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An Emerging Strategy for Grading Chun Mee Green Tea by Combination of Metabolites, Sensory Properties, and Bioactivities

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ABSTRACT: Chun Mee green tea (CMGT), one of the major export teas from China, has a comprehensive classification (grading) system. However, the chemical constituents underlying grade differentiation, as well as their associations with astringency and bioactivity, remain unclear. In this study, we integrated quantitative descriptive analysis (QDA), tea-mucin complex analysis, *in vitro* bioactivity evaluations, and metabolomics, supported by Random Forest (RF) modeling and correlation analysis. QDA demonstrated that higher-grade samples exhibited superior overall sensory quality and stronger astringency. Tea-mucin complex analysis (turbidity, particle size, SEM) confirmed reduced aggregation in lower grades, consistent with weaker astringency. Quantitative analysis revealed a significant decrease in major flavor compounds (EGCG, caffeine, and amino acids) as the grade declined. Liquid chromatography-mass spectrometry (LC-MS)-based metabolomics further classified samples into high-grade (T1, T2, T3) and low-grade (T4, T5, T6, 1, 2) clusters, identifying 68 grade-differentiating markers. High-grade teas exhibited stronger antioxidant activity and inhibition effects on α -amylase and α -glucosidase. RF and correlation analysis revealed that catechins, phenolic acids, hydrolysable tannins, and acylated quercetin glycosides were critical for grading, with *trans-p*-coumaroylquinic acid and its derivatives contributing significantly to both astringency and antioxidant capacity. In addition, a grade estimation model based on four compounds (5-galloylquinic acid, EGCG, ECG, GCG) was constructed using elastic net and ridge regression, achieving high accuracy ($R^2 = 0.995$, RMSE = 0.168). Commercial samples verification confirmed the model's reliability for ranking CMGT grades from the same factory. These findings provide an objective approach for grade evaluation and may facilitate the refinement of CMGT grading standards.

Keywords: Chun Mee green tea; grade; metabolomics; astringency; mucin; antioxidant; machine learning

1. Introduction

Green tea is one of the most popular beverages in the world, deeply ingrained in long-term dietary habits and cultural traditions^[1]. Chun Mee green tea (CMGT) is one of the main green teas exported from China, primarily to Africa, Central Asia, and Europe, and is widely favored for its heavy and thick taste^[2].

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Unlike other green teas, CMGT processing adds refining processes (such as cutting and blending) after the general green tea processing (fixation, rolling, drying). Currently, the grading of CMGT is usually decided by the sensory evaluation of infusion characteristics (aroma, taste, color) and the tenderness of fresh tea leaves used for producing CMGT. However, sensory evaluation has inherent drawbacks such as strong subjectivity and unstable results. This may lead to instability in tea quality, thereby affecting the economic benefits of exports, as the grade of tea directly affects its economic value^[3]. In order to mitigate the limitations of sensory evaluation, researchers have attempted to develop methods for objectively estimating tea quality grades based on their chemical or physical properties^[4]. In recent years, technologies such as near-infrared (NIR) have been attempted for CMGT grading, but it has not been possible to identify the key chemical components that affect tea quality^[5-6]. Hence, developing an objective grading method based on chemical components is an urgent requirement for CMGT quality control.

Taste evaluation by tea specialists is one of the most important attributes regarding the grade classification of tea products. Tea's taste is mainly derived from the combined effect of its complex non-volatile composition. Usually, catechins, caffeine, phenolic acids, and flavonol glycosides are the main astringent and bitter compounds in green tea infusion^[7], while *L*-theanine is an important umami factor for the fresh taste of green tea^[8]. However, there is still a scarcity of research on the key taste compounds of CMGT, especially the key astringent compounds. Quantitative descriptive analysis (QDA) is an effective method for foodstuff sensory evaluation, enabling the analysis, description, and quantification of flavor characteristics across different grades of CMGTs^[9]. Astringency is a core factor in green tea quality. It was suggested that the formation of astringency was related to physical, physiological, and psychological factors, with salivary protein aggregation being one of the most important astringency formation mechanisms^[10-11]. In a previous study, a correlation between the turbidity of mucin-polyphenol and astringent intensity was built and applied in the screening of potential astringent compounds of black tea^[12]. Therefore, characterization of tea-mucin complexes may provide a new strategy for studying the differences in astringency across different grades of CMGTs. In addition, certain chemical compounds such as catechins and flavonol glycosides in green tea have been reported to exhibit particular health effects, including inhibition of α -glucosidase and α -amylase, decrease of lipid accumulation, and antioxidant^[13-15]. Studies have confirmed that different grades of green tea, black tea, and oolong tea have different hypoglycemic effects and antioxidant capacity^[16-18]. However, it is unclear whether the different grades affect the biological activity of CMGT.

With the development of spectroscopic techniques, high-throughput qualitative and quantitative analysis of metabolites has been developed by using liquid chromatography-mass spectrometry (LC-MS)^[19]. Currently, LC-MS-based untargeted metabolomics has been used to distinguish grades and explore grade-related critical compounds of green tea^[17,20]. Analytical techniques incorporating multivariate statistical analysis and machine learning can provide robust data processing and reporting capabilities^[21].

The aim of this study was to establish a CMGT grading framework based on chemical components, combining quality and bioactivity analysis. First, integrated QDA, tea-mucin complexes characterization, LC-MS untargeted metabolomics, and *in vitro* activity testing to analyze and compare the quality characteristics, chemical profiles, and bioactivity of CMGTs of different grades. Second, Random Forest (RF) combined with Pearson correlation analysis was used to analyze the relationship between grade marker compounds and astringency and antioxidant activity. Finally, machine learning was used to construct a regression equation for grade estimation, and practical utility assessment was conducted using commercial samples.

2. Materials and methods

2.1 Samples and chemicals

Eight different grades of Chun Mee green tea (*Camellia sinensis* var. *sinensis*) produced in 2023 were purchased from Ningqing Tea Factory in Xuancheng City, Anhui Province, China. From high to low, there were six Special Chun Mee green teas (T1, T2, T3, T4, T5, and T6 grades) and two ordinary Chun Mee green teas (1 and 2 grades). Detailed information of these tea samples is listed in Table S1.

Standards (purity > 98%) of gallic acid (GA), caffeine (CAF), theobromine (THB), (+)-catechin (C), (–)-epicatechin (EC), (–)-gallocatechin (GC), (–)-epigallocatechin (EGC), (–)-gallocatechin gallate (GCG), (–)-epigallocatechin gallate (EGCG), (–)-epicatechin gallate (ECG), mucin (Bovine submaxillary gland), 4-nitrophenyl- β -D-glucopyranoside (pNPG), 3,5-Dinitrosalicylic acid (DNS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), total antioxidant capacity (T-AOC) detection kit (ABTS microplate method and FRAP microplate method), vitamin C (Vc), α -amylase and α -glucosidase were purchased from Shanghai Yuanye Biotechnology Company (Shanghai, China). Standards of *L*-theanine (Thea), *L*-serine (Ser), *L*-glutamic acid (Glu), *L*-phenylalanine (Phe), *L*-threonine (Thr), *L*-aspartic acid (Asp), *L*-tyrosine (Tyr), *L*-tryptophan (Trp), γ -aminobutyric acid (GABA), *L*-lysine (Lys), *L*-valine (Val), *L*-asparagine (Asn), and *L*-glutamine (Gln) were acquired from Merck Chemical Technology Co., Ltd. (Shanghai, China). Methanol (HPLC-grade), formic acid (MS-grade), and acetonitrile (MS-grade) were purchased from TEDIA Company Inc. (Fairfield, OH, USA). Other reagents were of analytical grade.

2.2 Quantitative descriptive analysis of tea quality

Before the sensory evaluation, ten panelists (3 males and 7 females, aged from 22 to 33 years old, with normal taste sense and no smoking habits) were trained for eight weeks, three times per week. The training included the basics of sensory analysis, discrimination of sensory attributes, and judgment of the intensity of taste attributes.

The formal evaluations of CMGT samples followed the Chinese sensory evaluation protocol for tea (GB/T 23776 – 2018). Eight CMGT samples were labeled with random three-digit codes. The trained panel evaluated quality attributes (appearance, aroma, taste, tea infusion, and brewed leaves) using a 100-point scale. The total scores were calculated with weighted coefficients: appearance (25%), aroma (25%), taste

(30%), tea infusion (10%), and brewed leaves (10%). Subsequently, the panel comparatively evaluated the intensities of astringency, bitterness, sweetness, and sourness of tea infusions. For each evaluation of taste, the panelists sucked in 20 mL of the tea infusion into their mouths and swirled it around to ensure that the tea infusion reached all parts of their tongues. After completing the evaluation of one sample, the mouth was rinsed with water and rested for 3 minutes to restore taste perception before proceeding to the evaluation of the next sample to ensure the accuracy of the evaluation. The panelists first ranked the samples according to the intensities of the taste attributes in each group and then scored them regarding the corresponding taste intensities of the food-grade standards, up to a maximum score of 10 points. The concentrations and corresponding scores of four standard taste solutions are listed in Table S2. All panelists refrained from communication during evaluations.

No human ethics committee is available in our institute, and the ethical approval of sensory evaluation is not required by national laws. The authors ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The appropriate protocols were used to protect the rights and privacy of all participants during the execution of the study, such as no coercion to participate, full disclosure of the study requirements and risks, written or verbal consent of participants, no release of participant data without their knowledge, and the ability to withdraw from the study at any time. Furthermore, the samples and standard solutions used in sensory training and evaluation were food-grade to ensure safety. Informed consent was obtained from all participants before sensory evaluation.

2.3 Characterization of tea-mucin complexes based on eight grades of CMGTs

The samples were prepared by aqueous extraction. A 0.10 g aliquot of each sample was weighed into a 5 mL centrifuge tube before adding 3 mL of distilled water. Each sample was extracted twice at 90 °C for 10 min and centrifuged for 10 min at 8000 rpm. The supernatants were collected and diluted with distilled water to 10 mL.

