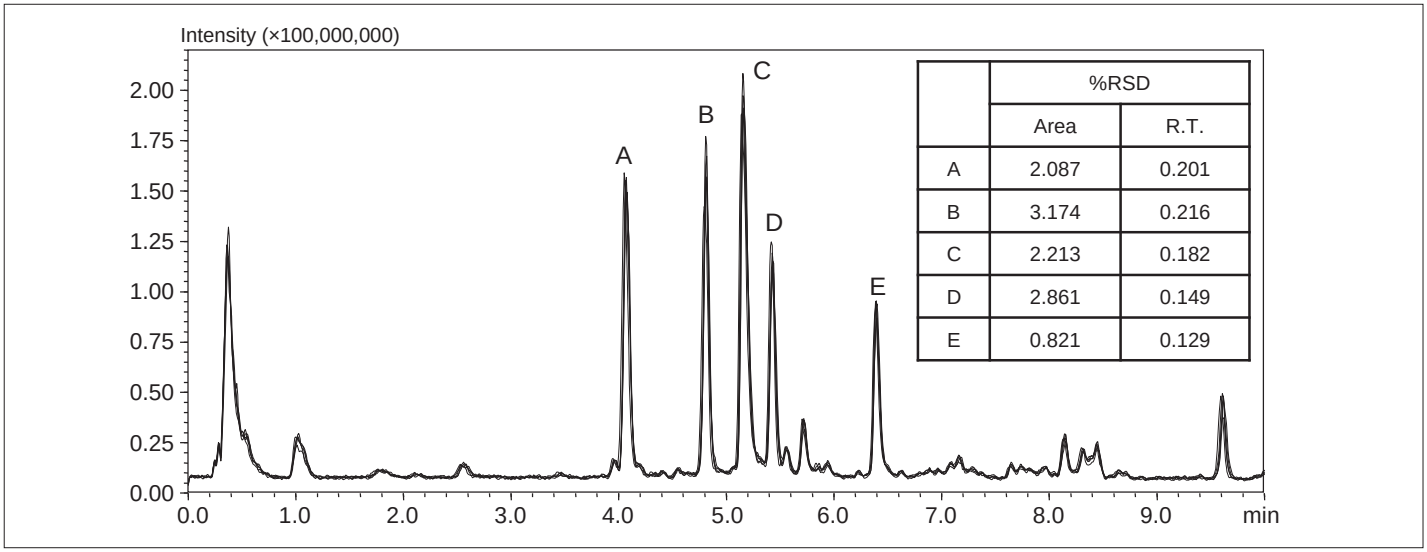


Fig. 5 Chromatogram of Caffeine, Theophylline, and Catechins in Commercially Available Green Tea Beverage



	%RSD	
	Area	R.T.
A	2.087	0.201
B	3.174	0.216
C	2.213	0.182
D	2.861	0.149
E	0.821	0.129

Fig. 6 Overlaid Chromatograms of Green Tea Leaf Sample (Analysis Results at 1st, 20th, 40th, 60th, 80th Measurement)

4.3.2 GC/MS Analytical Results

Fig. 7 shows the TIC chromatogram of a green tea leaf sample. Of the approximately 100 peaks detected, Table 4 shows the compounds identified using the GC/MS Metabolite Database and NIST library. Seventy-one saccharide, amino acid and other organic acid compounds were identified.

