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NMR Metabolomic Profiles for Quality Control of Korean Green Tea (*Camellia sinensis*) Classified by the Plucking Season

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Abstract The plucking season of green tea leaves is one of the important parameters that decide their metabolic diversity, quality, and prices. The effects of plucking sghlwleasons on green tea metabolites were investigated through metabolite profiling by ^{1}H NMR spectroscopy. The orthogonal projection on latent structure-discriminant analysis (OPLS-DA) showed clear discriminations of green teas by three different grades depending on plucking seasons: Ujeon, Sejak, and Jungjak. These results suggested that the nine peak groups could be used for diagnostics for identification of high quality Ujeon grade of green tea.

Keywords NMR, Metabolomics, Green Tea, Metabolite Profiling, Quality Control, Plucking Season

Introduction

Tea is the most widely consumed drink in the world and consists of three major types: green tea (unfermented), oolong tea (semi-fermented), and black tea (fermented).[1,2] Green tea contains a variety of natural medicines such as polyphenols, caffeine, theanine, and vitamins.[1-3] The composition of the green tea metabolites depends on several factors including genetic strain, climatic conditions (temperature, sun exposure, etc), soil, and growth altitude.[2] The plucking season of leaves also influences the metabolic diversity of green tea.[2] Generally, as the leaves are plucked earlier, the flavour and aroma of green tea are thought to be better and then the grade of green tea becomes higher.[2]

The quality of a green tea, which is mainly assessed through its color, flavour, and aroma, depends on the content of catechins, caffeine, and amino acids.[2,4,5] Many characteristics is considered for judgement of the tea quality that are reflected in the price of a tea.[2] It is reported that the Japan green tea of highest quality contains a high amount of amino acid but low amounts of catechins.[2,4] The plucking season of leaves is one of the important parameters that decide the qualities and prices of green teas. The best green teas are usually plucked during the first flush in April or May rather than summer and autumn. In Korea, green tea is classified as four grades which depend on the plucking timing of leaves: “Ujeon” (meaning before April rainy day, plucked before 20th, April), “Sejak” (meaning tiny leaves, plucked in late April or early May), “Jungjak” (meaning middle-sized leaves, plucked in the middle of May), and “Daejak” (meaning large-sized leaves, plucked in late May or early June) (Fig. 1). In Korea, the Ujeon grade is the green tea with highest quality and is the most expensive grade of green teas.

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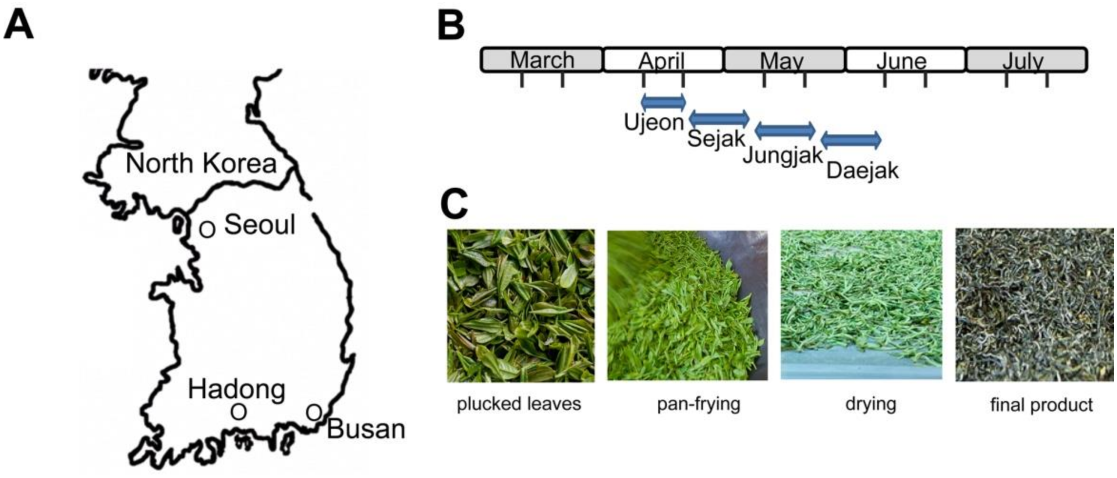