2.3.1 Turbidity analysis

The turbidity values were evaluated using a Hach TU5200 turbidimeter (Hach Lange, Switzerland) according to our published method^[12] with some modifications. Before testing, the turbidimeter was calibrated using three standard solutions (10, 20, and 600 nephelometric turbidity units (NTU)). Then, 2 mL of the sample solution (10 mg/mL) and 3 mL of the mucin solution (10 mg/mL, in distilled water) were mixed in a 15 mL centrifuge tube. The system was eventually diluted with water to 10 mL and then incubated at 37 °C for 20 min before being transferred to a sample test vial. The turbidity values of the sample solution, mucin solution, and the mixtures were recorded as T_S , T_M , and T_{test} , respectively. The turbidity (T) value of the sample-mucin adduct was calculated as follows:

$$T = T_{test} - (T_S + T_M)$$

2.3.2 Particle size and scanning electron microscopy (SEM) analysis

To further validate the results of turbidity, the microstructure and particle size of the tea-mucin adduct were examined using SEM (Hitachi SU8600, Japan) and a nanometer particle size analyzer (Beckman Coulter Inc., Brea, CA, USA). With the preparation of mixed samples, in brief, 1 mL sample solution (10 mg/mL) and 1 mL mucin solution (10 mg/mL, in distilled water) were combined in a 5 mL centrifuge tube and thoroughly incubated at 37 °C for 2 hours to facilitate the reaction. Before particle size analysis, the mixed samples were diluted (1 – 100) with distilled water to avoid multiple scattering effects. The method for particle size measurement was similar to a previous study^[22]. For microstructural observations, the prepared mixed samples were centrifuged at 8000 rpm for 10 minutes to allow the formed aggregates to settle. The sediment was then washed twice with distilled water to eliminate residual tea extract. The resulting precipitate samples were freeze-dried and observed by SEM.

2.4 Quantitative analysis of eight grades of CMGTs

2.4.1 Content determination of catechins, gallic acid, and purine alkaloids

To determine the contents of catechins (EGCG, EGC, ECG, GCG, EC, GC, and C), GA, and purine alkaloids (CAF and THB) in the samples, an ultra-high performance liquid chromatography (UHPLC, Thermo Scientific Dionex UltiMate 3000, USA) consisting of a binary pump, an integrated vacuum degasser, an autosampler, a thermostatic column chamber, and a diode array detector (DAD) was used for detection. The samples were prepared according to the method published by Zhou et al.^[23] in 2023. Briefly, 0.10 g of each tea powder was extracted with 70% methanol (v/v) at 25 °C to a final volume of 10 mL using an ultrasound cleaner (JK-5200B, Kinnick Machinery Manufacturing Co., Ltd., Hefei, Anhui, China). The extraction procedure was performed two more times to obtain each sample in three replicates. Before analysis, each extract was filtered through a 0.22 µm membrane. Chromatographic separation of CMGT extracts was conducted using an Acquity UPLC[®] BEH Shield RP₁₈ column (2.1 × 50 mm, 1.7 µm, Waters, USA) at a flow rate of 0.25 mL/min. The column oven temperature was set at 40 °C and the injected volume was 1 µL. The relevant analytical procedure was carried out strictly in line with Wen et al.^[24] in 2023.

2.4.2 Content determination of free amino acids

The quantitation of free amino acids in CMGT samples was conducted using an external standard method. Samples were prepared in triplicate using the aqueous extraction method (detailed in Section 2.3), then diluted tenfold and filtered through a 0.22 µm membrane. A triple quadrupole linear ion trap mass spectrometer (API 5500 QTRAP[™] MS system from AB Sciex, Concord, ON, Canada) was used to analyze thirteen amino acids in samples under multiple reaction monitoring (MRM) mode. The mass spectrometry conditions were set as follows: turbo spray temperature at 500 °C, ion spray voltage at 5500 V, nebulizer gas (GS 1) at 50 psi, heater gas (GS 2) at 50 psi, and curtain gas (CUR) at 30 psi. For the separation, a universal column (250 × 4.6 mm, 2.7 µm, Comixshell CARP, Phenomenex, Torrance, CA, USA) was employed. The flow rate was 1 mL/min, the column oven temperature was maintained at 35 °C, and the injection volume was 5 µL. The mobile phases consisted of ultrapure water with 0.02% formic acid (v/v, mobile phase A) and

acetonitrile with 0.2% formic acid (v/v, mobile phase B). Other chromatographic and mass parameters were applied according to the optimized method reported by Wang et al.^[25].

2.5 LC-Q-TOF-MS-based untargeted metabolomics analysis

An Agilent 1290 liquid chromatography system coupled to a quadrupole time-of-flight mass spectrometer (LC-Q-TOF-MS) (Agilent Technologies, Palo Alto, CA, USA) was used in the analysis of samples. The extraction method and chromatography conditions were the same as those described in Section 2.4.1. The gas temperature was set as 350 °C, with the gas flow set as 8 L/min, the nebulizer set as 35 psi, and the sheath gas flow set as 11 L/min. Metabolites were detected in negative ion mode by full scan mass analysis in the range of m/z 100 – 1700. The injection volume was set to 2 μ L. Each sample was analyzed three times, and a quality control (QC) sample was also prepared.

Mass spectral data were converted by Abf conversion software and then processed using MS-DIAL software (version 4.9) following our previous method^[26]. The analysis was then performed by unsupervised principal component analysis (PCA) and hierarchical cluster analysis (HCA) by Simca-P software (version 14.1, Umetrics, Umea, Sweden). Subsequently, the samples were further analyzed statistically by partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) according to PCA and HCA results. In the OPLS-DA analysis of high-grade (T1, T2, T3) and low-grade (T4, T5, T6, 1, 2) CMGT samples, the integration of Variable Importance in Projection (VIP) and loading S -plot results was used to search for key marker compounds associated with grade classification. Finally, the heatmap analysis was illustrated using TBtools software (version 2.0, Bioinformatics Lab, South China Agricultural University, China). In the heatmap analysis, columns and rows represented samples and compounds, respectively, while color represented the metabolite's peak area after Z-score normalization.

2.6 Determination of antioxidant activity and anti-hyperglycemic effects in vitro

2.6.1 Antioxidant activity assays

As described by Yang et al.^[27], the antioxidant capacity of different grades of samples was assessed using the DPPH, ABTS, and FRAP methods. Samples were prepared in triplicate using the aqueous extraction method (detailed in Section 2.3) and then diluted to five gradient concentrations (0.25, 1, 5, 10, and 25 μ g/mL for the DPPH assay; 0.5, 1, 1.5, 2, and 3 mg/mL for the ABTS assay; 0.05, 0.1, 0.2, 0.35, and 0.5 mg/mL for the FRAP assay). A positive control sample was prepared by diluting VC to the same concentration as the tea samples.

For the DPPH assay, 100 μ L of the sample or VC was mixed thoroughly with 100 μ L of the DPPH working solution (0.0178 mM) in a 96-well plate. After 30 min, the absorbance was detected at 517 nm using a Molecular Devices Enzyme Labeling Analyzer (Molecular Devices Co., Ltd.). For the control and blank groups, equi-voluminal anhydrous ethanol was utilized in place of the DPPH working solution and sample solution, respectively. For the ABTS assay, 3.5 μ L of sample solution or VC was mixed with 140 μ L of ABTS working solution. After 6 min, the absorbance was detected at 734 nm. The DPPH and ABTS free radical scavenging rates were calculated referring to the published article^[28].

The FRAP assay was performed according to the guidelines of the determination kit and the published method^[27]. A mixture of 88 μL of FRAP working solution and 10 μL of the tea samples was incubated at 37 °C for 30 minutes, and the absorbance value was measured at 593 nm. The ferric reduction capacity of these samples was calculated by an analytical curve constructed by ferrous standard solution ($y = 0.1474x + 0.0491$, $R^2 = 0.9979$). The antioxidant capacity differences of eight grades of CMGTs were represented by the IC_{50} values, which were calculated using GraphPad Prism software (version 9.3.1).

2.6.2 α -Amylase and α -glucosidase inhibition assays

Samples were prepared in triplicate using the aqueous extraction method mentioned in Section 2.3 with some modifications. Briefly, 0.30 g of each tea powder was extracted to a final volume of 10 mL (making the final solid-liquid ratio 30 mg/mL). Samples were subsequently diluted to different concentrations (10, 20, and 30 mg/mL for α -amylase; 100, 200, 300, and 400 $\mu\text{g/mL}$ for α -glucosidase). The α -amylase and α -glucosidase inhibition rate was detected by a SpectraMax M2 microplate reader (Molecular Devices, San Jose, CA, USA).

The α -amylase inhibitory activity was assayed as follows. First, 300 μL of sample solution was mixed with 300 μL of α -amylase solution (1 U/mg, pH 6.8) and incubated at 37 °C for 5 min. Then, 300 μL of 1% (m/v) soluble starch solution was added, and the mixture was further incubated at 37 °C for 25 min. After adding 500 μL of DNS solution, the reaction system was heated at 100 °C in a water bath for 5 min. Subsequently, the mixture was diluted with water to a final volume of 5 mL, and the absorbance was measured at 520 nm. The α -glucosidase inhibitory activity was determined using a similar protocol. Briefly, 50 μL of sample solution was incubated with 100 μL of α -glucosidase solution (1 U/mg, pH 6.8) at 37 °C for 10 min. Then, 50 μL of $p\text{NPG}$ solution was added and mixed thoroughly. The absorbance of the resulting solution was measured at 405 nm. The inhibition ratios for both enzymes were calculated according to our previous report^[29].

2.7 Modeling method based on elastic net and ridge regression

The grade estimation model was constructed using R software (version 4.4.1) with the caret (v7.0.1) and glmnet (v4.1.9) packages^[30]. Elastic net methodology was applied with 100 bootstrap samples to select variables. For each bootstrap iteration, the optimal α (mixing parameter, tested from 0.1 to 1.0) and λ (penalty parameter) values were determined via cross-validation. Compound selection stability was assessed by calculating selection frequency across all bootstrap samples. Key compounds were identified based on selection frequency ($> 70\%$ threshold). Subsequently, a ridge regression model was built using these selected compounds. The optimal λ value was determined by leave-one-out cross-validation (LOOCV). Analytical reproducibility was ensured by setting a random seed.

2.8 Statistical analysis

Results were expressed as mean $\pm$ standard deviation (SD). Quantitative data were further analyzed using a one-way analysis of variance (ANOVA) followed by the Duncan post hoc test, and $P < 0.05$ was considered statistically significant using SPSS software (IBM, Armonk, NY, USA).

RF regression and Pearson correlation analyses were performed in R using the rfPermute (v2.5.5) and psych (v2.5.6) packages. The RF models (1000 trees) quantified variable importance using %IncMSE (Percentage Increase in Mean Squared Error), tested with 1000 permutation trials. Pearson correlation analysis was performed to investigate the correlations between the compounds and these response metrics. The above analysis employed a fixed random seed for reproducibility.

