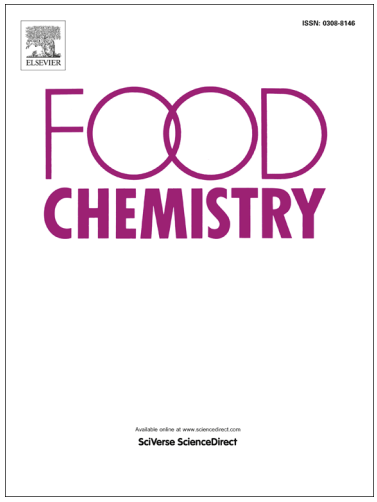
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Variation of theanine, phenolic, and methylxanthine compounds in 21 cultivars of *Camellia sinensis* harvested in different seasons

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ABSTRACT

This is the first study to use chemometric methods to differentiate among 21 cultivars of *Camellia sinensis* from China and between leaves harvested at different times of the year using 30 compounds implicated in the taste and quality of tea. Unique patterns of catechin derivatives were observed among cultivars and across harvest seasons. *C. sinensis var. pubilimba* (You 510) differed from the cultivars of *C. sinensis var. sinensis*, with higher levels of theobromine, (+)-catechin, gallocatechin, gallocatechin gallate and theasinensin B, and lower levels of (−)-epicatechin, (−)-epigallocatechin (EGC) and (-)-epigallocatechin gallate (EGCG), respectively. Three cultivars of *C. sinensis var. sinensis, Fuyun 7*, *Qiancha 7* and *Zijuan* contained significantly more caffeoylquinic acids than others cultivars. A Linear Discriminant Analysis model based on the abundance of 12 compounds was able to discriminate amongst all 21 tea cultivars. Harvest time impacted the abundance of EGC, theanine and afzelechin gallate.

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Keywords: *Camellia sinensis*; Chinese tea cultivars; cross-validation; Linear Discriminant Analysis, mode of variation; QDA, RPCA; Stepwise classification; Tukey's HSD.

Chemical compounds studied in this article:

Gallocatechin (PubChem CID: 9882981); Gallocatechin gallate (PubChem CID: 199472); Catechin (PubChem CID: 9064); Epicatechin (PubChem CID: 72276); Epigallocatechin (PubChem CID: 72277); Epigallocatechin gallate (PubChem CID: 65064); Epicatechin gallate (PubChem CID: 107905)

1. Introduction

Tea produced from *Camellia sinensis* (L.) Kuntze is an important non-alcoholic beverage. It is consumed globally in many different forms, from un-oxidised green leaf to fermented black tea leaf infusions and is drunk either hot or cold. It is reported to be first discovered by the Chinese emperor Shen Nong in 2737 B.C. and the book《茶经》(Cha Jing) written by Lu Yu in the 7th century is recognized as the first monograph of tea (Jiang & Jiang, 2006). Until the middle of the 19th century all teas consumed in the West were produced in China (Wan, Li, & Zhang, 2009). China is still the world's largest tea producer with an output of 1.9 million tons, which in 2013 was 38% of the world's total tea production of 5 million tons. The UK is the biggest importer of tea in Europe with 139,800 tonnes imported in 2013 (FAO, 2014). In China, there are 112 officially approved tea cultivars grown in 15 provincial regions (Yang, 2014). These teas are recognised as having distinct flavour characters. These flavours will be underpinned by differences in chemical composition. However, there is very little information about the variability in chemistry among different varieties and cultivars of *C. sinensis* and how the composition of the leaves is influenced by growing conditions and

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processing methods. The diversity of *C. sinensis* germplasm within China has been recognized as an important resource for crop improvement. However, because knowledge about the chemistry of this germplasm is limited its optimum use is constrained.

Numerous compounds have been implicated in the taste, quality and health benefits of tea. These include the amino acid theanine, methylxanthines (caffeine and theobromine), quinic acid esters as well as the diverse range of catechins and other flavonoids. Theanine accounts for about 60-70% of the total amino acid content in tea leaves and it stimulates the T1R1 and T1R3 umami taste receptors, thus it has an important effect on tea taste and quality(Chen, Apostolides, & Chen, 2013; Narukawa, Toda, Nakagita, Hayashi, & Misaka, 2014). The bitter-tasting caffeine and theobromine are associated with a diverse range of health benefits, although more studies are needed to support the efficacy of these compounds (Judelson, et al., 2013; Mitchell, et al., 2011). Of the quinic acid esters found in tea, chlorogenic acids have been intensively investigated for their health benefits and for the role they play in modulating insect behaviour (Chen & Wu, 2014; Hamamura, 1970; Mubarak, et al., 2012). Many studies have shown that green tea is rich in catechins, which can be up to 30% of the dry mass of the leaf (Goto, Yoshida, Kiso, & Nagashima, 1996). The diversity and abundance of catechins play an important role in the characteristic taste of some tea cultivars (Balentine Douglas, 1992). For example, it has been estimated that 70-75% of the bitterness and astringency of green tea is associated with these flavan-3-ol epimers (Zhen, Chen, Cheng, & Chen, 2002). The two flavan-3-ol epimers (+)-catechin and (–)-epicatechin differ in their oral astringency and bitterness. A higher (–)-epicatechin concentration imparts significantly more bitterness and astringency than an equal concentration of (+)-catechin. Furthermore, the bitterness and astringency of gallate type catechins are greater than that of free catechins (Kallithraka, Bakker, & Clifford, 1997). These flavan-3-ol compounds are also reported to exhibit a range of beneficial properties for health (Howes & Simmonds, 2014). For example, a metadata

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analysis of 13 studies suggested that green tea and its associated catechins can improve blood pressure, reduce total cholesterol and low-density lipids (Khalesi, et al., 2014).

This study investigated the abundance of 30 compounds from 21 cultivars of *C. sinensis* from China grown in a single plantation (under the same environmental conditions) and harvested in spring (March), summer (May) and late summer (September) in 2013. The 30 selected compounds are implicated in the taste, quality and health benefits of tea. They were studied using liquid chromatography-mass spectrometry and analysed using different chemometric methods. A preliminary study was undertaken on the leaves collected in March to evaluate whether the drying method (freeze-dried or oven-dried) influenced the profile of compounds. Evaluation of the variation in chemistry among cultivars as well as leaves harvested at different times of the year can support the selection of different cultivars for future breeding programmes and for selecting when best to harvest tea to optimise a particular attribute.

