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Simultaneous determination of caffeine, gallic acid, theanine, (−)-epigallocatechin and (−)-epigallocatechin-3-gallate in green tea using quantitative ^{1}H-NMR spectroscopy

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Green tea consists of the dried leaves of *Camellia sinensis*, and enjoys great popularity all over the world due to its pleasant taste and positive impact on human health. It has also been regarded as a natural medicine containing great amounts of caffeine (CA), gallic acid (GA), theanine (TH), and tea polyphenols, mainly including epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epigallocatechin-3-gallate (EGCG). In the present study, ^{1}H-NMR spectroscopy is utilized for chemical characterization along with simultaneous determination of CA, GA, TH, EGC and EGCG in commercial green tea. Signal assignment for representative samples was facilitated by reference compounds and comparing with information in the literature. On the other side, the diagnostic singlet signals at δ 7.68, 7.14, 6.59 and 6.62 in the ^{1}H-NMR spectra were selected as quantitative peaks for CA, GA, EGC and EGCG, respectively, while the triplet signal at δ 1.12 with a coupling constant (J) of 7.26 Hz was chosen for the determination of TH. TSP-d_4 was adopted as the internal standard (IS) and the reference chemical shift of δ 0.00. The limits of detection (LODs) and limits of quantitation (LOQs) were measured as 28.9 and 57.8 μg mL^{-1} for CA, 18.7 and 37.4 μg mL^{-1} for GA, 23.4 and 46.8 μg mL^{-1} for TH, 28.1 and 56.2 μg mL^{-1} for EGC, and 28.1 and 56.2 μg mL^{-1} for EGCG, respectively. The relative standard deviation of both precision and repeatability assays were lower than 4.5%. The mean recoveries of high, medium and low concentration levels for each analyte were among the range of 93.1–106.3%. The contents of CA, GA, TH, EGC and EGCG were measured among the ranges of 3.72–8.38 mg g^{-1}, 0.34–1.88 mg g^{-1}, 1.56–4.48 mg g^{-1}, 2.96–8.50 mg g^{-1} and 2.78–10.60 mg g^{-1}, respectively, in 9 batches of green tea. Above all, ^{1}H-NMR spectroscopy is proven as a reliable tool not only for metabolic characterization, but also for simultaneous determination of effective components in green tea.

1. Introduction

Green tea is made solely from the dried leaves of *Camellia sinensis* by minimal oxidation and fermentation during processing (unfermented). Originating in China, it has been consumed as a popular drink throughout the world for thousands of years with a high reputation of having health-promoting effects.[1] It has also become the raw material for the extracts which are used in various beverages, health foods, dietary supplements, and cosmetic items.[2] Over the past few decades, this drinking herb has been screened using many scientific and medical evaluations to determine the extent of its long-purported health benefits, and the results suggest that regular green tea drinkers have a lower risk of developing heart disease and certain types of cancers, such as skin, esophagus, colon, pancreas, stomach, lung, bladder, prostate and breast cancers.[3–5] In addition, the extract of green tea has been reported to exhibit diversely pharmacological activities, including, but not limited to, anti-inflammatory, anti-microbial, anti-tumour, anti-oxidative and anti-aging effects.[6] Although green tea could not raise the metabolic rate significantly to afford immediate weight loss, polyphenols, theanine (TH) and caffeine (CA), all of which are extensively present in the green tea extract, have been demonstrated to induce thermogenesis and to stimulate fat oxidation by boosting the metabolic rate without increasing the heart rate.[7]

Varieties of green tea products have been produced in locations where it is grown. Thus, the quality of these various products can differ substantially due to variable growing

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conditions, horticulture, production processing, and harvesting time. Up to now, the quality of green tea is mainly assessed through its appearance (color, color intensity, and cloudiness), flavor (astringency, bitterness, and sweetness), and aroma (floral, sweet, grassy, *etc.*).[8] However, modern evaluations have shown that the green tea quality exhibits high correlation with the contents of amino acids (in particular TH), gallic acid (GA), catechins [such as epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG)], CA and some other components.[8] Therefore, it is crucial to determine the quality of green tea by the performance of both global metabolic characterization and simultaneous quantitation of the effective constituents mentioned above.