3. Results and discussion

3.1 Quantitative descriptive analysis of different grades of CMGTs

To minimize the impact of processing variations, eight different grades of CMGTs from the same tea factory and batch were collected. Their quality differences were assessed by a trained panel evaluating appearance, aroma, taste, tea infusion, and brewed leaves. Figure 1A showed noticeable differences in the color of dried tea leaves, infused leaves, and infusions across grades. The dried tea leaves exhibited a chromatic gradient from dark green (highest grade) to dry yellow (lowest grade), while the infused leaves transitioned from yellowish green (highest grade) to dull green (lowest grade). Correspondingly, infusions for grades T1 – T4 were light yellow, contrasting with the dark yellow of grades T5, T6, 1, and 2.

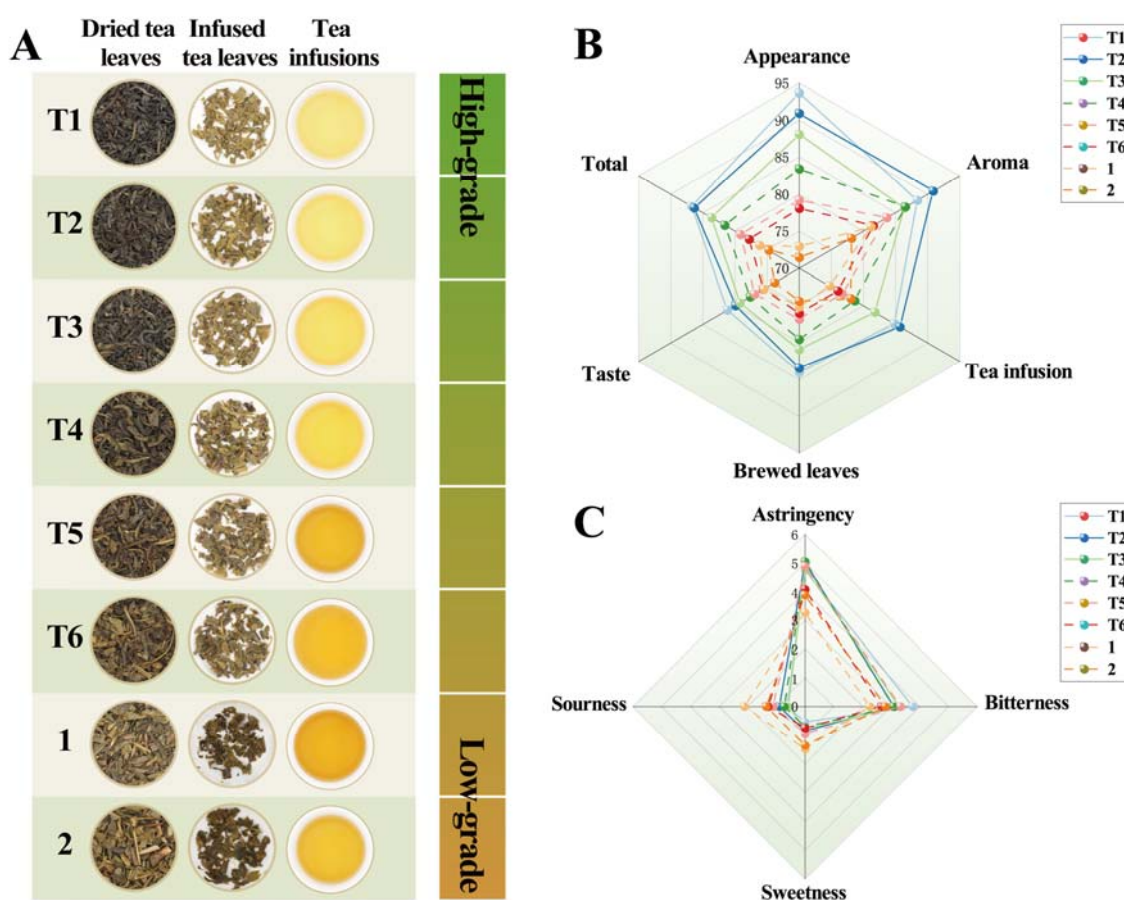


Figure 1 The quality parameters of different grades of CMGT samples. (A) Photos of dried tea, infused leaves, and tea infusions; (B) The scores on the appearance, taste, aroma, brewed leaves, and tea infusion according to sensory analysis; (C) The intensity of bitterness, astringency, sourness and sweetness according to sensory analysis.

Sensory scores showed that scores for appearance and internal quality decreased with lower grades (Figure 1B). Appearance differences were most pronounced: higher scores (88.1 – 93.7) for T1 – T3 grades

were due to their even and smooth appearance, while lower scores (71.5 – 83.4) for T4 – T6, 1, and 2 grades were attributed to visible stems and flakes. T1 – T3 grades also outperformed in aroma (86.3 – 90.8) and tea infusion (81.8 – 84.8), characterized by tender aroma and clear infusion. The softness of the brewed leaves gradually decreased from the highest grade (score of 84.0) to the lowest (score of 74.4), indicating a reduction in the tenderness of the fresh tea leaves used. Taste scores, evaluated based on mellowness and thickness per the national standard, also declined from the highest grade (81.5) to the lowest (73.8), indicating a reduction in these key taste attributes.

Taste is a critical sensory attribute during tea drinking. This study further evaluated the intensity of bitterness, astringency, sourness, and sweetness in the samples through comparative tasting analysis (Figure 1C). The results showed that CMGT presented strong astringency and bitterness, while sweetness and sourness were relatively weak. Astringency scores were closer for T1 – T5 grades (4.74 – 5.04) but significantly higher than T6, 1, and 2 grades (3.25 – 4.07). Bitterness scores showed no significant difference ($P > 0.05$). These results highlight that astringency is a more critical factor than bitterness in distinguishing the flavor quality of different grade CMGTs.

3.2 Analysis of tea-mucin complexes based on different grades of CMGTs

Astringency is a critical sensory attribute that significantly influences consumer acceptance and the perceived quality of tea. Understanding the mechanism of astringency is essential to decoding the overall flavor perception of tea, particularly in strong-flavored varieties such as CMGTs. The mechanism of astringency was thought to be related to lubrication failure, resulting in friction, dryness, and puckering^[31]. One of the existing models to explain oral astringency suggests that astringent agents can interact with human saliva, thereby inducing aggregate formation with the salivary proteins^[32]. Given the higher perceived astringency of higher-grade CMGTs, we characterized their interaction with mucin, a key salivary protein, through turbidity, particle size distribution, and high-resolution SEM.

3.2.1 Turbidity and particle size analysis

As shown in Figure 2B, the turbidity of tea infusions alone was low (6.38 – 8.68 NTU), while that of mucin was 35.12 NTU. After mixing, the turbidity values increased to more than double the original values, which indicated that protein precipitation took place. Critically, the turbidity of tea-mucin mixtures decreased gradually with decreasing tea grades, with grade T1 being the highest (113.80 NTU) and grade 2 being the lowest (91.46 NTU). This suggested that higher-grade teas contain more compounds capable of precipitating mucin. This finding aligns with previous studies^[12], which have linked increased turbidity to higher concentrations of specific flavan-3-ols, such as EGCG and ECG.

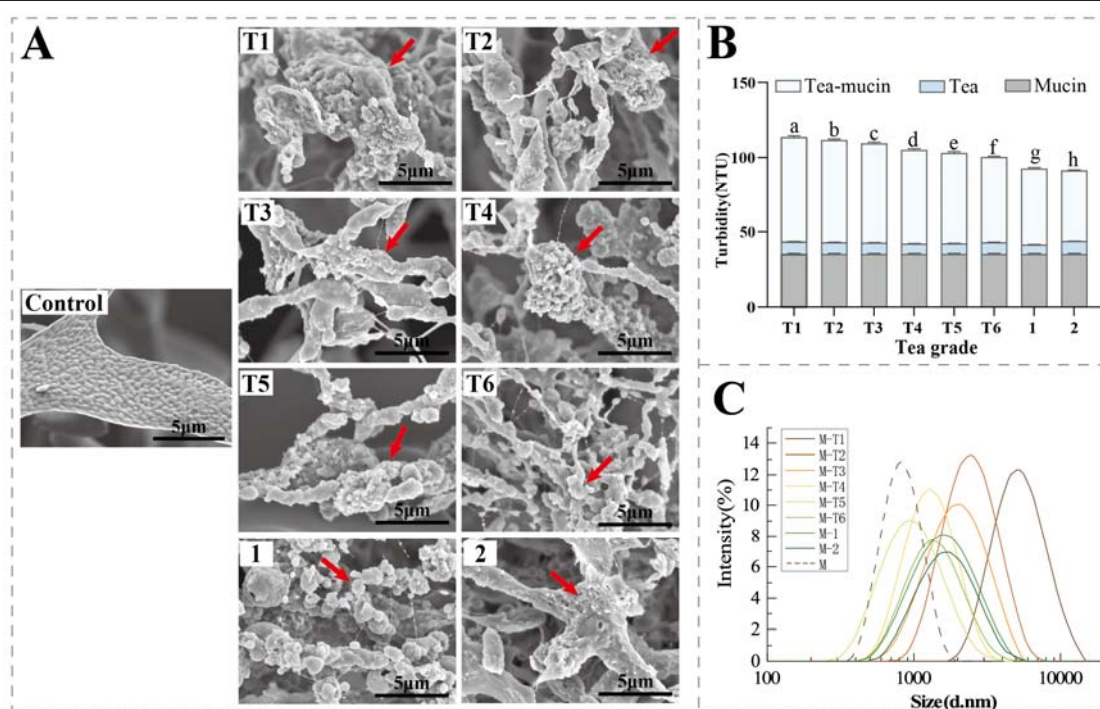


Figure 2 Characterization of tea-mucin complexes based on eight grades of CMGTs. (A) SEM observation ($\times 8,000$) of the mucin after exposure to tea infusions. Scale bar = 5 μm ; (B) Turbidity values of mucin mixed with tea infusions; (C) Impact of the tea infusions on mucin size. Error bars represent mean $\pm$ SD. Different letters in the exact figure indicate statistically significant differences between grades as determined by one-way ANOVA with Duncan post hoc test ($P < 0.05$).

Particle size analysis provided further insight (Figure 2C). Free mucin showed a sharp peak near 700 nm. In contrast, all tea-mucin complexes exhibited a broad size distribution extending into the micrometer range (1000 – 10000 nm), confirming the formation of large aggregates. The peak position of M-T1 was the most to the right, followed by M-T2, indicating that most of the complexes formed by samples of T1 and T2 grades have larger particle sizes than those of the other grades. Additionally, complexes from grades T3 – T5 showed significantly higher peak intensities than those from T6, 1, and 2 grades, reflecting a higher relative abundance of aggregated particles in the former system. Together, these findings align with the turbidity results and point to a positive correlation between tea grade and the extent and size of mucin aggregation, as previously demonstrated by Ye et al.^[33]. In their study, increased EGCG concentration led to the formation of EGCG-mucin complexes, elevated system turbidity, and the formation of larger particles and precipitates.