2. Materials and methods

2.1. General Instrumentation

LC-MS/MS analysis was carried out with a Thermo Scientific 'Accela' LC-system (autosampler, pump and photodiode array detector) coupled to a Thermo Scientific 'LTQ-Orbitrap XL' hybrid linear ion trap-orbitrap mass analyser fitted with an 'Ion-Max' electrospray ionisation (ESI) source. Samples (5 µl) were injected onto a RP C18 column (Phenomenex Luna C18(2), 150 × 3 mm i.d., 3 µm particle size) and eluted at 0.4 mL min^{-1} and 30 ^{o}C using a linear gradient of MeOH, H_2O and MeCN with 1% formic acid (0:90:10 – 90:0:10 v/v over 30 min) followed by a 5 min column wash (90:0:10) and equilibration to start conditions for 3 min before the next injection. For analyses to investigate compound identity a shallower gradient (0:95:5 – 45:50:5 v/v over 50 min) of the same mobile phase was

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employed using the same column conditions. MS1 spectra at 30,000 resolution were recorded in the range *m/z* 125–2000 by the Orbitrap (FTMS) in positive mode. Simultaneously with the high resolution FTMS analysis, the linear ion-trap (ITMS) recorded low resolution MS1 (*m/z* 125–2000), MS2 and MS3 spectra in both positive and negative modes. A 4 m/z ion isolation window and relative collision energy of 35% was used for all MS2 and MS3 spectra.

2.2. Sample Preparation

The 21 cultivars of tea were provided by Tea Research Institute, Chinese Academy of Agricultural Sciences (TRICAAS). Tea cultivars were selected by TRICAAS based on their differing shoot colour, size of leaves and reported taste. Samples were all propagated at the TRICAAS tea plantation in Fujian, China, under the same environmental conditions. A voucher of each of the teas has been retained at Royal Botanic Gardens, Kew. The accession number and background information about the 21 cultivars are shown in Table 1 (Jiang, et al., 2013; Xing-rong, Yi-ping, Mei, Yun-xiu, & Chun-lin, 2013; Yang, 2014). *Camellia sinensis* var. *sinensis* 'Fuding-dabaicha' (FD) is a famous old Chinese cultivar with over 150 years of use and is usually used as a benchmark for tea research in China (Yang, 2014). Six of the 21 cultivars are not yet officially registered by National Crop Cultivar Approval Committee but they are under development at TRICAAS (Table 1). Fresh tea shoots (200 g), consisting of one apical bud and two adjoining leaves were hand-plucked from each of the 21 cultivars at three commercial harvest seasons (the end of March, May and September) in 2013. In order to investigate the influence of the drying method on the chemical composition of the shoots, the March samples were separated into 2 subsets with one set being oven-dried (at 120 ^{o}C for 5 min then 100 ^{o}C for 24 hrs) and the other set being freeze-dried. May and September collections were freeze-dried; the May collection of cultivar Baijiguan (BJ) was not available. Samples were extracted in 80% methanol at a ratio of 50 mg sample per ml solvent. Each

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sample was extracted in triplicate, thus the total number of extracts analysed by LC-MS/MS was 249 (21 cultivars × 4 treatments ×3 repeats, minus 3 BJ samples).

2.3. Data set preparation and analysis

The 30 compounds (V1 to V30) selected for analysis were identified in the LC-MS/MS analyses either by comparison with standards or from interpretation of chromatographic and mass spectrometry data (Table 2) (Spiller, 1998; Yao, et al., 2004). Most peak areas of the ion species given in Table 2 were measured in ion chromatograms of FTMS. Due the saturation of EGCG (V8) and ECG (V11) signals from the mass spectrometer, their peak areas were measured from their UV chromatograms at wavelengths of 320 nm and 315 nm, respectively. This provided a range of meaningful variation in their peak area values as relative abundance for all tea samples (Figure 1). Peak V25 was a mixture of quercetin-3-*O*-glucoside and qercetin-3-*O*-galactoside, as revealed by LC-MS/MS analysis, but was considered as one variable for the purpose of multivariate analysis. The configuration and conformation of (+)-catechin (V7) and (−)-epicatechin (V9) has been thoroughly reviewed by Birch et al (Birch, Clark-Lewis, & Robertson, 1957). The structures of the flavan-3-ol (Figure S2 in supplementary material) were based on results from a conformational study of green tea catechins (Niemeyer & Brodbelt, 2007).

All statistical analyses was undertaken on the peak areas. The analyses were carried out using packages from the R statistical programming language (Van Amelsvoort, et al., 2001). A normality test was applied to the data set using Anderson-Darling test from the package 'nortest' prior to analysis of variance. Most samples had a normal distribution (Anderson-Darling $p \geq 0.05$). Analysis of variance is generally robust to non-normality, which could reduce the power of the F test, but the equality of the sample sizes mitigated this, and the data were analysed using the function *aov* in package 'stats' without transformation. Simultaneous

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confidence intervals on the differences between the means were created from the fitted *aov* models using the function *TukeyHSD*. The intervals are based on the Studentised range statistic, known as Tukey's 'Honest Significant Difference' method. This method avoids type I errors (false positives) which would arise from using the t-test and the algorithm employed is robust enough to deal with small departures from a balanced design (Leroy, 2011). Correlations between compounds were analysed using 'corrplot' package.

Exploratory Principal Components Analysis (PCA) was carried out with the function *princomp* and used the covariance matrix produced from the scaled data (i.e. each variable transformed to μ=0 or σ=1). The principal components provided low-dimensional representation of the variation in the original data, without being influenced by variables with the most variance in the original data. In case of outliers, another PCA was carried out on a robust estimate of the covariance matrix which was produced using MCD (Minimum Covariance Determinant) estimator in the package 'MASS'. Several supervised classification methods were used to identify marker compounds that could be used to discriminate between the tea cultivars and harvest seasons, such as linear discriminant analysis (LDA) and quadratic discriminant analysis (QDA) in the package 'MASS' and NaiveBayes classifier in package 'e1017'. Cross validated variable selection was carried out using the function *stepclass* in the package 'klaR'.

3. Results and discussion

3.1. Influence of drying method

Taste and quality of tea could be affected by preparation methods. In order to evaluate the influence of the drying procedure on the chemistry of the leaves the profile of the 30 selected compounds in the leaves of the 21 cultivars harvested in March were compared using two drying methods (oven-dried and freeze-dried). Overall, the abundance of 11 of the 30

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compounds varied significantly between the two drying methods (ANOVA: GCG (V10), chlorogenic acid (V14), 3-O-(E)-coumaroylquinic acid (V17), 5-O-(E)-coumaroylquinic acid (V18) and the three apigenin-*C*-glycosides (V21-V23), $p<0.001$; theobromine (V2), $p<0.01$; GC (V4), rutin (V24) and kaempferol 3-O-rhamnosyl-(1-6)-glucoside (V28), $p<0.05$). The level of GC (V4) and GCG (V10) were significantly higher in oven-dried samples compare to freeze-dried samples. This could be due to the epimerization between 2,3-*cis* configuration (e.g. EGC (V5), EGCG (V8)) and 2,3-*trans* configuration (e.g. GC (V4), GCG(V10)) that takes place during oven-drying process with heat (Chen, Zhu, Tsang, & Huang, 2000; Seto, Nakamura, Nanjo, & Hara, 1997). The abundance of the three apigenin-*C*-glycosides (V21-23) were significantly higher in oven-dried samples than freeze-dried samples. In contrast, the levels of rutin (V24) and kaempferol 3-*O*-rhamnosyl-(1-6)-glucoside (V28) were higher in freeze-dried samples than oven-dried material. In spite of these differences the RPCA biplot of 126 samples from leaf material collected in March shows that the variance due to drying-methods is less than the variance among cultivars (Figure S3 in supplementary material). Therefore, to decrease the impact of processing it was decided to use freeze-dried samples to investigate variations associated with harvest time and cultivar. Using freeze-dried samples would decrease the impact of heat on the compounds. It is known that post-harvest processing can impact the quality and taste of tea. For example, some methods used to process green tea avoid the oxidation of flavanols by rapid inactivation of enzymes either with steam in a rotating cylinder or with dry heat (Spiller, 1998). On the other hand, black tea has a low content of catechins because they are oxidized during the 'fermentation' process, whereas with Oolong tea a modified fermentation method is used resulting in a moderate amount of catechins that falls between levels found in green and fermented teas (Chen, Zhu, Tsang, & Huang, 2000).

