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Profiling of Japanese Green Tea Metabolites by GC-MS

GC/MS Technical Report No.1

GC/MS Metabolomics & Life Science Project
(Yuki Sakamoto, Katsuhiro Nakagawa, Shuichi Kawana, Novalina Lingga, Hui-Loo Lai Chin, Haruhiko Miyagawa)
Tairo Ogura

Abstract

Metabolites from nine varieties of high-grade Japanese green tea leaves which had been ranked in a tea competition were extracted and analyzed by GC-MS. Multivariate data analysis was applied to the GC/MS results; the compounds characterizing the rankings could be verified using a score plot and loading plot.
Moreover, a model for predicting rankings was created using PLS regression analysis.

Keywords: GC/MS, food, metabolomics, green tea leaves, amino acids, organic acids, sugars

Introduction

Metabolome analysis (metabolomics) refers to the in-depth analysis of metabolites by various techniques, and is used in a wide range of fields, including diagnostic marker search and etiology analysis in medical treatment, biomarker search for revealing efficacy and toxicity in drug manufacturing, and quality control in food processing[1].
Various combinations of analytical instruments consisting of a mass spectrometer coupled with a chromatograph such as LC or GC can be used for metabolome analysis. GC-MS distinguishes itself among other types of chromatography because of its excellent separation capacity, and its ability to generate a mass spectrum for each compound, allowing separation and detection of many metabolites. In addition, metabolite identification is simplified when standardized retention indices and mass spectra are registered in a database.
In order to verify the effectiveness of GC-MS for metabolome analysis, we investigated the rankings that had been assigned at a Japanese green tea competition and the substances that contributed to those rankings, as well as the effectiveness of a model for predicting the competitive rankings using the Shimadzu GCMS-QP2010 Plus, Shimadzu GC/MS Metabolites Spectral Database and SIMCA-P (multivariate data analysis software; Umetrics Inc.).

Experimental

Reagents

Ribitol was dissolved in Milli-Q water to prepare an internal standard solution with a concentration of 0.2 mg/mL. The methoxyamine solution used for methyloximation derivatization was prepared by dissolving methoxyamine hydrochloride (Wako Pure Chemical Industries, Ltd. ; Osaka, Japan) in pyridine to obtain a concentration of 20 mg/mL. The MSTFA (N – Methyl – N – trimethylsilyl trifluoroacetamide) used for trimethylsilyl (TMS) derivatization was obtained from GL Sciences Inc. (Tokyo, Japan).

Equipment and Software

The Shimadzu GCMS-QP2010 Plus was used for GC/MS analysis; GCMSsolution software was used for data processing, and Shimadzu GC/MS Metabolites Spectral Database and NIST 2008 mass spectral library were used as the mass spectral libraries. In addition, SIMCA-P software Ver. 11.0.0.0 (Umetrics Inc, Umeå, Sweden) was used for multivariate data analysis.

Preparation

The green tea leaves used for the experiment had been ranked according to 9 levels in the competition. The 9 rankings assigned to the tea leaves were from 5th to 45th place, in increments of 5 points. The tea leaves were prepared according to the preparation procedure previously reported [2]. First, a 30 mg tea leaf sample was crushed under liquid nitrogen; an extraction solvent consisting of a mixture of water / methanol / chloroform (1/2.5/1) and the ribitol internal standard were added to the crushed sample. After thorough mixing and centrifugation, 900 µL of the liquid phase was withdrawn. Milli-Q water (400 µL) was added to the retained liquid phase to separate the mixture into 2 phases (water / methanol phase and chloroform phase). After centrifugation, 400 µL of the water / methanol phase was removed. Next, application of partial vacuum and centrifugation were conducted to remove the methanol from solution, and the remaining liquid was freeze-dried. The freeze-dried residue was subjected to methyloximation derivatization and trimethylsilyl (TMS) derivatization; and this derivatized sample was used for GC/MS analysis. This preparation procedure was conducted in triplicate for each of the tea leaf samples. The GC/MS analytical conditions are shown in Table 1.

