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^{1}H NMR Based Metabolic Profiling in the Evaluation of Japanese Green Tea Quality

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Classification of tea quality is now mainly performed according to the sensory results by professional tea tasters. However, this evaluation method is inconsistent in differentiating their qualities. A combination of a ^{1}H NMR technique and a multivariate analysis was introduced to the quality evaluation of green tea by means of a metabolomic technique. A broad range of metabolites were detected by ^{1}H NMR spectrometry. The principal component analysis (PCA) was used to reduce the complexity of the ^{1}H NMR spectra data set and provided the quality discrimination result. It offered an extensive clue for classification and quality assessment without any prepurification method. A set of green teas from a Japanese tea contest were analyzed by ^{1}H NMR to classify the quality with respect to that judged by tea tasters and to conceive a quality prediction model. Metabolic profiling and fingerprinting of ^{1}H NMR spectra of green teas with different quality were studied. PCA showed a separation between the high- and the low-quality green teas. The taste marker compounds contributing to the discrimination of tea quality were identified. Reliable prediction models were obtained by the partial least-squares projection to latent structure (PLS) analysis together with a preprocessing filter of both orthogonal signal correction (OSC) and a combination between OSC and wavelet transform algorithms.

KEYWORDS: Metabolomics; ^{1}H NMR; metabolic profiling; metabolic fingerprinting; quality assessment; green tea; *Camellia sinensis*

INTRODUCTION

The fresh infusion of dried young leaves of tea from *Camellia sinensis* has become a highly consumed and desirable drink. Hundreds of teas are generally sorted into three main categories depending on their fermentation process: green (unfermented), oolong (partially fermented), and black (fermented). Green and oolong teas are mostly consumed in Asia and Northern Africa, while black tea is a worldwide drink (*1*). The chemical constituents of tea, which relate straightly to quality, are variable depending on several factors such as species, environment, growth, and storage conditions as well as tea leaf quality (*1*). In general, the quality of tea is assessed through its appearance, scent, and flavor. The taste quality is one of the key criteria of professional tea tasters to evaluate the tea quality. The four sensory words, bitterness, astringency, sweetness, and umami (a brothy or savory taste), are the characteristic flavors and are commonly used by tea tasters to describe the quality of tea infusion (*2–4*). Bitterness and astringency are attributed from alkaloid caffeine and catechins, respectively (*5, 6*), while umami-like taste is due to amino acids, especially theanine or 5-*N*-ethylglutamine (*7, 8*). However, the chemical compositions used to determine the tea quality are different between black and green teas. The quality of green tea is usually described by the high content of amino acids, caffeine, and some catechins (*9–11*).

Nowadays, the classification of tea quality is mainly performed according to the sensory result by professional tea tasters. However, this evaluation method is inconsistent in differentiating tea quality. Analytical techniques including mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectrometry offer a wide range of metabolites resulting in complex data sets. This information, in combination with metabolomics, has a potential to provide a reliable and conclusive picture of the composition of green tea related to its quality. Many attempts have been done by means of chromatographic and spectroscopic techniques to determine qualitatively the active compounds involved in the quality assessment of green tea (*1, 8, 10, 12–14*). Recently, chemometric methods including principal component analysis (PCA) and partial least-squares projection to latent structure (PLS) analysis based on gas chromatography/mass spectrometry (GC/MS) have been successfully relevant to quality control of

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the primary metabolites in green tea (*15*). In this new approach chemical fingerprinting is used instead of specific marker compounds to predict the quality of green tea, which gives a fast and more reliable result compared to that obtained from sensory test by tea tasters. On the basis of our knowledge, the consistent chemometric method used to determine the quality/grade of green tea is limited only to primary metabolites by GC/MS.

In this study, a combination of nontarget ^{1}H NMR based metabolomics and a pattern recognition technique will be established for the quality prediction of Japanese green tea. The ^{1}H NMR metabolic profiling and fingerprinting are expected to give a wealth of information in a broad range of metabolites but simplify sample preparation and short analysis time. In addition, green tea series with known ranking judged by professional tea tasters from the Kansai tea contest in Japan were employed to make a quality regression model in order to evaluate unknown green tea samples.

MATERIALS AND METHODS

Materials. The 53 ranked samples of dried first-crop green tea leaves or Ichiban cha in Japanese obtained from the commercial tea contest in the Kansai area of Japan were used in this study and were purchased by the Tea Branch of the Nara Prefecture Agricultural Experiment Station. The sensory quality of tea was scored and judged by professional tea tasters on the basis of its appearance, aroma, and flavor.

Chemicals and Reagents. All standard compounds used for ^{1}H NMR assignments were analytical grade with purity higher than 90%. Deuterium oxide (D_2O, D 99.9 atom %) purchased from Cambridge Isotope Laboratories, Inc., was used as solvent and 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS, 97%) from Aldrich was used as internal standard for all ^{1}H NMR measurements. Phosphate buffer solution (1.0 M, pH 7.4) used in this experiment was obtained from Sigma.