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3.2. Abundance of the 30 selected compounds in 21 tea cultivars

An overview of the relative abundance of the 30 selected compounds in the 21 cultivars is presented in Figure 1. In most cases the abundance of the 30 compounds in the 186 freeze-dried samples of tea (21 cultivars, 3 seasons and 3 replicates per treatment, minus the three May BJ samples) differed among the cultivars tested, and was also influenced by harvesting time (ANOVA with post hoc Tukey's HSD, $p<0.05$). The number of compounds that differed significantly between each pair of tea cultivars is summarized in Table S1. There were only two pairs of cultivars Jiukengzao (JK) versus Longjing-changye (LN) and JK versus Maoxie (MO) that did not differ significantly in their chemical profiles. You 510 (YU) was the only cultivar of *C. sinensis var. pubilimba* tested and the results showed that the abundance of compounds in this cultivar are very different from the 19 cultivars of *C. sinensis var. sinensis.* This difference is clearly seen in the PCA biplot, where the YU samples are displaced from the other tea cultivars (Figure 2). In addition, the profile of compounds in Zijuan (ZJ), the only cultivar of *C. sinensis var. kitamura* tested, did differ from most of the cultivars of *C. sinensis var. sinensis,* although not as much as YU. Some cultivars of *C. sinensis var. kitamura* are known to contain anthocyanins (Jiang, Shen, Shoji, Kanda, Zhou & Zhao, 2013), compounds not included in the 30 compounds selected for this study and not usually very abundant in cultivars of *C. sinensis var. sinensis*. It is also worth noting that of the 19 cultivars of *C. sinensis var. sinensis* analysed the cultivar that differed the most in abundance of compounds from the other cultivars was Qiancha 7 (QA). It was characterised by high levels of caffeoylquinic acids (V14-16) and low levels of coumaroylquinic acids (V17-19) and apigenin glycosides (V21-23), see Figure 1. As indicated earlier the cultivar FD is often taken as a standard for tea breeding, and the cultivars of *C. sinensis var. sinensis* that had the greatest difference in the abundance of the 30 compounds compared to this cultivar were MO (Maoxie), Zhenghe-dabaicha (ZE) (both with 14 compounds that differed from FD) and QA

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(13 compounds) (Table S1). In contrast, the chemical profile of Qingxin 1 (QN) was the most similar to FD, although this cultivar had greater amounts of GCG (V10), vitexin (V22), isovitexin (V23) and less ECG (V11) than FD.

Compared to the other cultivars, YU samples contained higher levels of theobromine (V2), the precursor of caffeine (V3), as well as GC (V4), theasinensin B isomers (V6), catechin (V7) and GCG (V10), but relatively low levels of EGC (V5), EGCG (V8) and EC (V9), as seen on the heat-map and PCA biplot (Figure 1 and 4). Polymerized flavan-3-ols, such as theasinensin B isomers (V6), were detected as trace levels in most cultivars but were more abundant in YU samples. An earlier study of an unnamed cultivars of *C. sinensis var. pubilimba* also reported higher levels of catechin (V7) and GCG (V10) than found in cultivars of *C. sinensis var. sinensis* (Jin, Ma, Ma, Yao, & Chen, 2014). It is of interest that overall, the abundance of caffeine (V3) did not vary significantly among the 21 cultivars.

These results illustrate the diversity in the abundance of the 30 compound in the cultivars selected for this study and these difference could contribute to differences in quality, taste, and resistance to pests and disease.

3.3. Seasonal variation associated with times of harvesting

In order to have an overview of the influence of seasonal and genotypic factors on the abundance of the 30 compounds, a robust version of PCA (based on an estimation of covariance matrix) was employed to accommodate the outlying YU population as an 'inherent variability'. The analysis was undertaken on the overall seasonal variation in abundance of the compounds in the 186 freeze-dried samples and there are some variations among the cultivars. The RPCA biplot showed that the profile of compounds in tea samples collected in March differed from those collected in May and September (Figure 3). Compounds that contributed to the separation of March harvest from the other two harvests, included higher levels of

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theanine (V1), theobromine (V2), afzelchin gallate (V12), theogallin (V13) and kaempferol coumaroyl hexoside (V30). The abundance of kaempferol derivatives (V26-V30) were higher in March than in September harvest (Figure 1). Tukey's HSD in conjunction with ANOVA analysis were used to evaluate whether the harvest time significantly influenced the abundance of each compound. As a result of this analysis the compounds were placed into five "groups" (Table 2): Theanine (V1) was the only compound in group A, it had a lower abundance in May compared to March and September (p<0.001) (Figure 1 and Table 2). Theanine (V1) is one of the major nitrogen-containing secondary metabolites in tea leaves. It is mainly synthesised in the root and transported to growing shoots and young leaves (Deng & Ashihara, 2015). Compounds in group B (e.g. theobromine (V2) and caffeine (V3) as well as the kaempferol-glycosides (V26-V30)) were more abundant in March than September. Those in group C (e.g. epicatechin (V9) and catechin (V7)) had a lower abundance in March than September. This group also included EGC (V5) whose abundance was most influenced by the time of harvesting. Those in group D (e.g. the isomers apigenin-8-*C*-glucoside (V22) and apigenin-6-*C*-glucoside (V23)) had a greater abundance in May compared to March and September. Compounds in group E such as EGCG (V8), 5-O-(E)-caffeoylquinic acid (V15), 5-*O*-(E)-coumaroylquinic acid (V18) or rutin (V24) did not differ in abundance among seasons (Table 2), although their abundance differed significantly among the 21 cultivars (p<0.001).