Figure 1. (A) Geographical information of Hadong county, Korea. (B) Representative picture for the grade of Korean green tea. The symbol arrows indicate plucking season of green tea leaves. (C) Photography of production of the green tea products: (left to right) plucked leaves (Ujeon grade), pan-frying of leaves, drying of leaves, and final products after eight cycles of stemming, pan-frying, and drying. NMR spectrum of an Ujeon grade Hadong green tea extract at 25 ℃.

Table 1. Summary of Hadong green tea products studied here.

Grade	Company	No. of samples	Price (per 100 g)
Ujeon	IHGT[1]	6	–
	Dosim[2]	2	$ 120
	Heesim	3	$ 100
	Soso	2	$ 100
Sejak	IHGT	6	–
	Dosim	2	$ 40
	Soso	3	$ 40
Jungjak	IHGT	6	–
	Dosim	2	$ 20
	Soso	2	$ 20

[1]IHGT: Institute of Hadong Green Tea.
[2]Special grade of Ujeon

NMR-based metabolomics is an important tool to identify the metabolic contents in various organisms, including animals, plants, and microbes.[6,7] In plant metabolomics, NMR has been used for studying quality control,[8,9] chemotaxonomy,[10,11] and analysis of genetically modified plants.[12] It has been reported in recent years that several NMR metabolomics studies elucidated the effects of climate, geography, and plucking position on the metabolite contents of green teas.[3,13,14]

Here, in order to understand the correlation between the metabolite contents and plucking time determining the quality of green tea, we have performed NMR experiments on the extract of three grades of the green tea cultivated in Hadong county, South Korea (Fig. 1). The metabolite profiling results provide the information required for quality control of the green tea products. In addition, this NMR study also shows the important chemical compounds of the green teas which are strongly correlated to the harvest timing of leaves and the grade (including flavour and aroma) of green teas.

Experimental Methods

Sample preparation- Hadong county (35° 08′ N, 127° 41′ E) is one of three famous area for green tea in Korea (Fig. 1). The commercial products of grean tea cultivated in Hadong county were purchased from Dosim Inc. (Hadong, Korea), Heesim Inc. (Hadong, Korea), or Soso Inc. (Hadong, Korea) or were gifted from Institute of Hadong Green Tea (IHGT) (Table 1). The three different grades (Ujeon, Sejak, and Jungjak) of green teas were studied here. 0.75 mL of