Sample Preparation for ^{1}H NMR Analysis. One milliliter of D_2O was added to 50 mg of dried, ground green tea leaves (ground with a Retsch ball mill at 20 Hz, 1 min) in a 1 mL Eppendorf tube. The mixture was continuously incubated in Thermomixer comfort (Eppendorf) at 60 °C and 1400 rpm for 30 min, followed by centrifugation at 25 °C and 16000*g* for 10 min (Centrifuge 5415 R, Eppendorf). The supernatant containing hydrophilic metabolites was filtered through a 0.45 μm PTFE membrane (Advantec). Four hundred microliters of filtrate was then pipetted and dissolved in 200 μL of 0.2 M buffer solution containing 3 mM DSS to make a total 600 μL solution reserved for NMR measurement. All samples were prepared one day prior to ^{1}H NMR analysis.

NMR Spectrometry. ^{1}H NMR spectra were recorded at 25 °C with a 750 MHz Varian Inova 750 spectrometer using a 5 mm ^{1}H{^{13}C/^{15}N} triple resonance indirect detection probe. D_2O and DSS were used as the internal lock signal and internal standard at chemical shift (δ) 0.0 ppm, respectively. The ^{1}H NMR measurement was carried out with 64 transients and 128K complex data points. The acquisition time and recycle delay were 6.257 and 3.743 s per scan, respectively, using a 30° pulse angle. The presaturation pulse sequence was applied to suppress the residual water signal. All spectra were Fourier transformed with 0.1 Hz line broadening prior to data reduction and preprocessing.

NMR Data Reduction and Preprocessing. All NMR spectra were first phased and baseline corrected by Chenomx NMR Suite4.6 software, professional edition (Chenomx Inc., Canada). Each NMR spectrum was reduced to a smaller number of variables by integrating regions of an equal bin size of 0.02 ppm over a range of 1.0–8.0 ppm, while those of the water signal between 4.6 and 5.0 ppm were eliminated. All bins were normalized to the area of the DSS methyl peak to provide absolute contributions of particular resonances to the spectrum prior to the conversion of Chenomx software to the Microsoft Excel format (*.xls). The Excel format was then imported to Pirouette software version 3.11 (Infometrix, Inc., Woodinville, WA) to change to the ASCII format (*.dat) and subjected to in-house software for spectra overlay and multiple baseline correction prior to multivariate analysis.

^{1}H NMR Assignment and Pattern Recognition. The chemical shifts of significant chemical constituents were assigned by comparing their resonances to those of authentic standard compounds including quinic acid, *p*-coumaric acid, *myo*-inositol, 2-*O*-β-L-arabinopyranosyl-*myo*-inositol, chlorogenic acid, L-arginine, (–)-epigallocatechin-3-gallate (EGCG), (–)-epicatechin-3-gallate (ECG), (–)-epigallocatechin (EGC), and (–)-epicatechin (EC). The Chenomx 750 MHz (pH 4–9) libraries provided from Chenomx were also used for peak assignments.

Principal component analysis (PCA), an unsupervised pattern recognition method, of the ^{1}H NMR spectra was performed by Pirouette software version 3.11. The significant intrinsic variation could be differentiated in simplified two- or three-dimensional forms by reducing the complexity of the data sets *via* the mapping method. In this analysis the mean center was used as a preprocessing method.

Partial least-squares (or projection to latent structure, PLS), orthogonal signal correction (OSC), and OSC wavelet compression (OSCW) were chosen to create a prediction model. PLS was calculated using SIMCA-P software version 11.0 (Umetrics AB, Umeå, Sweden), which can be described as the regression extension of PCA. It derives latent variables which maximize the covariation between measured data (X) and the response variable (Y) regressed against instead of describing the maximum variation in the X matrix used in PCA (*16*). The mean center was used as a preprocessing method for all data sets before analysis.

Orthogonal signal correction (OSC) is normally used to remove the uncorrelated variables or orthogonal to Y from X using the nonlinear iterative partial least-squares (NIPALS) algorithm (*17, 18*). This approach will cope also with moderate amounts of missing data. The residual data from this orthogonal model are obscured the variation in the data set. In this study, the mean center was applied as the preprocessing method for OSC analysis.

Another approach used in this experiment was a combination between OSC and wavelet transform named OSCW, which tended to compress and denoise complicated signals (*19*). The wavelet transform uses a mother wavelet as a basis function with a certain scale to investigate the time scale properties. The detail properties of either sharp or coarse signals are captured by varying the width of window function. The wavelet transform technique and wavelet function used in this study were discrete wavelet transform (DWT) and Daubechies-10, respectively.