To conclude, the chemometric analysis of 30 compounds in 21 tea cultivars has highlighted that the abundance of many of the compounds does vary with harvesting time. These differences in chemistry between harvesting times will influence the flavour of the tea. In China tea harvested in spring is considered as the premium quality tea (He, 2013). This could be because the leaves collected in spring usually contain more theanine and less polyphenols, hence the spring tea tastes more umami, refreshing and less bitter (Wang, Li,

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Ding, Zhang, & Wang, 2009). The observed seasonal variation of theanine (V1) and catechins (V5–V11) is consistent with this preference (Figure 1 and Table 2). A previous study on seasonal effects on tea grown in Australia demonstrated higher levels of EGC (V5) in the cooler months, and significantly higher levels of EGCG (V8), ECG (V11) were found in warmer months (Yao, et al., 2005). In contrast, overall the levels of EGC (V5) in the tea cultivars from China increased from March to September when temperatures in Fujian increase. This was true for 12 of the 21 cultivars, whereas 9 of the 21 cultivars did not show significant higher levels in September. Thus the trend was for EGC (V5) to be greater in September. The overall trend in the Chinese cultivars showed ECG (V11) was higher in May than that in March and September and levels of EGCG (V8) did not vary between harvests. This illustrates that not all cultivars will respond in the same way. The climatic conditions in the geographical origin of the cultivar might influence some of the observed seasonal changes.

3.4. Identification of marker compounds to differentiate between cultivars and seasons

Despite some of the differences in the chemistry of the freeze-dried and oven-dried samples the data from the 186 freeze-dried samples and 63 oven-dried samples were combined to evaluate whether the diversity and abundance of the 30 compounds could be used to discriminate among the cultivars. These data were included because, although there were significance differences between the drying methods the differences between cultivars was greater and combining the data makes the rest of the LDA's more robust.

A LDA model that included 12 compounds (theobromine (V2) + GC (V4) + ECG (V11) + chlorogenic acid (V14) + 5-*O*-(E)-caffeoylquinic acid (V15) + 3-*O*-(E)-coumaroylquinic acid (V17) + myricetin 3-*O*-hexoside (V20) + iso/vitexin rhamnoside (V21) + rutin (V24) + quercetin-*O*-hexosides (V25) + kaempferol 3-*O*-rhamnosyl-(1-6)-galactoside (V26) +

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kaempferol 3-*O*-rhamnosyl-(1-6)-glucoside (V28)) was able to differentiate among the cultivars with an accuracy of 94%. Although the best model built by Naivebayes was different from the LDA model, chlorogenic acid (V14) was identified as an important marker compound by both methods with a correctness rate of 20% (Naivebayes) and 22% (LDA). An independent LDA biplot was constructed with 30 compounds (Figure S1), which indicated caffeoylquinic acids (V14-16) were characteristic of the three tea cultivars FY, QA and ZJ (Figure 1). Hence the LDA plot supports the selection of chlorogenic acid (V14) and 5-*O*-(E)-caffeoylquinic acid (V15) as part of a chemical model that can be used to differentiate amongst the cultivars. This illustrates the power of chemometric analysis to identify differences in the chemical profile of related cultivars. Chlorogenic acid (V14) is reported to have health benefits and it is also known to modulate the feeding behaviour of insects such that cultivars with higher levels of this compound could be less susceptible to insect herbivory (Chen & Wu, 2014; Hamamura, 1970; Mubarak, et al., 2012). Thus, it would be interesting to evaluate whether the three cultivars FY, QA and ZJ with higher levels of this compounds are more resistant to pests than those cultivars with lower levels such as AJ, LN and JK.

Advanced data-mining methods were also used to identify compounds that could be used to differentiate between the three harvests. This involved a stepwise classification coupled with 10-fold cross-validation procedure on the 186 freeze-dried samples using LDA, QDA and NaiveBayes methods. The results of these three methods indicated that theanine (V1) + EGC (V5) + afzelechin gallate (V12) could be used as markers to predict the harvest seasons with an accuracy of 75%. Of the three compounds, EGC (V5) is the most influenced by harvest time and EGC (V5) alone was able to predict up to 61% correct seasonal classification within this set of data.

3.5. Correlations between abundance of compounds

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Because the profile of compounds in YU differed from the other cultivars, the correlations between the abundance of compounds were analysed twice; once with and once without YU. The results of the Pearson correlation are presented in Figure 4. Overall, the correlation plots undertaken with the YU and without the YU samples were similar; however, when the YU samples were removed the correlation coefficients associated with the catchins (V4 -V10) changed significantly. For instance, GC (V4) became positively correlated with EGC (V5), and theobromine (V2) was no longer correlated with (+)-catechin (V7) and GCG (V10). Nevertheless, the correlations in abundance of some other compounds stayed the same (with or without the YU samples). For example, the abundance of apigenin-*C*-glucosides (V22-V23) negatively correlated to theobromine (V2), (+)-catechin (V7) and ECG (V11), whereas caffeoylquinic acids (V14-V16) positively correlated with two flavonol rutinosides rutin (V24) and kaempferol-3-*O*-rhamnosyl-(1-6)-glucoside (V28). Moreover, as expected, the abundance of these two flavonol rutinosides were also positively correlated.

There were a few positive correlations within groups of isomers, such as caffeoylquinic acids (V14-V16), coumaroylquinic acids (V17-V19) and between apigenin-*C*-glycosides (V22-V23). Overall, significant positive correlations were observed between catechins GC (V4), EGC (V5), (+)-catechin (V7) and EC (V9). These compounds share the same enzymes in their biosynthesis, such as leucoanthocyanidin-4-reductase (LAR), anthocyanidin synthase (ANS) and anthocyanidin reductase (ANR) (Punyasiri, et al., 2004). However, the catechin derivatives (V4, V7 and V10) were much higher in YU samples, whilst their epimers (V5, V8 and V9) showed much lower abundance than other cultivars (Figure 1). These differences could reflect variations in the expression of enzymes between cultivars. This observation suggests that the activity of LAR, that controls production of GC (V4) and catechin (V7) (Punyasiri, et al., 2004), could be higher in YU than the other cultivars. A study of *Camellia sinensis* from Sri Lanka demonstrated that ANR was essential to the dominance of

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epicatechin and its derivatives in tea, and it had seven times higher activity than the combined activity of dihydroflavonol 4-reductase (DFR) and LAR (Punyasiri, et al., 2004). Hence differences in the expression of genes for these enzymes, especially LAR, ANS and ANR in the case of YU cultivar, are likely to be a key to the diversity and abundance of the catechin derivatives in these cultivars.

A negative correlation between GC (V4) and afzelechin gallate (V12), together with a negative correlation between myricetin 3-O-hexoside (V20) and afzelechin gallate (V12) support the biosynthesis of tea flavonoids proposed by Punyasiri et al. (Punyasiri, et al., 2004), and suggested that F3′5′H (flavonoid 3′5′-hydroxylase) might play a key role in regulating the balance between galloyl-type catechins (such as GC, EGC and EGCG) and non-galloyl-type catechin derivatives (including afzelechin derivaties). The expressions of gene CsF3′5′H enables dihydrokaempferol to be hydroxylated at both 3′ and 5′ positions of the B-ring by a flavonoid 3′5′- hydroxylase (F3′5′H) (Rani, Singh, Ahuja, & Kumar, 2012), this leads to the production of flavanol with 3 hydroxy groups on the “B ring” (Figure S2). Correlation based on structure similarity was also found between flavonol-*O*-glycosides (V24-V28). They share flavanone 3β-hydroxylase (FHT) and flavonol synthase (FLS) in the biosynthetic pathway of their aglycones.