3.2.2 SEM analysis

In order to investigate the effect of green tea on the structure of mucin, we observed the microstructure of tea-mucin complexes from eight different grades of CMGTs using SEM. As shown in Figure 2A, in the control condition, mucin (no tea infusion) exhibited a thin, smooth lamellar structure, consistent with previous reports^[34]. Upon exposure of the mucin to tea infusions, the structure was clearly disrupted, and larger aggregates (see arrows in Figure 2A) formed, which probably correspond to tea-mucin aggregates. Notably, the degree of aggregation diminished with decreasing tea grade, providing a visual explanation for the sensory scores. Large, dense, and rough irregular aggregates observed in the T1 – T5 grades were likely perceived as a dry and gritty sensation (astringency) reported by panelists, as their rough surfaces increase

oral friction^[32]. Conversely, complexes from T6 and 1 grades were smaller and smoother, with some near-round particles (Figure 2A). This morphology aligns with their lower astringency scores, as these particles may roll more easily, thereby reducing the oral coefficient of friction^[32]. The mucin structure was minimally affected by grade 2, with no noticeable particle aggregates, indicating an extremely low content of mucin-precipitating compounds in this lowest grade sample. In summary, SEM imaging visually links higher-grade CMGTs to the formation of larger, irregular mucin aggregates that are sensorially perceived as stronger astringency.

3.3 Quantitative analysis of main taste components on CMGT with different grades

3.3.1 Contents of catechins, gallic acid, and purine alkaloids

The flavor of tea infusion resulted from the synergistic effects of multiple compounds. The major compounds of CMGT were determined by UHPLC. As shown in Table 1, the concentrations of the major galloylated catechins, EGCG and ECG, progressively decreased with decreasing grades. The levels of EGCG and ECG ranged from 54.32 – 84.17 mg/g and 11.31 – 17.17 mg/g, respectively. Catechins mainly contribute to the astringency and bitterness of green tea infusion, with galloylated catechins exhibiting stronger taste intensity than non-galloylated ones^[35]. Additionally, increased catechin content may enhance the kokumi flavor intensity in tea infusion^[36]. Similarly, the bitter compounds caffeine and theobromine followed the same decreasing trend. In contrast, the contents of other measured compounds, including GA and non-galloylated catechins, remained relatively stable across the grades. These results suggested a gradual decrease in the major taste-active and potentially astringent compounds of CMGT with decreasing grades, and provided a clear chemical basis for their strong flavor profile.

Table 1 The content of major compounds of different grades of CMGT samples (mean $\pm$ SD, mg/g).

Compounds	Grades							
	T1	T2	T3	T4	T5	T6	1	2
GA	2.33 $\pm$ 0.02 ^a	2.24 $\pm$ 0.03 ^b	2.23 $\pm$ 0.01 ^b	2.12 $\pm$ 0.02 ^c	2.15 $\pm$ 0.01 ^c	2.08 $\pm$ 0.01 ^d	1.94 $\pm$ 0.01 ^e	1.98 $\pm$ 0.04 ^e
THB	5.06 $\pm$ 0.12 ^a	4.61 $\pm$ 0.16 ^b	4.08 $\pm$ 0.04 ^c	3.46 $\pm$ 0.01 ^d	3.08 $\pm$ 0.09 ^e	2.83 $\pm$ 0.05 ^f	2.52 $\pm$ 0.03 ^g	2.23 $\pm$ 0.03 ^h
CAF	36.56 $\pm$ 0.42 ^a	35.34 $\pm$ 0.51 ^b	33.77 $\pm$ 0.15 ^c	29.63 $\pm$ 0.35 ^d	27.69 $\pm$ 0.28 ^e	26.65 $\pm$ 0.30 ^f	23.25 $\pm$ 0.08 ^g	23.16 $\pm$ 0.36 ^g
GC	3.46 $\pm$ 0.09 ^b	3.69 $\pm$ 0.02 ^{ab}	3.73 $\pm$ 0.03 ^{ab}	3.94 $\pm$ 0.05 ^a	3.50 $\pm$ 0.51 ^b	2.94 $\pm$ 0.02 ^c	3.87 $\pm$ 0.03 ^a	3.73 $\pm$ 0.03 ^{ab}
EGC	23.12 $\pm$ 0.47 ^b	23.65 $\pm$ 0.48 ^{ab}	24.13 $\pm$ 0.06 ^a	23.41 $\pm$ 0.28 ^b	22.28 $\pm$ 0.33 ^c	21.64 $\pm$ 0.22 ^{de}	21.39 $\pm$ 0.36 ^e	22.10 $\pm$ 0.51 ^{cd}
C	2.07 $\pm$ 0.09 ^{ab}	2.18 $\pm$ 0.05 ^{ab}	2.14 $\pm$ 0.08 ^{ab}	2.18 $\pm$ 0.09 ^{ab}	2.09 $\pm$ 0.09 ^{ab}	2.02 $\pm$ 0.01 ^{ab}	2.19 $\pm$ 0.16 ^a	2.00 $\pm$ 0.06 ^b
EC	7.83 $\pm$ 0.20 ^{bc}	7.79 $\pm$ 0.17 ^{bc}	7.77 $\pm$ 0.05 ^c	8.08 $\pm$ 0.06 ^a	7.36 $\pm$ 0.08 ^d	7.84 $\pm$ 0.01 ^{bc}	7.71 $\pm$ 0.07 ^c	7.96 $\pm$ 0.10 ^{ab}
EGCG	84.17 $\pm$ 1.00 ^a	81.35 $\pm$ 1.84 ^b	78.28 $\pm$ 0.55 ^c	69.66 $\pm$ 0.67 ^d	65.09 $\pm$ 0.65 ^e	62.46 $\pm$ 0.36 ^f	57.24 $\pm$ 0.16 ^g	54.32 $\pm$ 1.18 ^h
GCG	3.48 $\pm$ 0.05 ^b	3.63 $\pm$ 0.12 ^a	3.38 $\pm$ 0.10 ^{bc}	3.29 $\pm$ 0.10 ^c	3.15 $\pm$ 0.02 ^d	3.16 $\pm$ 0.04 ^d	2.84 $\pm$ 0.03 ^e	2.84 $\pm$ 0.10 ^e
ECG	17.17 $\pm$ 0.25 ^a	16.46 $\pm$ 0.37 ^b	15.91 $\pm$ 0.08 ^c	15.18 $\pm$ 0.12 ^d	13.36 $\pm$ 0.16 ^e	13.66 $\pm$ 0.15 ^e	12.43 $\pm$ 0.05 ^f	11.31 $\pm$ 0.29 ^g

Note: GA, gallic acid; THB, theobromine; GC, (–)-gallocatechin; CAF, caffeine; EGC, (–)-epigallocatechin; C, (+)-catechin; EC, (–)-epicatechin; EGCG, (–)-epigallocatechin gallate; GCG, (–)-gallocatechin gallate; ECG, (–)-epicatechin gallate. Different letters in the same row indicate statistically significant differences between grades as determined by one-way ANOVA with Duncan post hoc test ($P < 0.05$).

However, the grade differences in catechin content of CMGT are contrary to those of certain green teas produced in China. For example, in Lu'an Guapian green tea and Longjing green tea, the contents of catechins increased with decreasing grades^[17,37], which are usually produced from a single tea cultivar. In contrast, CMGT production involves blending multiple crude teas (green tea after primary production) in

specific proportions to achieve the target flavor^[2]. These blended crude teas may originate from different cultivars, different geographic origins, or different harvest seasons. The chemical composition of tea is known to be heavily influenced by these variables, particularly cultivar and harvest season^[38]. As a result, the chemical characteristics of CMGT are not directly comparable to those of unblended green teas.

3.3.2 Contents of free amino acids

Furthermore, the contents of thirteen free amino acids of different grades of CMGTs were determined (Table 2). Quantitative analysis revealed that *L*-theanine was the most abundant free amino acid, consistently accounting for over 60% of the total. Its concentration was highest in the premium grades T1 and T2 (8.07 – 8.09 mg/g), followed by grade T3 (7.67 mg/g), while the lower grades (T4, T5, T6, 1, 2) contained significantly reduced levels (5.34 – 5.99 mg/g). Based on the published literature, *L*-theanine was the main contributor to the umami taste in green tea infusion and could weaken the astringency of EGCG^[39]. The contents of other amino acids (excluding *L*-glutamic acid) showed a similar trend, being higher in grades T1 – T3 than in the other grades. These results indicated that higher-grade CMGT (T1 – T3) possesses a significantly richer free amino acids, which are crucial contributors to the desirable umami taste and complex mouthfeel of premium green tea^[40].

Table 2 The content of amino acids of different grades of CMGT samples (mean $\pm$ SD, mg/g).