RESULTS AND DISCUSSION

Identification of Chemical Constituents in Dried Green Tea Leaves. To determine the hydrophilic compounds imparted in the quality of green tea, the aqueous solution was extracted with D_2O at 60 °C for 30 min. The ^{1}H NMR spectrum of water-soluble compounds extracted from the best quality tea evaluated by tea taster ranking as no. 1, which was selected as an example for signal assignment, is shown in **Figure 1**. The resonances of metabolites were assigned by comparison to those of the well-defined standard compounds run in the same condition as green tea as well as the Chenomx Suite4.6, 750 MHz (pH 4–9), library database. The corresponding resonances were in good agreement with standard signals. However, some resonances were slightly different from those assigned in the Chenomx library database due to the difference of measurement condition. About 10 major compounds were identified. Theanine (δ 1.10, 2.12, 2.39, 3.19 ppm), a predominant amino acid in green tea (*20*), and quinic acid (δ 1.88, 1.97, 2.05 ppm) were mostly observed in the low-frequency region at δ 0.5–3.0 ppm (**Figure 1A**). By excluding common sugar signals such as sucrose and fructose, the signals due to caffeine (δ 3.27, 3.43, 3.89 ppm), arginine (δ 3.22, 3.47 ppm), *myo*-inositol (δ 3.30 ppm), chlorogenic acid (δ 3.89, 4.22 ppm), and quinic acid (δ 3.56 and 4.01 ppm) were clearly presented in the middle-frequency region at δ 3.0–4.5 ppm (**Figure 1B**). Signals presented in the high-frequency region at

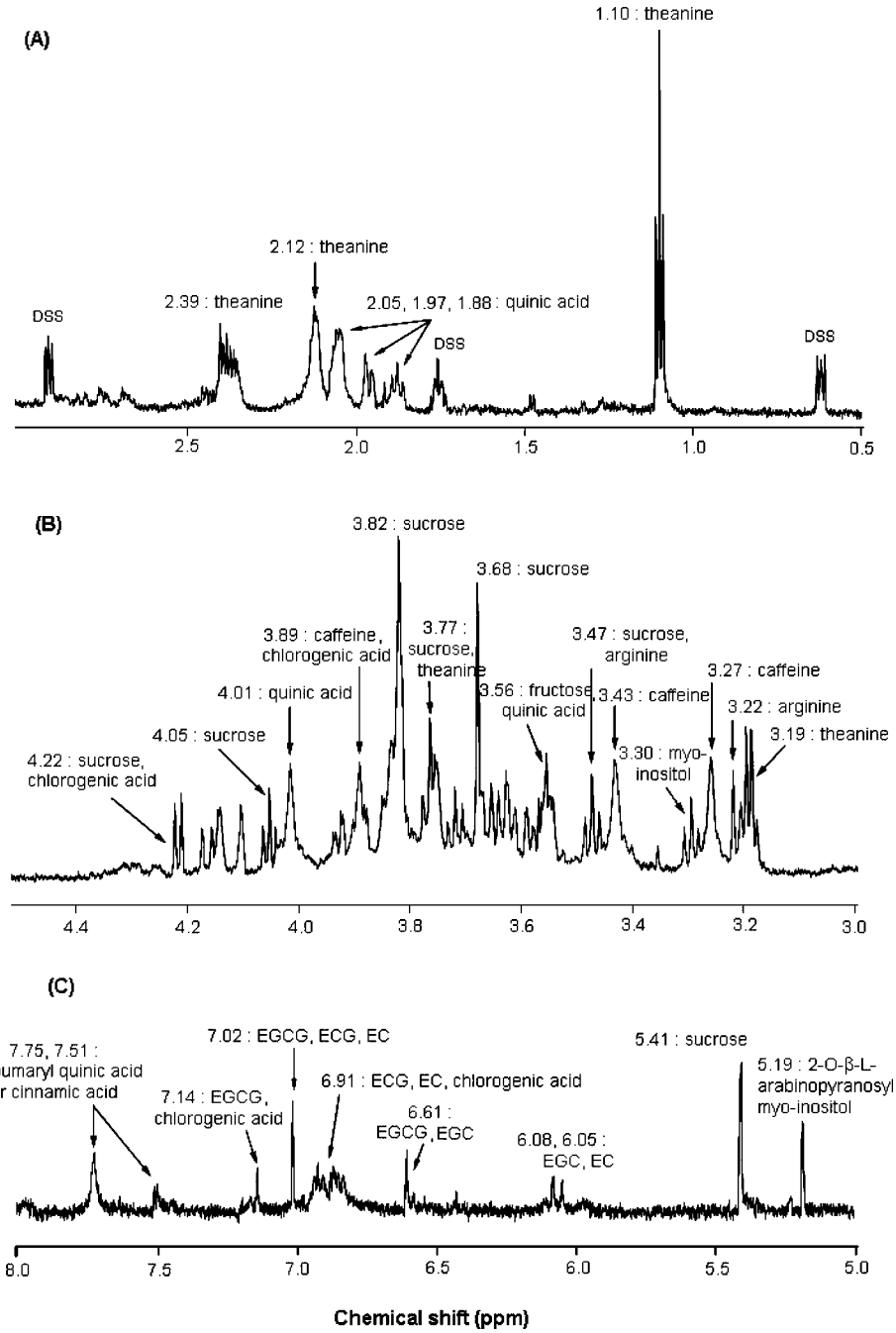

Figure 1. ^{1}H NMR spectra (750 MHz, D_2O) of green tea extract from the highest quality sample ranking no. 1 in **(A)** high-, **(B)** middle-, and **(C)** low-frequency regions, measured at 25 °C.