Comparing with routine analytical techniques, such as capillary electrophoresis (CE), high performance liquid chromatography (HPLC) or ultra performance liquid chromatography (UPLC) coupled with diode array detection (DAD) or tandem mass spectrometry (MS/MS), proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy has been widely proved as a more attractive method with an "all in one" feature being able to provide not only qualitative but also quantitative information for a wider range of chemical metabolites with simple sample preparation and fast acquisitions, yet without a further time consuming purification process.[9] This technology has been utilized to characterize metabolic profile and simultaneous determination of a group of catechins in green tea.[10–15] The assignment of signals for the main constituents present in green tea has been accomplished, and the results indicate a possibility for the simultaneous determination of a set of active components using quantitative ^{1}H-NMR spectroscopy. In addition, liquid chromatography coupled with time-of-flight mass spectrometry (TOF-MS) has been applied for the metabolomic study of green tea,[16,17] and EC, EGC, ECG, EGCG, CA, TH, myricetin and theogallin were picked out as the chemical biomarkers for different cultivars of green tea. However, to the best of our knowledge, ^{1}H-NMR method has not been proposed for the simultaneous determination of active components in green tea at the same time of characterizing the major constituents. Moreover, the simultaneous determination of CA, GA, TH, ECG and EGCG, of which the latter three are known to be the active components in green tea and may be used as quality indicators,[18,19] has not been achieved either. Therefore, in the present study, we aim to propose a practical method for the simultaneous determination of CA, GA, TH, ECG and EGCG (Fig. 1) in crude green tea extract on the basis of comprehensive chemical characterization using ^{1}H-NMR spectroscopy. The findings obtained in this paper are expected to prove this method as a meaningful choice for the quality control of green tea.

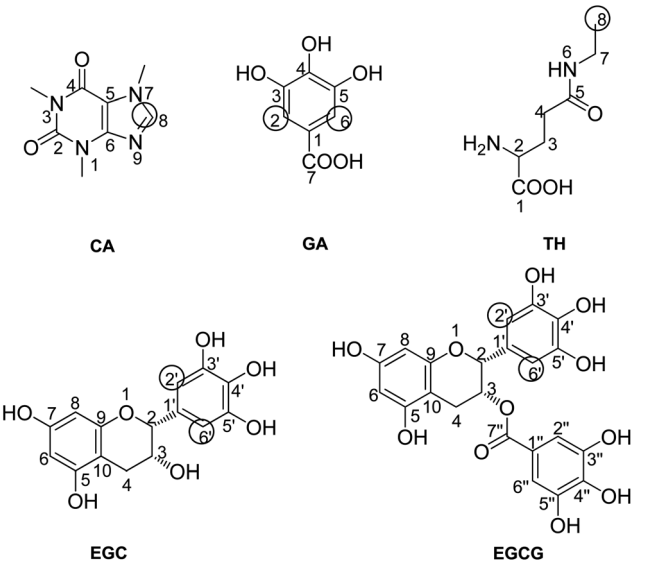

Fig. 1 Chemical structures of caffeine (CA), gallic acid (GA), theanine (TH), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG). The protons indicated with circles are chosen for quantitative analysis.

2. Materials and methods

2.1 Chemicals and reagents

Deuterium oxide (D_2O, 99.9 atom % D) was purchased from Cambridge Isotope Laboratories (Andover, Massachusetts, USA). TSP-d_4 [3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid, sodium salt] (purity = 99%), which was used as the internal standard, was obtained from NORELL (Landisville, NJ, USA). Analytical grade CA, GA, TH, EGC, EGCG, K_2HPO_4 and NaOH were supplied by Sigma-Aldrich (St. Louis, MO) or Merck (Darmstadt, Germany). Ultra-pure water was prepared in house using a Milli-Q plus water purification system (Millipore, Bedford, MA, USA).

2.2 Preparation of TSP-d_4–D_2O solution

Phosphate buffer (50 mmol L^{-1}, pH 6.5) was prepared by adding 0.68 g KH_2PO_4 and 0.1 mol L^{-1} NaOH into Milli-Q water. 0.06 g TSP-d_4 was dissolved in the phosphate buffer to obtain TSP-d_4 stock solution. 250 μL TSP-d_4 stock solution and 7.0 mL D_2O were transferred into a 10.0 mL volumetric flask and mixed thoroughly, and then the mixture was made up to 10.0 mL with the phosphate buffer. The final contents of TSP-d_4 and D_2O were 0.15 g L^{-1} and 70% (v/v) in the prepared TSP-d_4–D_2O solution, respectively.

2.3 Preparation of green tea extract

Various types of commercial green tea samples (*C. sinensis*) were collected from a local supermarket (Guangzhou, China) in May 2012, including (1) *Tieh-Kuan-Yin*, (2) *BiLuoChun*, (3) *Huangshan Maofeng*, (4) *Xinyang Maojian*, (5) *Saiqing Maocha* and (6) *Kejia Chaoqing*. The voucher specimens of all nine batches are deposited at the Key Laboratory of Natural Pesticide and Chemical Biology of Ministry of Education, South China Agricultural University. The detailed description of the samples is summarized in Table 1.