Amino acid	Grades							
	T1	T2	T3	T4	T5	T6	1	2
Thea	8.07 $\pm$ 0.13 ^a	8.09 $\pm$ 0.41 ^a	7.67 $\pm$ 0.05 ^b	5.94 $\pm$ 0.09 ^c	5.99 $\pm$ 0.06 ^c	5.86 $\pm$ 0.15 ^c	5.34 $\pm$ 0.24 ^d	5.73 $\pm$ 0.05 ^c
Ser	0.32 $\pm$ 0.01 ^{ab}	0.33 $\pm$ 0.01 ^a	0.34 $\pm$ 0.01 ^a	0.28 $\pm$ 0.03 ^c	0.28 $\pm$ 0.05 ^{bc}	0.23 $\pm$ 0.02 ^{cd}	0.23 $\pm$ 0.02 ^d	0.23 $\pm$ 0.02 ^d
Glu	0.86 $\pm$ 0.04 ^b	0.88 $\pm$ 0.04 ^b	0.98 $\pm$ 0.02 ^a	0.79 $\pm$ 0.03 ^c	0.79 $\pm$ 0.04 ^c	0.85 $\pm$ 0.04 ^c	0.85 $\pm$ 0.04 ^b	1.01 $\pm$ 0.02 ^a
Phe	0.19 $\pm$ 0.01 ^a	0.17 $\pm$ 0.01 ^b	0.16 $\pm$ 0.00 ^c	0.12 $\pm$ 0.00 ^d	0.10 $\pm$ 0.00 ^e	0.09 $\pm$ 0.00 ^f	0.08 $\pm$ 0.00 ^g	0.08 $\pm$ 0.00 ^g
Thr	0.18 $\pm$ 0.01 ^a	0.17 $\pm$ 0.02 ^a	0.18 $\pm$ 0.01 ^a	0.13 $\pm$ 0.01 ^{bc}	0.14 $\pm$ 0.01 ^b	0.13 $\pm$ 0.00 ^{bc}	0.12 $\pm$ 0.02 ^c	0.14 $\pm$ 0.01 ^b
Asp	0.81 $\pm$ 0.08 ^{ab}	0.83 $\pm$ 0.09 ^{ab}	0.91 $\pm$ 0.06 ^a	0.71 $\pm$ 0.00 ^c	0.78 $\pm$ 0.07 ^{bc}	0.74 $\pm$ 0.01 ^{bc}	0.74 $\pm$ 0.05 ^{bc}	0.73 $\pm$ 0.01 ^{bc}
Tyr	0.18 $\pm$ 0.01 ^a	0.17 $\pm$ 0.01 ^b	0.16 $\pm$ 0.00 ^b	0.13 $\pm$ 0.00 ^c	0.11 $\pm$ 0.00 ^d	0.11 $\pm$ 0.00 ^d	0.09 $\pm$ 0.01 ^e	0.11 $\pm$ 0.00 ^d
Trp	0.27 $\pm$ 0.00 ^a	0.25 $\pm$ 0.02 ^b	0.24 $\pm$ 0.00 ^b	0.19 $\pm$ 0.00 ^c	0.16 $\pm$ 0.00 ^d	0.15 $\pm$ 0.01 ^e	0.13 $\pm$ 0.00 ^f	0.15 $\pm$ 0.00 ^{de}
GABA	0.18 $\pm$ 0.01 ^a	0.17 $\pm$ 0.01 ^a	0.15 $\pm$ 0.00 ^b	0.11 $\pm$ 0.00 ^d	0.12 $\pm$ 0.00 ^{cd}	0.11 $\pm$ 0.01 ^{de}	0.10 $\pm$ 0.00 ^e	0.13 $\pm$ 0.00 ^c
Lys	0.52 $\pm$ 0.05 ^a	0.45 $\pm$ 0.04 ^b	0.42 $\pm$ 0.00 ^{bc}	0.30 $\pm$ 0.00 ^d	0.29 $\pm$ 0.02 ^d	0.26 $\pm$ 0.00 ^d	0.30 $\pm$ 0.07 ^d	0.37 $\pm$ 0.01 ^c
Val	0.16 $\pm$ 0.00 ^a	0.16 $\pm$ 0.01 ^a	0.16 $\pm$ 0.00 ^a	0.13 $\pm$ 0.01 ^b	0.10 $\pm$ 0.00 ^c	0.10 $\pm$ 0.00 ^{cd}	0.09 $\pm$ 0.00 ^d	0.09 $\pm$ 0.00 ^d
Asn	0.36 $\pm$ 0.01 ^a	0.32 $\pm$ 0.05 ^b	0.37 $\pm$ 0.02 ^a	0.27 $\pm$ 0.01 ^c	0.23 $\pm$ 0.01 ^d	0.22 $\pm$ 0.02 ^{de}	0.18 $\pm$ 0.01 ^f	0.19 $\pm$ 0.02 ^{ef}
Gln	0.48 $\pm$ 0.05 ^a	0.43 $\pm$ 0.04 ^{ab}	0.40 $\pm$ 0.02 ^b	0.30 $\pm$ 0.01 ^{cd}	0.28 $\pm$ 0.01 ^{cd}	0.25 $\pm$ 0.00 ^d	0.31 $\pm$ 0.08 ^c	0.38 $\pm$ 0.01 ^b

Notes: Thea, *L*-theanine; Ser, *L*-serine; Glu, *L*-glutamic acid; Phe, *L*-phenylalanine; Thr, *L*-threonine; Asp, *L*-aspartic acid; Tyr, *L*-tyrosine; Trp, *L*-tryptophan; GABA, γ -aminobutyric acid; Lys, *L*-lysine; Val, *L*-valine; Asn, *L*-asparagine; Gln, *L*-glutamine. Different letters in the same row indicate statistically significant differences between grades as determined by one-way ANOVA with Duncan post hoc test ($P < 0.05$).

3.4 Analysis of non-volatile compounds by LC-MS-based metabolomics

Usually, the chemical characteristics or chemotaxonomy are used in the classification of plant relationships, including tea types. This work aimed to classify different grades of green tea through their non-volatile compounds. Accordingly, the non-volatile metabolite profiles of eight distinct grades of green tea samples were characterized via UPLC-Q-TOF-MS/MS. A total of 4794 metabolites in negative ion mode were detected. To obtain a more accurate classification of CMGT samples with different grades, multivariate statistical analysis was performed for the detected metabolites. Following data pretreatment, unsupervised

principal component analysis (PCA) and hierarchical clustering analysis (HCA) were conducted. PCA results (Figure 3A) showed that the first two principal components explained 59.6% of the total variance. Notably, the PCA score plot revealed a distinct, grade-dependent sequential distribution of the samples (Figure 3A). This suggested that the metabolites of CMGT may change gradually as the grade decreases. Meanwhile, HCA distinctly separated the samples into two groups: high-grade (T1, T2, T3) and low-grade (T4, T5, T6, 1, 2), indicating a significant difference in metabolites between these categories (Figure 3B). The same classification was also observed in partial least squares data analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) of LC-MS metabolomics data (Figure 3C & 3D). Collectively, these unsupervised and supervised analyses confirm that the non-volatile metabolite profile serves as a robust and reliable basis for differentiating green tea grades.

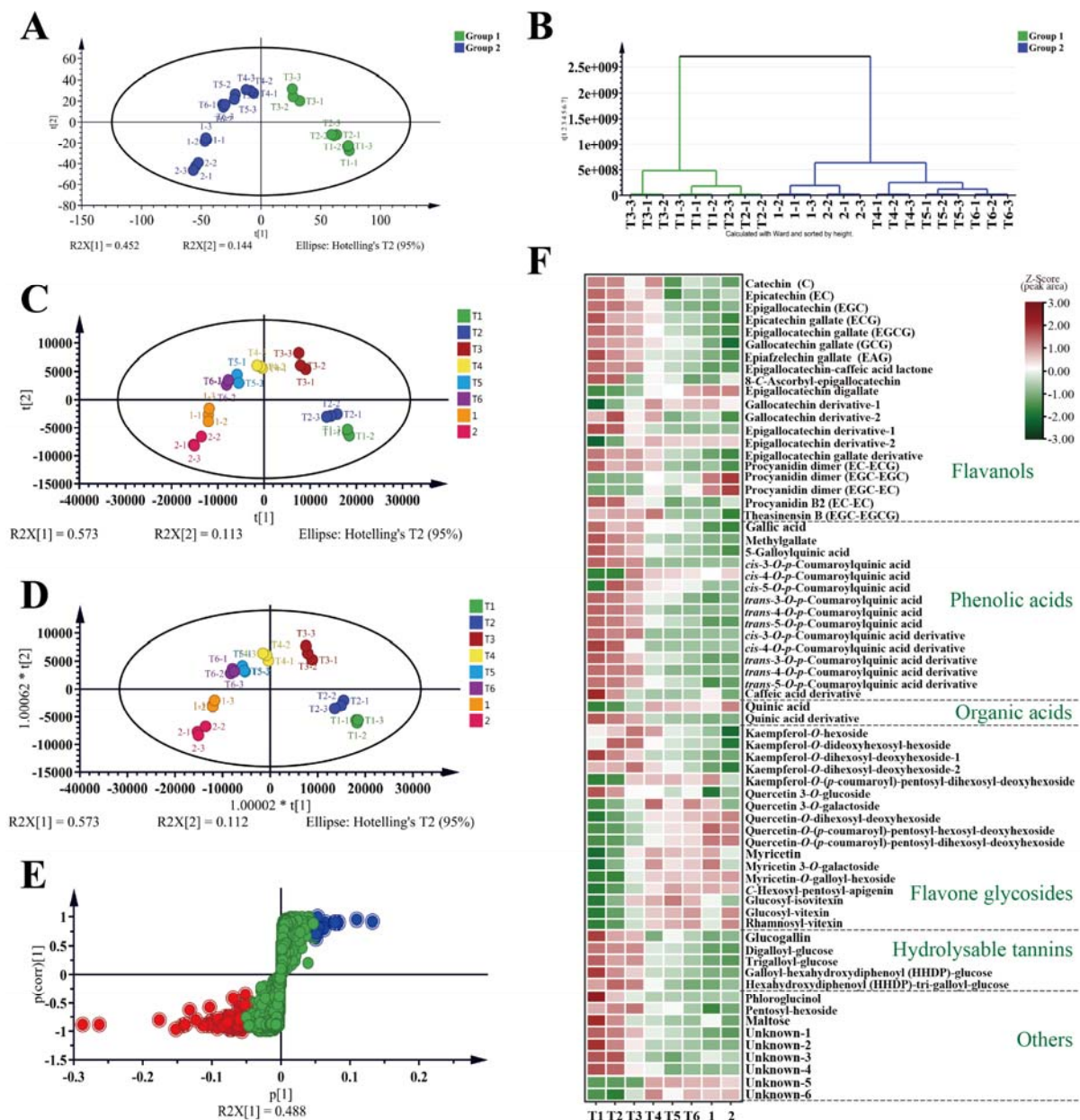


Figure 3 The metabolomics and multivariate analysis for different grades of CMGTs by LC-MS. (A) PCA; (B) HCA; (C) PLS-DA scattering plot; (D) OPLS-DA; (E) S-plot by OPLS-analysis of high- and low-grade green teas (T1, T2, T3 vs T4, T5, T6, 1, 2); (F) Heatmap showing the variation of marker compounds (VIP > 1) of high- and low-grade CMGTs based on their peak area values.

To chemically depict the important compounds responsible for the classification of high- and low-grade green tea, the *S*-plot (Figure 3E) was presented under OPLS-DA analysis of high- and low-grade CMGTs. Metabolites with a VIP value > 1 were selected as potential chemical markers. These compounds were putatively identified based on authentic standards, retention time, accurate mass measurements, and MS² fragmentation patterns referring to the tea metabolite database. As shown in Table 3, a total of sixty-eight marker compounds were listed, including flavan-3-ols (20), flavone glycosides (17), phenolic acids (15), hydrolysable tannins (5), organic acids (2), and others (9). In addition, the differences of these marker compounds in different grades of CMGTs were profiled by the heatmap (Figure 3F).