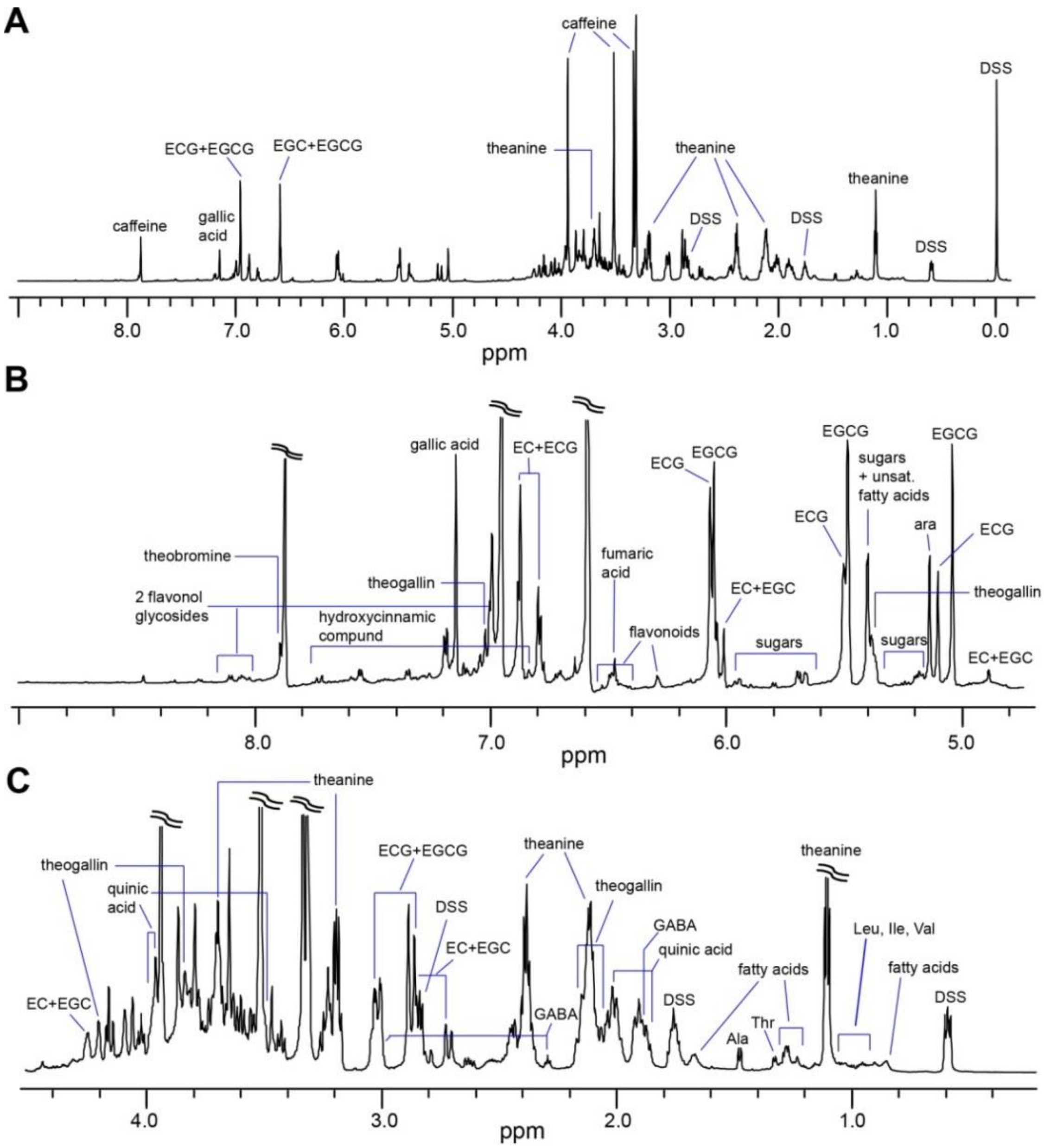

Figure 2. Details of 1D NMR spectrum of an Ujeon grade Hadong green tea extract at 25 ℃. (A) Full spectrum, (B) expansion of low field region, (C) expansion of high field region.

CH_3OH-d_4 and 0.75 mL of 10 mM KH_2PO_4 buffer in D_2O (pH = 6.0) containing 2-2-dimethyl-2-silapentane-5-sulfonate (DSS) (final concentration: 1.27 mM) were added to 50 mg of well-ground green tea leaves. The mixture was ultrasonicated for 20 min at room temperature and centrifugated at 12,000 rpm for 10 min. Each NMR sample consisted of 0.5 mL of supernatant, which was stored at 4 ℃.

NMR experiments- All NMR experiments were performed on an Agilent DD2 700MHz NMR spectrometer (GNU, Jinju, Korea) equipped with a cold probe at 25 °C. Residual water signal of one-dimensional (1D) NMR spectra was suppressed by using a pre-saturation water suppression pulse sequence. 1D NMR data were processed with the program Chenomx NMR Suite 7.1 (Chenomx, Alberta, Canada). 2D nuclear Overhauser effect spectroscopy (NOESY), rotating-frame NOE spectroscopy (ROESY), total correlation spectroscopy (TOCSY), and J-resolved spectra were acquired by using standard Agilent pulse sequences with pre-saturation water suppression. 2D NMR data were processed with the program NMRPipe[15] and analyzed with the program Sparky.[16] DSS was used for the ^1H reference (0 ppm).