Approximately 50 mg pulverized green tea was weighed in 2 mL Eppendorf tube. Following the addition of 1.5 mL Milli-Q water, tubes were tightly closed with the plastic cap and then kept in a 70 °C water bath with continuous shaking for 25 min. After each extract was cooled to room temperature, around 1.0 mL clarified supernatant was harvested by centrifugation at

Table 1 The information of the nine batches of green tea and the contents (mg g^{-1}) of the five analytes in the aqueous extracts[a]

		CA		GA		TH		EGC		EGCG	
No.	Name	Con.	RSD (%)	Con.	RSD (%)	Con.	RSD (%)	Con.	RSD (%)	Con.	RSD (%)
1	*Tieh-Kuan-Yin*	3.72	1.37	0.34	2.34	1.56	2.03	3.85	1.36	8.81	1.24
2	*BiLuoChun* (1)	5.47	1.49	1.58	1.78	2.94	1.39	3.97	2.01	3.13	1.13
3	*Huangshan Maofeng* (1)	7.84	1.02	1.88	1.65	4.48	1.97	8.50	0.77	10.6	0.87
4	*Xinyang Maojian* (1)	5.02	0.95	1.31	2.78	3.74	2.57	3.18	2.44	3.78	1.21
5	*Saiqing Maocha*	3.77	1.45	1.73	1.48	1.94	2.35	2.96	1.86	3.37	1.39
6	*Kejia Chaoqing*	5.20	1.32	1.63	1.99	1.83	2.21	6.75	2.28	2.78	2.77
7	*BiLuoChun* (2)	5.90	0.76	1.51	2.05	3.13	1.65	3.58	2.73	5.82	1.84
8	*Xinyang Maojian* (2)	5.07	1.22	1.17	1.34	2.92	2.89	7.83	1.72	8.76	2.55
9	*Huangshan Maofeng* (2)	8.38	1.38	1.45	2.31	4.24	3.04	7.50	2.13	9.63	2.97

[a] CA: caffeine; GA: gallic acid; TH: theanine; EGC: (−)-epigallocatechin; EGCG: (−)-epigallocatechin-3-gallate; Con.: content.

13 000 rpm for 20 min. Subsequently, an aliquot of 600 μL supernatant was mixed with 100 μL prepared TSP-d_4–D_2O solution. The obtained mixture was transferred into 5 mm tubes (Norell ST500-7) and immediately subjected to ^{1}H-NMR measurement. Reference compounds mixture were prepared and analyzed in parallel at the same time. All experiments were carried out in triplicate and variation was expressed with relative standard deviation (RSD, %).

2.4 ^{1}H-NMR spectroscopy

All ^{1}H-NMR spectra were recorded on a Bruker AVANCE II 600 spectrometer at 600.13 MHz proton frequency (Bruker, Karlsruhe, Germany) equipped with TCI cryoprobe and *Z*-gradient system at 297 K.

To determine the longitudinal relaxation time (T_1) of these CA, GA, TH, EGC and EGCG, the inversion recovery pulse sequence method was applied for the selected proton signals using T_1 cal Bruker program. Then, the measured data was put into the exponential equation: $I = I_0 + P\exp(-\gamma/T_1)$, in which I is the intensity of each proton resonance at inversion delay (γ) and I_0 at the equilibrium state, and P is a constant. A series of inversion delays were fixed as 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 15.0, 30.0 and 60.0 s.

For each sample, 128 scans of 38 460 data points were acquired with a spectral width of 9600 Hz (16 ppm), pulse width of 12.34 μs, acquisition time of 4.0 s, relaxation delay time (d_1) of 10 s, flip angle of 90° and constant gain of 181. All the data was obtained under an automatic procedure, requiring about 19.4 min per sample. Solvent suppression was achieved using the Watergate pulse sequence.[20] D_2O served as the field frequency lock, and the chemical shifts of all the spectra were calibrated using the signal from TSP-d_4 at δ 0.00.

Initialized data processing was carried out with Bruker TOPSPIN 2.1 software (Bruker, Karlsruhe, Germany). An exponential function with LB as 0.3 Hz was applied, and the data were zero-filled to give at least five data points above the half width for each resonance to allow for precise and reliable integration. The Free Induction Decay (FID) signals were Fourier transformed (FT) and all the spectra were manually phased and automated polynomial baseline correction was applied to improve the accuracy of the integral. Data analysis was carried out with MestReNova 5.3.1 software package (Mestrelab Research SL). All selected NMR peaks were integrated manually with the same integral limits being used for a given peak in all spectra.

2.5 Simultaneous determination using quantitative ^{1}H-NMR spectroscopy

The contents of these five analytes in green tea samples were calculated using the following equation:[21]

$$\text{content}\ (\text{mg g}^{-1}) = \frac{A_x}{A_{IS}} \times \frac{N_{IS}}{N_x} \times \frac{M_x}{M_{IS}} \times \frac{W_{IS}}{W_x} P_{IS}$$

where A_x and A_{IS} represent the integral areas of the analyte and internal standard (IS), respectively; N_{IS} and N_x correspond to the proton numbers of internal standard and the analyte, respectively; M_x and M_{IS} are the molecular weights of the analyte and IS, respectively; W_{IS} is the weight of IS, while W_x is the weight of the analyte; P_{IS} stands for the purity of the standard (99%).

