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ARTICLE

Metabolomics Analysis Reveals the Compositional Differences of Shade Grown Tea (*Camellia sinensis* L.)

Kang Mo Ku,[†] Jung Nam Choi,[†,∥] Jiyoung Kim,[†] Jeong Kee Kim,[‡] Lang Gook Yoo,[‡] Sang Jun Lee,[‡] Young-Shick Hong,[§] and Choong Hwan Lee*,[†]

[†]Department of Bioscience and Biotechnology and Bio/Molecular Informatics Center, Kon-Kuk University, Seoul 143-701, Republic of Korea, [‡]Food Research Institute, AmorePacific R&D Center, Yongin-si, Gyeonggi-do, Republic of Korea, and [§]School of Life Science and Biotechnology, Korea University, Seoul 136-701, Republic of Korea. [∥]J.-N. C. contributed equally to this work.

The different cultivation methods affect tea quality by altering the basic metabolite profiles. In this study, the metabolome changes were investigated in green tea and shade cultured green tea (tencha) by liquid chromatography−mass spectrometry (LC-MS) and gas chromatography−mass spectrometry (GC-MS) coupled with a multivariate data set. The principal component analysis (PCA) and orthogonal projection to latent structures discriminate analysis (OPLS-DA) of green tea clearly showed higher levels of galloylquinic acid, epigallocatechin, epicatechin, succinic acid, and fructose, together with lower levels of gallocatechin, strictinin, apigenin glucosyl arabinoside, quercetin *p*-coumaroylglucosyl-rhamnosylgalactoside, kaempferol *p*-coumaroylglucosylrhamnosylgalactoside, malic acid, and pyro-glutamic acid than tencha. The effects of some seasonal variations were also observed in the primary metabolite concentrations such as amino acids and organic acids. In addition, green tea showed stronger antioxidant activity than tencha in both April and July. The antioxidant activity of green tea samples were significantly correlated with their total phenol and total flavonoid contents. This present study delineates the possibility to get high umami and less astringent green teas in shade culture. It highlights the metabolomic approaches to find out the effect of cultivation methods on chemical composition in plants and the relationship with antioxidant activity.

KEYWORDS: Green tea; tencha; antioxidant; shading culture; multivariate analysis; metabolomics

INTRODUCTION

Green tea (*Camellia sinensis* L. (Kuntze)) belongs to the Theaceae family and is a popular nonalcoholic healthy beverage that is consumed in all regions of the world. Recent studies have revealed that green tea has many beneficial health effects such as antioxidant activity (*1*), cancer-preventive activity (*2*), and protection activity against nitric oxide toxicity (*3*).

The four types of teas most commonly found on the market are black tea, oolong tea, green tea, and white tea. Unlike black and oolong teas, green tea does not blend well with many additives such as sugars, lemon, or milk and as a result is usually consumed plain. Thus, when it comes to green tea, the material contained in the tea itself is important for tasting. The chemical composition of green tea depends on several factors: genetic strain, climatic conditions, soil, growth altitude, and horticultural practices, the plucking season, sorting (grading) of the leaves, processing, storage, etc. Some parameters are more important than others. For example, the best green teas are usually plucked during the first flush in April or May (*4*).

The highest quality green tea from Japan called matcha (fine powder made from tencha) is grown in the shade and is reported to contain high amino acid but low catechin content (*5*, *6*). Sweetness is attributed to amino acids, especially theanine, which has a taste that is described as umami or brothy, while catechins and caffeine contribute to the astringency. Matcha has not only a unique taste but also a high content of chlorophylls (*7*). Generally, unlike other green teas, tencha (raw material of matcha) is grown under 60−90% shade for about 7−10 or 21 days before harvest depending on the region, farmer's experience, and tea tree ages. This shade treatment makes green teas greener in color, sweeter, and less astringent in taste. The effect of shading culture on chemical composition has been studied; however, these studies mostly focused on catechins (*8*), amino acids, and pigments, in a targeted manner. Until now, research to understand the effect of shading culture on the metabolome of green tea has not been performed. If we understand the effect of shading treatment on the compositional changes of green tea, it could be applied to produce better green tea.

With the recent developments in plant metabolomic techniques, it is now possible to detect several hundred metabolites simultaneously and to compare samples reliably to identify differences and similarities in an untargeted manner (*9*). Previous metabolomic studies have demonstrated that this technique can be effectively used to differentiate phytochemical compositions among different origins (*10*) or varieties (*11*).

*Corresponding author. Phone: 82-2-2049-6177. Fax: 82-2-455-4291. E-mail: chlee123@konkuk.ac.kr.

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This study aims to understand the effect of shading treatment on chemical composition changes in green tea using metabolomics approaches such as liquid chromatography−mass (LC-MS) and gas chromatography−mass (GC-MS) coupled with multivariate statistical analysis. LC-MS is a powerful tool to study metabolomics not only because it gives us information for identification but also because it detects a broad group of metabolites (*12*). However, it is hard to detect the primary metabolites which were also very important in characterizing green tea. Thus, to compensate for the drawback of LC-MS, GC-MS was also used to compare differences in composition between green tea and tencha. However, these different analytical methods generate a complex data sheet that is hard to understand and visualize without multivariate statistical analyses such as principal component analysis (PCA), partial least-squares to latent structure (PLS), and orthogonal PLS discriminant analysis (OPLS-DA) (*13*). In the present study, PCA and OPLS models were employed to identify the significantly different metabolites between green tea and tencha. In addition, the correlation between the antioxidant activity of green teas cultivated under different conditions and their antioxidant compounds was investigated.

MATERIALS AND METHODS

Cultivation of Green Tea. Green teas and tencha samples were provided by Amore Pacific Co. (Republic of Korea). The green tea leaves were collected from a 20-year-old Yabukita (*Camellia sinensis* var. Yabukita) cultivar in Hannam-ri Namwon-eup, Seogwipo Si, Jeju-Do, Republic of Korea (latitude: 33°18′21.21′′N, longitude: 126°17′22.51′′E). Before plucking tencha leaves, green tea leaves were grown under 80% shade by directly covering with black polyethylene (100 m × 2 m) for 10 days. Green tea and tencha were harvested in April and July in 2008.