Statistical analysis- All NMR spectra were phased and baseline-corrected with the program Chenomx NMR Suite 7.1 (Chenomx, Alberta, Canada). Principal component analysis (PCA) was carried out in SIMCA P+ version 12 (Umetrics, NJ, USA). Furthermore, orthogonal projection on latent structure-discriminant analysis (OPLS-DA) was used to analyze the NMR binning data with class information.

Results and Discussion

Resonance Assignments- Fig. 1 shows the ^1H NMR spectra of an Ujeon grade of green tea selected as a typical example for detailed resonance assignments. The overall view in Fig. 1A also illustrates the relative vertical scales of the expansions shown in Figs. 1B and 1C. Fig. 1 summarizes the resonance assignments of green tea metabolites from 2D NOESY, ROESY, TOCSY, and J-resolved NMR spectra. The resonances of theanine, which is the predominant amino acid present, were assigned by the analysis of TOCSY spectra. Similarly, the signals of several amino acids, such as alanine, leucine, threonine, and valine, were assigned by TOCSY cross-peaks from their methyl groups. Fumaric acid, gallic acid, caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine) are assigned by comparison with previous report.[2] Resonance assignments of theogallin, γ-aminobutyric acid (GABA), quinic acid are made by the analysis of their TOCSY spectra. The major polyphenols of green tea are catechins and their derivatives such as epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epigallocatechin-3-gallate (EGCG). Resonance assignments of four catechin derivatives, EC, EGC, ECG, and EGCG are based on previous reports.[2,3] Signals of fatty acids, sugars, phenolics, and flavonoids can be observed (Figure 1).

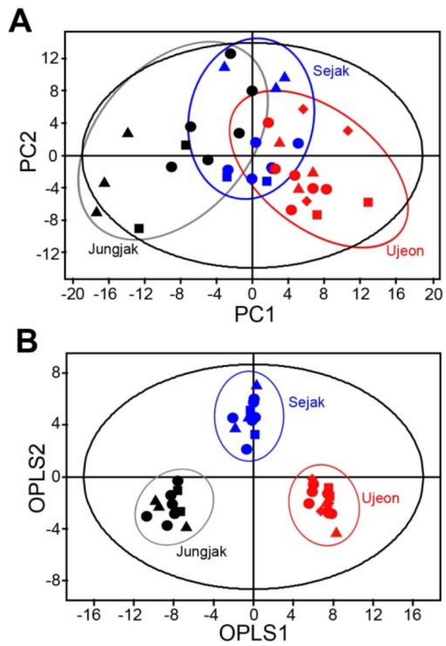

Figure 3. (A) PCA and (B) OPLS-DA score plots derived from 1H NMR spectra of green tea from three different grades classified by plucking seasons. Color used to illustrate green tea grades: red, Ujeon; blue, Sejak; black, Jungjak. Symbols indicate the origin of green tea samples: circle, IHGT; triangle, Soso Inc.; square, Dosim Inc.; diamond, Heesim Inc.

Principal component analysis (PCA)- NMR data binning was performed from 0 to 9 ppm with a bin size of 0.04 ppm. The spectral region of the suppressed water (4.60 ~ 5.00 ppm) and DSS signals