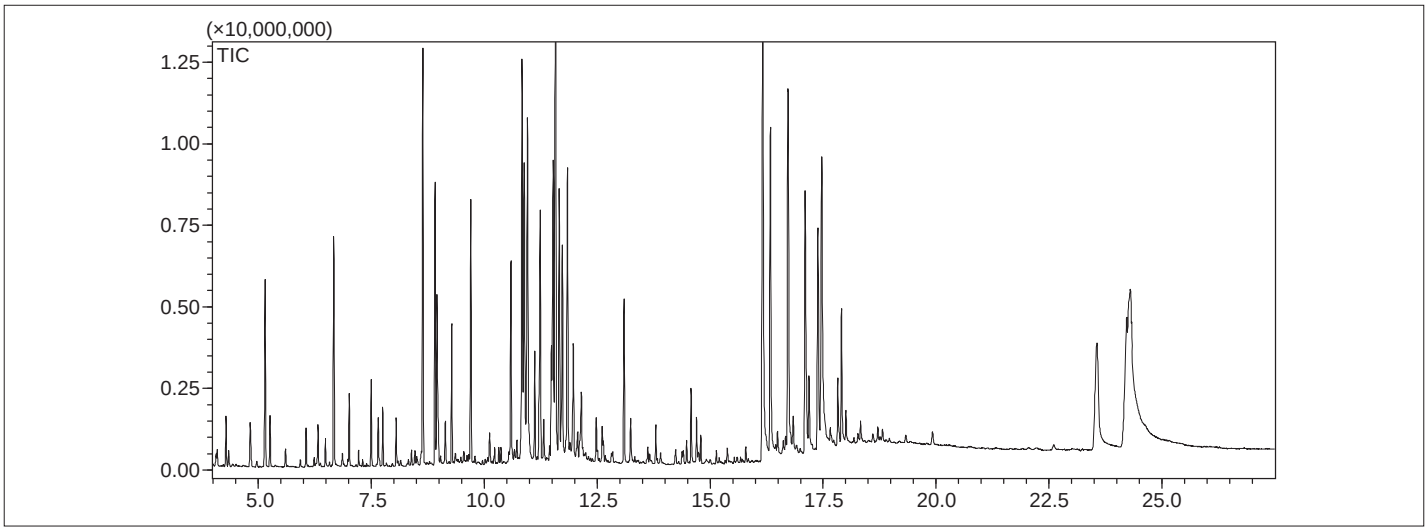


Fig. 7 TIC Chromatogram of Green Tea Leaf Extract Sample

Table 4 Identified Compounds and their Retention Times

ID	Compound Name	RT	ID	Compound Name	RT
1	L-Alanine-2TMS	4.82	37	Lyxose methyloxime-TMS 1	10.11
2	Oxalic acid-2TMS	5.15	38	Asaparagine-3TMS	10.11
3	Malonic acid-2TMS	5.93	39	Lyxose methyloxime-TMS 2	10.22
4	Urea-2TMS	6.27		Ribitol-5TMS (IS)	10.58
5	Serine-2TMS	6.48	40	L-Glutamine-4TMS	10.58
6	Methyl 5-oxo-2-pyrrolidinecarboxylate	6.56	41	Xylonic acid-3TMS	10.66
7	Ethanolamine-3TMS	6.58	42	Theanine	10.83
8	L-Leucine-2TMS	6.63	43	L-Glutamine-3TMS	10.87
9	Phosphoric acid-3TMS	6.67	44	Shikimic acid-4TMS	11.11
10	L-Isoluecine-2TMS	6.85	45	Citric acid-4TMS	11.23
11	L-Threonine-2TMS	6.86	46	Caffeine	11.51
12	L-Proline-2TMS	6.89	47	Quinic Acid- TMS	11.57
13	Glycine-3TMS	6.98	48	Fructose-Methyloxime-5TMS 1	11.65
14	Succinic acid-2TMS	7.01	49	Fructose-Methyloxime-5TMS 2	11.72
15	Glyceric acid-3TMS	7.22	50	Glucose-Methyloxime-5TMS	11.83
16	Fumaric acid-2TMS	7.31	51	L-Lysine-4TMS	11.89
17	L-Serine-3TMS	7.50	52	Mannose-Methyloxime-5TMS	11.96
18	3,4-Bis[(trimethylsilyl)oxy]dihydro-2(3H)-furanone-2TMS	7.65	53	L-Tyrosine-3TMS	12.02
