JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Metabolic Dependence of Green Tea on Plucking Positions Revisited: A Metabolomic Study

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ABSTRACT: The dependence of global green tea metabolome on plucking positions was investigated through ^{1}H nuclear magnetic resonance (NMR) analysis coupled with multivariate statistical data set. Pattern recognition methods, such as principal component analysis (PCA) and orthogonal projection on latent structure-discriminant analysis (OPLS-DA), were employed for a finding metabolic discrimination among fresh green tea leaves plucked at different positions from young to old leaves. In addition to clear metabolic discrimination among green tea leaves, elevations in theanine, caffeine, and gallic acid levels but reductions in catechins, such as epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epigallocatechin-3-gallate (EGCG), glucose, and sucrose levels were observed, as the green tea plant grows up. On the other hand, the younger the green tea leaf is, the more theanine, caffeine, and gallic acid but the lesser catechins accumlated in the green tea leaf, revealing a reverse assocation between theanine and catechins levels due to incorporaton of theanine into catechins with growing up green tea plant. Moreover, as compared to the tea leaf, the observation of marked high levels of theanine and low levels of catechins in green tea stems exhibited a distinct tea plant metabolism between the tea leaf and the stem. This metabolomic approach highlights taking insight to global metabolic dependence of green tea leaf on plucking position, thereby providing distinct information on green tea production with specific tea quality.

KEYWORDS: Green tea, leaf, metabolomics, NMR, plucking position

INTRODUCTION

As green tea has been characterized as a natural medicine containing bioactive components such as tea polyphenols, caffeine, theanine, and vitamins,[1] numerous studies reported the characterization of tea chemical compositions and their variations according to product processes,[2] growing conditions,[3] climatic conditions,[4] and plucking season.[5] However, the variations in the tea chemical compositions are very often subtle, especially with respective to plant physiology. Owuor and Obanda[6] have reported the dependencies of catechins and caffeine levels in black tea on plucking standards. However, although the green tea samples were plucked at different positions of tea plants, the standard samples included tea stems and were also mixed with bud leaf and thus might not provide representive results on chemical compositions of individual green tea leaves. Although as green tea plant grows up, elevated contents of theanine and ECG together with reduced contents of EGCG have been reported in the study with fresh green tea leaves plucked at different positions,[7] it is more likely that theanine levels are reversly associated with catechin levels in green tea since the *N*-ethyl group of theanine is incorporated into catechins as green tea plant grows.[8] These subtle results may be caused by inappropriate collections of the samples for characterizing the dependence of chemical constituents of individual green tea leaves on plucking positions. Moreover, the plucking in one shoot from the single fresh green tea leaf might lead to the subtle results due to lack of statistical significance.[7] Therefore, studies on the influences of plucking positions on green tea constituents are strongly needed by an appropriate sample collection of fresh green tea leaf together with statistical analysis. Our previous studies[2,4] showed a simultaneous detection of main tea metabolties, such as catechins, theanine, caffeine, amino acids, quinic acid, and gallic acid, and exhibited their strong geographical dependences in green tea plucked at different growing areas,[4] and also marked changes in the tea metabolites during tea fermentation,[2] which were carried out through ^{1}H NMR-based metabolomic approach. Moreover, these ^{1}H NMR-based metabolomic approaches have been used successfully to find out a relationship between green tea quality and tea metabolite.[9,10] The present study was aimed at providing a better understanding of the green tea quality through insight into the metabolic dependence of green tea leaf on plucking position. Metabolites of green tea leaves plucked at different positions and corresponding stems were profiled comprehenisvely, and the global dependences of green tea metabolites on the plucking positions were assessed by using a ^{1}H NMR-based metabolomic approach.

MATERIALS AND METHODS

Chemicals. All chemical reagents were of analytical grade. All standard reagents, CD_3OD (99.9%), D_2O (99.9%), and TSP (97%) were purchased from Sigma (St. Louis, MO).