Preparation of Samples. Dried tea leaves were ground with mortar, and 400 mg of the ground tea leaves were extracted with 10 mL of solvent, a single-phase solvent mixture of MeOH, H_2O, and $CHCl_3$ at a ratio of 2.5/1/1 (v/v/v), respectively. The mixture was incubated for a day and was centrifuged at 16 000*g*, for 3 min. Subsequently, 900 μL of the supernatant was transferred to a 1.5-mL Eppendorf tube. After adding 400 μL of distilled water to the Eppendorf tube and centrifuging, 400 μL of the polar phase was then transferred to another 1.5-mL Eppendorf tube capped with a pierced cap. The extracts were filtered through a 0.2 μm PTFE filter for LC-MS analysis.

Chemicals and Reagents. (+)-Catechin, (−)-epicatechin-3-gallate, NaOH, $AlCl_3$, sodium carbonate, gallic acid, Folin−Ciocalteu's phenol reagent, potassium persulfate, 2,2′′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-*s*-triazine (TPTZ), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), $FeCl_3 \cdot 6H_2O$, $C_2H_3NaO_2 \cdot 3H_2O$, dimethyl sulfoxide (DMSO), and formic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Acetonitrile and water used in LC-MS were of the optima grade from Fisher Scientific (Pittsburgh, PA). For GC-MS, methanol and chloroform used as extraction solvents and pyridine used as a solvent, and methoxyamine hydrochloride, and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Sigma Chemical Co. (St. Louis, MO).

Trolox Equivalent Antioxidant Capacity (TEAC). The TEAC assay was conducted as described by Re et al. (*14*). Briefly, 7 mM ABTS ammonium was dissolved in a potassium phosphate buffer (pH 7.4) and treated with 2.45 mM potassium persulfate. The mixture was then allowed to stand at room temperature for 12−16 h until it turned dark blue. The solution was then diluted with potassium phosphate buffer until the absorbance reached 1.0 ± 0.02 at 734 nm. The latter was measured using a BioTek EL 808 microplate reader (Biotek Instruments Inc., Power Wave XS, Winooski, VT). Subsequently, 190 μL of the solution was mixed with 10 μL of the sample. The absorbance was recorded at room temperature after 6 min. Results were expressed in millimols of Trolox equivalent concentration per gram of green tea. The concentration of the standard solutions ranged from 0.25 to 4 mM. Experiments were carried out in triplicate.

Determination of Antioxidant Activity by the DPPH Free Radical Scavenging Assay. The DPPH assay was conducted as described, with minor modifications, by Dietz et al. (*15*). Reaction mixtures containing test samples (10 μL) and 190 μL of a 200 μM DPPH ethanol solution were incubated at room temperature for 30 min in 96-well plates. The absorbance of the DPPH free radical was measured at 515 nm with a microplate reader. Results were expressed in millimols of Trolox equivalent concentration per gram of green tea. The concentration of the standard solutions ranged from 0.156 to 2.5 mM. Experiments were carried out in triplicate.

Ferric Reducing/Antioxidant Power (FRAP) Assay. The antioxidant capacity of each standard was estimated according to the procedure described by, with minor modifications, Benzie and Strain (*16*). Briefly, 300 μL of FRAP reagent, freshly prepared and warmed at 37 °C, was mixed with 10 μL of test sample of solutions as appropriate for the reagent blank. The absorbance was measured at 593 nm after 6 min using a microplate reader. The FRAP reagent contained 2.5 mL of a 10 mmol/L TPTZ solution in 40 mmol/L HCl, 2.5 mL of 20 mmol/L $FeCl_3 \cdot 6H_2O$, and 25 mL of 0.3 mol/L acetate buffer at pH 3.6. Results were expressed in millimols of Trolox equivalent concentration per gram of green tea. The concentration of standard solutions ranged from 0.25 to 2 mM. Experiments were carried out in triplicate.

Determination of Total Polyphenol Content (TPC) and Total Flavonoid Content (TFC). TPC was analyzed using the method of Isabelle et al. (*17*). The assay conditions were as follows: a 20 μL sample was added to 0.2 N Folin−Ciocalteu's phenol reagent (100 μL) in 96 wells. After 3 min, 80 μL of a saturated sodium carbonate solution was added to the mixture and subsequently incubated at room temperature for 1 h. The resulting absorbance of the mixture was measured at 750 nm using a microplate reader. The total phenol content was calculated on the basis of a standard curve with gallic acid. The standard solution concentrations ranged from 6.25 to 200 μg/mL. Results were expressed in milligrams of gallic acid equivalent (GAE) per gram of green tea. Experiments were carried out in triplicate.

TFC was analyzed using the methods described by Yoo et al. (*18*), with minor modifications. Twenty microliters of the samples or standard solutions of (+)-catechin was added to each well in the 96-well plates. The standard solution concentrations ranged from 100 to 800 μg/mL. Distilled water (40 μL) and 6 μL of 5% (w/v) sodium nitrite were added to each well. After 5 min, 12 μL of 10% (w/v) $AlCl_3$ was added. After 6 min, 40 μL of 1 M NaOH was added to the mixture followed by 42 μL of distilled water. Absorbance was measured at 515 nm using a microplate reader, and the flavonoid content was expressed as milligrams of (+)-catechin equivalent per gram of green tea. Experiments were carried out in triplicate.

Determination of Total Chlorophyll Content (TCC) and Total Amino Acid Content (TAA). To measure the total chlorophyll content, 100 mg of tea leaves was extracted using 20 mL of acetone at 4 °C for a day. The content of chlorophyll was calculated using the equation derived from Arnon's method (*19*). The total amino acid content was determined using the ninhydrin method. One hundred microliters of the water extract (10 mg/mL) was added to a 1.5-mL Eppendorf tube and mixed with 1 mL of 3.5% (w/v) ninhydrin solution. The mixtures were incubated in a heat block at 90 °C for 15 min. The total amino acid content was calculated on the basis of a standard curve determined using glycine standard solutions (37.5−375 μg/mL).