2.6 Quantitative ^{1}H-NMR method validation

The proposed quantitative ^{1}H-NMR (q^{1}H-NMR) method was validated using the calibration curves, precision, accuracy, repeatability, limit of detection (LOD) and limit of quantitation (LOQ) assays.[22]

Accurately weighed CA, GA, TH, EGC and EGCG (around 1.65 mg for each) were mixed and dissolved in 600 μL Milli-Q water and diluted using 100 μL of prepared TSP-d_4–D_2O solution to obtain stock solution. Then, the mixture was stepwise diluted by Milli-Q water : TSP-d_4–D_2O solution (v/v, 6 : 1) to yield calibration samples at a series of concentration levels. At least six concentration levels of the calibration samples were analyzed in triplicate, and then the calibration curves were constructed by plotting the ratios of the peak areas of each standard detected by q^{1}H-NMR to internal standard (IS, TSP-d_4) *versus* the theoretical concentration levels of each analyte over the corresponding calibration concentration range. LOD is the lowest concentration of an analyte that can be detected, while limit of quantification LOQ is defined as the lowest concentration of a targeted analyte that can be accurately and precisely quantified. Typically,

they are three and ten folds of the noise level (S/N = 3, 10), respectively. For each targeted ingredient, both LOD and LOQ were determined by serial dilution of stock solution under the described q^{1}H-NMR parameters.

Precision, repeatability and stability assays were assessed by the relative standard deviation (RSD, %). The *BiLuoChun* (1) sample was chosen to measure the six replicates continuously to achieve the precision assay. Stability study was carried out by detecting the same sample at different time points within 24 h during the storage at 4 °C. Repeatability was determined by testing six separately prepared *BiLuoChun* (1).

The recovery was used to evaluate the accuracy of the method and was determined by adding the mixed standard solutions with three different concentration levels (high, middle and low) to the known amounts of *BiLuoChun* (1). The mixture was extracted and analyzed following the procedures described above. Three replicates were performed at each level. The percentage recoveries were calculated according to the following equation: recovery (%) = (amount found − original amount) × 100%/amount spiked. Variations were expressed by RSD (%).

3. Results and discussion

3.1 Optimization of q^{1}H-NMR experimental conditions

In accordance with usual performance, in this paper, hot water was selected as the extraction solvent for pulverized green tea sample. After optimizing extraction time and temperature, 70 °C and 25 min duration were chosen to process each batch of green tea samples. Furthermore, the cycle of extraction was optimized as one.

The T_1 values of the selected working proton signals were measured as 3.735 s for CA, 7.140 s for GA, 2.546 s for TH, 2.647 s for EGC, 3.404 s for EGCG and 3.483 s for the internal standard (TSP-d_4). If 5-fold T_1 relaxation delay time was adopted as the relaxation delay time (d_1, about 36 s) for the simultaneous determination, the single measurement time would be too long for the routine analysis. For time-saving measurement, we compared the response of GA, which afforded the longest T_1, under different relaxation delay times (from 1 s to 36 s), and the results revealed that no significant change was observed for the peak area of GA after 10 s of relaxation delay (data not shown). Therefore, 10 s was chosen in the current study, which is three times more than the average T_1. In fact, some examples that the d_1 was set less than 5-fold T_1 could be found in the literature.[20] Owing that increasing the scanning numbers is helpful to improve the signal–noise (S/N) ratio, which has a potent effect on the integral value, in particular for that of trace components, 128 scans were finally selected in this NMR analysis.