Table 3 The marker compounds (VIP > 1) contributing to the re-classification of high-grade and low-grade CMGTs (T1, T2, T3 vs T4, T5, T6, 1, 2).

Peak No.	t _R (min)	m/z	VIP	MS/MS	Identification	Classification
2815	8.97	457.0776	18.21	305.0666, 169.0142, 125.0247	Epigallocatechin gallate (EGCG)	Flavanols
2650	10.65	441.0829	12.18	289.0715, 169.0138, 125.0244	Epicatechin gallate (ECG)	Flavanols
1665	7.58	337.0931	10.52	191.0542, 173.0457	<i>trans</i> -4- <i>O</i> - <i>p</i> -Coumaroylquinic acid	Phenolic acids
1392	5.81	305.0670	9.87	287.0555, 261.0767, 219.0666, 179.0352, 125.0248, 111.0455	Epigallocatechin (EGC)	Flavanols
1719	2.24	343.0687	9.57	191.0564, 85.0292	5-Galloylquinic acid	Phenolic acids
590	0.71	191.0579	9.17	111.0090, 87.0088, 85.0299, 57.0347, 67.0187	Quinic acid	Organic acids
2818	9.82	457.0788	9.11	305.0663, 169.0139, 125.0245	Gallocatechin gallate (GCG)	Flavanols
4615	7.04	633.0723	7.79	463.0510, 300.9998, 275.0192	Galloyl-hexahydroxydiphenoyl (HHDP)-glucose	Hydrolysable tannins
2631	0.78	439.0870	7.28	191.0185, 96.9701	Unknown-1	Others
2449	7.58	422.0698	6.35	337.0927, 173.0454, 163.0392, 61.9880	<i>trans</i> -4- <i>O</i> - <i>p</i> -Coumaroylquinic acid derivative	Phenolic acids
5491	10.84	755.2026	5.76	285.0404	Kaempferol- <i>O</i> -dihexosyl-deoxy hexoside-1	Flavone glycosides
4384	10.92	609.1475	5.74	457.0779, 169.0136	Epigallocatechin digallate	Flavanols
1704	0.65	341.1097	5.49	191.0557, 179.0563, 119.0349, 113.0248, 101.0248, 89.0249	Maltose	Others
5598	10.62	771.2001	5.39	609.1440, 301.0349, 300.0280, 151.0045	Quercetin- <i>O</i> -dihexosyl-deoxyhexoside	Flavone glycosides
6541	9.04	937.1425	5.3	479.0605, 309.0388, 169.0140, 125.0247	Hexahydroxydiphenoyl (HHDP)-tri-galloyl-glucose	Hydrolysable tannins
1247	7.34	289.0719	5.26	245.0821, 203.0711, 125.0248, 109.0298, 123.0455, 97.0293	Epicatechin (EC)	Flavanols
4642	9.53	635.0884	5.26	465.0671, 313.0544, 169.0139, 125.0242	Trigalloyl-glucose	Hydrolysable tannins
5492	11.3	755.2045	5.15	593.0523, 285.0402	Kaempferol- <i>O</i> -dihexosyl-deoxy hexoside-2	Flavone glycosides
3039	9.23	479.0578	4.76	341.0273, 309.0383, 287.0547, 169.0150, 125.0246	8- <i>C</i> -Ascorbyl-epigallocatechin	Flavanols
3461	0.67	515.2111	4.65	191.0518, 173.0929, 111.0095	Quinic acid derivative	Organic acids
3209	13.38	493.2315	4.43	315.1782, 179.0560, 149.0454, 119.0344, 89.0247	Unknown-2	Others
538	4.73	183.0307	4.38	124.0174, 125.0204, 78.0111	Methylgallate	Phenolic acids
2100	0.65	387.1151	4.35	179.0560, 119.0350, 89.0246	Caffeic acid derivative	Phenolic

						acids
2480	11.94	425.0892	4.26	273.0765, 255.0673, 169.0133, 125.0237, 97.0307	Epiafzelechin gallate (EAG)	Flavanols
1661	5.65	337.0921	4.22	191.0563, 163.0391, 119.0495, 93.0344	<i>trans</i> -3- <i>O</i> - <i>p</i> -Coumaroylquinic acid	Phenolic acids
2020	0.82	377.0858	4.08	341.1092, 191.0175, 96.9602, 88.9872	Unknown-3	Others
462	1.71	169.0142	4	125.0246, 81.0340, 79.0191	Gallic acid	Phenolic acids
1659	5.16	337.0920	3.94	191.0549, 163.0386	<i>cis</i> -3- <i>O</i> - <i>p</i> -Coumaroylquinic acid	Phenolic acids
4074	7.8	577.1351	3.91	425.0878, 289.0717, 125.0247	Procyanidin B2 (EC-EC)	Flavanols
2872	10.71	463.0641	3.54	301.0352, 300.0284, 271.0252, 178.9983, 151.0034	Quercetin 3- <i>O</i> -glucoside	Flavone glycosides
1992	5.91	373.0533	3.5	261.0767, 219.0661, 165.0192, 125.0249	Epigallocatechin derivative-1	Flavanols
3048	10.08	479.0831	3.49	316.0234, 317.0296, 169.0150, 125.0246	Myricetin 3- <i>O</i> -galactoside	Flavone glycosides
1834	7.34	357.0590	3.34	245.0816, 179.0350	Unknown-4	Others
2875	11.23	463.0881	3.32	300.0279, 271.0596, 169.0138, 125.0246	Quercetin 3- <i>O</i> -galactoside	Flavone glycosides
6909	14.35	1049.2835	3.24	903.2398, 301.0308, 300.0265	Quercetin- <i>O</i> -(<i>p</i> -coumaroyl)-pentosyl-dihexosyl-deoxyhexoside	Flavone glycosides
3088	6.75	483.0768	3.21	313.0561, 271.0450, 211.0229, 169.0139, 125.0234	Digalloyl-glucose	Hydrolysable tannins
6367	10.62	899.1695	3.14	743.6206, 729.1365, 577.1249, 457.0792, 441.0825, 169.0140	Epigallocatechin gallate derivative	Flavanols
3389	12.63	511.0555	2.80	431.0982, 311.0554, 283.0612, 96.9603	Unknown-5	Others
2919	16.06	467.1353	2.77	321.0971, 173.0449, 163.0402, 119.0500	Epigallocatechin-caffeic acid lactone	Flavanols
3949	8.85	563.1440	2.71	545.1276, 519.2363, 503.1168, 473.1088, 383.0764, 353.0663, 297.0769	C-Hexosyl-pentosyl-apigenin	Flavone glycosides
1623	1.24	331.0692	2.64	191.0580, 169.0139, 125.0246, 61.9885	Glucogallin	Hydrolysable tannins
1668	8.37	337.0940	2.62	191.0565, 173.0448, 163.0404	<i>trans</i> -5- <i>O</i> - <i>p</i> -Coumaroylquinic acid	Phenolic acids
4376	6.16	609.1246	2.5	441.0793, 423.0732, 305.0642, 209.0451, 177.0186, 125.0246	Procyanidin dimer (EGC-EGC)	Flavanols
1243	6.14	289.0711	2.45	245.0821, 203.0711, 125.0248, 109.0298, 123.0455, 97.0293	Catechin (C)	Flavanols
1797	7.34	352.0674	2.42	336.0515, 173.0432	Unknown-6	Others
4587	5.82	631.1098	2.38	317.0133, 305.0665	Myricetin- <i>O</i> -galloyl-hexoside	Flavone glycosides
2448	6.71	422.0700	2.35	337.0927, 173.0454, 93.0344	<i>cis</i> -4- <i>O</i> - <i>p</i> -Coumaroylquinic acid derivative	Phenolic acids
4220	6.88	593.1301	2.28	425.0863, 423.0698, 407.0753, 305.0661, 289.0704, 177.0187, 125.0240	Procyanidin dimer (EGC-EC)	Flavanols
2706	12.1	447.0942	2.26	285.0391, 284.0327, 255.0304, 227.0347, 125.0248	Kaempferol- <i>O</i> -hexoside	Flavone glycosides
6304	14.63	887.2264	2.18	301.0348, 300.0266, 145.0291	Quercetin- <i>O</i> -(<i>p</i> -coumaroyl)-pentosyl-hexosyl-deoxyhexoside	Flavone glycosides
2446	5.17	422.0695	2.17	337.0945, 248.0164, 163.0398, 119.0501, 61.9880	<i>cis</i> -3- <i>O</i> - <i>p</i> -Coumaroylquinic acid derivative	Phenolic acids
5377	11.56	739.2073	2.14	285.0387	Kaempferol- <i>O</i> -dideoxyhexosyl-hexoside	Flavone glycosides
1669	7.68	337.0942	2.04	191.0557, 173.0456, 119.0521	<i>cis</i> -5- <i>O</i> - <i>p</i> -Coumaroylquinic acid	Phenolic acids
1666	6.86	337.0933	2.02	191.0558, 173.0456, 119.0497,	<i>cis</i> -4- <i>O</i> - <i>p</i> -Coumaroylquinic	Phenolic

				93.0344	acid	acids
2135	4.15	390.0444	2	305.0646, 261.0759, 137.0252, 125.0245	Gallocatechin derivative-1	Flavanols
1448	0.62	311.0978	1.96	179.0550, 161.0443, 125.0243, 85.0301	Pentosyl-hexoside	Others
2445	5.71	422.0690	1.96	337.0924, 248.0164, 163.0396	<i>trans</i> -3- <i>O</i> - <i>p</i> -Coumaroylquinic acid derivative	Phenolic acids
4085	10.33	577.1587	1.96	493.0752, 413.0906, 293.0452, 169.0135, 125.0237	Rhamnosyl-vitexin	Flavone glycosides
2137	5.89	390.0465	1.92	305.0645, 261.0757, 137.0253, 125.0246	Epigallocatechin derivative-2	Flavanols
2452	8.37	422.0717	1.81	337.0923, 173.0455	<i>trans</i> -5- <i>O</i> - <i>p</i> -Coumaroylquinic acid derivative	Phenolic acids
155	1.38	125.0244	1.8	108.0207, 107.0140, 67.0183, 79.0187, 51.0235	Phloroglucinol	Others
5323	10.13	729.1475	1.75	407.0762, 305.0640, 289.0723, 169.0135, 125.0236	Procyanidin dimer (EC-ECG)	Flavanols
5517	8.08	761.1340	1.66	593.0957, 423.0719, 305.0671, 177.0197, 125.0252	Theasinensin B (EGC-EGCG)	Flavanols
4228	9.85	593.1527	1.57	413.0888, 293.0479	Glucosyl-vitexin	Flavone glycosides
1500	0.82	317.0555	1.53	225.0055, 128.9782, 95.9505, 80.9653	Myricetin	Flavone glycosides
6857	14.84	1033.2810	1.38	451.1437, 285.0414, 146.9656	Kaempferol- <i>O</i> -(<i>p</i> -coumaroyl)- <i>p</i> -pentosyl-dihexosyl-deoxyhexoside	Flavone glycosides
1991	4.15	373.0531	1.3	261.0768, 165.0193, 125.0250	Gallocatechin derivative-2	Flavanols
4223	8.07	593.1511	1.2	473.1084, 383.0788, 353.0640, 125.0245	Glucosyl-isovitexin	Flavone glycosides

Flavan-3-ols, particularly EGCG and ECG (VIP > 12), were pivotal in distinguishing high-grade from low-grade CMGTs. In addition, except for major catechins (*e.g.*, EGC, GCG, and epiafzelechin gallate), several catechin derivatives were also detected, such as epigallocatechin digallate, 8-*C*-ascorbyl-epigallocatechin, and epigallocatechin-caffeic acid lactone. Heatmap results (Figure 3F) showed that most flavanols were enriched in high-grade teas and decreased with lower grade, consistent with the quantitative analysis results.