δ 5.0–8.0 ppm were mainly accounted for by 2-*O*-β-L-arabinopyranosyl-*myo*-inositol (δ 5.19 ppm), *p*-coumaryl quinic acid and/or cinnamic acid (δ 7.51, 7.75 ppm), EGCG (δ 6.61, 7.02, 7.14 ppm), and ECG (δ 6.91, 7.02 ppm). Signals of EGC (δ 6.05, 6.08, 6.61 ppm) and EC (δ 6.05, 6.08, 6.91, 7.02 ppm) were insignificantly observed in this region due to its lower water solubility.

The quality evaluation of 53 green tea samples has been judged by professional tea tasters from a green tea contest in 2005. The best quality was defined as no. 1, while the worst one was assigned as no. 53, ranked in order. In this study, these 53 samples were divided into two groups: high quality (green tea nos. 1–25) and low quality (green tea nos. 26–53). The overlaid ^{1}H NMR spectra in different chemical shift regions of all green tea samples are shown in **Figure 2** in which the high- and low-quality teas are tinted as blue and pink, respectively. By comparing these ^{1}H NMR spectra, no significant difference between the high- and the low-quality teas was observed in low- (**Figure 2A**) and high- (**Figure 2C**) frequency regions. In the middle-frequency region (**Figure 2B**), on the contrary, ^{1}H NMR spectra of the high-quality teas differed from the low-quality ones, most particularly in the caffeine, a major xanthine in green tea. Caffeine signals at δ 3.27 and 3.43 ppm were rich in the high-quality tea, but most were absent in the low-quality one. The relationship between tea quality and chemical components in green tea has been studied (*1, 10, 11, 21–23*). Theanine, catechins, caffeine, and gallic acid are frequently used as taste markers of tea infusion. The important role of caffeine in the black tea quality characteristics has been reported (*24*). It contributes toward the bitter taste of black tea. In addition, a complex of caffeine and theaflavin kinds of polyphenols, makes a distinct brisker taste characteristic which contributes positively

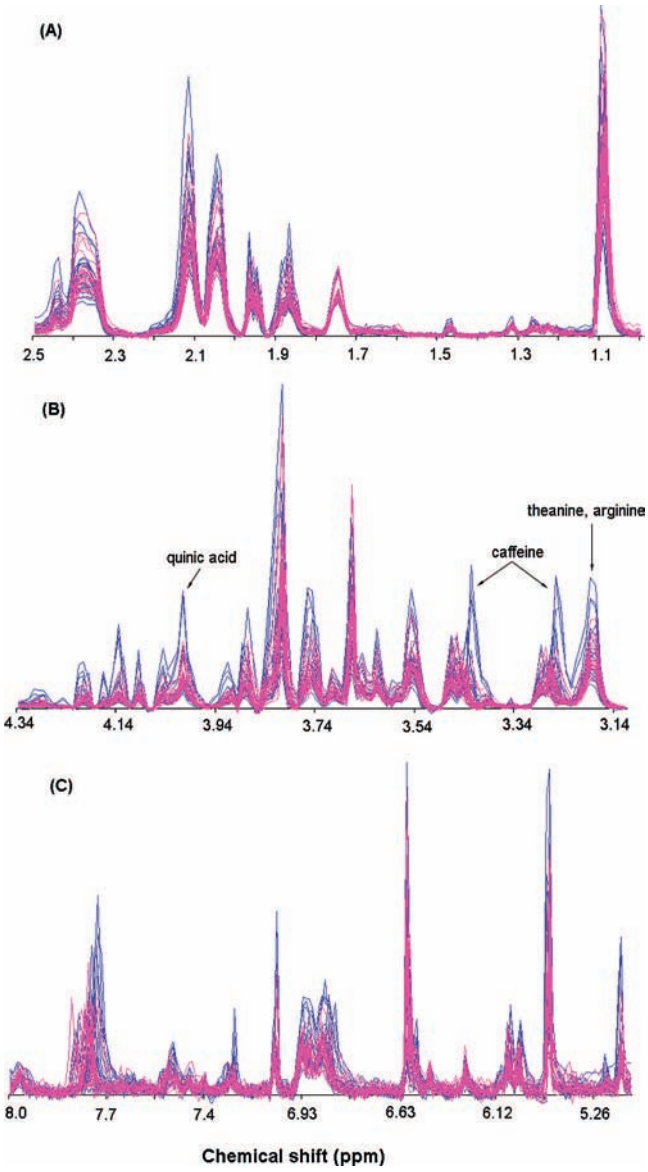

Figure 2. ^{1}H NMR spectra (750 MHz, D_2O) of green tea extract in **(A)** high-, **(B)** middle-, and **(C)** low-frequency regions, measured at 25 °C. Each spectrum is colored by the category of tea quality: high-quality (blue) and low-quality (pink) teas. Green tea nos. 2 and 8 were omitted from the spectrum overlay due to a baseline problem.

to black tea evaluation (*25*). The similar role was expected to be able to be valid through green tea infusion as well.