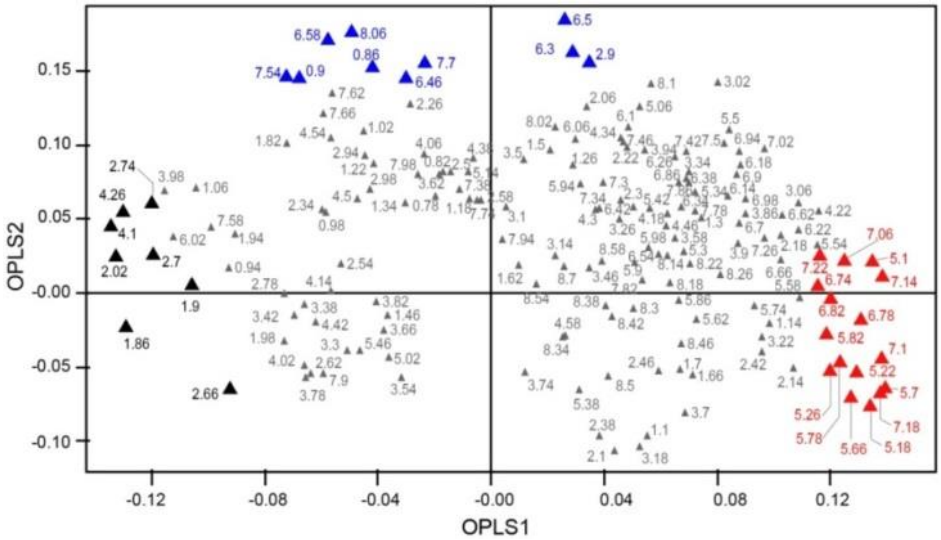

Figure 4. OPLS-DA loading plot derived from 1H NMR spectra of Hadong green tea extracts from three different grades classified by plucking seasons. Color used to illustrate biomarks for specific green tea grades: red, Ujeon; blue, Sejak; black, Jungjak.

(2.80 ~ 2.88, 1.72 ~ 1.82, 0.54 ~ 0.64, and 0 ~ 0.04 ppm) were excluded from the binning process. PCA was applied to the NMR binning data in order to obtain maximum variation among the green tea samples. PCA with the eleven principal components (PC1 - PC11) explaining 91 % of the variance did not show clear differentiation among the green tea samples. For example, although the PC1 component shows a slight difference among three classes of green tea extracts, these three classes were not clearly differentiated from other samples in the PCA score plots with first two components (Fig. 3A).

Orthogonal projection on latent structure-discriminant analysis (OPLS-DA)- To maximize the differences between classes, OPLS-DA was applied to the NMR binning data with the class information. OPLS-DA models were constructed with two predictive components (OPLS1 and OPLS 2) and five orthogonal components. Cumulative R^2x value of two predictive components (OPLS1 and OPLS2) and five orthogonal components is 70.0 %. In contrast to PCA, the OPLS-DA score plots showed clear differentiation among the green tea samples from the different plucking season (Fig. 2B). The loading plot obtained from OPLS-DA using OPLS1 and OPLS2 indicated that some NMR binning data show clear differentiations in their coefficients for OPLS1 and OPLS2 components among the three grades of green teas (Fig. 3).

Discrimination of Ujeon grade of green tea- In loading plot, some binning data have significantly larger coefficients for OPLS1 component compared to other grades (red in Fig. 4). The clear differentiation of the 5.10-ppm binning data implies that the H2 resonances of ECG at 5.11 ppm in the Ujeon grades exhibit significantly stronger peak intensities than those of the Sejak and Jungjak grades (I in Fig. 5). In contrast, the EGCG-H2 resonances at 5.04 ppm show no clear differences in the peak intensities among three grades (Fig. 5), consistent with relatively smaller OPLS1 coefficients for 5.02- and 5.06-ppm binning data (Fig. 4). The NMR binning data including other ECG resonances did not show clear differentiation because these resonances are partially overlapped with very strong resonances of EGCG (Fig. 2). The H2 resonances of gallic acid