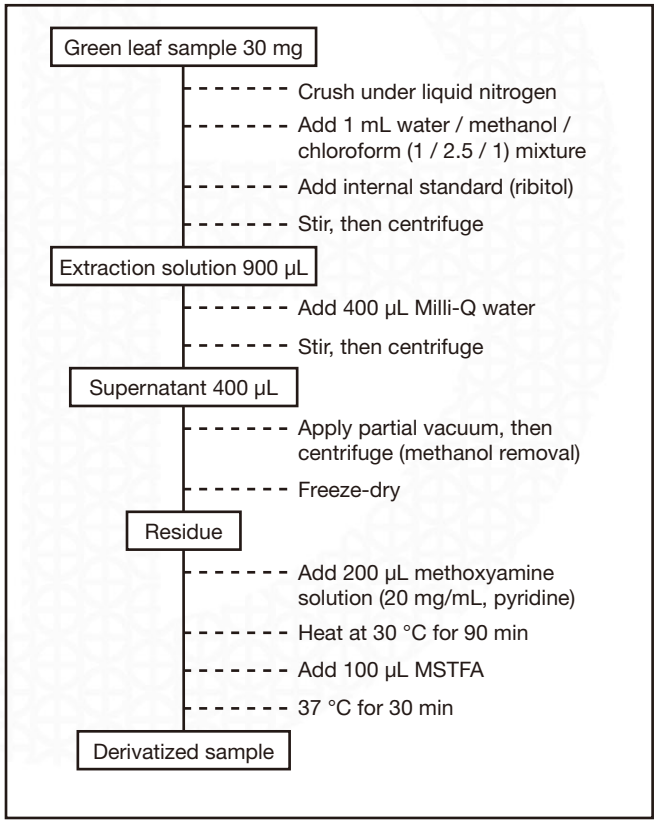

Fig. 1: Sample preparation flow chart

Table 1: Analytical conditions

Instruments	
GC-MS	: GCMS-QP2010 Plus
Auto-Injector	: AOC-20i + s
Column	: Rtx®-5MS (30 m x 0.25 mm I.D. df=0.25 µm, Restek Corporation)
Analytical Conditions	
GC	
Injection temp.	: 250 °C
Column temp.	: 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	
Ion source temp.	: 200 °C
Interface temp.	: 250 °C
Scan range	: *m/z* 50 – 1000
Event time	: 0.2 sec
Scan speed	: 5000 *u*/sec

Identification of Compounds

The peaks were detected on the total ion chromatogram and mass chromatograms, and the detected peaks were identified using the GC/MS Metabolites Spectral Database and NIST 2008 mass spectral library. Based on the identification results, the quantitation ion, reference ion(s), retention time, mass spectrum and retention index for each compound were registered in the compound table. That table was used to automatically detect each compound, automatically calculate the peak area of each detected compound, and calculate the relative concentration (area ratio) to the area of the internal standard (ribitol).

Multivariate Data Analysis

Multivariate data analysis was conducted using SIMCA-P software.

Results

Analytical Results

Fig. 2 shows the total ion chromatogram of a green tea leaf sample; about 100 peaks were detected. The detected peaks were identified using the GC/MS Metabolites Spectral Database and NIST 2008 mass spectral library. The results are shown in Table 2. Seventy-one compounds, including sugars, amino acids, and the organic acids, were identified.