Cell Culture. The mouse macrophage cell line (RAW 264.7, KCLB, Seoul, South Korea) was cultured in a high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Gaithersburg, MD), 4 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco, Gaithersburg, MD). Cells were grown in a humidified incubator at 37 °C under 5% CO_2.

Cell Protective Activity Using Raw 264.7 Cell Lines. Cell protective activity was analyzed using the method of Hu et al. (*20*). Macrophages were cultured in 96-well (1×10^5/mL) plates for a day. The cell cultures were preincubated with the green tea extracts for 1 h, and then hydrogen peroxide was added to a final concentration of 100 μM in medium for 24 h. Cell protective activity was determined using the MTT assay, which yields a blue formazan product in living cells but not in dead cells or their

Table 1. Bioactivities and the Total Compound Content of Green Tea and Tencha[a]

		April		July	
	negative control	green tea	tencha	green tea	tencha
TEAC (TE mM/g)[b]		785 ± 14 [ns]	745 ± 32	926 ± 24***	838 ± 17
FRAP (TE mM/g)		784 ± 5***	720 ± 36	1006 ± 41***	841 ± 37
DPPH (TE mM/g)		807 ± 1***	778 ± 21	863 ± 30***	815 ± 21
cell protective activity (%)[c]	25.4 ± 2.1	40.5 ± 2.6***	37.1 ± 2.8	42.7 ± 4.7[ns]	40.6 ± 3.6
TPC (GAE mg/g)[d]		152 ± 3[ns]	140 ± 16	223 ± 15**	180 ± 17
TFC (CE mg/g)[e]		262 ± 4[ns]	257 ± 16	324 ± 26*	284 ± 23
TCC (mg/g)[f]		4.33 ± 0.02	5.65 ± 0.02***	4.36 ± 0.01	5.19 ± 0.01***
TAA (GE mg/g)[g]		22.3 ± 1.5	27.6 ± 0.7***	9.0 ± 0.2	10.0 ± 0.3***

[a] Significance was evaluated by Student's *t*-test using STATISTICA. ***, **, and * indicate significant difference at 5%, 1%, and 0.1%, respectively. [ns], non-significance. [b] Trolox equivalent mM/g of green tea. [c] Cell protective activity was present as the percentage against the control (without 100 μM H_2O_2) and negative control, and samples were treated with 100 μM H_2O_2 [d] Total phenol content (TPC) was presented as gallic acid equivalent mg/g of green tea. [e] Total flavonoid content (TFC) was presented as catechin equivalent mg/g of green tea. [f] Total chlorophyll content (TCC). [g] Total amino acids (TAA) were presented as glycine equivalent mg/g of green tea.

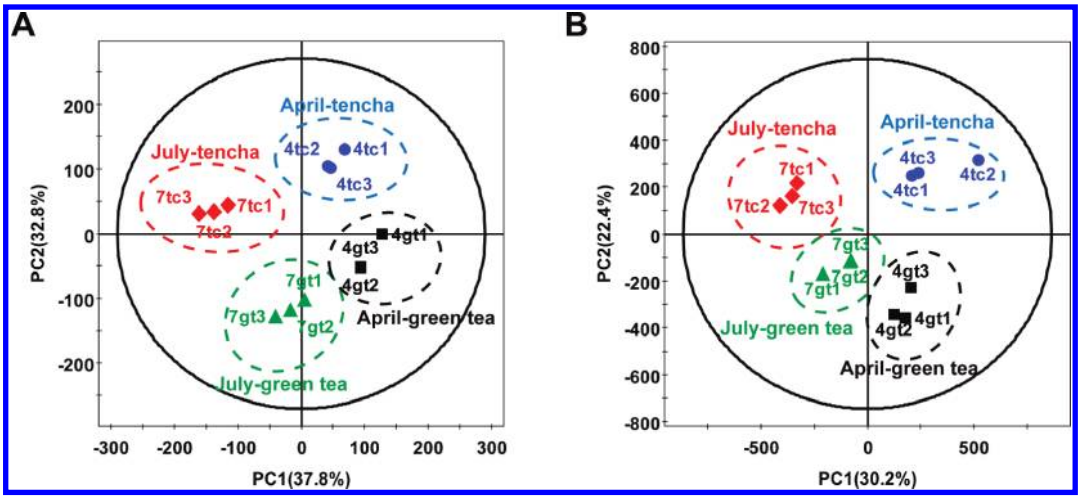

Figure 1. Principle component analysis (PCA) score plots derived from LC-MS (**A**) and GC-MS (**B**) data sets of green tea and tencha harvested in April and July, showing that the metabolomes are clearly different between green tea and tencha depending on harvesting time.

lytic debris. Ten microliters of MTT (5 mg/mL) dissolved in phosphate buffered saline was added at the end of incubation and then incubated further at 37 °C for 4 h. The resultant formazan product was dissolved with 200 μL of DMSO and detected using a microplate reader at 570 nm.

Liquid Chromatography−Photodiode Array−Electrospray Ionization−Mass Spectrometry (LC-PDA-ESI/MS) Conditions. We used a 212-LC Binary Solvent Delivery System, a MetaTherm HPLC Column Heater, a Prostar 410 AutoSampler, a Prostar 335 photodiode array detector, and a 500-ion trap mass spectrometer from Varian Technologies (Palo Alto, CA). The system was operated using MS workstation software (version 6.9.2, Varian, Inc., Palo Alto, CA). Chromatographic separation was performed on a ChromSep HPLC column SS 100 × 2.0 mm i.d., 3 μm particle size (Varian Inc., Lake Forest, CA) with a ChromSep guard column PurSuit XRs 3-C18 (Varian Inc., Lake Forest, CA) at a column oven temperature of 45 °C. A flow rate of 0.2 mL/min was used during separation. The mobile phase consisted of a combination of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient was increased linearly from 15 to 30% B (v/v) at 15 min, to 100% B at 30 min, and held at 100% B up to 35 min, after which it was decreased linearly to 15% at 35.01 min and held at 15% for 40 min until injection of the next analytical sample. The UV spectrum was set at 280 nm for real-time monitoring of the peak intensities. A photo diode array was used to continuously record the absorbance from 200 to 600 nm for tea component identification. Mass spectra were simultaneously acquired using electrospray ionization in negative ionization (NI) modes at 70 V over the range of m/z 50−1000. A drying gas pressure and temperature of 30 psi and 350 °C, respectively, a nebulizer pressure of 50 psi, and capillary voltages of 70 V for NI mode were used. The MS^n analysis was performed with Turbo DDS, data dependent scanning for a 500-MS system, at the same conditions used for metabolite full scanning.