3.2 Signal assignment of ^{1}H-NMR spectra

Table 2 summarizes the assignment of proton signals of the main components that were tentatively identified from green tea. Firstly, the signals of CA, GA, TH, EGC and EGCG were assigned using reference compounds which were measured in parallel. CA exhibited three obvious signals at δ 3.23 (s), 3.42 (s) and 3.79 (s), corresponding to the three *N*-methyl groups at N-1, N-7 and N-3 positions, respectively, and one single peak at δ 7.65 (s) that was definitely assigned to the aromatic proton (H-8) (Fig. 2). GA solely afforded a singlet peak at δ 7.16 (s), which was generated by the protons of H-2 and H-6 (Fig. 2). Five signals were yielded by TH, including one intense triplet signal at δ 1.12 (t, J = 7.26 Hz) vesting to 8-CH_3, one triplet signal at δ 3.85 (t, J = 6.06 Hz, H-2) for H-2 and three multiplet signals at δ 2.20 (m), 2.45 (m) and 3.27 (m) belonging to the methylene groups at C-3, C-4, and C-7 positions, respectively. Five signals were observed for EGC, including a singlet peak at δ 6.59 (s) corresponding to the aromatic protons of H-2′ and H-6′, a multiplet peak at 4.32 (m) belonging to H-3, and two multiplet signals at δ 2.81 (m) and 2.93 (m) being respectively assigned to H-4eq and H-4ax. EGCG generated seven proton signals in the ^{1}H-NMR spectrum, including two singlet signals at δ 6.62 (s) and 6.87 (s) that corresponded to the aromatic protons of H-2′ and H-6′, and H-2″ and H-6″, respectively; one doublet peak at δ 6.09 (d, J = 2.28 Hz) for aromatic protons of H-6 and H-8; two multiplet signals at δ 2.93 (m, H-4ax) and 3.08 (m, H-4eq) and two multiplet ones at δ 5.03 (m); 5.50 (m) yielded by H-2 and H-3.

In general, ^{1}H-NMR spectroscopy based quantification requires at least one non-overlapping signal for each molecule for easy integration and quantification. The signal exhibited at 0.00 ppm of TSP-d_4 was used as internal standard. The triplet signal at δ 1.12 with a coupling constant (J) as 7.26 Hz was selected to determine TH's concentration (Fig. 2), while the diagnostic single signals at δ 7.65, 7.16, 6.62 and 6.59 in the proton NMR spectra were chosen as quantitative peaks for CA, GA, EGCG and EGC (Fig. 2B), respectively. The protons indicated with circles were chosen for quantitative analysis because the corresponding signals afford intense signal or triplet and do not overlap with the other signals (Fig. 1).

Besides the observation of EGC and EGCG with the assistance of reference compounds, some other catechin derivatives were also tentatively identified in the ^{1}H-NMR spectra by comparing with spectroscopic values in the literature. The presence of epicatechin (EC) and epicatechin-3-gallate (ECG) was obviously indicated by the observation of two sets of signals, which were δ 2.77, 2.95, 4.27, 4.81, 5.90, 6.83, 6.92, 7.02, and δ 2.89, 3.03, 5.09, 6.94, 7.04 (Table 2).[14,23] Meanwhile, as the biosynthesis precursor of polyphenols in green tea, catechin was also identified based on the signals at δ 2.57, 2.87, 4.63 and 6.85.[13,24] In addition, the signals at δ 6.57, 6.84 and 6.96 tentatively accounted for the existences of gallocatechin-3-gallate (GCG), gallocatechin (GC) and catechin-3-gallate (CG), respectively (Table 2).[13,24] Flavonoids were responsible for the signal at the low field around 8.00 ppm, and the flavonoids could be apigenin glucoside, quercetin glucoside and/or kaempferol glucoside on the basis of the information in literature.[13]

Sugar compounds were mostly observed in the region between δ 3.00 and 5.50. Sucrose was the major disaccharide (nonreducing sugar) in green tea having resonances at δ 3.43, 3.53, 3.76, 3.80, 3.84, 3.88, 4.05, 4.19, and 5.42.[13,14] Signals belonging to monosaccharides (reducing sugar) including fructose, α-glucose and β-glucose clearly resonated at δ 3.56, 3.70, 3.79, 3.88, 4.00, 4.10; δ 3.50, 5.20; δ 3.50, 5.20, and δ 3.21, 4.58, respectively (Table 2).[13]

Table 2 Assignment of proton signals in the representative ^{1}H-NMR spectrum (10% D_2O, δ in ppm, J in Hz)

