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High-resolution ^{1}H NMR Spectroscopy of Green and Black Teas

Ji-Ho Jeong, Hyun-Jun Jang, and Yongae Kim*
Department of Chemistry, Hankuk University of Foreign Studies, Yongin 17035, Korea.
**E-mail: yakim@hufs.ac.kr*
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ABSTRACT. High-resolution ^{1}H NMR spectroscopic technique has been widely used as one of the most powerful analytical tools in food chemistry as well as to define molecular structure. The ^{1}H NMR spectra-based metabolomics has focused on classification and chemometric analysis of complex mixtures. The principal component analysis (PCA), an unsupervised clustering method and used to reduce the dimensionality of multivariate data, facilitates direct peak quantitation and pattern recognition. Using a combination of these techniques, the various green teas and black teas brewed were investigated via metabolite profiling. These teas were characterized based on the leaf size and country of cultivation, respectively.

Key words: Multivariate analysis, ^{1}H NMR, Green tea, Black tea, Classification

INTRODUCTION

Tea is the most popular beverage and consumed worldwide. The five main types of tea including white, green, oolong, black and pu-erh were classified according to their fermentation type (Balentine et al., 1997; Ohno et al., 2011). Although they were derived from the same plant species, *Camellia sinensis*, their flavor and taste as well as metabolites varied according to the fermentation process. Currently, taste is one of the most important criteria of professional tea tasters to determine the quality of tea. The characteristic flavors include: bitterness, astringency, sweetness, sourness, saltiness and savory (Teranish, 1983; Tarachiwin et al., 2007). The caffeine and catechin content determine the bitterness and astringency, respectively, as well as the savory taste due to the amino acid composition (Wright et al., 2000; Kaneko et al., 2006). Since the sensory results of evaluation of various types of tea by professional tea tasters do not facilitate the classification of the tea quality and types, the analytical techniques and chemometry focus on tea metabolites after brewing. The chemical composition of tea is complex: polyphenols, alkaloids (caffeine, theophylline and theobromine), amino acids, carbohydrates, proteins, chlorophyll, volatile compounds, minerals, trace elements and other unidentified compounds (Jian et al., 2004; Wu et al., 2003; Zhang et al., 2002). The chemopreventive effects of polyphenol compounds in green tea (catechin, epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG)) on cancer and heart disease, and their antiallergic and antimicrobial properties have been studied (Setiawan et al. 2001; Ito et al. 2008; Karori et al., 2007). Black tea, more oxidized than oolong and green tea, exhibits a strong flavor and contains more caffeine than green tea (Fujita, & Yamagami, 2008; Davies et al., 2003). Several health benefits of drinking green tea and black tea have been widely associated with a lower risk of cardiovascular disease (Fujiwara et al., 2003; Mineharu et al., 2011). The content and type of metabolites in various teas differ according to the differences in their growing environment such as climate, altitude, humidity and date of cultivation (Ohno et al., 2011). Since the NMR spectra of samples reflect not only major chemical components but also minor ingredients, the NMR spectroscopy is increasingly popular in the field of metabolomics. Although NMR spectroscopy has been criticized for its low sensitivity, it is nondestructive, revealing nuclei of interest, with precise insight into spectrum and facilitates rapid analysis of the sample. It is also an appropriate technique to define metabolite fingerprinting. These characteristics of NMR spectroscopy contribute to its utility in profiling of a wide range of metabolites (Son et al., 2009; Dixon et al., 2006; Larive et al., 2015). However, it is difficult to distinguish intuitively NMR spectra with a similar appearance. In order to determine the subtle differences between metabolomic profiles and ^{1}H NMR spectra, a chemometric analysis was carried out via multivariate statistical modeling procedures, for example, principal component analysis (PCA) for the remapping of the original ^{1}H NMR dataset in a reduced novel multivariate coordinate dimension (Kellogg et al., 2017; Ku et al., 2010).

In this paper, the various metabolites of green tea and black tea were identified and the quantitative changes in

metabolites with brew times were observed using high resolution ^{1}H NMR coupled with multivariate statistical analysis. In addition, the qualities of green tea and black tea were classified according to growth environments.