Sample Derivatization for GC-MS Analysis. The green extracts were dried in a freeze dryer until dryness (overnight). For derivatization, 50 μL of methoxyamine hydrochloride in pyridine (20 mg/mL) was added as a first derivatizing agent. The mixture was incubated at 75 °C for 30 min. A second derivatizing agent, 100 μL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), was added and incubated at 75 °C for 30 min. One microliter of sample was injected in split mode (25:1, v/v).

GC-MS Analysis. To pinpoint the differences in primary compounds between green tea and tencha, the gas chromatograph used in this study was a 30 m × 0.25 mm i.d. fused silica capillary column coated with 0.25 μm low bleed, FactorFour column (Varian Inc., Palo Alto, CA) coupled with a Varian 4000 mass spectrometer (Varian, Inc., Palo Alto, CA), and a Varian CP-8400 injector (Varian, Inc., Palo Alto, CA) as an autosampler. GC-MS conditions were set on the basis of a previous study (*21*). The injection temperature was 230 °C. The helium gas flow rate through the column was 1 mL/min. The column temperature was held at 80 °C for 2 min isothermally and then raised at 15 °C/min to 300 °C, and held there for 6 min isothermally. The transfer line and the ion source temperatures were 250 and 230 °C, respectively. Ions were generated by a 70 kV electron impact (EI) and were recorded over the mass range 50−650 m/z.

Data Processing. Data preprocessing was performed with Varian MS Workstation 6.9 software (Palo Alto, CA). The LC-MS raw data files were converted into netCDF (*.cdf) format with Vx Capture (version 2.1, Laporte, MN) for further analysis. After conversion, automatic peak detection and alignment were performed by XCMS. R-program version 2.9.0 (The R project for statistical computing, www.r-project.org) and XCMS version 1.16.3 were used. The XCMS parameters for the R language were performed by simple commands as XCMS's default settings with the following: (http://masspec.scripps.edu/xcms/documentation.php). The data files from GC-MS analyses were deconvoluted using

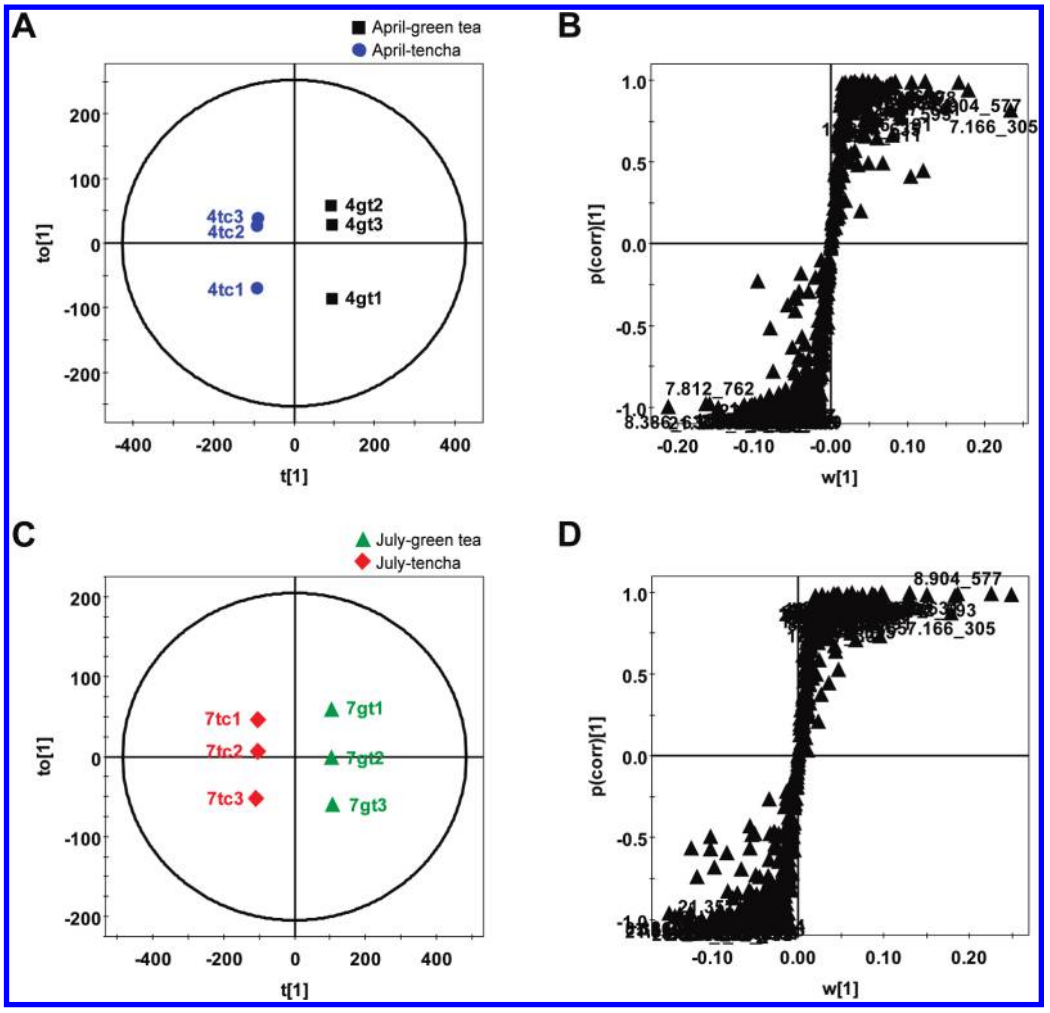

Figure 2. OPLS-DA score plots (**A** and **C**) and loading S-plots (**B** and **D**) derived from an LC-MS data set of green tea and tencha harvested in April and July. The S-plot shows the covariance *w* against the correlation *p* (corr) of the variables of the discriminating component of the OPLS-DA model. Cut-off values for the $p < |0.01|$ were used; the variables thus selected are highlighted in the S-plot with retention time/*m*/*z*.