Phenolic acids play an important role in the flavor of coffee, cocoa, and tea^[7]. In the present study, 15 phenolic acids were identified as marker compounds. These mainly included gallic acid, methylgallate, caffeic acid derivative, 5-galloylquinic acid, and various hydroxycinnamoyl quinic acids and their derivatives (*e.g.*, *trans*-5-*O*-*p*-coumaroylquinic acid & *trans*-4-*O*-*p*-coumaroylquinic acid). Notably, over half of the phenolic acids detected were hydroxycinnamoyl quinic acids and their derivatives (Table 3), and their abundance varied significantly between high- and low-grade CMGTs. According to the literature, hydroxycinnamoyl quinic acid had significant biological activity and was a crucial active component in tea^[12]. 5-Galloylquinic acid was the main scavenger of reactive oxygen species (ROS) in green tea^[41]. These phenolic acids (except *cis*-4-*O*-*p*-coumaroylquinic acid) were enriched in high-grade teas and decreased with decreasing grade (Figure 3F). Hence, phenolic acids may contribute significantly to the enhanced taste quality and bioactivity of high-grade CMGTs.

Five hydrolysable tannins were identified as marker compounds, namely glucogallin, digalloyl-glucose, trigalloyl-glucose, galloyl-hexahydroxydiphenoyl (HHDP)-glucose, and hexahydroxydiphenoyl

(HHDP)-tri-galloyl-glucose. The heatmap results showed that all hydrolysable tannins were enriched in high-grade teas. This is consistent with the study of Han et al.^[20] on Huangshan Maofeng tea (a renowned Chinese green tea). They demonstrated that hydrolysable tannins were not only the marker compounds for high-grade green tea, but also positively correlated with the sweet aftertaste of green tea infusion. Therefore, hydrolysable tannins may not only serve as reliable chemical markers for premium teas but also may be key contributors to superior taste profiles of CMGT.

Flavones and their glycosides play a crucial role in constructing the flavor of the final tea products^[42]. In the present study, 17 flavone glycosides (including kaempferol glycosides, quercetin glycosides, myricetin glycosides, vitexin glycosides, glucosyl-isovitexin, and C-hexosyl-pentosyl-apigenin) were identified as marker compounds. These flavone glycosides (except kaempferol glycosides) were enriched in low-grade CMGTs (Figure 3F). This is in agreement with Han et al.^[20] and He et al.^[43], who reported that flavone glycosides were mainly negatively correlated with the quality grade of green tea. In addition, several *p*-coumaroyl-flavone glycosides were also detected in this study and identified as marker compounds (Table 3), such as quercetin-*O*-(*p*-coumaroyl)-pentosyl-dihexosyl-deoxyhexoside and kaempferol-*O*-(*p*-coumaroyl)-pentosyl-dihexosyl-deoxyhexoside. These *p*-coumaroyl-flavone glycosides were also enriched in low-grade teas.

3.5 *In vitro* bioactivity analysis of different grades of CMGTs

Chemical analysis revealed distinct compositional differences among tea grades, notably in known bioactive compounds like catechins and phenolic acids. We hypothesized that these chemical differences would result in significant variations in health-related bioactivities. To test this, we assessed the *in vitro* antioxidant activity and inhibitory effects on α -amylase and α -glucosidase across the grades.

The antioxidant activity of different grades of CMGTs was assessed using three independent assays: DPPH, ABTS, and FRAP. As shown in Figure 4A-F, the radical scavenging activity (or reducing power) of all samples increased in a dose-dependent manner. Statistical analysis of the IC₅₀ values from the three assays showed that the IC₅₀ values of high-grade teas were significantly lower than those of low-grade teas. These results indicated that the *in vitro* antioxidant capacity of high-grade teas was significantly stronger than that of low-grade teas. The fact that the green tea with higher catechin content presented higher antioxidant activity is in agreement with the data obtained by Carloni et al.^[14]. Likewise, it is apparent that the inhibitory effect of high-grade teas on α -amylase and α -glucosidase was stronger than that of low-grade teas (Figure 4G-H). α -Amylase and α -glucosidase are key enzymes in carbohydrate metabolism^[44]. Based on the published literature, catechins and procyanidins inhibited their activity through competitive binding to enzymes^[29,45]. In summary, high-grade CMGTs exhibited superior *in vitro* bioactivities, which are linked to their richer polyphenols like catechins. These findings demonstrated that their premium quality is characterized not only by superior sensory attributes but also by enhanced health benefits.

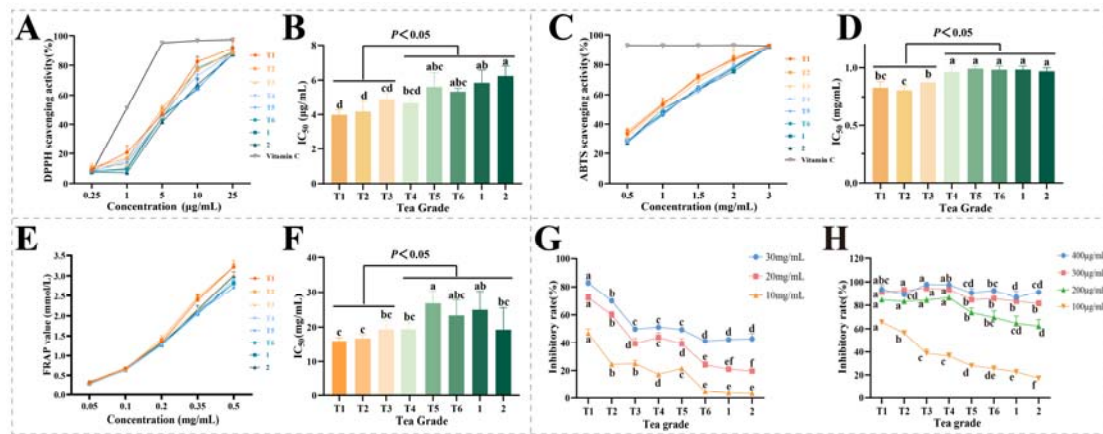


Figure 4 The **antioxidant** activity (A-B, DPPH test; C-D, ABTS test; E-F, FRAP test) and **inhibition of α -amylase (G) and α -glucosidase (H) of different grades of CMGTs**. Error bars represent mean $\pm$ SD. Different letters in the exact figure indicate statistically significant differences between grades as determined by one-way ANOVA with Duncan post hoc test ($P < 0.05$). The significant differences in IC_{50} values between high-grade and low-grade CMGTs were determined by the Independent Samples T-test.

3.6 Chemicals of CMGT in relation to grade, astringency, and antioxidant activity

Given the observed connections among chemical composition, sensory quality, and bioactivity, we aimed to identify the key compounds responsible. Our strategy combined RF machine learning and Pearson correlation analysis for this purpose. The RF is capable of handling massive data dimensionality and multicollinearity while remaining less susceptible to noise and overfitting^[46]. In a study by Zan et al.^[40], key taste compounds in green tea were successfully identified by RF combined correlation analysis. In the present study, this combined strategy was applied to marker compounds to elucidate their specific associations with tea grade, astringency (both astringency scores and turbidity values), and antioxidant activity (IC_{50} values), as visualized in Figure 5.

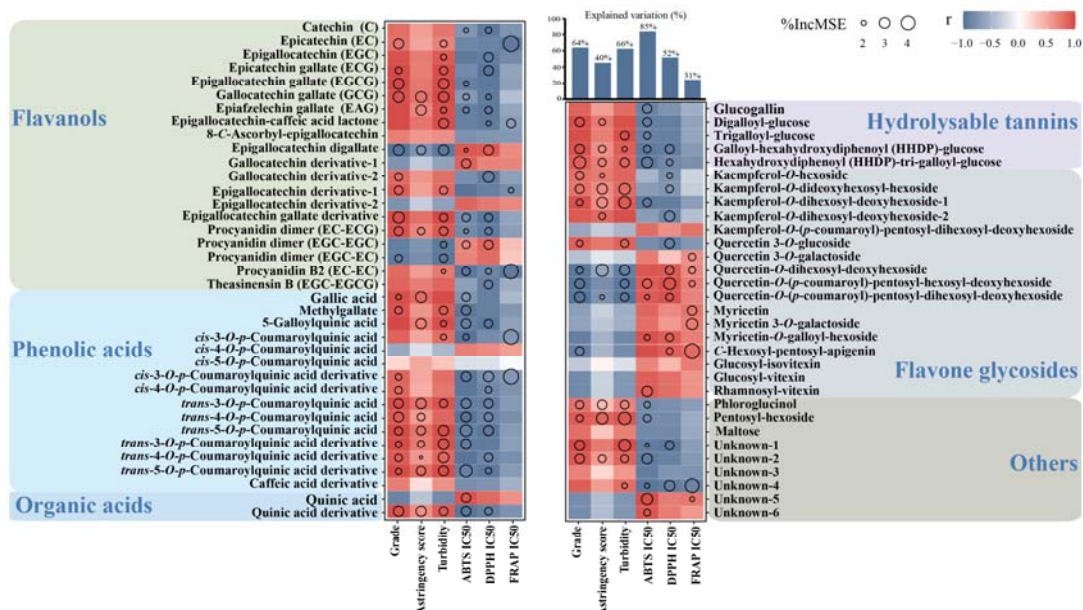


Figure 5 Correlation and Random Forest regression model of marker compounds (VIP > 1) with grade, **astringency scores, turbidity values, and antioxidant capacity (IC_{50} values)**. Column chart was used to response the cumulative contribution of marker compounds on explained variables, heatmap was used to present the correlation strength between marker compounds and grade, **astringency scores, turbidity values, and antioxidant capacity**. The circle size indicated %IncMSE (*i.e.*, importance of the variable), and the colors represent Pearson's correlation coefficients.