EXPERIMENTAL

Materials

Green teas used in this work were made from Korea and Japan. Green tea leaves were plucked over the course of several months (April~July) and had different taste according to harvest time. After plucked, leaves were dried to prevent fermentation and steamed. And then the rolling process made leaves shape tight. The final drying improved the leave's flavor and taste. Many classifications were possible according to various criteria, we classified leaves with their size; cultivated time. Woojeon and Gyokuro were harvested from the tender first bud on end of April (before Gogu; one of the 24 seasonal days in Korea). They were the highest grade and had rich savoury taste. Also they had intense flavor because small leaves had many catechins. Jaksul and Sejak were harvested from the bud and a little leaf on early May (after Gogu). They looked like a Eurasian Tree Sparrow's small beak and had smell of dried straw and cooked asparagus. Joongjak and Daejak were harvested from young leaves and mature leaves respectively on May to June. They had robust, deep and rich flavors because they grew under the strong sunlight on summer solstice.

Black teas were made from India, Sri Lanka and China. Black teas grown Darjeeling and Assam tea garden in India contained tannin with a slightly bitter taste and malt flavor respectively. They also had the flavor of tinge of astringent tannic characteristics and a musky spiciness. Black teas from Sri Lanka (Ceylon, Uva and Dimbula) had various taste according to grown altitudes and cultivated season. They had reddish amber color and slightly fruity flavor. Using 'contour planting' method black tea leaves were cultivated and harvested. And then leaves were spread out on ground known as 'process of withering'. Finally, tea leaves met with fermentation and drying. The black teas from China in this experiment were 'Keemun' and 'Lapsang Souchong' had relatively low level of caffeine content rather than from India. Keemun leaves characterized by low temperature and high humidity and these conditions lead to them fruity, sweet and mellow taste. Keemun leaves were picked in spring and summer and went through and an elaborate fermentation process. Lapsang Souchong leaves were plucked and then withered over cypress or pine wood fires. After withered leaves had smoky aroma and dried on bamboo trays over smoky pine fires.

Sample Preparation

A set of 10 commercial green teas and 16 commercial black tea samples were analyzed. All kinds of dried tea samples of 500 mg were added to 50 mL de-ionized hot water at 85 °C for 5 to 10 min and 2 aliquots of 500 μL each of brewed tea were transferred to the 1.5 mL micro tube followed by lyophilization. The lyophilized tea samples were re-dissolved in 500 μL deuterated water (D_2O) for ^{1}H NMR spectroscopy.

^{1}H NMR Spectroscopy

All ^{1}H NMR experiments were acquired at 9.4 T Ascend^{TM} using a Bruker Avance III HD 400MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with the z-gradient unit and all data were collected under the control of the Topspin 3.2 software (Bruker BioSpin, Rheinstetten, Germany). The field-lock frequency was used as deuterium frequency (61.4 MHz in 9.4T magnet) of D_2O solvent. Universal parameters of all ^{1}H spectra were used as 16 k complex data points to cover a typical spectral width of 6000 Hz, sweep width of 15 ppm, 256 scans, 2 dummy scans, 6.5 μs pre-scan delay, 90° tip angle and 1 s relaxation delay at 300 K. In order to suppress the residual water signal, the pre-saturation pulse technique was applied and a 0.3 Hz line broadening function was used for all spectra.

Multivariate Analysis

To maximize the separation between samples (5, 10 min brewed green tea and brewed black tea) PCA, an unsupervised pattern recognition was carried out using the software package AMIX 3.9.15 (Bruker BioSpin, Rheinstetten, Germany). The bucket method used for these samples was based on normal rectangular bucketing with total intensity except for classification of black teas with growing region. After data processing the spectra were analyzed by PCA to compare the quality of green and black tea. In order to distinguish green and black teas, specific regions of the ^{1}H NMR spectra were designated such as 6.45 to 7.02 ppm with 0.04 ppm bucket width. The green tea ^{1}H NMR spectra were segmented into 16 regions of 0.04 ppm with range of 6.02 to 6.65 ppm for classifying based on leaf size. The black tea ^{1}H NMR spectra were subdivided into regions with point-wise bucketing over a chemical shift range of 1.70 to 4.25 ppm to classify black teas according to the growing region.