4. Conclusions

This study is the first to use chemometric tools to investigate how the abundance of key compounds that contribute to the taste of tea vary among cultivars and how these data can be used to differentiate among cultivars. The fact that some of the relationships between compounds differed among the cultivars all grown in the same environmental conditions indicates that genetic variation is influencing the synthesis and accumulation of phenolics. This justifies further study in which genomic and metabolomics data are combined. The

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influence of harvesting time on the profile of compounds among cultivars is also worth further research as this impacts taste and indicates that climatic conditions can impact the quality of tea and this can vary among cultivars. Many of the 30 compounds studies not only influence the taste of the tea but could also play a role in the resilience of the cultivars to pests and pathogens.

Acknowledgment

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Conflicts of Interest

Two of the authors, Drs Mark Berry and Sally Redfern are employed by Unilever R&D Colworth. However, none of the authors gain financially from the results of this paper and the cultivars studied are not commercialised by Unilever.

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Figure Legends:

Figure 1: Heatmap of data set with 30 compounds for 83 tea samples, from 21 cultivars of *Camellia sinensis* harvested in 3 seasons (MR: March, MY; May, SP: September). One set of March collection was oven dried (MRO) for study of drying-method effect; relative abundance level plot (red=high, blue=low) is produced based on means of triplicate LC-MS tests of each treatment. See Table 1 and 2 for name of cultivars and compounds, respectively.

Figure 2. PCA based on correlation matrix of freeze-dried samples to detect outliers in 21 cultivars of *Camellia sinensis* collected at three times of the year. Codes of the cultivars and compounds (Vxx) in Table 1 and 2, respectively.

Figure 3. Robust principal component analysis (RPCA) using a robust estimate of the covariance matrix to show seasonal impact on production of 30 compounds detected in

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cultivars of *Camellia sinensis*. Codes of the cultivars and compounds are given in Table 1 and 2, respectively.

Figure 4. Correlations between abundance of 30 compounds, which are calculated based on a data set with all cultivars including *C. sinensis var. pubilimba cv. You 510* (YU) samples (upper diagonal) and a data set without YU samples (lower diagonal). Size of circle represent the Pearson product-moment correlation coefficient (black indicate positive correlation and grey indicate negative correlation, empty cell means no significant correlation at $p<0.001$ level). See Table 2 for details of compounds V1 to V30.

Supplementary Materials

Figure S1: Linear discriminant analysis (LDA) for classification of cultivars of *Camellia sinensis* using randomly selected training dataset (75% of 249 observations). The correct prediction achieved by LDA with 30 compounds was 98%. See Table 1 and 2 for name of cultivars and compounds, respectively.

Figure S2. Structures of tea catechins in cultivars of *Camellia sinensis*

Table S1. Pair-wise comparisons between cultivars of *Camellia sinensis* showing number of compounds with significant differences in their abundance (confidence level: 95%, $p<0.05$), this is a cross tabulation based on ANOVA's post-hoc test Tukey's HSD results (data not provided). Table 1 provides information about the cultivars.

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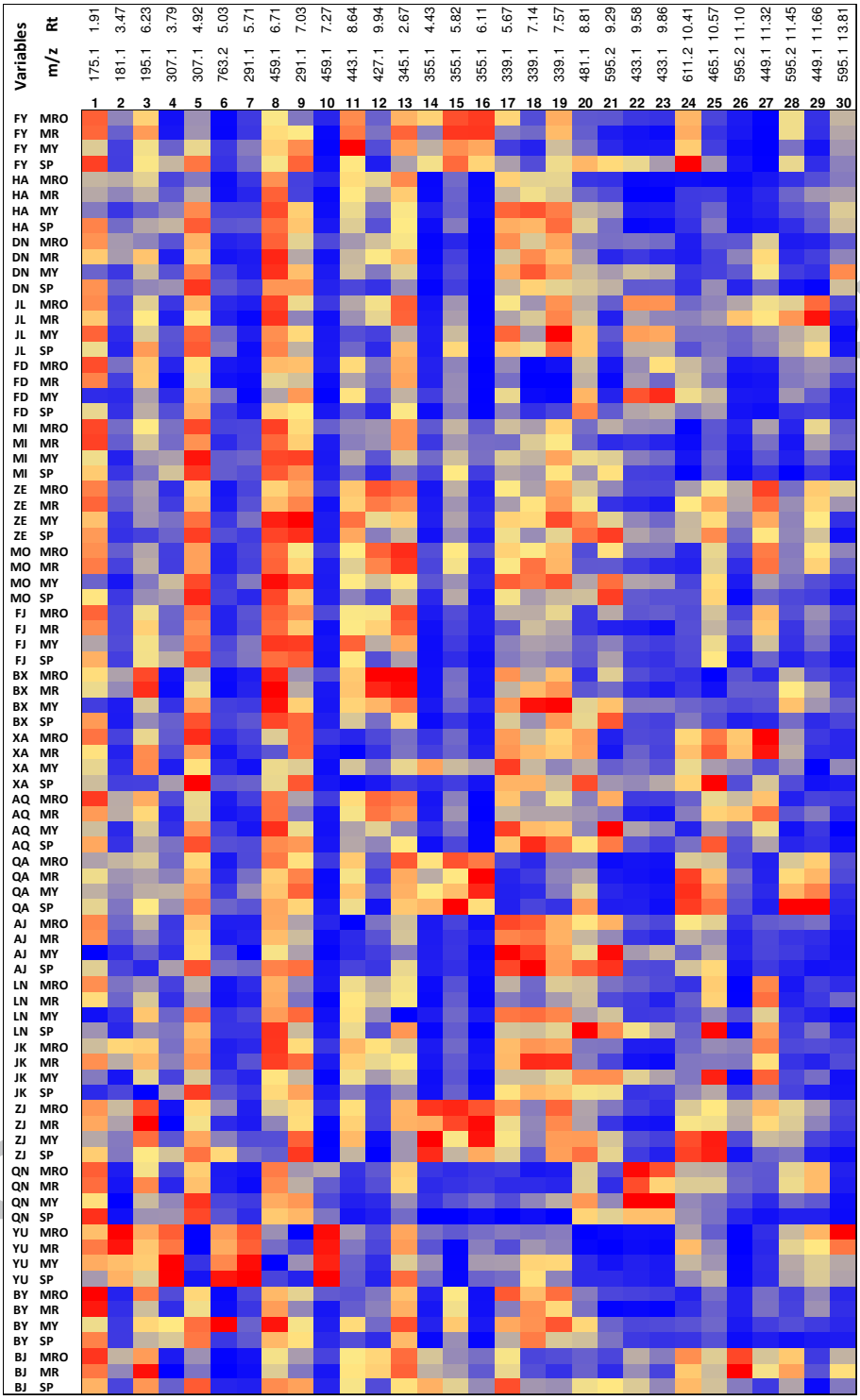