Received: June 10, 2011
Accepted: September 8, 2011
Revised: August 5, 2011
Published: September 08, 2011

dx.doi.org/10.1021/jf202304z | *J. Agric. Food Chem.* 2011, 59, 10579–10585

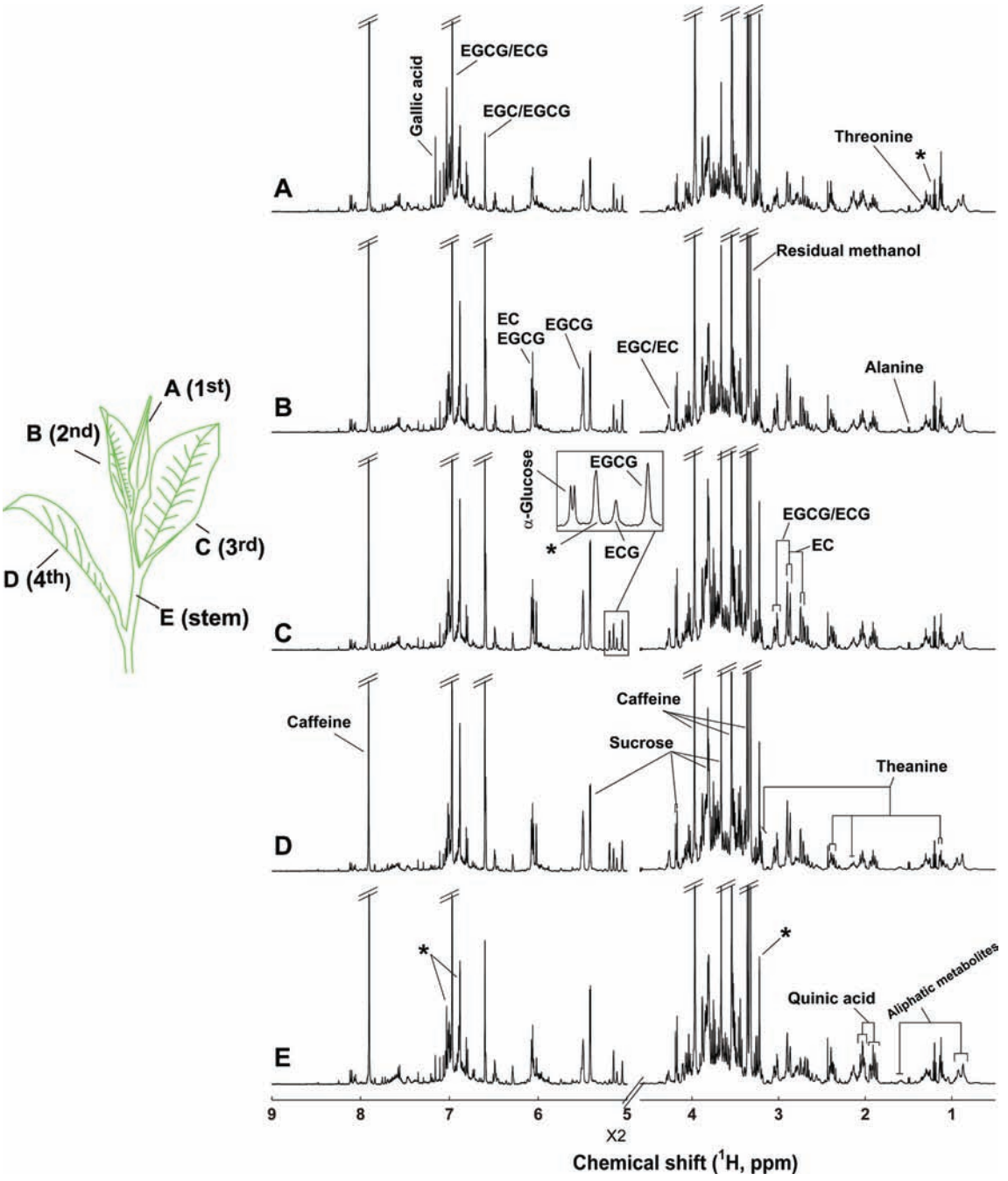

Figure 1. Illustration of green tea leaf (left) and representative ^{1}H NMR spectra (right) of green tea leaves plucked at different positions from the youngest or the first (A) to the oldest or the fourth (D) leaves and stem (E). Asterisks (*) denote unknown compounds.