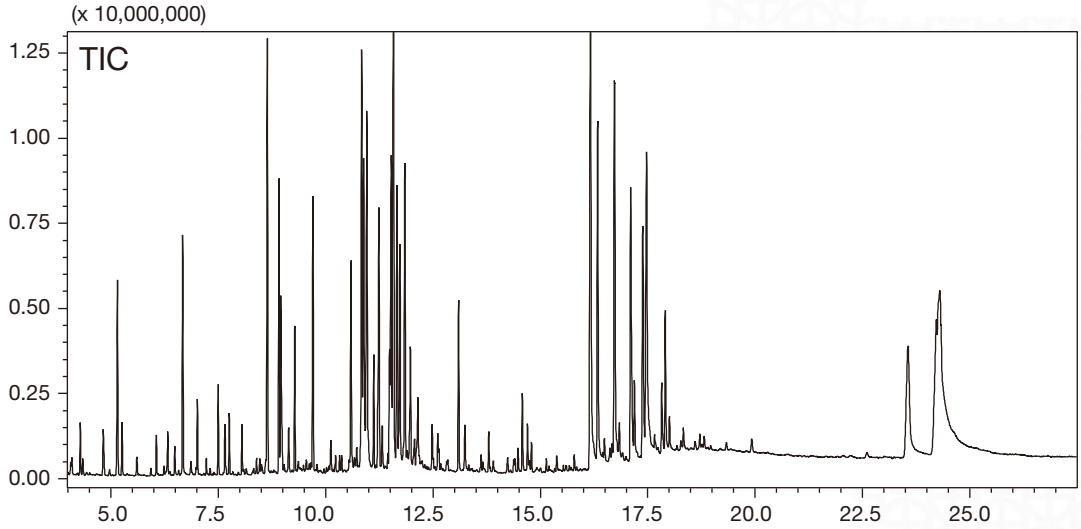

Fig. 2: Total Ion Chromatogram of Green Leaf Extract

Table 2: Identified compounds and their retention time

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

Multivariate Data Analysis

Fig. 3 shows the results of examination of the differences among green tea leaves of different ranks using a principle component analysis (PCA) score plot. The numerals in the figure indicate the rankings that had been assigned in the competition. With respect to principle component 1, the higher ranked tea leaves and the lower ranked tea leaves are positioned separately to the right and left sides of the graph, clearly indicating the differences in ranking. Furthermore, the R2 and Q2 values are 0.7505 and 0.6182, respectively.

A loading plot was created based on the results of the score plot in order to investigate the relationships between compounds from rank to rank. The results are shown in Fig. 4. The numerals in the figure indicate the component IDs (Table 2). The results obtained suggest that tea leaves that had been ranked high in the competition contain large amounts of glutamine, arabinopyranose and caffeine, while lower ranked tea leaves contain large amounts of sucrose, theanine, quinic acid, fructose and glucose.