Analyte	Assignment of proton signals
Caffeine[a]	3.23 (s, N_1-CH_3); 3.42 (s, N_7-CH_3); 3.79 (s, N_3-CH_3); **7.65 (s, H-8)**[b]
Gallic acid[a]	**7.16 (s, H-2,6)**
Theanine[a]	**1.12 (t, J = 7.26 Hz, 8-CH_3)**; 2.20 (m, 3-CH_2); 2.45 (m, 4-CH_2); 3.27 (m, 7-CH_2); 3.85 (t, J = 6.06 Hz, H-2)
Epigallocatechin[a]	2.81 (1H, m, H-4eq); 2.93 (1H, m, H-4ax); 4.32 (1H, m, H-3); 4.92 (1H, m, H-2); **6.59 (s, H-2′,6′)**
Epigallocatechin-3-gallate[a]	2.93 (m, H-4ax); 3.08 (m, H-4eq); 5.03 (m, H-2); 5.50 (m, H-3); 6.09 (d, J =2.28 Hz, H-6,8); **6.62 (s, H-2′,6′)**; 6.87 (s, H-2″,6″)
Epicatechin	2.77, 2.95, 4.27, 4.81, 5.90, 6.83, 6.92, 7.02
Epicatechin-3-gallate	2.89, 3.03, 5.09, 6.94, 7.04
Catechin	2.57, 2.87, 4.63, 6.85
Catechin-3-gallate	6.96
Gallocatechin	6.57
Gallocatechin-3-gallate	6.57, 6.84, 6.98
Flavonoids	8.00
p-Coumaryl quinic acid	7.51, 7.72
Theobromine	7.74
Theogallin	2.02, 2.15, 2.20, 7.15
2-*O*-Arabinopyranosyl-myo-inositol	3.26, 3.61, 3.68, 3.89, 3.97, 4.18, 5.14
Sucrose	3.43, 3.53, 3.76, 3.80, 3.84, 4.05, 4.19, 5.42
Fructose	3.56, 3.70, 3.79, 3.88, 4.00, 4.10
α-Glucose	3.50, 5.20
β-Glucose	3.21, 4.58
Arginine	1.73, 3.27
Threonine	1.35, 4.22
Alanine	1.57, 3.71
Valine	0.97, 1.02
Leucine	0.98
Isoleucine	0.92, 0.99
γ-Amino butyric acid	1.92, 2.34, 3.03
Acetate	1.98
Glutamine	2.13, 2.43, 3.70
Quinic acid	1.90, 1.92, 2.03, 3.98, 4.05
Fatty acid	0.88, 1.28, 1.60

[a] Signals were assigned using reference compounds. [b] Signals indicated in bold were chosen for quantitation.

Six types of amino acids including arginine (1.73 and 3.27 ppm), alanine (1.57 and 3.71 ppm), valine (0.97 and 1.02 ppm), threonine (1.35 and 4.22 ppm), leucine (0.98 ppm) and glutamine (2.13, 2.43 and 3.70 ppm) were also detected in the spectra (Table 2).[13]

As the biosynthesis pioneer of caffeine, the existence of theobromine was confirmed by the signal at δ 7.74.[12,13] *p*-Coumaryl quinic acid was identified using the signals at δ 7.51 and 7.72, while quinic acid afforded the signals at δ 1.90, 1.92, 2.03, 3.55, 3.98 and 4.05.[13] Some other components including γ-amino butyric acid (1.92, 2.34 and 3.03 ppm), fatty acid (0.88, 1.28 and 1.60 ppm), 2-*O*-arabinopyranosyl-myo-inositol (3.26, 3.61, 3.68, 3.89, 3.97, 4.18 and 5.14 ppm), theogallin (2.02, 2.15, 2.20 and 7.15 ppm) and acetate (1.98 ppm) were also assigned in the spectra (Table 2).[13]

3.3 Validation of quantitative method

In fact, it is not really necessary for the quantitative analysis of individual compounds to establish regressive calibration curves since the content of the analyte was strictly proportional to the peak area of corresponding signal in the ^{1}H-NMR spectrum. However, depending on the different concentrations, the accuracy evaluation of this method may be determined by establishing calibration curves for each compound determined over a large concentration range. For the calibration equations, the correlation coefficients of the five compounds were higher than 0.999, while the high values obtained indicated a good linearity response within the concentration range studied (Table 3).

A signal-to-noise (S/N) ratio of 3 is used to determine LODs. For the five measurements performed on diluted standard solution with known concentration, the LODs obtained were 28.9 μg mL^{-1} for CA, 18.7 μg mL^{-1} for GA, 23.4 μg mL^{-1} for TH, 28.1 μg mL^{-1} for both EGC and EGCG, respectively. The LOQs were 57.8, 37.4, 46.8, 56.2, 56.2 μg mL^{-1} for CA, GA, TH, EGC and EGCG, respectively (Table 3).

The RSDs of precision and repeatability tests are both below 4.5% (Table 4). The stability results proved that the sample could keep stable within 24 h. The recovery tests were analyzed by the method described above. As shown in Table 5, the mean recoveries of the five compounds are in the range of 93.1–106.3% over the three concentration levels.

These data prove that the developed approach is precise, accurate and sensitive enough for simultaneous quantitative determination of these five compounds in green tea samples.