Samples and Extraction. Fresh green tea (*Camellia sinensis* var. Yakukita) leaves were plucked from 10 different parcels (30 cm × 30 cm) throughout a green tea garden located at Seogwang (33° 18′ 17.67″ N, 126° 17′ 42.97″ E) area, Jeju, South Korea, on June 25, 2010, which is one of the green tea gardens described in our previous study on the geographical dependence of green tea metabolite.[4] Freshly plucked green tea samples were kept in dry ice following separations into first (the youngest or a bud), second, third, and fourth (the oldest) leaves and corresponding stems (Figure 1), sent to Korea University, and stored at −80 °C until analysis. Sample extraction was carried out according to Kim et al.[11] Frozen tea leaves and stems were ground with a pestle and mortar under liquid nitrogen. The ground samples were transferred into a plastic tube using a spatula, kept in a deep freezer for 24 h, and then dried by a freeze dryer for 48 h. Freeze-dried samples of 10 mg were dissolved in mixture of methanol-d_4 (CD_3OD, 400 μL), potassium phosphate buffer (320 μL, pH 6.4, 100 mM, in D_2O), and deionized water (80 μL, included 5 mM TSP) in a 1.5 mL Eppendorf tube. The mixture was sonicated to extract tea metabolites at 25 °C for 20 min and then centrifuged at 13000 rpm for 15 min.

^{1}H NMR Spectroscopic Analysis of Green Tea Extracts. The supernatant was transferred into 5 mm NMR tubes. D_2O and TSP provided a field frequency lock and a chemical shift reference (^{1}H, δ 0.00), respectively. ^{1}H NMR spectra were acquired on a Bruker Avance 500 spectrometer (Bruker Biospin, Rheinstetten, Germany), operating

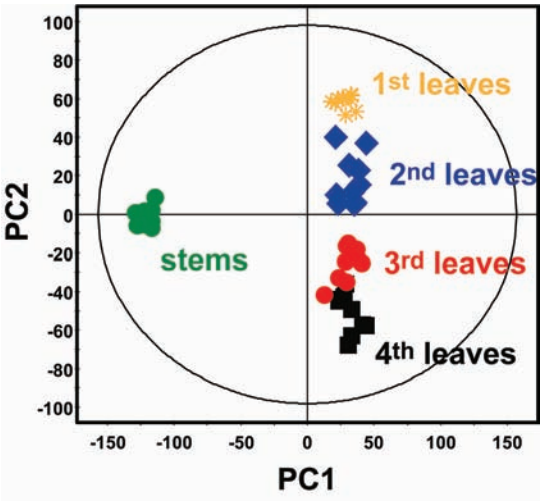

Figure 2. Principal component analysis (PCA) score plots derived from ^{1}H NMR spectra of green tea plucked at different positions from the first (1st, the youngest or a bud) to the fourth (4th, the oldest) leaves and of stems, showing a strong dependence of green tea metabolome on plucking positions and a clear metabolic difference between green tea leaves and stems.

at 500.13 MHz ^{1}H frequency and a temperature of 300 K, using a cryogenic triple-resonance probe and a Bruker automatic injector. For efficient water suppression, one-dimensional nuclear Overhauser effect spectrometry (NOESY) pulse sequence with water presaturation was applied. For each sample, 64 transients were collected into 32K data points using a spectrum width of 6510.4 Hz with a relaxation dealy of 2.0 s and a mixing time of 100 ms. A 0.3 Hz line-broadening function was applied to all spectra prior to Fourier transformation. Signal assignment for respresentative samples was facilitated by two-dimenstional (2D) total correlation spectrocopy (TOCSY), heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum correlation (HSQC), spiking experiments, and comparisons to our previous studes.[2,4] Furthermore, 1D Statistical TOCSY[12] was also used for the signal assignment.