Table 2. Biomarkers Related to the Variation between Green Tea and Tencha Identified by LC-MS

no	t_R (min)	UV λ_{max} (nm)	$[M-H]^-$ (*m*/*z*)	MS^n fragment ions (*m*/*z*)	tentative identification	references
1	2.86	210, 264	191	172, 127, 110, 85	quinic acid	*26* and *30*
2	3.89	225, 273	343	191, 169	galloylquinic acid	*27*−*30*
3	4.51	222, 272	609/305[a]	261, 221, 179, 165, 137	gallocatechin	*27*, *28* and *30*
4	7.16	239, 271	305	263, 221, 179, 165, 137	epigallocatechin	*26* and *29*
5	8.38	244, 272	633	463, 301, 275, 256, 229	strictinin	*27* and *30*
6	8.90	240, 272	577/289	245, 231, 205, 179	epicatechin	*28*−*30*
7	12.18	222, 276	635	480, 313, 271, 234,	trigalloylglucose	*28*
8	13.24	224, 269, 341	563	503, 473, 443, 383, 353	apigenin glucosyl arabinoside	*28*
9	14.16	223, 257, 354	771	609, 463, 301	quercetin glucosyl rutinoside	*28* and *30*
10	15.77	222, 266, 347	755	593, 447, 285	kaempferol glucosyl rutinoside	*28* and *30*
11	17.07	224, 266, 349	593	447, 285	kaempferol rutinoside	*28*−*30*
12	20.86	311	917	752, 301	quercetin *p*-coumaroyl glucosylrhamnosylgalactoside	*28* and *30*
13	21.35	270, 311	901	755, 285	kaempferol *p*-coumaroyl glucosylrhamnosylgalactoside	*28* and *30*

[a] $2[M-H]^-/[M-H]^-$.

NIST's Automated Mass Spectral Deconvolution and Identification System (AMDIS). Then, SpectConnect (http://spectconnect.mit.edu/index.php) was used to automatically list and track otherwise unidentifiable conserved metabolite peaks across sample replicates and different sample conditions groups without the use of reference spectra (*22*). With mass/retention time pairs, the corresponding peaks were confirmed in the original chromatogram and compared to the NIST mass spectral database (National Institute of Standards and Technology, FairCom Co., USA).

Statistical and Multivariate Analysis. Statistical analyses were performed on all continuous variables using SIMCA-P+ (version 12.0, Umetrics, Umeå, Sweden). Univariate statistics was performed by breakdown and one-way ANOVA using SPSS (version 15.0, SPSS Inc., Chicago, IL) and STATISTICA (version 7.0, StatSoft Inc., Tulsa, OK). Unsupervised principal component analysis (PCA) was run for obtaining a general overview of the variance of metabolites, and supervised orthogonal projection to latent structure discriminant analysis (OPLS-DA) was performed to obtain information on differences in the metabolite composition of samples. Biomarkers for the difference between green tea and tencha were subsequently identified by analyzing the S-plot, which was declared with covariance (*p*) and correlation (*p*corr). All variables were