The presence of caffeine signals mainly in the high-quality green tea was in good agreement to the previous black tea studies indicated elsewhere. The positive correlation between caffeine content and taster's preferences demonstrated clearly that caffeine was a key constituent used by professional tea tasters to rank the quality of green tea.

Metabolite Fingerprinting of Green Tea Extracts. Fingerprinting overlooks the problems due to complicated signal assignments. Instead, multivariate analysis is used to compare sets of spectra by sorting data sets into categories (*26*). PCA was used as the pattern recognition method analyzing individually in the different chemical shift regions: the low (δ 1.0–2.5 ppm), middle (δ 3.12–4.34 ppm), and high (δ 5.15–6.68 ppm) frequencies. The results of the PCA score and loading plots of green tea extracts in the middle and high frequencies are shown in **Figures 3** and **4**, respectively; however, data of those in the

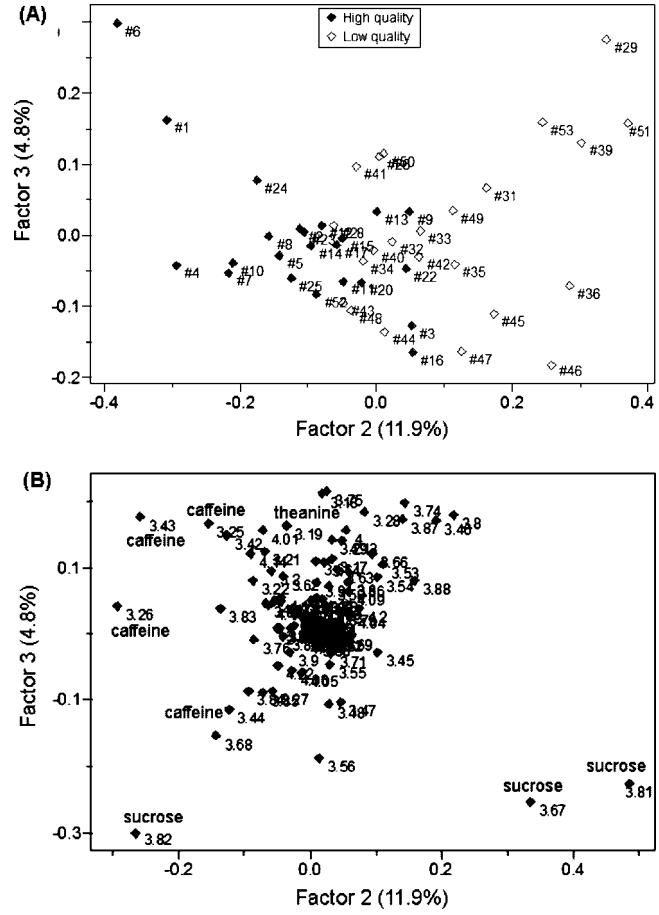

Figure 3. PCA of green tea NMR profiles in the middle-frequency region between δ 3.12 and 4.34 ppm. PCA shows a separation of clustering between high-quality (♦) and low-quality (◇) teas. **(A)** PCA score plot of the second and third PCs; **(B)** PCA loading plot responsible for PCA classification. The number in parentheses describes the fraction of the variance in the NMR data set explained by each component.

low-frequency region were not shown here due to the fact that no significant discrimination was observed.

The PCA score plots (**Figures 3A** and **4A**) showed clustering of green tea samples related to the high and low qualities in the second and third PCs. The corresponding loading plots (**Figures 3B** and **4B**) showed that the ^{1}H NMR signals from caffeine, theanine, EGCG, ECG, EGC, and EC, after ignoring the overlapped sugar peaks, contributed most to the cluster separation, which was in good agreement to the previous studies. These compounds have been reported to be rich in the high-quality tea and corresponded to the major tea taste metabolites that are usually used to evaluate the quality of tea, except EC and EGC (*1, 10, 11, 21–23*). It should be noted that EGC and EC had less hydrophilicity compared to EGCG and ECG; thus EGCG and ECG were predominant catechins and played a more important role in quality assessment when the water-soluble metabolite was concerned. The significant compounds used to create a quality prediction model obtained from ^{1}H NMR were similar to that of GC/TOF-MS (*15*). It showed that sugars, quinic acid, caffeine, and theanine were the significant compounds used to create a quality prediction model (*15*); however, EGCG, EGC, ECG, and EC were not detected by GC/TOF-MS, indicating that the result obtained from ^{1}H NMR gained more qualitative information compared to that obtained from the GC/MS technique.