RESULT AND DISCUSSION

^{1}H NMR Spectroscopy

The total of 52 ^{1}H NMR spectra were obtained from the green and black teas with 5 and 10 min of brewing time. The ^{1}H spectra of green tea with 5 min of brewing time and 10 min brewing time were shown in *Fig*. 1(A) and (B) respectively. The ^{1}H spectra of black tea with 5 min of brewing time and 10 min brewing time were shown in *Fig*. 1(C) and (D) respectively. There were no big differences at ^{1}H spectra of 5 min brewing time and 10 min brewing time except spectral intensity. And there was a little difference between the green tea spectrum and black tea spectrum in a view. The representative ^{1}H NMR spectra with a large number of isolated and overlapping resonances of green and black tea are shown in *Fig.* 1 and detailed signal assignments are shown in *Table* 1 (Le Gall et al., 2004; Yuan et al., 2014). Several overlaps of ^{1}H NMR resonances from chemical compounds in green tea and black tea were detected in all the spectral windows. However, the signals from catechins about 5 to 8 ppm showed different patterns between green and black tea. The difference between green tea and black tea is related to withering, processing and oxidation, and therefore, result in further darkening of the black tea leaves yielding strong flavor. The down field of ^{1}H NMR spectrum of green tea in *Fig*. 1(A) and (B) revealed multiple resonances from four major catechins; EC (6.04, 6.11, 6.50, 6.87, 6.99 ppm), ECG (4.88, 6.50, 6.95, 7.00 ppm), EGC (6.06, 6.55, 6.80 ppm), and EGCG (4.94, 5.56, 6.08, 6.59, 6.78, 6.92 ppm). However, relatively small signals were observed from ^{1}H NMR spectrum of black tea in *Fig*. 1(C) and (D). Many