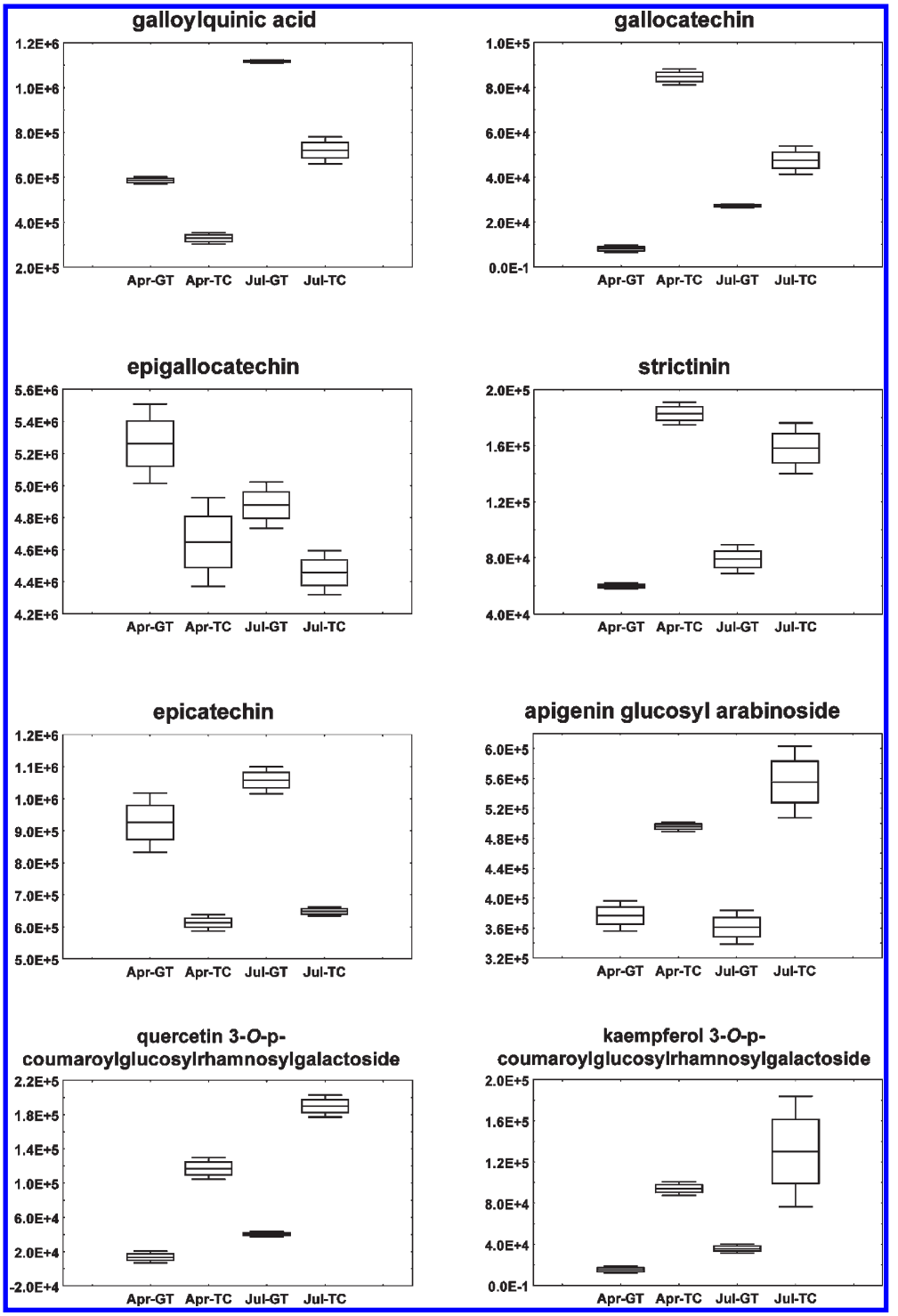

Figure 3. Significantly different secondary metabolites between green tea and tencha. These secondary metabolites were identified by LC-MS and are responsible for the differentiation in the OPLS-DA model. (Line, mean; box, standard error; whisker, standard deviation.)

mean centered and scaled to Pareto variance for both PCA and OPLS-DA of metabolites from LC-MS and GC-MS in a column-wise manner.

RESULTS AND DISCUSSION

Bioactivities and Total Compound Content of Differently Cultivated Green Teas. As summarized in **Table 1**, the antioxidant activity of green teas (harvested both in April and July) was significantly higher than that of tencha in three different antioxidant assays. In the case of green teas, the antioxidant activities were significantly stronger than those of tencha. The cell protective activities of green tea were also higher than those of tencha in different seasons, but only the green tea from April showed statistical difference. TPC and TFC of green tea were significantly higher than those of tencha, but the samples harvested in April were not statistically different. The high content of antioxidant compounds such as polyphenol and flavonoid was probably responsible for the cell protective effect against H_2O_2-induced toxicity or the scavenging effect against hydroxyl radical. The TPC and TFC of the tencha samples were lower than those in green tea, probably due to shading cultivation, which lowers the biosynthesis of catechins through decreased expression of the genes encoding for chalcone synthase and phenylalanine