19	L-threonine-3TMS	7.75	54	Benzoic acid-4TMS	12.14
20	L-Aspartic acid-2TMS	8.05	55	Gluconic acid-5TMS 1	12.47
21	4-Ketoglucose methyloxime-3TMS	8.50	56	Palmitic acid-TMS	12.60
22	2-Methylmaric acid-3TMS	8.52	57	Gluconic acid-5TMS 2	12.62
23	Arabino-Hexos-2-ulose-4TMS	8.61	58	Inositol-6TMS	13.08
24	Malic acid-3TMS	8.64	59	Galactose-Methyloxime-5TMS	13.32
25	L-Aspartic acid-3TMS	8.91	60	Phosphoric acid propylester-4TMS	13.65
26	Pyroglutamic acid-2TMS	8.95	61	Stearic acid-TMS	13.79
27	4-aminobutyric acid-3TMS	8.98	62	L-Tryptophan-3TMS	13.82
28	L-Norvaline-3TMS	9.13	63	Xylopyranose-4TMS	14.37
29	L-Threonic acid-4TMS	9.28	64	Glucose-6-phosphate-Methyloxime-5TMS 1	14.40
30	Isopropylmalic acid-3TMS	9.40	65	Glucose-6-phosphate-Methyloxime-5TMS 2	14.47
31	Glycerol-3TMS	9.54	66	Glucoheptulose-Methyloxime-TMS	14.74
32	Ornithine-3TMS	9.65	67	Di-n-octyl phthalate	15.53
33	L-Glutamic acid-3TMS	9.70	68	Maltose-8TMS	15.79
34	Phenylalanine-2TMS	9.79	69	Sucrose-8TMS	16.16
35	Asparagine-4TMS	9.81	70	Arabinopyranose-4TMS	16.33
36	2,3,4,5-Tetrahydroxypentanoic acid-1,4-lactone-3TMS	10.01	71	Raffinose-8TMS	19.92