Multivariate Data Analysis. All NMR spectra were corrected for phase and baseline distortions manually and then converted to ASCII format. The ASCII format files were imported into MATLAB (R2008a, The Mathworks, Inc., Natick, MA). Probabilistic quotient normalization of the spectra using the median spectrum to estimate the most probabilistic quotient was carried out after total integral normalization to avoid dilution effects of samples and effects of metabolites in massive amounts on changes on the overall concentration of samples.[13] The spectra were then aligned by the recursive segment-wise peak alignment (RSPA) method to reduce variablitiy in the peak position.[14] Regions corresponding to water (4.69−4.8 ppm), TSP (−0.5−0.5 ppm), and residual methanol (3.29−3.34 ppm) and ethanol (1.16−1.23 and 3.6−3.7 ppm) were removed prior to the normalization and spectrum alignment. The resulting data were then imported into SIMCA-P version 12.0 (Umetrics, Umeå, Sweden) and applied to a mean centering scaling method for multivariate statistical analysis. At first, principal component analysis (PCA), an unsupervised pattern recognition method, was performed to examine intrinsic variation in the data set. Furthermore, a supervised pattern recognition method, orthogonal projection on latent structure-discriminant analysis (OPLS-DA),[15] was used to extract maximum information on discriminant compounds from the data using MATLAB (The Mathworks, Inc.) with scripts developed in-house at Imperial College London. For visualization purposes, the OPLS coefficient indicating variables or metabolites responsible for the differentiation or classification in the model were back transformed as described by

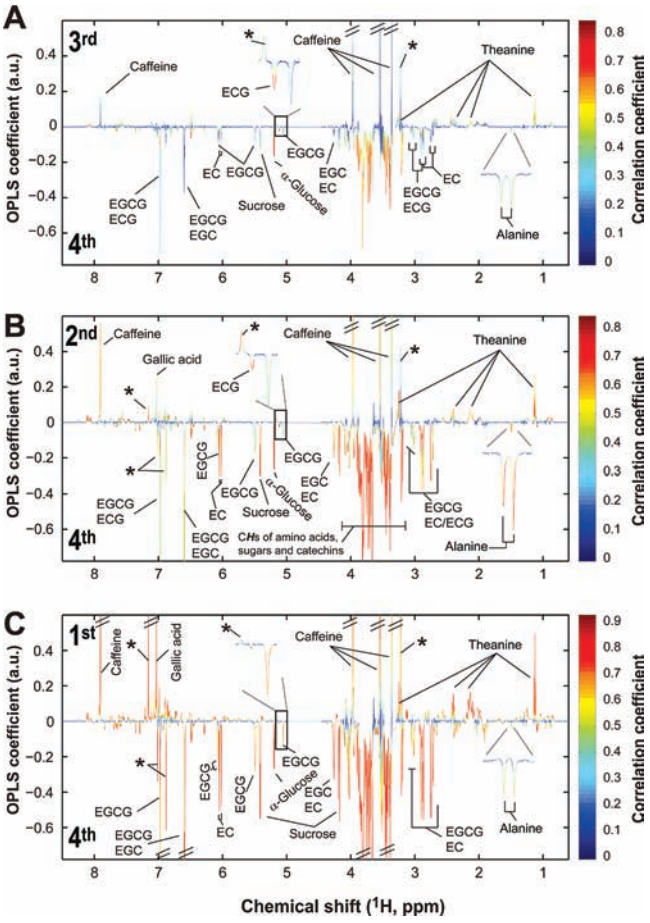

Figure 3. OPLS loading plots derived from ^{1}H NMR spectra of green tea leaves, providing a pairwise comparison among green tea leaf extracts obtained from different plucking positions of the first (1st), second (2nd), third (3rd), and fourth (4th) leaves. The color code in the loading plot corresponds to the correlation of the variables. Asterisks (*) denote unknown compounds.