By comparison to the result obtained from individual ^{1}H NMR analysis, a combination between ^{1}H NMR and multivari-

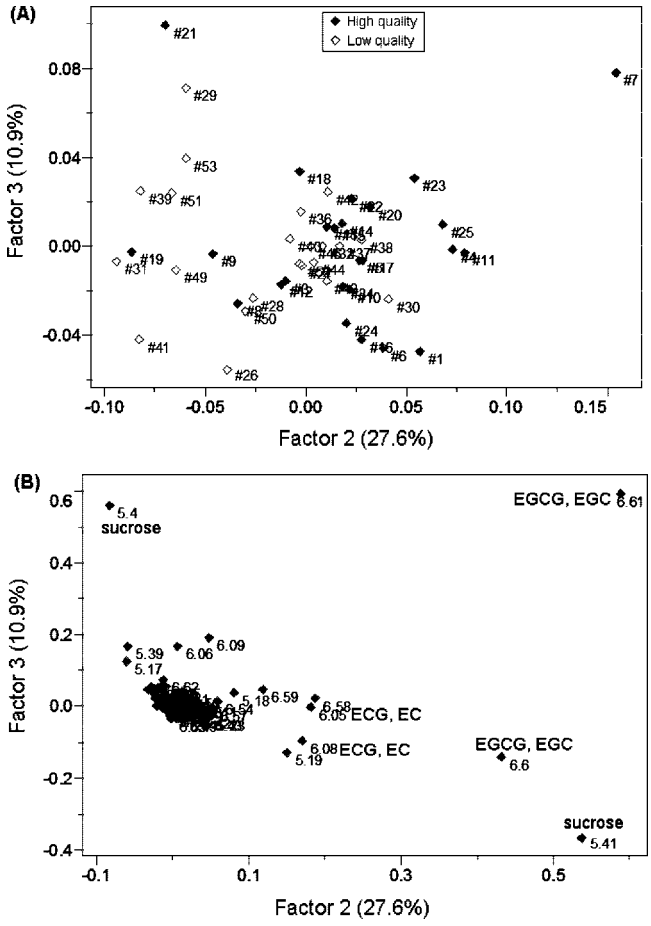

Figure 4. PCA of green tea NMR profiles in the high-frequency region between δ 5.15 and 6.68 ppm. PCA shows a separation of clustering between high-quality (♦) and low-quality (◊) teas. **(A)** PCA score plot of the second and third PCs; **(B)** PCA loading plot responsible for PCA classification. The number in parentheses describes the fraction of the variance in the NMR data set explained by each component. Key: EGCG, (–)-epigallocatechin-3-gallate; EGC, (–)-epigallocatechin; EC, (–)-epicatechin; ECG, (–)-epicatechin-3-gallate.

ate analyses allowed the comprehensive identification of key metabolites which help to assess the quality determination in tea infusion.

Quality-Predictive Model. Any regression models are built mainly to predict the dependent (response) variables *Y* from independent (predictor) variables *X* (*27*). The regression is made by creating the mathematical model based on the system behavior, followed by the determination of optimal values for model parameters with respect to training samples. Then, values of unknown independent variables are predicted by using the resulting training model (*27*). PLS regression is one of the chemometric projection methods relating both *X* and *Y* variables *via* a linear multivariate model (*28*) and was applied to the quality predictive of green tea. In this study, the tea quality ranking judged by professional tea tasters was used as a dependent variable. The entire data set was divided into two parts: a training set which was used to create a model and a test set that was used to verify the model's predictive ability and not included in the regression model. The samples ranked nos. 5, 15, 25, 35, and 45 were used as a test set for model validation. The data were centered and scaled to unit variance before analysis without any transform was applied.

The PLS relationship between measured and predicted values of green tea samples is presented in **Figure 5**. The quality of