4.3.3 Search for Constituents that Contribute to Quality using Principal Component Analysis

Fig. 8 shows the results of obtained from conducting principle component analysis (PCA) using the lists of metabolite peaks detected in LC/MS and GC/MS analysis. The numbers inside the score plot show the rankings according to the green tea quality selection contest, with the high-ranked teas and low-ranked teas positioned respectively on the right and left sides of the graph. The contribution of each component to the quality is clear from the loading plot of the first principal ingredient axis (PC1). Compounds present in large quantity in high-ranked green tea leaves are plotted on the positive side of the axis, while compounds present in large quantities in low-ranked green teas are plotted on the negative side.
These results confirmed that organic acids detected in GC/MS, and catechins, etc. detected in LC/MS are substances that contribute greatly to green tea product quality.
After this, the compound candidate Peak X was predicted as a representative of unknown components that have a high contribution to tea product quality.

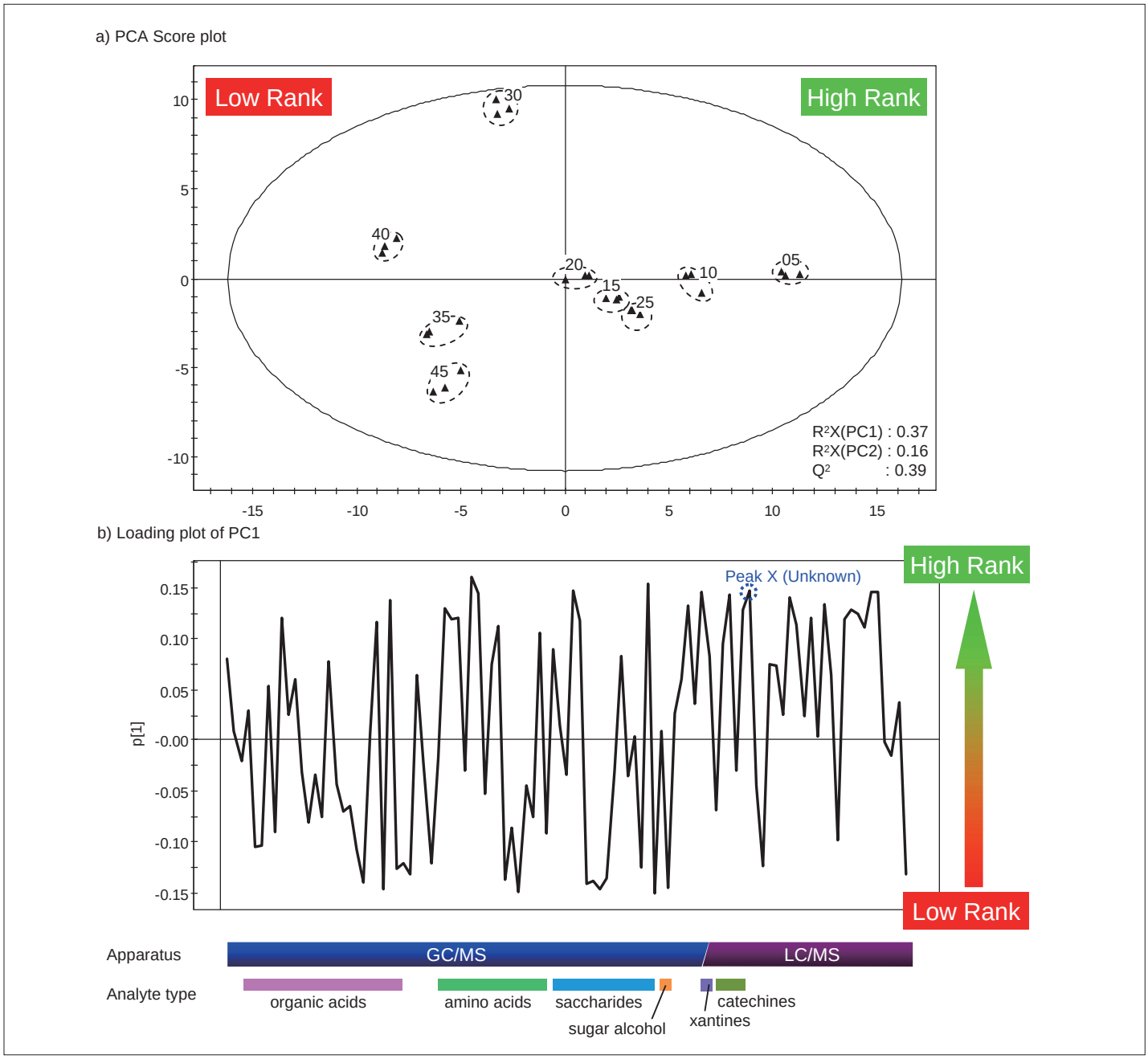


Fig. 8 Principle Component Analysis (PCA) Results for Green Tea Samples Analyzed by LC/MS and GC/MS

4.3.4 Composition Prediction of Unknown Compound

Compound composition was predicted from accurate mass information using MS^3 analysis data for Peak X. The results are shown in Fig. 9.

Using the composition prediction software, the composition formula for Peak X was determined to be $C_{14}H_{16}O_{10}$.