Cloarec et al.[16] Validation of the model was conducted using 7-fold cross-validation and permutation tests with 200 times. Q^2 values generated from the permutation test were compared to the Q^2 values of the real model. If the maximum value $Q_{max}{}^2$ from the permutation test was smaller than the Q^2 of the real model, the model was regarded as a predictable model. $R_X{}^2$ was used to evaluate possible overfitting of the model. Hence, the quality of the models is described by $R_X{}^2$ and Q^2 values. $R_X{}^2$ is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, and Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability.

Statistical Analysis. The statistical analysis system (SAS package ver. 9.20) was used for data analysis by anaylsis of variance and Duncan's multiple range test. In particular, the paired Student's t test was performed for the significance analysis of each metabolite with integral area in pairs of loading plots of OPLS models.

RESULTS

Figure 1 shows typical ^{1}H NMR spectra of fresh green tea leaves plucked at the different positions from the first (the youngest or a bud) and fourth (the oldest) leaves and of green tea plant stem. A total of 12 green tea metabolites, including theanine, quinic acid, threonine, alanine, caffeine, sucrose, glucose, gallic

acid, epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC), were identified by ^{1}H NMR spectroscopy.

To provide comparative interpretations for dependences of green tea metabolites on plucking positions, a series of pattern recognition methods were employed. As described in the Materials and Methods, PCAs were initially applied to the entire NMR spectral data to visualize metabolic differentiation among the green tea leaves plucked at different positions and the tea plant stems and simultaneously to find out an outlier. Interestingly, all of the leaves were differentiated in the PCA score plot, as well as stems (Figure 2), indicating strong dependences of green tea leaf metabolites on plucking positions and their marked differences with stem metabolites. To identify the metabolites responsible for these differentiations, OPLS-DA models were generated with one predictive and one orthogonal component in the pairwised comparisons. All OPLS models shown in Figure 3 exhibited a strong predictability (Q^2) and high goodness of fit (R^2_X), as calculated to 0.64 and 0.48 between the third and fourth leaves, 0.91 and 0.80 between the second and fourth leaves, and 0.98 and 0.91 between the first and fourth leaves, respectively. Furthermore, all of these models were validated by permutation test with 200 times as described in our previous study (data not shown).[4] The upper sections of the OPLS loading plots represent metabolites that were higher in the first, second, and third leaves, as compared to the fourth leaves, whereas the lower sections reveal metabolites that were lower (Figure 3). The colors on the statistical OPLS loading plot are associated with the significances of differences in metabolites contributing to the differentiations between two classes. In the present study, the correlation coefficients in the OPLS loading plots were considered to be significant when >0.45, which corresponded to the critical value of a correlation coefficient at $P < 0.05$ verified by Student's t test with integral areas of NMR spectra corresponding to individual metabolites. The differentiations of the third green tea leaves from the fourth leaves were caused by increased levels of theanine and caffeine and decreased levels of alanine, EC, EGCG, ECG, EGC, glucose, and sucrose (Figure 3A). Moreover, increased theanine, caffeine, and gallic acid levels, together with decreased alanine, EC, EGCG, ECG, EGC, (mono- or disaccharide), glucose, and sucrose in the second green tea leaves contributed to the differentiations from the fourth green tea leaves (Figure 3B). Increases in theanine, caffeine, and gallic acid levels and decreases in EGCG, EC, ECG, EGC, glucose, and sucrose levels in the first green tea leaves were more marked than in the second and third leaves, which compared to the fourth leaves (Figure 3C).