3.6.1 Key compounds associated with grade in CMGT

The marker compounds explained 64% of the grade variance (Figure 5). Most compounds were positively correlated with tea grade, including the majority of catechins and their derivatives, procyanidin dimer, phenolic acids (except for *cis*-4-*O*-*p*-coumaroylquinic), all identified hydrolysable tannins, and kaempferol glycosides. In contrast, quinic acid and most flavone glycosides were negatively correlated with the grade. Based on variation importance (%IncMSE > 2) and correlation (absolute values of correlation coefficients > 0.96), important contributors to CMGT grading were selected. The positive contributors included EGCG, ECG, epigallocatechin gallate derivative, *trans*-3-*O*-*p*-coumaroylquinic acid and its derivative, *trans*-5-*O*-*p*-coumaroylquinic acid and its derivative, digalloyl-glucose, and gallic acid. The negative contributors included epigallocatechin digallate, quercetin-*O*-(*p*-coumaroyl)-pentosyl-dihexosyl-deoxyhexoside, and quercetin-*O*-(*p*-coumaroyl)-pentosyl-hexosyl-deoxyhexoside. In summary, this analysis depicted two contrasting chemical characteristics of CMGT quality. Positively correlated catechins, phenolic acids, and hydrolysable tannins were markers for tea quality, contributing to the desirable taste of high-grade teas. Conversely, the negative correlation of specific acylated quercetin glycosides suggested they may serve as chemical indicators of lower-quality leaf material.

3.6.2 Key compounds associated with astringency in CMGT

It was proposed that the turbidity of mucin could explain a portion of astringent compounds, particularly for those polyphenols that demonstrated a strong correlation between turbidity generation and sensory astringency^[33]. Correlation analysis showed that all compounds positively correlated with sensory astringency scores were also positively correlated with turbidity values, such as GCG, 5-galloylquinic acid, *trans*-3-*O*-*p*-coumaroylquinic acid, and *trans*-5-*O*-*p*-coumaroylquinic acid. Notably, the model's explanatory power for turbidity (66%) was substantially higher than for astringency scores (40%), and the correlation coefficients with turbidity were generally stronger (Figure 5). This disparity underscored the complementarity of the two methods.

Accordingly, we identified two distinct sets of key contributors. Those most critical to the astringency score were gallic acid, *trans*-3-*O*-*p*-coumaroylquinic acid and its derivative, as well as kaempferol-*O*-dihexosyl-deoxyhexoside-2 ($r > 0.8$, $P < 0.05$). As it was already reported by other authors^[12,47-48], these key astringency score contributors have a lower astringency threshold than catechins and bind weakly to saliva proteins. On the other hand, the key contributors influencing turbidity were galloylated catechins (GCG, EGCG, ECG), *trans*-5-*O*-*p*-coumaroylquinic acid and its derivative, epigallocatechin-caffeic acid lactone, and 5-galloylquinic acid ($r > 0.96$, $P < 0.05$). The result aligned with prior findings that the galloyl moiety was a key determinant for strong salivary protein binding^[47]. In summary, our results revealed that the astringency of CMGT is an integrated perception driven by distinct compounds. Sensory perception was significantly influenced by phenolic acids and kaempferol glycosides

with lower astringency thresholds, whereas the mucin aggregation (turbidity) was primarily driven by galloylated catechins with high protein affinity.

3.6.3 Key compounds associated with antioxidant activity in CMGT

To identify the metabolites driving the superior antioxidant capacity of high-grade CMGTs, we correlated marker compound levels with IC₅₀ values from three antioxidant assays. For the ABTS and DPPH radical scavenging assays, which share similar experimental principles, seven compounds exhibited high importance and strong negative correlations ($r < -0.85$). These were primarily phenolic acids, including 5-galloylquinic acid and multiple *trans-p*-coumaroylquinic acid and their derivatives (*trans*-3-*O-p*-coumaroylquinic acid, *trans*-4-*O-p*-coumaroylquinic acid, *trans*-5-*O-p*-coumaroylquinic acid and its derivatives), alongside epiafzelechin gallate and galloyl-HHDP-glucose. For the FRAP assay, which measures reducing power, EC and procyanidin B2 (EC-EC) may be the most potent reducing agents, as they showed high importance (%IncMSE > 4) and strong negative correlations ($r < -0.92$) (Figure 5).

3.7 Construction of a compound-based grade estimation model

3.7.1 Elastic net screening of key grade predictors

Correlation analysis of marker compounds and tea grades revealed collinearity among compounds. Including collinear indicators in the estimation model would cause information redundancy and reduce estimation capability. To address this, elastic net regression was applied to extract key grade predictive features, thereby simplifying the model and enhancing robustness. The elastic net algorithm uses L1-penalty and L2-penalty to shrink some regression coefficients toward zero, satisfying model selection criteria^[49]. Additionally, selection stability was reinforced through 100 Bootstrap resampling. The selection frequency of each compound across bootstrap samples was shown in Figure S1. 5-Galloylquinic acid exhibited the highest selection frequency (95.0%), confirming its stable grade estimation ability. Based on a > 70% frequency threshold, four key predictors were identified: 5-galloylquinic acid, EGCG, ECG, and GCG (selection frequencies of 95.0%, 84.0%, 84.0%, 74.0%, respectively). Notably, these four compounds also serve as markers of CMGT quality, having previously been shown to correlate positively with grade and contribute significantly to taste and bioactivity.

3.7.2 Ridge regression model construction

Using the four selected predictors, a ridge regression model was constructed to estimate tea grade. The fundamental concept of ridge regression is to acquire more reliable regression coefficients by adding an L2 penalty to the loss function. Tea grades were assigned as continuous variables from one (highest grade) to eight (lowest grade). Model training utilized LOOCV for λ optimization. Performance metrics (Table S3) demonstrated that the coefficient of determination (R^2) was 0.995, the Root Mean Square Error (RMSE) for LOOCV was 0.168, and the Mean Absolute Error (MAE) was 0.139, indicating that the model had a high prediction accuracy. The negative regression coefficients logically indicated that higher compound levels

predict lower (*i.e.*, better) estimated grade values (Table S3). The regression equation for grade estimation of CMGT was:

$$\text{Grade-estimated} = 18.054 - 3.125 \times 10^{-7} \times 5\text{-Galloylquinic acid} - 7.539 \times 10^{-8} \times \text{EGCG} - 5.982 \times 10^{-8} \times \text{ECG} - 6.993 \times 10^{-8} \times \text{GCG}$$

3.7.3 Graded verification with commercial samples

To assess the grade differentiation ability of key predictors obtained using elastic net regression, we randomly selected three tea factories from different regions and purchased four grades of tea samples from each factory to form three verification sets (noted as H1-4, Z1-4, S1-4; Table S4). The peak areas of the predictors were obtained through a testing procedure consistent with the above eight-grade samples, and then input into the ridge regression equation for grade estimation. The results showed that the estimated values perfectly matched the labeled grade sequences within each factory set (*e.g.*, H4 > H3 > H2 > H1 in Figure 6), confirming its robustness in distinguishing relative grades from the same origin. In addition, differences in estimates across factories may be attributed to differences in the fresh tea leaves used for production (*e.g.*, cultivar and geographic origin) or processing techniques.

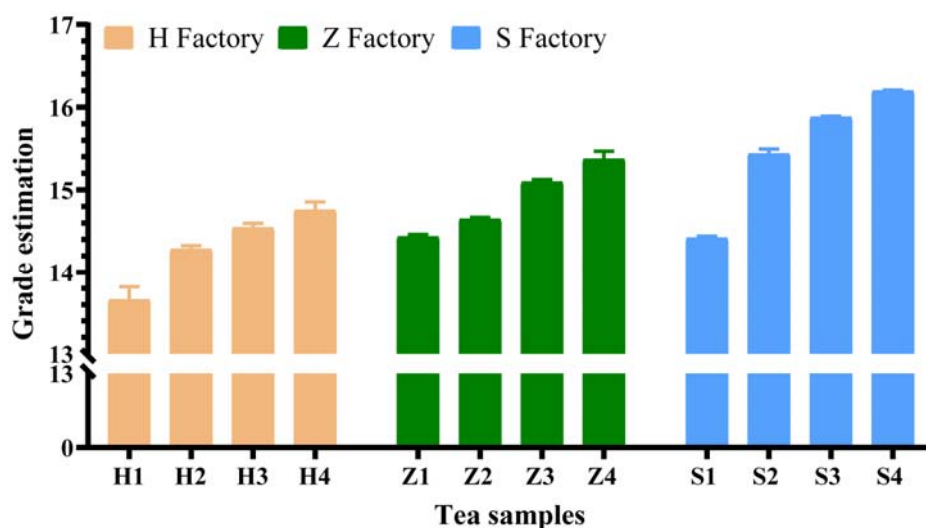


Figure 6 Grade estimation of the verification set (tea samples of different grades from three tea factories).

4. Conclusion

This study established a chemistry-driven framework for grading CMGT by systematically linking its non-volatile metabolite profile to sensory and bioactivity attributes. The results confirmed that high-grade CMGT possesses greater astringency, bioactivity, and metabolite richness. Moreover, specific phenolic acids, particularly *trans-p*-coumaroylquinic acid and its derivatives, were identified as key contributors to quality, as their levels were positively correlated with tea grade, astringency, and antioxidant activity via RF and correlation analysis. Subsequently, a machine learning regression model based on four predictive compounds (5-galloylquinic acid, EGCG, ECG, and GCG) was constructed and successfully validated for accurately ranking CMGTs from the same manufacturer. The proposed model complements traditional subjective grading, offering a novel and objective chemical grading strategy for CMGT and other similar blended teas.

Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Data including detailed information on the CMGT samples (Table S1); The gradient concentrations and scores of taste standard compounds (Table S2); Ridge regression analysis between MS peak areas of grade predictors and CMGT grades (Table S3); Detailed information on verification set samples (Table S4); Variables with frequencies $\geq 40\%$ were selected based on elastic net regression in 100 Bootstrap resampling (Figure S1).

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