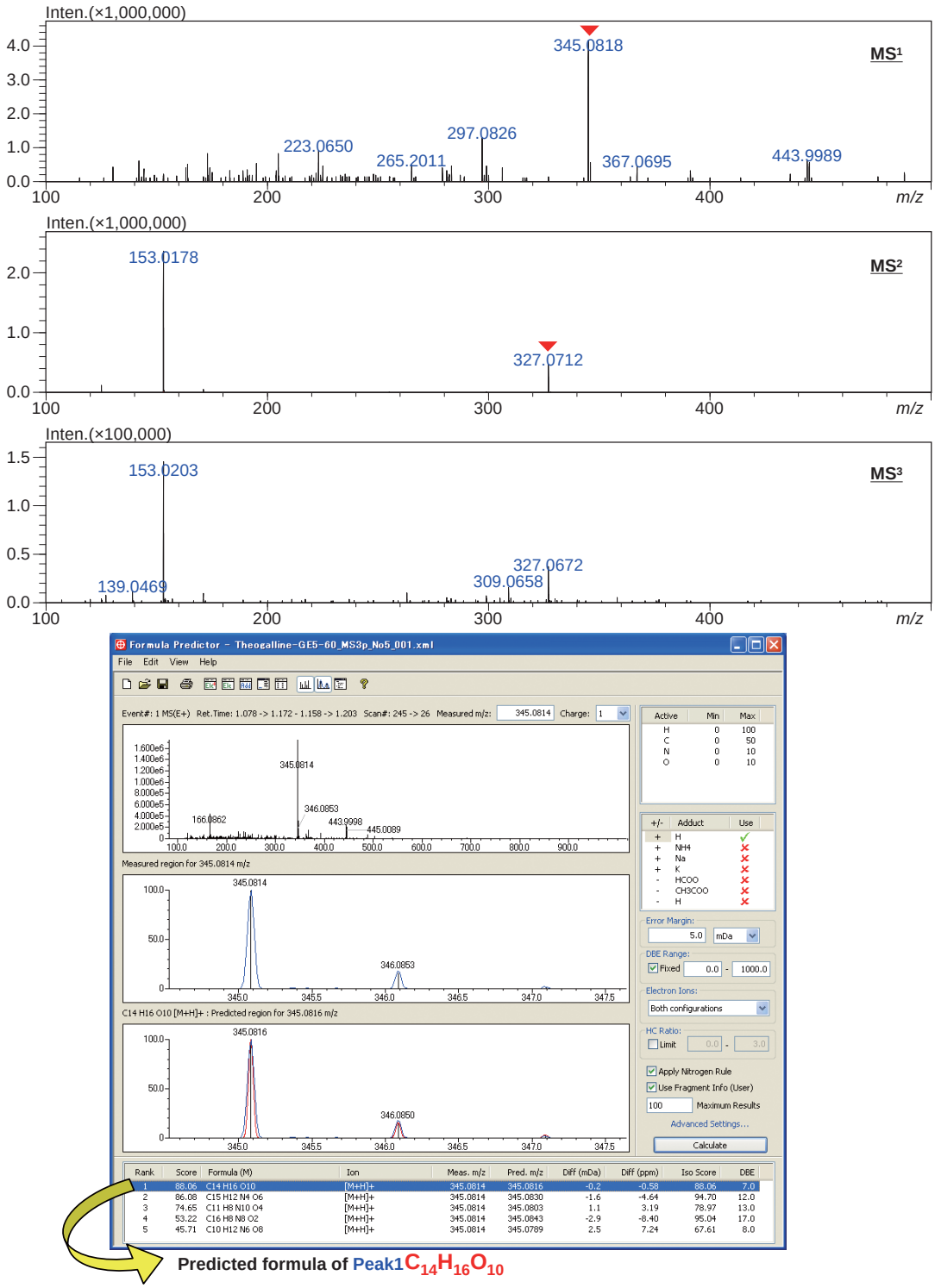


Fig. 9 Mass Spectrum of Peak X and Composition Prediction Results

4.3.5 Compound Candidate Prediction for Unknown Compound

The composition formula "$C_{14}H_{16}O_{10}$" obtained using the Composition Prediction Software introduced in Section 4.3.4 was submitted for a compound search in a web-based publically-available database. The search results indicated the possibility of the compound being theogallin, a type of polyphenol.
When the mass spectrum is assigned based on the theogallin structure, the results suggested that Peak X is theogallin. (Fig. 10)