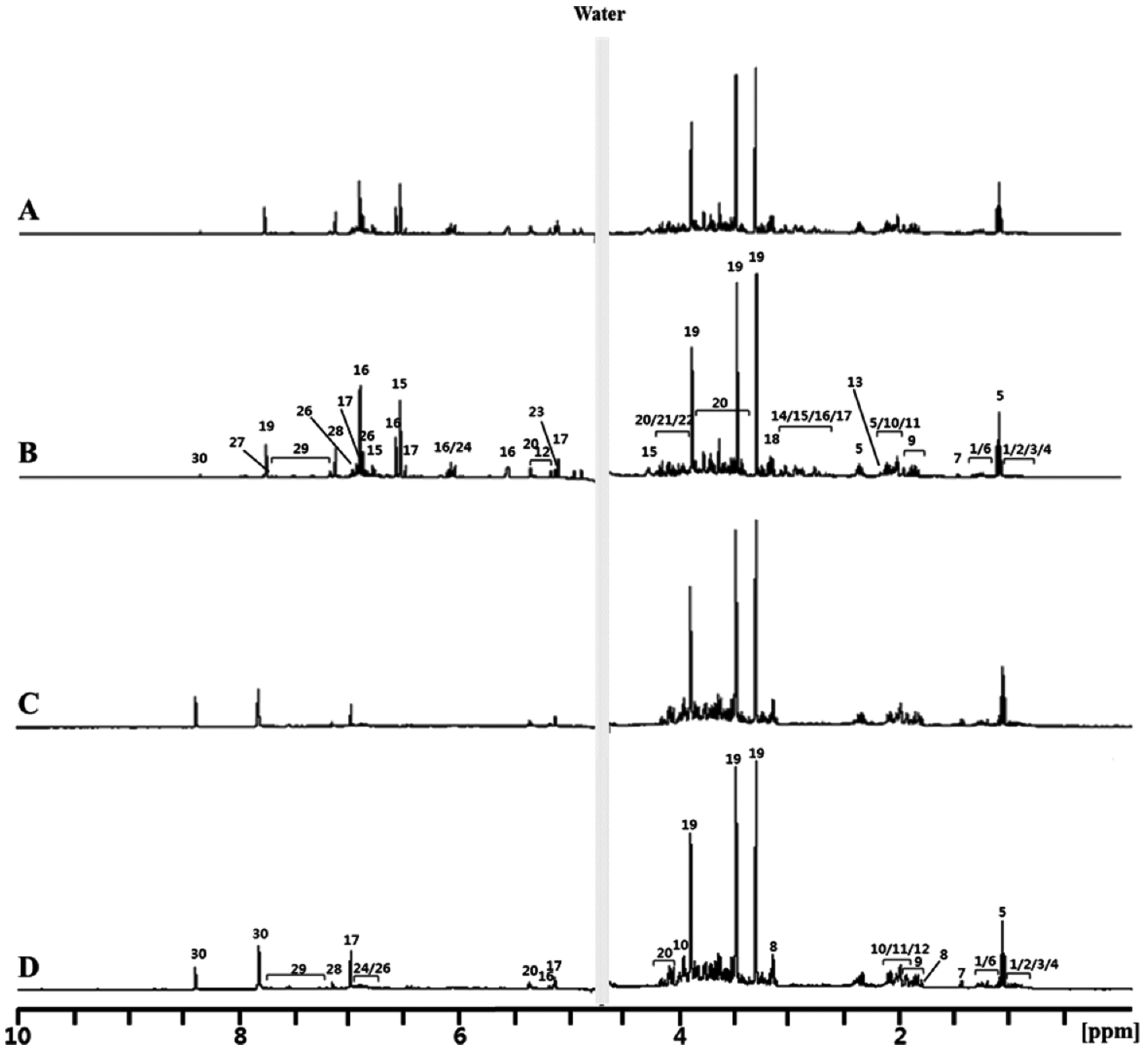

***Figure* 1.** ^{1}H NMR spectra of green tea brewed 5 min (A), brewed 10 min (B), black tea brewed 5 min (C) and brewed 10 min (D). The detailed ^{1}H NMR peak assignments of compounds from green tea and black tea are shown in *Table* 1.

***Table* 1.** ^{1}H NMR chemical shift data for green tea

Green Tea		
No.	Component	Chemical shifts (ppm)
1	Fatty acid	0.85–0.90, 1.21, 1.54–1.65
2	Isoleucine	0.92, 0.99
3	Valine	0.96, 1.01
4	Leucine	0.97
5	Theanine	1.04, 2.07, 2.32, 3.10–3.17
6	Threonine	1.26
7	Alanine	1.42
8	Acetate	1.78
9	Quinic acid	1.81, 1.85, 1.88, 1.92
10	Theogallin	1.98, 2.03, 3.95
11	Glutamine	2.01
12	α-glucose	5.18–5.28
13	γ-amino butyric acid	2.13
14	Catechin	2.42–2.62
15	EGC	2.62–2.71, 4.27, 6.06, 6.55, 6.80
16	EGCG	2.72–3.08, 4.94, 5.56, 6.08, 6.59, 6.78, 6.92
17	ECG	2.72-3.08, 4.88, 6.50, 6.95, 7.00
18	β-glucose	3.10–3.17
19	Caffeine	3.27, 3.45, 3.86, 7.79
20	Sucrose	3.46–3.84, 4.09–4.20, 5.30–5.38
21	Quinic acid	3.99
22	Fructose	4.12
23	2-O-arabinopyranosyl-myo-inositol	5.10
24	EC	6.04, 6.11, 6.50, 6.87, 6.99
25	Gallocatechin	6.75
26	Gallocatechin-3-gallate	6.90, 6.98
27	Theobromine	7.8
28	Gallic acid	7.15
29	p-coumaryl quinic acid	7.2–7.7
30	Flavonoid	8.39

frequencies from other components in the up field of both ^{1}H NMR spectra are hard to distinguish intuitively. The ^{1}H NMR spectra of 3 types of green teas, Woojeon, Jaksul and Daejak, were shown in *Fig*. 2(A), (B) and (C) respectively. All resonances in 3 types of spectra were similar except the flavonoid resonance at 8.39 ppm. It was increased depending on the cultivated time since Jaksul and Daejak grew under the strong sunlight on summer solstice than Woojeon.

Multivariate Analysis

A PCA score plot indicates clear differentiation of green tea (circle) and black tea (triangle) suggesting metabolic differences between the tea types in *Fig*. 3 and the scatter plot scores of the first PCA expressing 75.5% of the total variability (PC1 = 65.6%, PC2 = 9.9%). The two clusters are well separated leading to a possible detailed characterization with this analysis. The region of ^{1}H spectra from 6.45 to 7.02 ppm associated with brewed green tea and black tea represents mostly catechins and the PCA analysis of this region in *Fig*. 3 indicated clear demarcation between the two types of tea. The negative PC1 score of black tea cluster suggests that the catechin content in black tea is lower than in green tea, which served as a useful biomarker to distinguish the two types of tea rapidly for accurate classification. The cumulative score of PC1 and PC2 was about 82%, which is a satisfactory level.