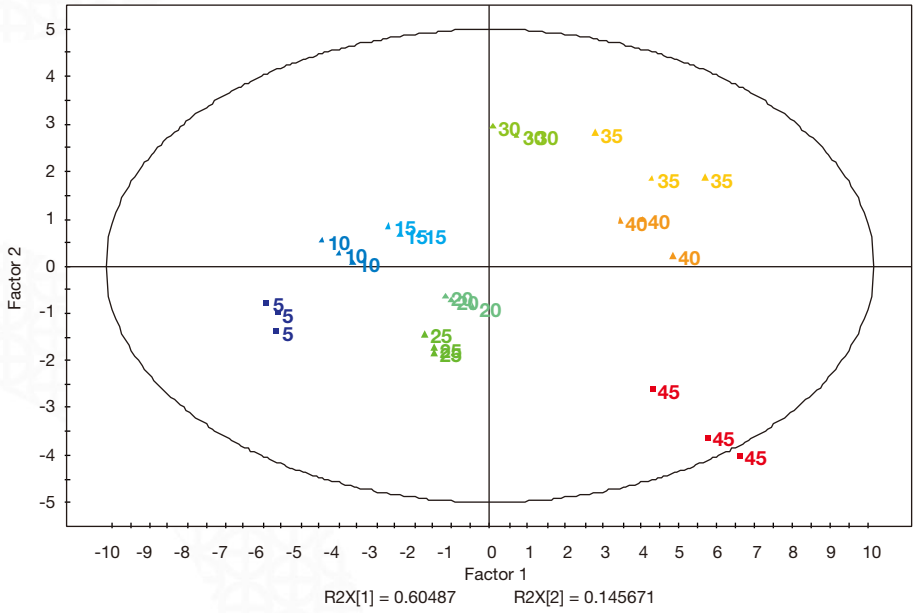

Fig. 3: Score plot result of principal component analysis

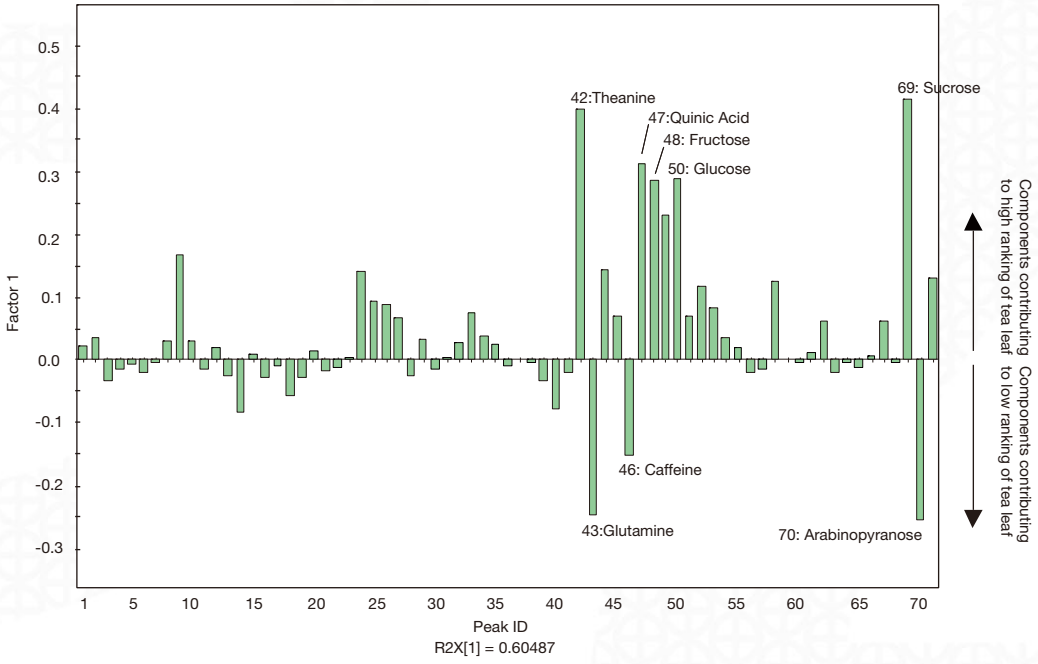

Fig. 4: Loading plot result of principal component analysis

A model for predicting rankings by PLS regression analysis was created, and the results of applying data to this model are shown in Fig. 5. The x-axis shows the predicted rankings using this model, and the y-axis shows the rankings assigned at the competition. The model was created using a training set of data comprising the ranking positions 5, 10, 20, 30, 40 and 45. The values obtained for R2Y, Q2 and RMSEE were 0.9766, 0.9721 and 2.3879, respectively. Next, the data corresponding to the positions 15, 25 and 35 were applied to the model to predict the rankings. The value for RMSEP calculated from the test set was 4.5584. These results suggest that this model is applicable for predicting rankings.