Figure 4B highlights the metabolite correlated among protons of quinic acid by 1D Statistical TOCSY (STOCSY) anslysis used to assign the NMR peaks having of a marked difference between the stems and the green tea leaves found in 1D NMR sepctra in Figure 4A. These quinic acid peaks were strongly responsible for the differentiations of the stems from the green tea leaves, revealing markedly higher levels of quinic acid in the green tea stems, as shown in the OPLS-DA model (Figure 5). Moreover, higher levels of theanine and alanine together with lower levels of EGCG, ECG, EC, caffeine, and sucrose in green tea stems contributed to the differentiation from green tea leaves.

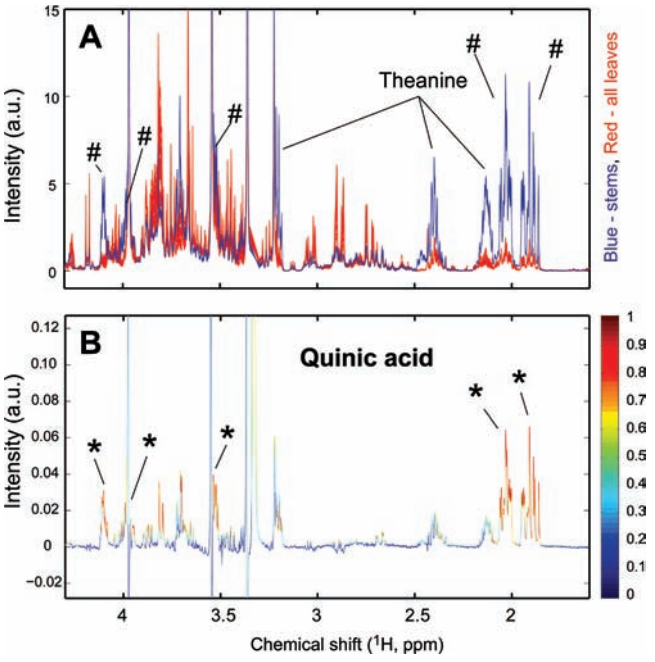

Figure 4. One-dimensional raw ^{1}H NMR spectra (A) and STOCSY analysis (B) for the quinic acid corresponding to δ 1.91, which were obtained from green tea leaves and stems. Stars represent the STOCSY correlations among all protons of quinic acid, showing the assignments of molecules indicated by # in the raw NMR spectra to quinic acid.

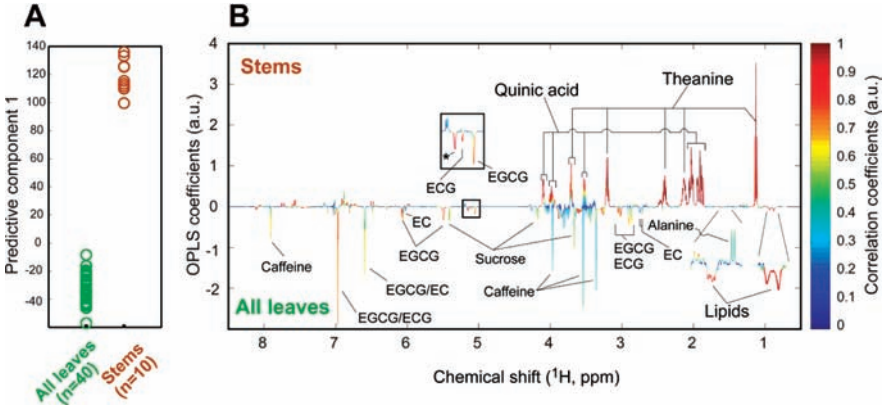

Figure 5. OPLS score (A) and loading (B) plots derived from ^{1}H NMR spectra of all green tea leaf and stem extracts, showing a clear differentiation between stems and all tea leaves. The color code in the loading plot corresponds to the correlation of the variables.