In *Fig*. 4, clear demarcations were found on PC1 with relatively large leaves (cultivated in June ~ July) displaying negative scores compared with other positive scores. The EGCG and EC sites appeared at 6.02 to 6.65 ppm suggested possible delineation between the 3 groups. In

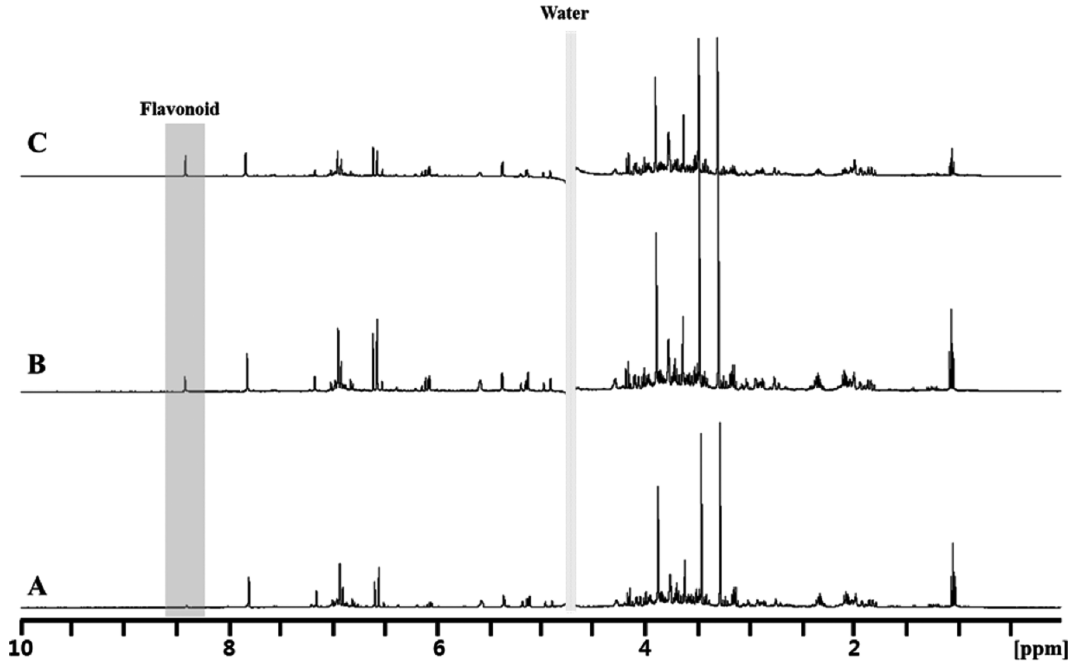

***Figure* 2.** ^{1}H NMR spectra of green tea of (A) Woojeon, (B) Jaksul and (C) Daejak. They were classified according to cultivated time.

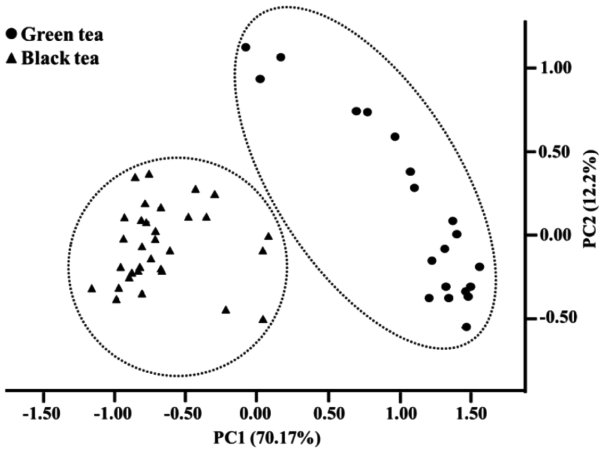

***Figure* 3.** PCA score plot for the ^{1}H NMR spectra of green tea (circles) and black tea (triangles). The variation of green tea is wider than black tea but the two clusters are discriminated clearly.