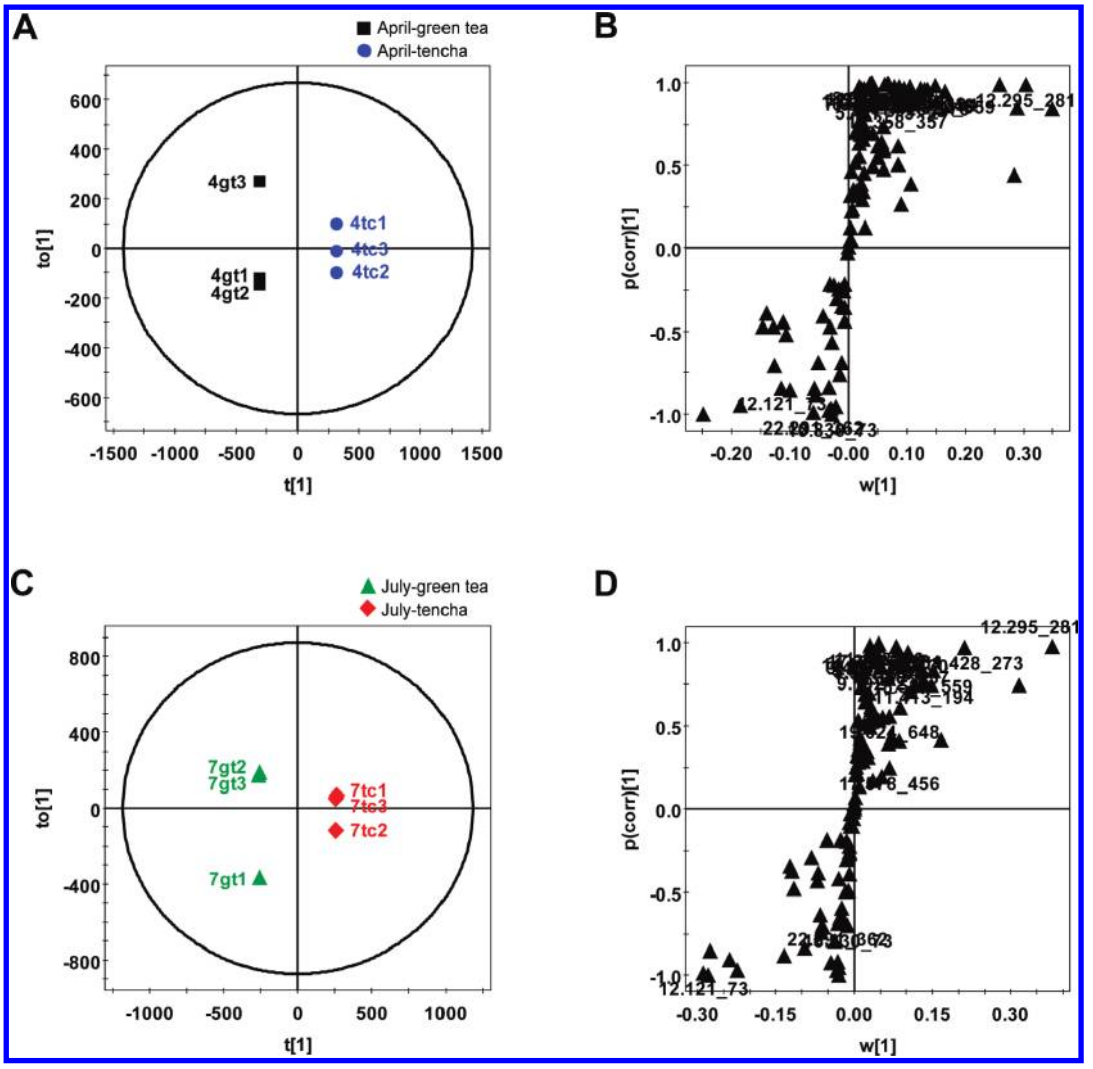

Figure 4. OPLS-DA score plots (**A** and **C**) and loading S-plots (**B** and **D**) derived from a GC-MS data set of green tea and tencha harvested in April and July. The S-plot shows the covariance *w* against the correlation *p* (corr) of the variables of the discriminating component of the OPLS-DA model. Cut-off values for the $p < |0.01|$ were used; the variables thus selected are highlighted in the S-plot with retention time/*m*/*z*.

ammonia-lyase (PAL) in the tea tree (*23*). The concentration of TCC and TAA in green teas from two different seasons was lower than that in tencha. TPC concentration was well correlated with the antioxidant assays, ABTS (r = 0.983**), DPPH (r = 0.981**), and FRAP (r = 0.985**). The concentration of TFC was also well correlated with the antioxidant assays, ABTS (r = 0.940**), DPPH (r = 0.901**), and FRAP (r = 0.953**). Thus, we think that TFC and TPC are the most important factors affecting the antioxidant activity of green tea. The three different antioxidant assays were well correlated with each other. This result was most likely due to the fact that these three different antioxidant assays are based on the same mechanism: the electron-transfer reaction (*24*).

Metabolite Profiling of Green Teas by LC-MS and GC-MS. From the 12 samples obtained from various different green teas, 477 peaks were extracted by XCMS with the LC-MS data set, and 118 peaks were extracted by SpectConnect with the GC-MS data set. Preliminary PCA analysis with mean centering was performed on all samples. As shown in the **Figure 1**, the PCA score plot derived from the LC-MS data set explained 70.6% of the total variance (R^2) with the prediction goodness parameter Q^2 = 50.6%. As a result, green tea and tencha were clearly separated by PC2 (32.8%) and also were discriminated by PC1 (37.8%) with seasonal variation between April and July (**Figure 1A**). The PCA score plot of the GC-MS data set explained 52.6% of R^2 with 50.0% of Q^2. Green tea and tencha were separated by PC2 (22.4%) and also discriminated between April and July samples by PC1 (30.2%) (**Figure 1B**). PCA score plots were obtained using Pareto scaling with mean-centering from the data set. The green tea and tencha of hot water extraction were also clearly discriminated in the PCA plot (further detailed in Supporting Information, Figure S1). Green teas, especially shade green tea (tencha), are taken not only as hot water extracts but also as whole powders, matcha (fine powder made from tencha). Therefore, the metabolome analysis based on mixed solvent extraction is useful for revealing the compositional differences of bioactive compounds and reflect the real life consumption of tea.

Multivariate Statistical Analysis Using LC-MS. To clearly find out the significantly correlated metabolites on shading effects, OPLS-DA modeling was applied to LC-MS data sets. These models constructed with data from shade cultivation methods separate tencha from green tea which was harvested the same season along the discriminating t[1]. The model showed one orthogonal component, with R^2 = 0.56 and Q^2 = 0.99 in April, and R^2 = 0.67 and Q^2 = 0.99 in July samples (**Figure 2A** and **C**). Potential biomarkers for separation by shade effects were subsequently identified using S-plots, which were represented with covariance (*p*) against correlation (*p*corr) (*25*). The S-plots of the

Table 3. Biomarkers Related to the Variation between Green Tea and Tencha Identified by GC-MS

no	name	retention time (min)	MS fragment ions (*m*/*z*)	derivatized[a]
1	succinic acid	6.62	147, 248	TMS (x2)
2	malic acid	8.91	147, 189, 217, 233, 245, 306	TMS (x3)
3	pyroglutamic acid	9.15	147, 156, 230, 258	TMS (x2)
4	threonic acid	9.44	147, 217, 220, 292	TMS (x3)
5	fructose	11.93	147, 217, 277, 307, 364	TMS (x5)
6	dihydroxyanthraquinone	17.12	179, 369, 383	TMS (x2)
7	terephthalic acid	17.16	219, 255, 346, 401	ND[b]

[a] Number of hydrogen atoms derivatized. [b] ND, not detected with the derivatized form.