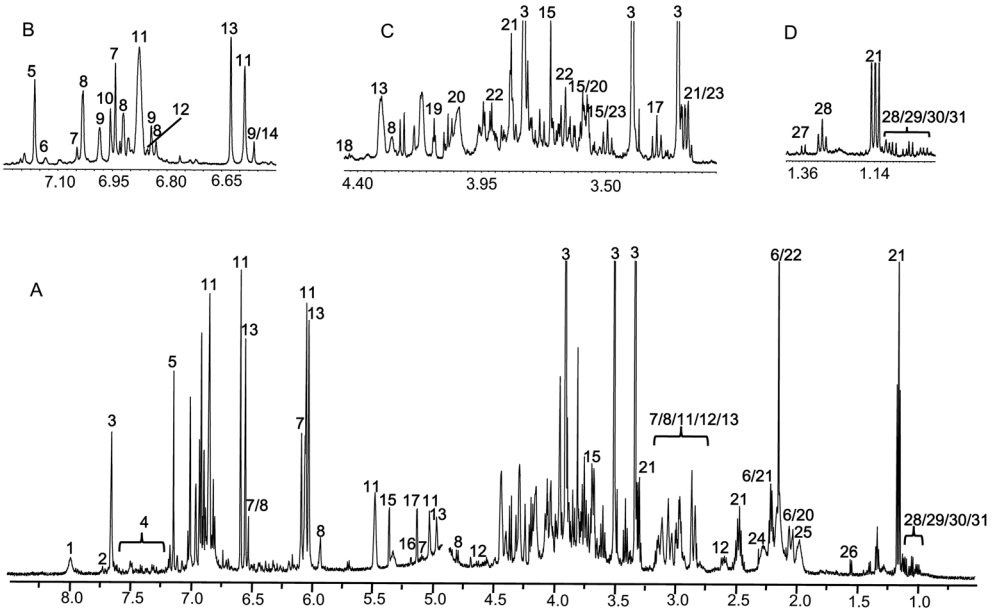

Fig. 2 Representative ^{1}H-NMR spectra of green tea in the range of δ −0.5 to 8.5 (A); expansions for the range of δ 6.5–7.3 (B), δ 3.2–4.5 (C), and δ 0.9–1.5 (D). (1) Flavonoid(s); (2) theobromine; (3) caffeine; (4) *p*-coumaryl quinic acid; (5) gallic acid; (6) theogallin; (7) epicatechin-3-gallate; (8) epicatechin; (9) gallocatechin-3-gallate; (10) catechin-3-gallate; (11) epigallocatechin-3-gallate; (12) catechin; (13) epigallocatechin; (14) gallocatechin; (15) sucrose; (16) α-glucose; (17) 2-*O*-arabinopyranosyl-myo-inositol; (18) β-glucose; (19) fructose; (20) quinic acid; (21) theanine; (22) glutamine; (23) arginine; (24) γ-amino butyric acid; (25) acetate; (26) alanine; (27) threonine; (28) fatty acid; (29) valine; (30) leucine; (31) isoleucine.

3.4 Simultaneous determination of CA, GA, TH, EGC and EGCG in commercial green tea samples

The contents of CA, GA, TH, EGC and EGCG in nine batches of commercial green tea were analyzed following the method described above, and the results are summarized in Table 1. The representative ^{1}H-NMR spectrum of green tea is shown in Fig. 2. All the contents of CA (7.84 mg g^{-1}), GA (1.88 mg g^{-1}), TH (4.48 mg g^{-1}), EGC (8.50 mg g^{-1}) and EGCG (10.6 mg g^{-1}) were detected in *Huangshan Maofeng* (1), and the other batch of *Huangshan Maofeng* (2) also contained high contents of these five analytes (CA, 8.38 mg g^{-1}; GA, 1.45 mg g^{-1}; TH, 4.24 mg g^{-1}; EGC, 7.50 mg g^{-1}; and EGCG, 9.63 mg g^{-1}), indicating a good active prospect for this type of green tea. For *Tieh-Kuan-Yin*, which was mainly cultivated in Fujian province, it exhibited low contents of GA and TH in all types. On the other hand, the content of EGCG in *Kejia Chaoqing* was detected much lower than the other type of green tea, while the lowest content of EGC was observed for *Saiqing Maocha*. Interestingly, big variations were found between the two batches of *Xinyang Maojian*, especially for the contents of EGC and EGCG. The content of EGCG in *BiLuoChun* (1) was quite different from that in *BiLuoChun* (2), and these two batches contained analogous contents of CA, GA, TH and EGC.

The results indicate that the contents of these five compounds exhibited big variations among the different types of plant materials. 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, the processing, storage, *etc.*[12] For example, the best green teas are usually plucked during the first flush in April or May (there are three main plucking times: spring, summer, and autumn).[25] Furthermore,

Table 3 Linear regression data, LOD, LOQ of the five analytes[a]

	Linear regression data				
Analytes	Regression equation	*R*	Test range (μg mL^{-1})	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
CA	$y = 0.0051x - 0.0078$	0.9995	28.9–1850	28.9	57.8
GA	$y = 0.0122x - 0.0875$	0.9995	18.7–1200	18.7	37.4
TH	$y = 0.0184x - 0.1099$	0.9995	23.4–1500	23.4	46.8
EGC	$y = 0.0043x - 0.1003$	0.9993	28.1–1800	28.1	56.2
EGCG	$y = 0.0044x - 0.0466$	0.9992	28.1–1800	28.1	56.2

[a] CA: caffeine; GA: gallic acid; TH: theanine; EGC: (−)-epigallocatechin; EGCG: (−)-epigallocatechin-3-gallate. *x* stands for the ratio of the peak areas of each analyte detected by q^{1}H-NMR to internal standard (IS, TSP-d_4); *y* stands for the concentration of the target analyte.