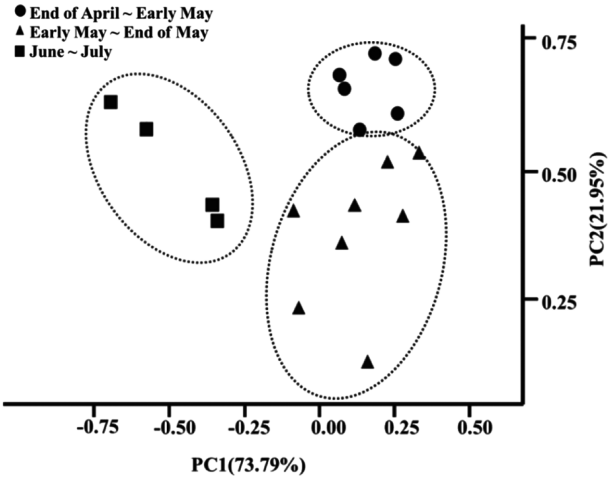

***Figure* 4.** PCA on the ^{1}H NMR spectra of green teas with different size of the leaves. Circles indicate that leaves were cultivated end of April to early May. Triangles and squares indicate leaves were cultivated early May to end of May and June to July respectively.

order to distinguish the metabolite profiles of brewed black tea leaves cultivated in three different countries (China, India and Sri Lanka), 11 different black tea samples were analyzed using ^{1}H NMR spectroscopy combined with PCA. It was used to identify the different metabolites in three groups and showed significant correlation between the components and the effect of cultivation in different countries.

Fig. 5 displays the PCA score plot of ^{1}H NMR spectra of black teas with specifically designated regions ranging from 1.07 to 4.25 ppm with point-wise bucketing. It showed distinct separation among three different countries with a total variance of about 50%. It was possible to classify black teas from different countries.

Currently, green tea or black tea is consumed by millions of people worldwide due to the various health benefits associated with specific chemical ingredients present in the tea. These ingredients depend on factors such as the

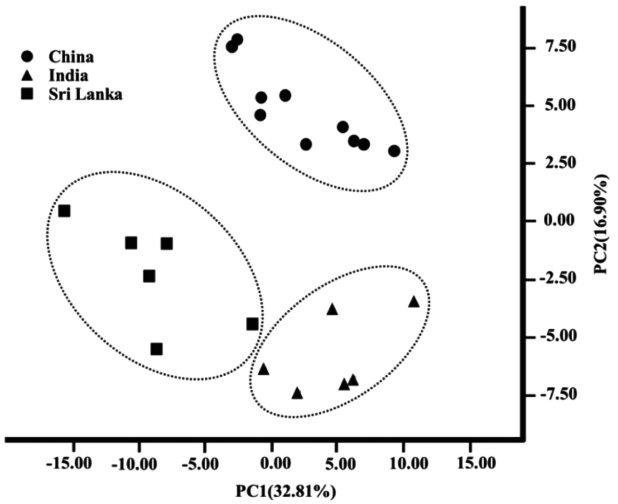

***Figure* 5.** PCA score plot on the [1]H NMR spectra of black teas cultivated at different countries. Circles indicate that leaves from China. Triangles and squares indicate leaves from India and Sri Lanka respectively. Three clusters are separated clearly with PC1 and PC2.

country of origin, the time of collection, and processing technique, all of which determine the quality of the tea. This difference in quality is reflected in the price of the tea. Therefore, it is important for the industry to evaluate the tea quality and distinguish the unique features. Previously, it was judged by professional tasters, and currently, it is analyzed using scientific measurements such as chromatography or near infrared spectroscopy (NIR) (Le Gall et al., 2004). The combination of NMR spectroscopy and multivariate analysis is used to control tea quality by simultaneously analyzing various markers such as catechins, amino acids and sugars in tea.

The results of the present study showed that the high-resolution [1]H NMR spectroscopy coupled with multivariate analysis were adequate to effectively classify green and black teas. The sensory quality control of green and black teas using these techniques is expected to be precise and based on one of the best methodologies. In particular, the chemometric study with multivariate analysis offered highly reliable results compared with the traditional sensory testing. Varying catechin content has been used to categorize various green and black teas. Specific areas of high resolution [1]H NMR spectra combined with multivariate analysis may facilitate tea characterization under each category.

CONCLUSION

The [1]H NMR spectra of green tea and black tea were monitored and analyzed by PCA. These spectra permitted simultaneous qualitative analysis of green tea and black tea. First, the [1]H NMR spectra of green tea and black tea were separated by using many catechins resonances in [1]H NMR spectra. The Black tea from China, India and Sri Lanka were separated using many resonances of metabolites. And the selections of variable contributing to discerning metabolites of green teas cultivated on diverse periods were helpful to clearly classify using PCA.

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