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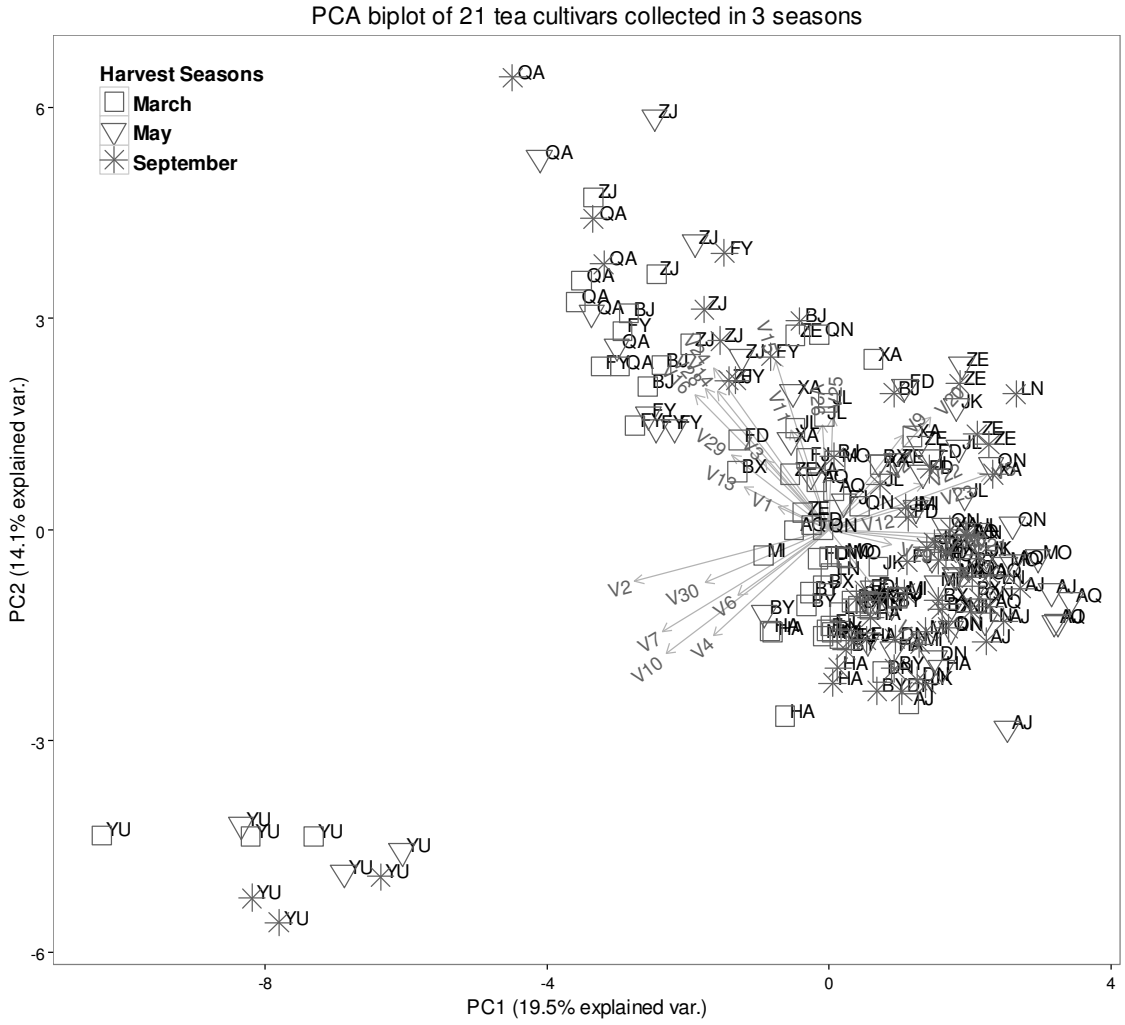


Figure 2. PCA based on correlation matrix of freeze-dried samples to detect outliers in 21 cultivars of *Camellia sinensis* collected at three times of the year. Codes of the cultivars and compounds (Vxx) in Table 1 and 2, respectively.

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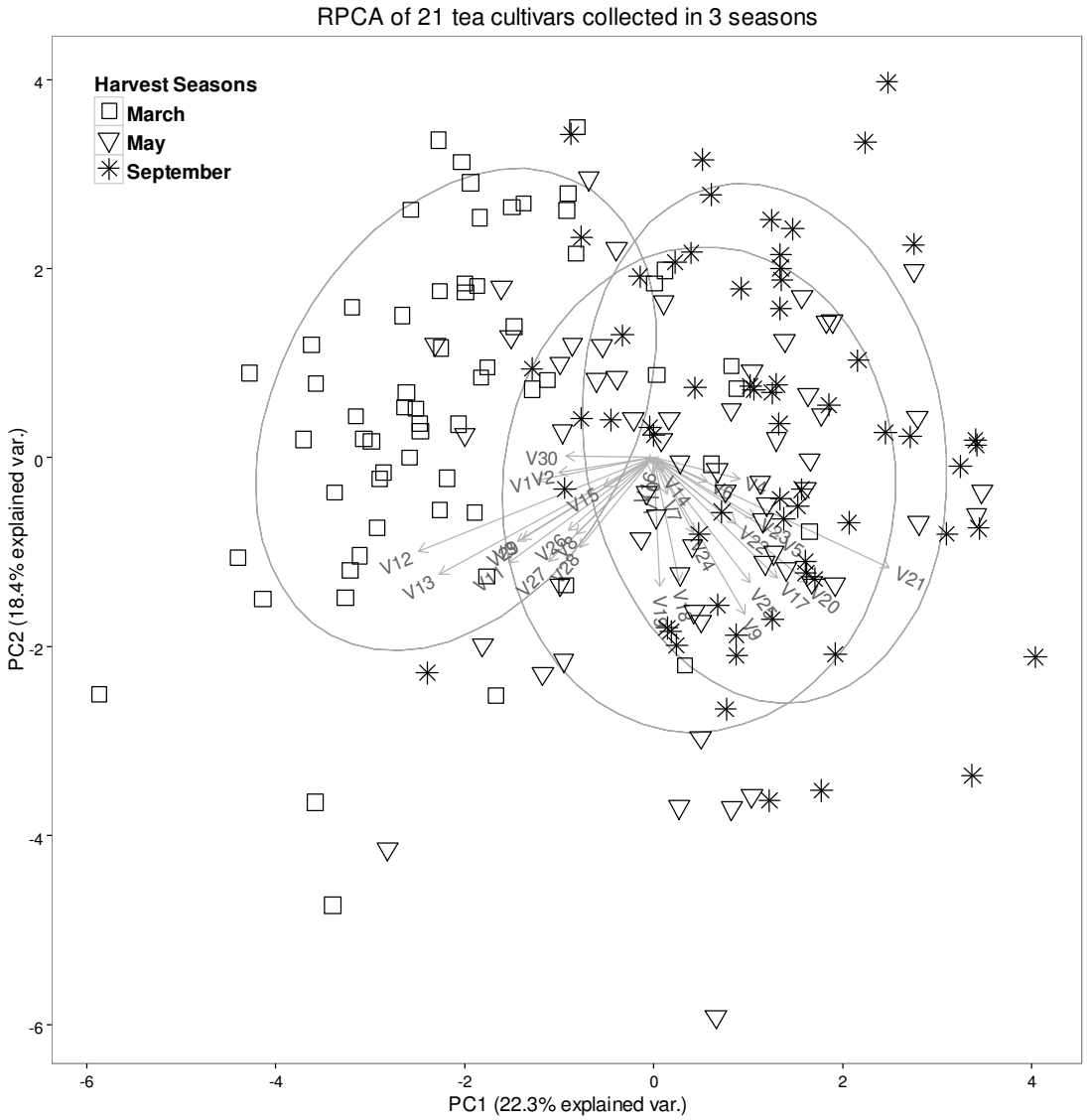


Figure 3. Robust principal component analysis (RPCA) using a robust estimate of the covariance matrix to show seasonal impact on production of 30 compounds detected in cultivars of *Camellia sinensis*. Codes of the cultivars and compounds are given in Table 1 and 2, respectively.