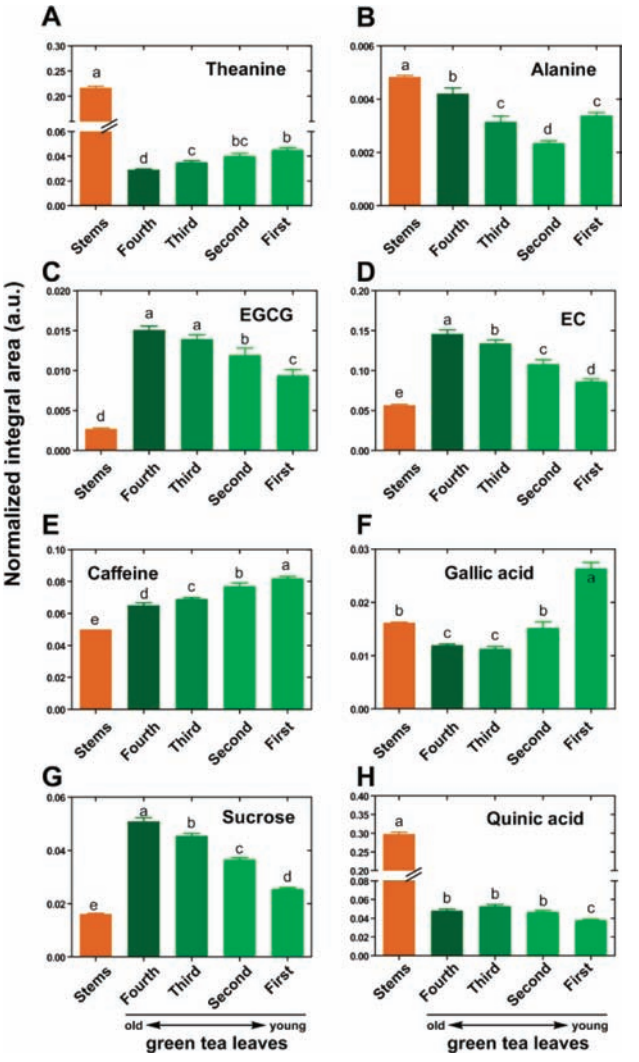

Figure 6. Relative changes in green tea metabolite levels of theanine (A), alanine (B), EGCG (C), EC (D), caffeine (E), gallic acid (F), sucrose (G), and quinic acid (H). Values with different letters in each panel are significantly different by Duncan's multiple test at $P < 0.5$. The error bars were based on 10 samples.

DISCUSSION

To investigate the metabolic dependence of green tea on plucking position, green tea leaf and stems were harvested or plucked from 10 different parcels in same tea garden. The plucked green tea samples were divided into the green tea leaves from the first (the youngest) and fouth (the oldest) ones as well as stem. Interestingly, strong metabolic dependences on plucking positions of green tea leaves were observed through ^{1}H NMR spectroscopy coupled with multivariate statistical analysis.

Theanine contents significantly varied among green tea leaves plucked at different positions and were largely higher in stems than in leaves (Figure 6A). Theanine is synthesized from glutamic acid and from ethylamine derived from alanine in all parts of seedlings of green tea *Camellia sinesis*.[17,18] Theanine is considered to have a unique taste characteristic of "umami",[19] reported as an antogonist against paralysis induced by caffeine, and is known to have a relaxation-inducing effect in human acting as a neurotransmitter in the brain.[20] A reversed relationship between theanine and catechin levels has been reported in the green teas grown in different geographical areas,[4] and the same result was observed in the green teas plucked at different positions in the present study, in which theanine contents decreased but catechin contents increased as green tea plant grows up, indicating the incorporation of the *N*-ethyl group of theanine into catechins.[8] Interstingly, it was therefore suggested that the greater incorporation of theanine into catechins occurs in green tea leaf rather than in stem, as indicated by relatively large levels of theanine and low levels of EGCG and EC in green tea stem (Figure 6).