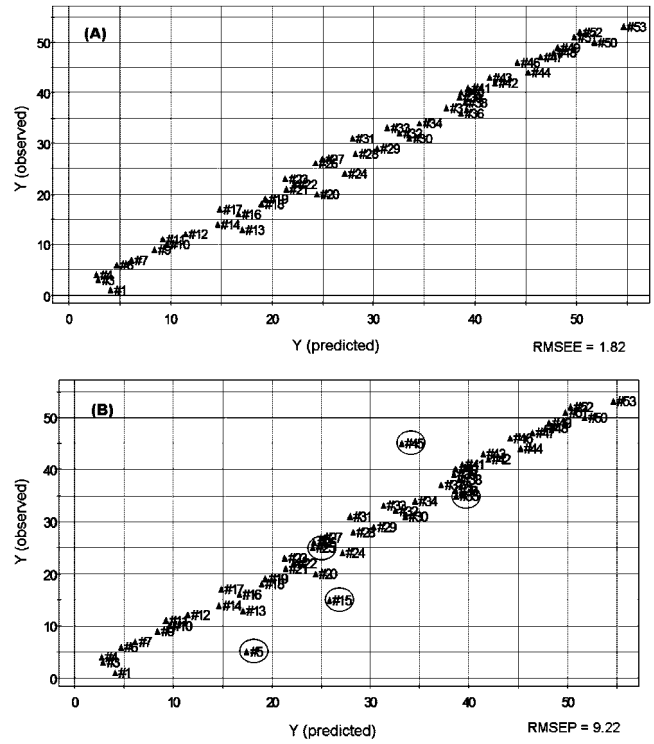

Figure 5. Observed and predicted green tea quality for the PLS model calculated from the ^{1}H NMR data set of **(A)** 46 tea samples as the training set and **(B)** 51 tea samples included in training and test (circle mark) sets. Green tea nos. 2 and 8 were omitted from the PLS model due to a baseline problem.

the PLS regression model can be verified by a correlation coefficient R^2 (goodness to fit) and a cross-validated correlation coefficient Q^2 (goodness of prediction), as well as the validation errors of estimation and that between measured and predicted values called root mean squared error of estimation (rmsEE) and prediction (rmsEP), respectively. Generally, R^2, which describes how well the data of the training set is mathematically reproduced, varies between 0 and 1, where 1 means a perfectly fitting model. A good prediction model is achieved when $Q^2 > 0.5$, and if $Q^2 > 0.9$, it is regarded as excellent predictive ability (*29*). The PLS regression model of green tea showed that $R^2 = 0.987$ and $Q^2 = 0.671$ with rmsEE $= 1.82$ (**Figure 5A**), indicating excellent fitting and prediction abilities. The predictability of the model to predict the ranking order of green tea was tested by subsequently subjecting the test set into the resulting PLS regression (**Figure 5B**). The prediction result was rather scattered from the ideal diagonal having rmsEP $= 9.22$. The large validation error of about 9.22 was expected to be due to uncorrelated *X* variables interrupting the prediction of *Y* variables, hence a distortion of model predictability.

The quality of PLS regression can be improved by simplifying the complexity of variations using the OSC approach which reduces the number of variables from the spectra matrix *X* by removing only the ones that are linearly unrelated (orthogonal) to the response matrix *Y* to be interpreted (*30*). By removing two OSC components from the prior PLS model, the predictability improved by 46% in which Q^2 increased from 0.671 to 0.982 as presented in **Figure 6A**. The remaining sum of squares of the PLS-OSC regression was 26.77% indicating that 73.23% of *X* variables did not correlate to *Y* and were subtracted. The predictability of the PLS-OSC regression model was again verified by a test set described above and is shown in **Figure**

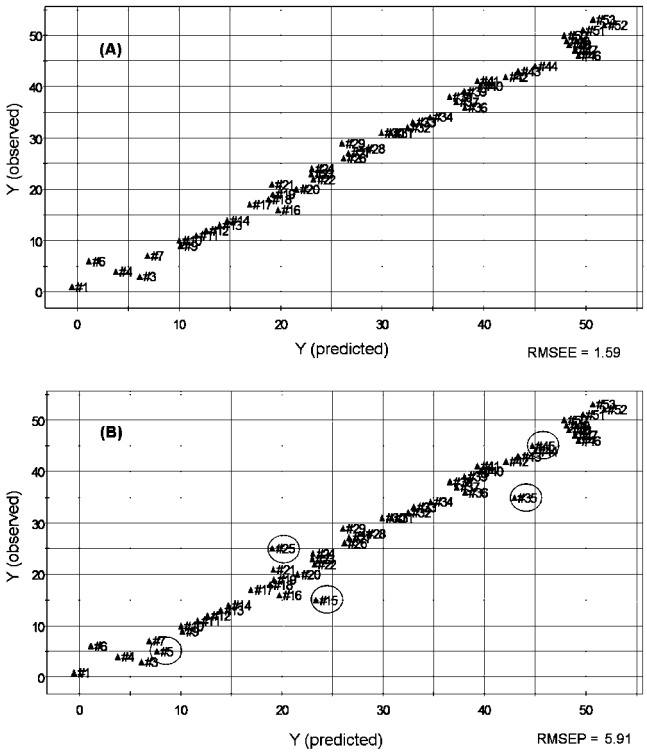

Figure 6. Observed and predicted green tea quality for the PLS model with the orthogonal signal correction (OSC) preprocessing method, calculated from the ^{1}H NMR data set of **(A)** 46 tea samples as the training set and **(B)** 51 tea samples included in training and test (circle mark) sets. Green tea nos. 2 and 8 were omitted from the PLS model due to a baseline problem.