Table 4 Results of precision, repeatability and stability assays of the five investigated components[a]

	Precision ($n = 6$)		Repeatability ($n = 6$)		Stability ($n = 6$)	
Analyte	Content (mg g^{-1})	RSD (%)	Content (mg g^{-1})	RSD (%)	Content (%)	RSD (%)
CA	5.46	2.04	5.47	0.69	5.47	3.21
GA	1.58	1.32	1.57	1.23	1.57	2.64
TH	2.94	2.09	2.93	4.34	2.94	4.32
EGC	3.98	2.54	3.97	1.62	3.97	2.06
EGCG	3.14	1.65	3.13	2.54	3.14	3.22

[a] CA: caffeine; GA: gallic acid; TH: theanine; EGC: (−)-epigallocatechin; EGCG: (−)-epigallocatechin-3-gallate.

Table 5 The recovery test results of the five investigated compounds[a]

		Found drug		
Analyte	Added drug (mg)	Average[b] (mg)	RSD (%)	Recovery[c] (%)
CA	0.3000	0.3470	2.74	93.1
	0.1500	0.2139	2.39	97.5
	0.0750	0.1431	3.47	100.5
GA	0.3600	0.4331	1.27	97.6
	0.1800	0.2571	2.33	97.3
	0.0900	0.1653	3.68	92.6
TH	0.3450	0.3958	1.49	98.4
	0.1725	0.2247	2.29	97.7
	0.0863	0.1376	3.13	94.3
EGC	0.2850	0.3276	2.03	102.1
	0.1425	0.1861	2.77	104.9
	0.0713	0.1124	2.98	106.3
EGCG	0.3150	0.4012	1.74	103.1
	0.1575	0.2375	1.79	102.3
	0.0788	0.1587	3.04	104.5

[a] CA: caffeine; GA: gallic acid; TH: theanine; EGC: (−)-epigallocatechin; EGCG: (−)-epigallocatechin-3-gallate. [b] The data is presented as average of three measurements. [c] Recovery (%) = (amount found − original amount) × 100%/amount spiked.

the plucking position also plays a key role for the quality of green tea.[10] In general, the youngest green tea leaves provide the highest quality of the tea, the highest levels of caffeine, gallic acid, and theanine yet the lowest level of EGCG.[10]

Some factors were widely believed to contribute to the LOD of quantitative ^{1}H-NMR, such as the magnetic field strength of the spectrometer, the scanning numbers, the type of probe, and the size of the tube. Generally speaking, the introduction of cryo-probes could strengthen the LOD obviously. In current case, a TCI cryoprobe was adopted. For the other factors, the magnetic field strength was selected as 600 MHz, and 5 mm tubes were chosen due to convenient process, while scanning number was optimized as 128 scans.

Quantitative ^{1}H-NMR has been introduced for the simultaneous determination of catechin derivatives,[11] however, DMSO-d_6 served as the solvent during NMR spectrum measurement in that case. As a common drinking herb, aqueous solvent should be more appropriate for the quality assessment of green tea. On the other hand, the separation between EGC and EGCG usually could not meet the demands of determination due to overlapping signals of these two analytes.[10,12,13] However, in the current study, baseline separation was achieved for EGC and EGCG, which should be accounted for the adoption of high-field NMR spectrometer and acidic buffer (pH 6.5). Moreover, the simultaneous quantitation of CA, GA and TH in green tea was carried out for the first time using quantitative NMR spectroscopy.

In addition, 31 components were tentatively identified in the representative ^{1}H-NMR spectra. Sucrose and some amino acids (alanine and threonine) were also obtained for baseline separation with the other signals in corresponding domains, indicating the potential for the determination of those components using ^{1}H-NMR spectroscopy with the conditions proposed in the current study.

4. Conclusion

In this study, ^{1}H-NMR spectroscopy was adopted for the simultaneous determination of the contents of CA, GA, TH, EGC and EGCG in nine batches of commercial green tea samples for the first time based on the comprehensive metabolic profiling. Validation assays proved that the developed quantitative ^{1}H-NMR method was of good accuracy, precision and repeatability, indicating it could be adopted as a simple, rapid, and powerful tool for effective quality assessment. The results obtained suggest that ^{1}H-NMR can be adopted as a reliable tool for rapid quality evaluation of green tea.

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