Gallic acid, a naturally occurring polyphenol antioxidant identified as an excellent free-radical scavenger, is regarded as an important constitutent responsible for the health benefits in many food sources.[21] Moreover, plant extracts rich in gallic acid were found to possess antidiabetic, antiangiogenic, and antimelanogenic effects, reduced heart infarction incidence, oxidative liver and kidney damage, and inhibited growth and induced apoptosis in various cancer cell lines such as prostate cancer.[22−27] Gallic acid is accumulated in oolong or fermented tea through releasing from EGCG by heat treatment and enzymatic reaction during producing green tea products.[2,28] Gallic acid in fresh green tea leaves was the highest in the youngest ones, indicating that gallic acid in green tea leaf is metabolized as tea plant grows up (Figure 6F). Therefore, gallic acid may contribute to high quality of green tea with respect to the bioactivity of its product.

The variations in catechin levels, such as EGCG and EC, in the green tea leaves in the present study were not consistent with the report that catechins levels were higher in fresh young green tea leaves than those in old ones.[29] However, the authors have compared the catechin amounts between the young leaves mixed with the apical bud and the two youngest leaves and the old leaves mixed with the tenth to the fifth leaves,[29] while, in this study, the fresh green tea leaves were plucked separately and collected individually from the first (the youngest), second, third, and fourth (the oldest) ones. Moreover, the authors have plucked the tea leaves in summer,[30] but we did between spring and summer. However, Lin et al.[30] have reported that the levels of EGCG, EGC, and EC in old leaves were higher than in young green tea leaves, showing a good agreement with the results in the present study. Therefore, both different plucking positions and seasons may cause different results on catechins contents of green tea leaves.

Quinic acid biosynthesis occurs widely in the plants, and quinic acid is a metabolite involved in the shikimate pathway.[31] Large quantities of quinic acid have been found in fruits and berries.[32,33] The efforts on chemical and microbial synthesis of quinic acid have been reported[34,35] since quinic acid has emerged as a versatile chiral starting material for the synthesis of neuraminidase inhibitors effective in the treatment of influenza[36] and is known as a biologically active component for the inhibition of the transcriptional regulator nuclear factor κB (NF-κB).[37] To date, quinic acid in green tea has rarely been considered due to its low amounts in green tea leaf. However, it was interesting to note that s markedly large amount of quinic acid was observed in green tea stems as compared to the respective tea leaves (Figures 4−6). Therefore, stem from green tea plant could be a good source of natural quinic acid. Moreover, the highest sucrose levels in the oldest green tea leaf observed in the present study may indicate its lowest green tea quality as it was reported that sucrose was negatively correlated with green tea quality.[9,10]

Caffeine, gallic acid, and theanine are believed to be possible quality markers of green tea, and thus, their high contents are responsible for high quality of tea.[7,38] Furthermore, green tea

with high quality contains less catechins.[39] These associations of tea chemical compositions with tea quality have also been reported in the studies for assessing green tea quality through metabolite profiling of commerical green tea using NMR spectroscopy.[9,10] Considered that, in general, the youngest green tea leaves provide the highest quality of the tea, the highest levels of caffeine, gallic acid, and theanine together with the lowest levels of catechins of EC and EGCG in the youngest tea leaf in the present study were in good agreement with those reports. Furthermore, markedly high levels of theanine and quinic acid in stems may provide useful information that the quality of the tea product would be adjusted by blending with tea leaf and stem for producing the product with different tastes.

In conclusion, the present study therefore highlights that metabolomics with the global profiling of green tea metabolites enable us to gain insight to the effective assessment of the quality of green tea made from leaves plucked at different positions.

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ACKNOWLEDGMENT

We thank Prof. Jeremy K. Nicholson at Imperial College London for help in using the in-house MATLAB script for application of the OPLS-DA algorithm.

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