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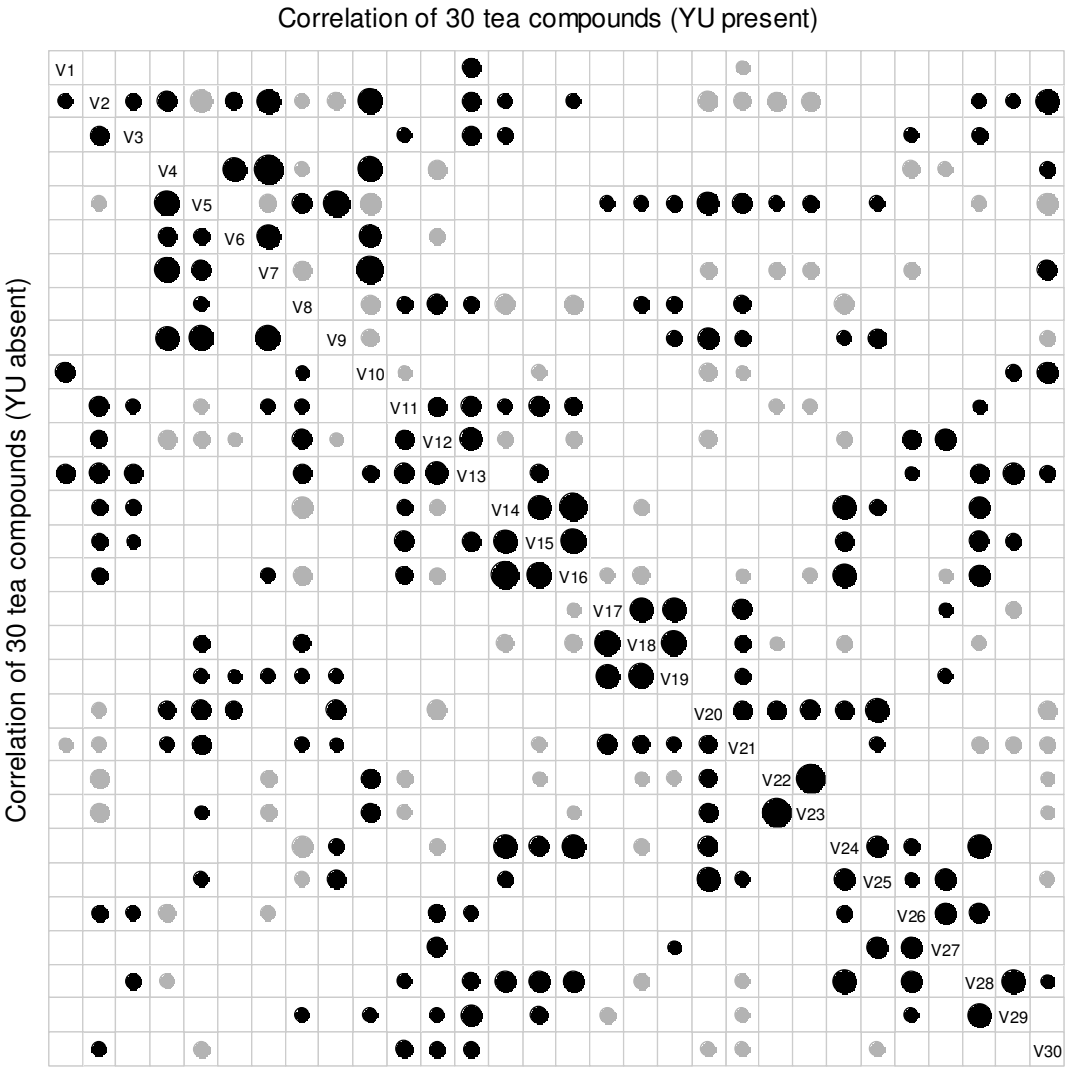


Figure 4. Correlations between abundance of 30 compounds, which are calculated based on a data set with all cultivars including *C. sinensis var. pubilimba cv. You 510* (YU) samples (upper diagonal) and a data set without YU samples (lower diagonal). Size of circle represent the Pearson product-moment correlation coefficient (black indicate positive correlation and grey indicate negative correlation, empty cell means no significant correlation at $p<0.001$ level). See Table 2 for details of compounds V1 to V30.

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Kew Accession Number	Code	Cultivar name	Chinese name	Registered number	Ploidy	Size of leaf*	Origin	Distribution (in China)	Leaf budding time
23766	FY	*C. sinensis var. sinensis cv. Fuyun 7*	福云 7 号	GS13004-1987	2*n*	L	Fujian TRICAAS	South China	Middle March
23769	HA	*C. sinensis var. sinensis cv. Huangguanyin*	黄观音	GS2002015	2*n*	M	Fujian TRICAAS	South China	Late March
23772	DN	*C. sinensis var. sinensis cv. Dangui*	丹桂	GS2010015	2*n*	M	Fujian TRICAAS	South China	Middle March
23775	JL	*C. sinensis var. sinensis cv. Jiulongpao*	九龙袍	闽审茶 2000002	2*n*	M	Fujian TRICAAS	Fujian only	Late March
23778	FD	*C. sinensis var. sinensis cv. Fuding-dabaicha*	福鼎大白茶	GS13001-1985	2*n*	M	Fujian	NA	Early March
23781	MI	*C. sinensis var. sinensis cv. Meizhan*	梅占	GS13004-1985	mixoploid	M	Fujian	South China	Late March
23784	ZE	*C. sinensis var. sinensis cv. Zhenghe-dabaicha*	政和大白茶	GS13005-1985	mixoploid	XL	Fujian	South China	Early April
23787	MO	*C. sinensis var. sinensis cv. Maoxie*	毛蟹	GS13006-1985	mixoploid	M	Fujian	South China	Late March
23790	FJ	*C. sinensis var. sinensis cv. Fujian-shuixian*	福建水仙	GS13009-1985	3*n*	L	Fujian	South China	Late March
23793	BX	*C. sinensis var. sinensis cv. Baxiancha*	八仙茶	GS13012-1994	2*n*	L	Fujian	South China	Early April
23796	BY	*C. sinensis var. sinensis cv. Baiya-qilan*	白芽奇兰	闽审茶 1996001	2*n*	M	Fujian	Fujian, Guangdong	Late March
23832	XA	*C. sinensis var. sinensis cv. Xiapu-yuanxiaocha*	霞浦元宵绿	闽审茶 99003	2*n*	M	Fujian	Fujian, Zhejiang	Early March
23805	AJ	*C. sinensis var. sinensis cv. Baiye 1*	白叶 1 号 (安吉白茶)	浙品认字第 235 号	2n	M	Zhejiang	South China	Early April
23808	LN	*C. sinensis var. sinensis cv.Longjing-changye*	龙井长叶	GS13008-1994	2*n*	M	Zhejiang	Fujian TRICAAS	Late March
23814	ZJ	*C.sinensis var. kitamura cv. Zijuan*	紫娟	国家林业局品种权号：20050031	NA	L	Yunnan	Yunnan only	Late February
23811	JK	*C. sinensis var. sinensis cv. Jiukengzao*	鸠坑早	Not registered	NA	L	Zhejiang	Developing	Late March
23817	QN	*C. sinensis var. sinensis cv. Qingxin 1*	青心 1 号	Not registered	NA	S	Guangdong	Developing	Middle March
23820	BJ	*C. sinensis var. sinensis cv. Baijiguan*	白鸡冠	Not registered	2n	M	Fujian TRICAAS	Developing	Early April
23823	YU	*C. sinensis var. pubilimba cv. You 510*	优 510	Not registered	NA	M	Fujian TRICAAS	Developing	Middle March
23799	AQ	*C. sinensis var. sinensis cv. Anqing 8902*	安庆 8902	Not registered	NA	NA	Anhui	Developing	NA
23802	QA	*C. sinensis var. sinensis cv. Qiancha 7*	黔茶 7 号	Not registered	NA	M	Guizhou	Developing	Middle March