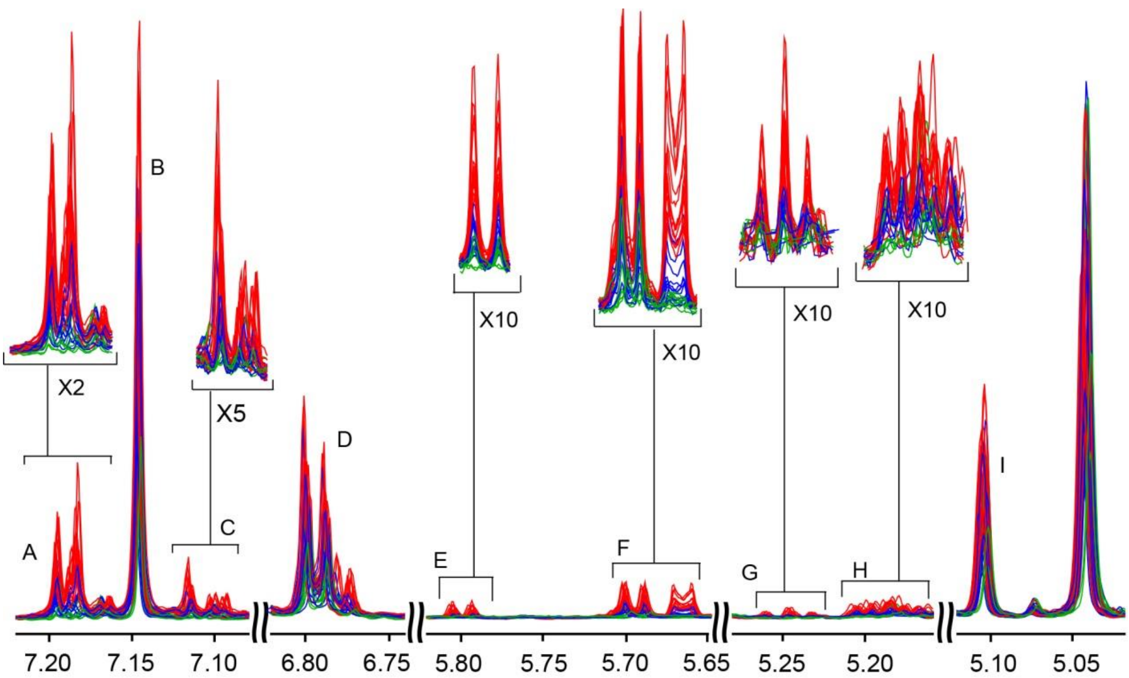

Figure 5. Superimposed ^{1}H NMR spectra of Hadong green tea extracts from three different grades classified by plucking seasons. Color used to illustrate green tea grades: red, Ujeon; blue, Sejak; black, Jungjak.

at 7.14 ppm in the Ujeon grade also have stronger peak intensities than those of other two grades (B in Fig. 5). Certain resonances from unknown hydroxycinnamic compounds (A and C in Fig. 5) and sugars (E to H in Fig. 5) also showed clear differences in their peak intensities among the three grades. From this approach, nine peak groups including ECG and gallic acid could be used for diagnostics for identification of Ujeon grade among three grades of green teas (A - I in Fig. 5).

Discrimination of Sejak and Jungjak grades of green tea- The Sejak grade of green tea exhibits significantly larger coefficients of some NMR binning data for OPLS2 component compared to other two grades (Fig. 4). From loading plot, resonances for hydroxycinnamic compounds at 7.72 and 7.55 ppm, flavonoids including fumaric acid at 6.46 ~ 6.56 ppm, and methyl groups of amino acids at 0.86 ~ 0.92 ppm could be used for diagnostics for identification of Sejak grade (Fig. 4).

The identification diagnostics for Jungjak grade are H2 (1.88 ppm), H3 (3.96 ppm), H4 (3.97 ppm), and H6 (2.00 ppm) resonances of quinic acids and H3 (4.25 ppm), H4 (2.71/2.74 ppm), and H6/H8 (6.02 ppm) resonances of EC and EGC (Figures 1 and 3).

In conclusion, we have performed the ^{1}H NMR metabolomics study to investigate the effects of plucking season on green tea metabolites. The OPLS-DA showed clear discrimination of green teas by three different grades depending on plucking seasons: Ujeon, Sejak, and Jungjak. These results suggested that the nine peak groups could be used for diagnostics for identification of high quality Ujeon grade of green tea.

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