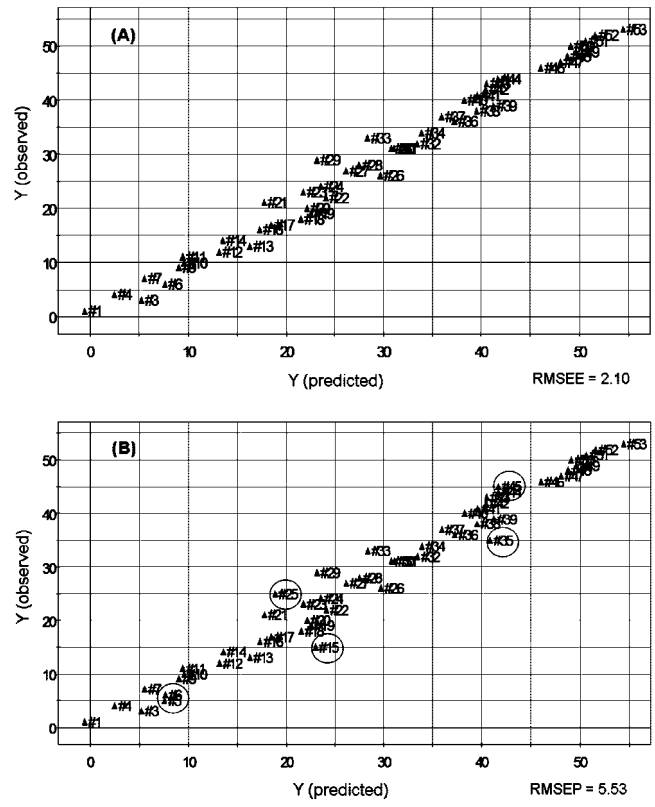

Figure 7. Observed and predicted green tea quality for the PLS model with a combination of the orthogonal signal correction preprocessing method and wavelet analysis (OSCW), calculated from the ^{1}H NMR data set of **(A)** 46 tea samples as the training set and **(B)** 51 tea samples included in training and test (circle mark) sets. Green tea nos. 2 and 8 were omitted from the PLS model due to a baseline problem.

6B. The rmsEP value significantly decreased from 9.22 to 5.91, accounting to 36% after OSC was applied. According to the regression plot shown in **Figure 6**, the increase of Q^2 and decrease of rmsEP indicated that the predictive model's ability was drastically improved by the removal of unwanted variations by signal correction. This meant that OSC was an effective filtering method to remove the anticipated variables and enhance the regression model accuracy.

Wavelet transform is a viable tool in signal processing used to compress and denoise complicated signals and extract only relevant information (*18*, *31*). Details of the wavelet transform approach can be cited from dedicated references (*18*, *19*, *31*). It has been reported that the combination of OSC for preprocessing and wavelet analysis for compression of spectral data (OSCW) improved the multivariate calibration quality without any loss of predictive power (*18*). Thus, OSCW was then applied through the PLS regression model of green tea quality prediction: 63.42% of uncorrelated *X* variables were removed by one-component OSC filtering; 506 wavelet coefficients were used to retain 99.50% of the total sum of squares. The resulting PLS-OSCW regression is displayed in **Figure 7A**, giving R^2 and Q^2 of 0.982 and 0.944, respectively. The predictive ability of the PLS regression model improved when OSCW was implemented compared to that obtained from OSC by reducing the rmsEP from 5.91 to 5.53 accounting for 6.43% when the test set was verified (**Figure 7B**). The number of wavelet coefficients used in the PLS model to capture the informative variables was very high, and only 0.5% of the uncorrelated variables were removed. This implied that almost all interesting *Y* variances were captured by OSC filtering and only small parts of the variation originating from noise remained. Wavelet analysis enhanced the preprocessing performance by removing those residual variables without any loss of the predictive ability.

By comparing the R^2, Q^2, and rmsEP values of PLS, PLS-OSC, and PLS-OSCW regression, both R^2 and Q^2 values were greater than 0.9 for all models, except the Q^2 value of the PLS regression, signifying that all multivariate calibrations could be used to predict the quality of green tea with a very good fitting and excellent predictability. However, the best quality predictive model with highest prediction accuracy was obtained from the PLS-OSCW regression, in which rmsEP was lowest when compared to the others.

On the basis of all of the above regression models, it implied that a combination of OSC filtering and wavelet transform was the most effective preprocessing method to build the best fit with the high accuracy PLS regression model for green tea quality evaluation.

The ^{1}H NMR based metabolomic study provided informative details on the quality evaluation of Japanese green tea with simple sample preparation and short analysis time. All metabolites could be identified within a single run, which differed from the previous studied in which specific key metabolites were focused on for specific purposes. As mentioned thus far, the sensory quality of green tea derived from several metabolites, so metabolomics was expected to be one of the best methodologies to fulfill either qualitative or quantitative intentions. A combination of metabolomics and multivariate analysis had an advantage over the ordinary sensory test, in which chemometric study offered more reliable results for classification and determination of the quality of Japanese green tea.

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