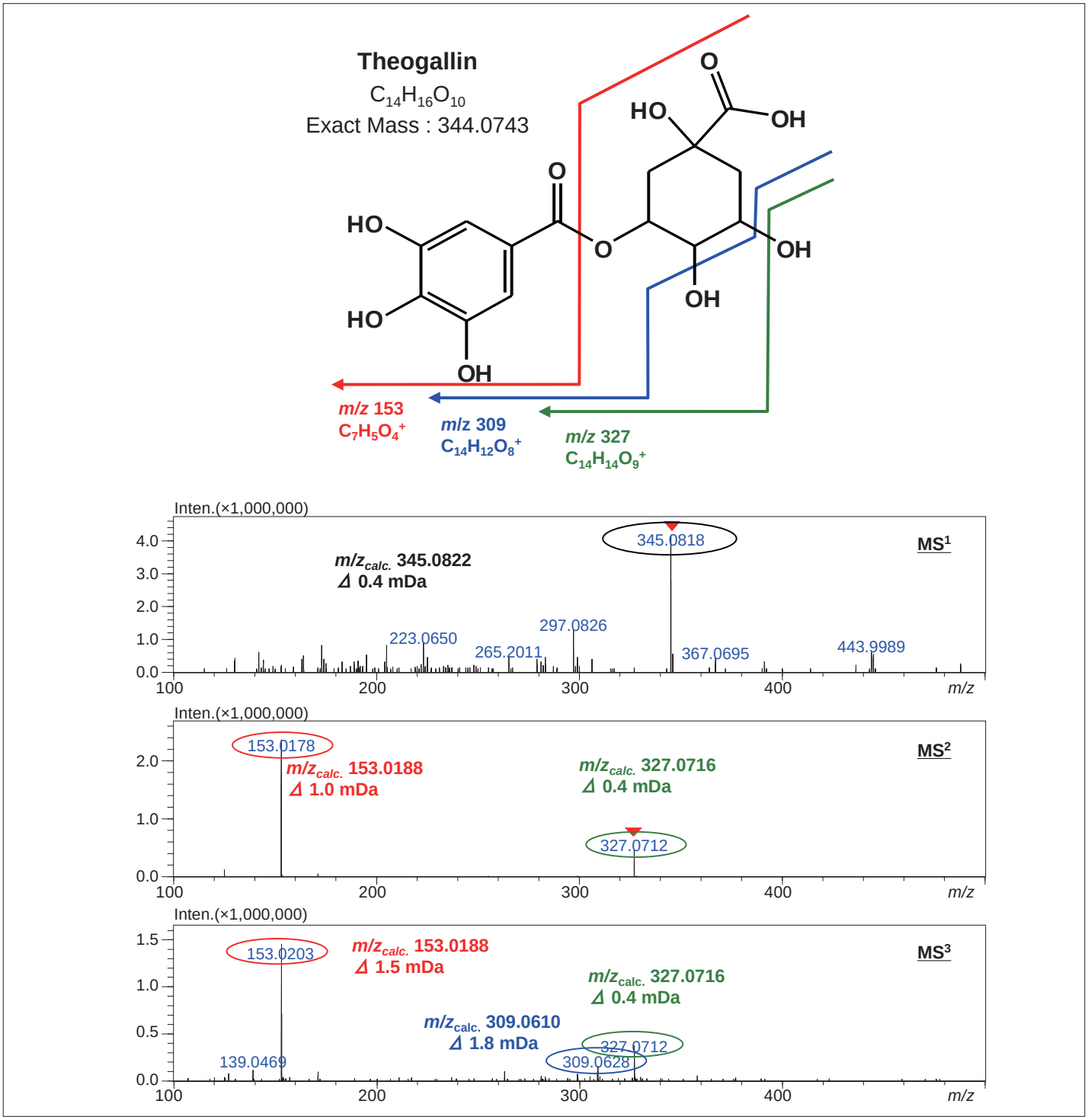


Fig. 10 Theogallin Structural Formula and Peak X Mass Spectral Assignment

5. Summary

In this Application Note, we introduced an overview of metabolomics and an actual example of quality evaluation of green tea leaves using the typical LC/MS and GC/MS analytical techniques.
LC/MS and GC/MS are both powerful analytical techniques, with GC/MS database search and the LCMS-IT-TOF composition prediction feature, as well as other powerful tools helping to advance progress in the field of metabolomics. Moreover, using these analytical techniques in combination makes possible the analysis of metabolites with a wide range of characteristics, allowing even a wider range and more in-depth analysis in metabolomics as well as other applications.

Acknowledgment :
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