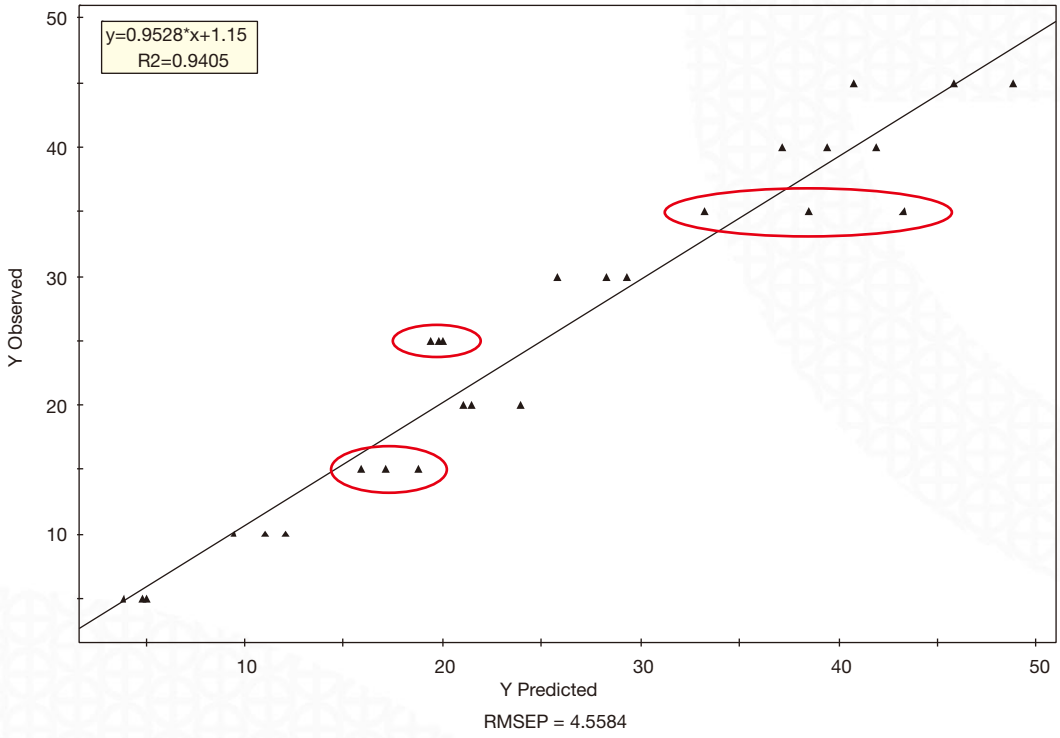

Fig. 5: PLS model of measured and predicted ranking

Conclusion

The metabolites from 10 varieties of green tea leaves that had been ranked in a tea competition were extracted and then analyzed using the GCMS-QP2010 Plus. Approximately 100 components were detected, and 71 of these were identified using the GC/MS Metabolites Spectral Database and the NIST 2008 mass spectral library. The results obtained from applying multivariate data analysis to the identified compounds using SIMCA-P, in addition to a score plot and loading plot enabled characterization of high-ranking and low-ranking tea leaves, which in turn indicated the expected metabolite compounds. In addition, a ranking prediction model was created using PLS regression analysis.

Acknowledgments

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References

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[2] Pongsuwan, W.; Fukusaki, E.; Bamba, T.; Yonetani, T.; Yamahara, T.; Kobayashi, A. Prediction of Japanese Green Tea Ranking by Gas Chromatography/Mass Spectrometry-Based Hydrophilic Metabolite Fingerprinting. J. Agric. Food Chem. 2007, 55, 231–236.

Shimadzu GC-MS and Metabolomics

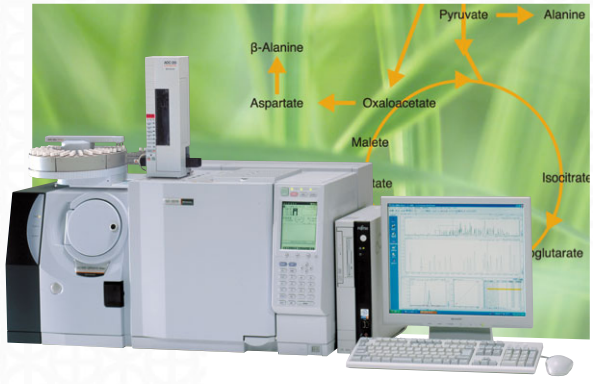
The Shimadzu GC-MS is used in advanced metabolomics research of congenital metabolic abnormalities, and is earning high acclaim internationally. A GC-MS must have the following functionality to be suitable for metabolite analysis and metabolomics analysis.

(1) Metabolites that deserve attention are not always present at high concentrations, so sufficiently high sensitivity for the detection of trace level metabolites is necessary.
(2) To enable an exhaustive metabolite search, it is important to minimize the loss of components during sample preparation. Sample cleanup is often omitted for this reason, resulting in analytical samples that contain significant interferences. This can be a problem when a GC-MS is used to analyze such samples due to contamination of the ion source. Therefore, an instrument that is resistance to contamination and which allows simple cleaning of the ion source even in the event of contamination is highly desirable.
(3) Since it is not uncommon for metabolites to have similar mass spectra, identification that is based on both the retention index and mass spectrum is required. Therefore, the data analysis software used for analysis should also support the use of retention indices.
(4) NIST and other mass spectral libraries do not contain entries for every metabolite. Therefore, specialized libraries for specific metabolites are required.

The Shimadzu GCMS-QP2010 Plus satisfies all of these conditions.

Gas Chromatograph / Mass Spectrometer GCMS-QP2010 Plus

Features of GCMS-QP2010 Plus
1. High sensitivity
2. Easy maintenance
3. Compound identification using retention indices



GC/MS Metabolites Spectral Database (Amino acids, fatty acids, organic acids)

The GC/MS Metabolites Spectral Database is a mass spectral library for the GCMSsolution workstation software which controls the GCMS-QP2010 series gas chromatograph / mass spectrometer. Use of a mass spectral library equipped with retention indices greatly reduces the number of candidate compounds to improve the reliability of search results.

This database consists of 4 different kinds of method files containing analytical conditions, mass spectra, retention indices, etc., and 4 kinds of libraries containing CAS numbers and other compound information, mass spectra and retention indices. A printed handbook containing the library information is also provided with the database.
The methods and libraries contain metabolite-related information for amino acids, fatty acids and other organic acids, including 261 electron ionization spectra and 50 chemical ionization spectra.

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SHIMADZU CORPORATION. International Marketing Division
3. Kanda-Nishikicho 1-chome, Chiyoda-ku, Tokyo 101-8448, Japan
Phone: 81(3)3219-5641 Fax. 81(3)3219-5710
URL http://www.shimadzu.com

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