Table 1. Information about 21 cultivars of *Camellia sinensis* from China. Background information acquired from TRICAAS and other publications.[4,21,22] *Range of leaf size: S<20 cm^2, M=20–39 cm^2, L=40–60 cm^2, XL>60 cm^2 .NA = no data available; Developing = new breed that is only available as few bushes at the location where it is originally found or created.

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Code	Assignment	Rt	Expt m/z	Formula	Identification	Group*
V1	theanine	1.91	175.108	$C_7 H_{15} O_3 N_2$	tentative	A
V2	theobromine	3.47	181.072	$C_7 H_9 O_2 N_4$	against standard	B
V3	caffeine	6.23	195.087	$C_8 H_{11} O_2 N_4$	against standard	B
V4	gallocatechin (GC)	3.79	307.081	$C_{15} H_{15} O_7$	tentative	C
V5	(-)-epigallocatechin (EGC)	4.92	307.081	$C_{15} H_{15} O_7$	against standard	C
V6	theasinensin B (three isomers)	5.03	763.150	$C_{37} H_{30} O_{18}$	tentative	C
V7	(+)-catechin	5.71	291.086	$C_{15} H_{15} O_6$	against standard	C
V8	(-)-epigallocatechin gallate (EGCG)	6.71	459.092	$C_{22} H_{19} O_{11}$	against standard	E
V9	(-)-epicatechin (EC)	7.03	291.086	$C_{15} H_{15} O_6$	against standard	C
V10	gallocatechin gallate (GCG)	7.27	459.092	$C_{22} H_{19} O_{11}$	tentative	C
V11	(-)-epicatechin gallate (ECG)	8.64	443.097	$C_{22} H_{19} O_{10}$	against standard	D
V12	afzelechin gallate	9.94	427.102	$C_{22} H_{19} O_9$	tentative	B
V13	theogallin	2.67	345.081	$C_{14} H_{17} O_{10}$	tentative	B
V14	chlorogenic acid (3-*O*-(E)-caffeoylquinic acid)	4.43	355.102	$C_{16} H_{19} O_9$	against standard	D
V15	5-*O*-(E)-caffeoylquinic acid	5.82	355.102	$C_{16} H_{19} O_9$	against standard	E
V16	4-*O*-(E)-caffeoylquinic acid	6.11	355.102	$C_{16} H_{19} O_9$	against standard	B
V17	3-*O*-(E)-coumaroylquinic acid	5.67	339.107	$C_{16} H_{19} O_8$	against standard	D
V18	5-*O*-(E)-coumaroylquinic acid	7.14	339.107	$C_{16} H_{19} O_8$	against standard	E
V19	4-*O*-(E)-coumaroylquinic acid	7.57	339.107	$C_{16} H_{19} O_8$	against standard	D
V20	myricetin 3-*O*-hexoside	8.81	481.097	$C_{21} H_{21} O_{13}$	tentative	C
V21	iso/vitexin rhamnoside	9.29	595.165	$C_{27} H_{31} O_{15}$	tentative	C
V22	vitexin (apigenin-8-C-glucoside)	9.58	433.113	$C_{21} H_{21} O_{10}$	against standard	D
V23	isovitexin (apigenin-6-C-glucoside)	9.86	433.113	$C_{21} H_{21} O_{10}$	against standard	D
V24	rutin (quercetin 3-*O*-rhamnosyl-(1-6)-glucoside)	10.41	611.160	$C_{27} H_{31} O_{16}$	against standard	E
V25	quercetin-*O*-hexosides (two isomers)	10.57	465.102	$C_{21} H_{21} O_{12}$	tentative	C
V26	kaempferol 3-*O*-rhamnosyl-(1-6)-galactoside	11.10	595.165	$C_{27} H_{31} O_{15}$	against standard	B
V27	kaempferol 3-*O*-galactoside	11.32	449.107	$C_{21} H_{21} O_{11}$	tentative	B
V28	kaempferol 3-*O*-rhamnosyl-(1-6)-glucoside	11.45	595.165	$C_{27} H_{31} O_{15}$	against standard	B
V29	kaempferol 3-*O*-glucoside	11.66	449.107	$C_{21} H_{21} O_{11}$	tentative	B
V30	Kaempferol coumaroyl hexoside	13.81	595.144	$C_{30} H_{26} O_{13}$	tentative	B

Table 2. Summary of 30 compounds detected in cultivars of *Camellia sinensis*. * Abundance of compounds sometimes varied among the three harvesting times. Variation in abundance for each of the 30 compounds could be allocated to five groups based on the significant difference in abundance between harvests (ANOVA and Tukey's HSD results; data not provided). Group of compounds A: March>May<Sept. (i.e. no difference between March and Sept.); B: March>Sept.; C: March<Sept.; D: March<May>Sept.; E: March=May=September.

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Highlights

- Chemometrics showed that 12 compounds could differentiate among 21 tea cultivars
- Harvest time of tea influenced the abundance of many of the 30 compounds analysed

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Variation of theanine, phenolic, and methylxanthine compounds in 21 cultivars of Camellia sinensis harvested in different seasons

Fang, Rui; Redfern, Sally P.; Kirkup, Don; Porter, Elaine A.; Kite, Geoffrey C. ...

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