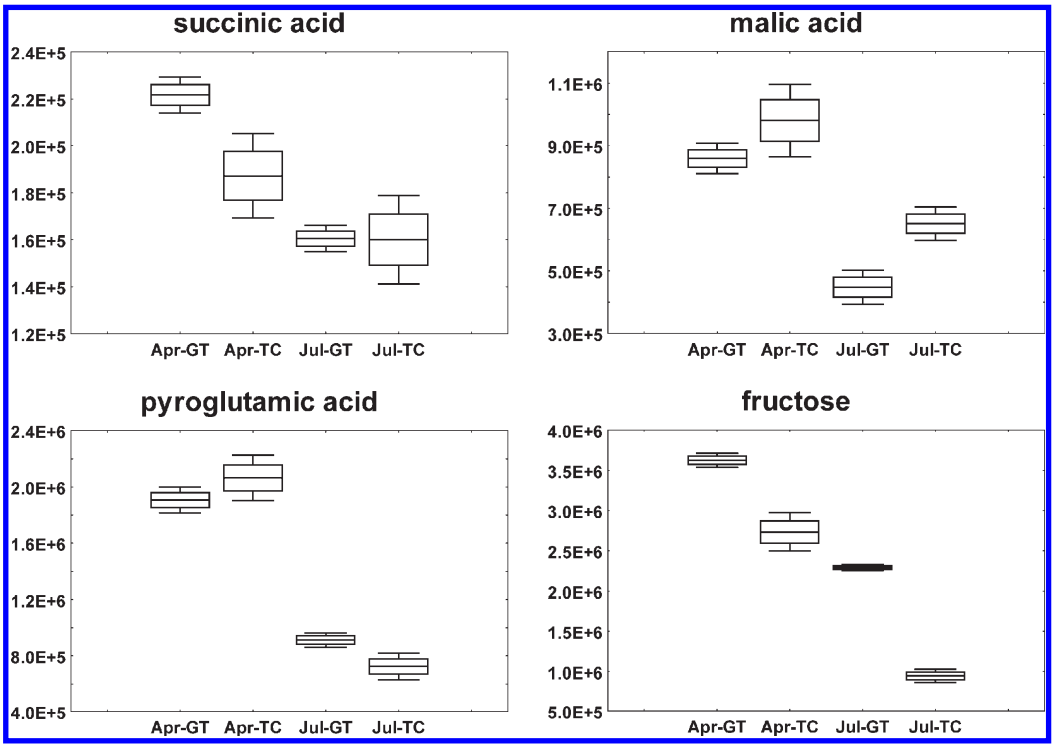

Figure 5. Significantly different primary metabolites between green tea and tencha. These primary metabolites were identified by GC-MS and are responsible for the differentiation in the OPLS-DA model. (Line, mean; box, standard error; whisker, standard deviation.)

OPLS-DA were proposed for the identification of potential biomarkers of group separation by Wiklund et al. (*13*). It shows the most relevant variables on the differentiation of two samples. For the indication of plots with retention time *m*/*z*, cutoff values $P < 0.01$ were used (**Figure 2B** and **D**). The variables accounting for biomarkers were identified using an in-house database (*26*) and cochromatography, the MS/MS spectral data, and the UV spectral data of the authentic compounds available and/or by comparison with the published literature (*27*−*30*).

The identified biomarkers are summarized in **Table 2**. The mass peak intensities of galloylquinic acid, gallocatechin, epigallocatechin, strictinin, epicatechin, apigenin glucosyl arabinoside, quercetin *p*-coumaroylglucosylrhamnosylgalactoside, and kaempferol *p*-coumaroylglucosylrhamnosylgalactoside were significantly different between green tea and tencha in both April and July (**Figure 3**). The level of galloylquinic acid, epigallocatechin, and epicatechin decreased when green tea was grown under shade. A recent study revealed that tea trees accumulated catechins to protect against damage from ultraviolet-B (*31*). Thus, epigallocatechin and epicatechin synthesis was decreased in tea leaves cultivated in the shade, which was due to their lower exposure to ultraviolet-B (*23*). In a previous study, galloylquinic acid, epigallocatechin, and epicatechin were identified as major antioxidant compounds in green tea (*32*). Thus, we speculate that the lower antioxidant activity of tencha was due to the low concentration of these compounds. It is worth noting that strictinin can be enhanced by shading treatment, which is important compound because it has immunostimulating activity (*33*), lipid peroxidation (*34*), and antiallergic activity (*35*).

Multivariate Statistical Analysis Using GC-MS. As shown in the PCA score plot (**Figure 1B**), significant differences were detected between green tea and tencha. The model showed one orthogonal component, with $R^2 = 0.45$ and $Q^2 = 1$ in April, and $R^2 = 0.43$ and $Q^2 = 0.99$ in July samples (**Figure 4A** and **C**). The S-plots showed the most relevant variables on differentiation between green tea and tencha. Also, to indicate plots with retention time *m*/*z*, cutoff values $P < 0.01$ were used (**Figure 4B** and **D**). Biomarkers related to the variation between green tea and tencha were identified using a NIST library and compared with the previous report (*21*), summarized in **Table 3**. Succinic acid, malic acid, pyroglutamic acid, and fructose were significantly different between green tea and tencha in both April and July (**Figure 5**). Green tea showed higher levels of succinic acid and fructose together with lower levels of malic acid and pyroglutamic acid than tencha.

The change in primary metabolites was influenced by shade cultivation and harvesting season (**Figure 5**). The samples harvested in April were found to have more primary metabolites than those in July. To analyze the effect of seasonal variation on the primary metabolites of green tea, more than 20 peaks on GC-MS

chromatograms were selected and identified using the NIST library and compared with the reference (*21*) (further detailed in Table S1, Supporting Information). With the result, the amino acids and organic acid composition were significantly changed by shade culture in the two seasons harvested (**Figure 5**). The increased amino acid content from shading seems to be dependent on the season since this increase was much higher in April. The compound that was most significantly increased in April was pyroglutamic acid, which was a derivative from glutamate, and contributes to the sour and umami taste of green tea (http://www.ajinomoto.co.jp/kfb/amino/e_aminoscience/bc/b-7.html). In addition, sugar content was decreased by seasonal variation as well as shading. We speculate that the decreased level of sugars was associated with a decrease in photosynthesis due to the amount of sunshine depending on the season.

To the best of our knowledge, this was the first time that a metabolomics approach was used to reveal compositional differences between green teas cultivated under different conditions. LC and GC-mass spectrometry coupled with multivariate analyses were used to obtain the experimental results, and interesting and meaningful differences between the various samples were identified. In summary, green teas have more catechins and sugars than tencha, whereas tencha samples have more flavonoid derivatives than green teas. Strictinin, gallocatechin, apigenin glucosyl arabinoside, quercetin *p*-coumaroylglucosylrhamnosylgalactoside, and kaempferol *p*-coumaroylglucosylrhamnosylgalactoside were identified as biomarkers of tencha. The levels of epicatechin, epigallocatechin, galloylquinic acid, succinic acid, and fructose found in the green tea samples were significantly higher than those found in the tencha samples. Since most of the catechin compounds have antioxidant capability, this could be one of the reasons why the tencha sample showed less antioxidant activity. In addition, we have demonstrated that metabolomics is a useful tool for analyzing the compositional pattern of green teas cultivated under different conditions.

Supporting Information Available: Primary metabolite identification of Green tea by GC-MS; PCA score plot derived from LC-MS and GC-MS data sets of green tea and tencha water extracts; and description of hot water extraction. This material is available free of charge via the Internet at http://pubs.acs.org.

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