LC-MS BASED METABOLOMICS AND SENSORY EVALUATION REVEAL THE CRITICAL COMPOUNDS OF DIFFERENT GRADES OF HUANGSHAN MAOFENG GREEN TEA

by

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ABSTRACT OF THE THESIS

LC-MS Based Metabolomics and Sensory Evaluation Reveal the Critical Compounds of Different Grades of Huangshan Maofeng Green Tea

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Green tea is a widely consumed tea category in China and many other countries worldwide. Usually, green tea only has few processing steps, which can mostly preserve the original compounds of fresh tea leave. Regarding the tea raw materials' tenderness, flavor, taste and appearance, for each kind of green tea, it can be classified as different grades. Huangshan-Maofeng (HSMF) tea is a very famous and typical pan-fried green tea in China. The fresh tea leaves go through three processes, as the order of fixation, rolling and drying to obtain the final green tea product. The best or high grade of HSMF tea is usually labelled as "tè" (T), which means the special high grade of tea. The HSMF tea could be rated as six grades, "T1, T2, T3, 1, 2, 3" ordered from the high to low grade. The price of HSMF tea is highly correlated to its grade, which mainly depends on the sensory evaluation.

Recently, we just studied the metabolomics of black tea and the chemical contributors of glucose-producing enzymes inhibition effect. Similarly, the metabolomics can also be used in exploring the marker compounds, especially the minor or trace composition of tea under different treatments, or from different geographical locations. Combining with the chemical standards, tea plant chemistry database, and mass fragment regulations of typical types of secondary metabolites of tea plant, many marker compounds have been identified from tea. To find the grade-related compounds of tea, metabolomics could also be a potent tool.

The taste of tea infusion is tightly correlated to the non-volatile compounds of tea. There have been some studies about the non-volatile flavor compounds of tea, such as L-theanine, galloylated catechins, caffeine, and flavonoid glycosides. Usually, caffeine is the major bitter compound of tea, while L-theanine is a vital umami factor for the fresh taste of green tea. In this decades, it was reported that flavonoids glycosides in addition contributed to the astringency of black tea, though galloylated catechins and procyanidins are also astringent compounds which can cause the strong mouth-drying feeling in some low-grade of tea. To connect the chemical composition and taste of tea infusion, except for the classic threshold studies, the metabolomics analysis combining with sensory evaluation could also be promising.

In this research, the different grades of HSMF green tea were studied by LC-MS based metabolomics, and then the critical compounds responsible for the classification of tea grades were identified by chemical standard or related database.

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Table of Contents

List of Figures

List of Tables

Introduction

1.1 Huangshan Maofeng Green Tea

Green tea was original in China, nowadays however, its manufacture has spread to a number of countries worldwide. Green tea is obtained from the parts of leaf and bud from the plant *Camellia sinensis* that mainly grows in areas with tropical and subtropical climates. Buds and leaves that used to produce green tea didn't experience the identical process of withering and oxidation which underwent in the production of black teas and oolong teas (Khan & Mukhtar, 2013). Usually, green tea only has few processing steps, which can mostly preserve the original compounds of fresh tea leaves (Wang, Kim & Lee, 2000). The fresh tea leaves go through about three processes, as the order of fixation, rolling and drying to obtain the final green tea product (Figure 1.1). There is green tea with several varieties, which differ basically by the variety of original plant used, production processing, growing conditions, horticultural methods and harvest time.

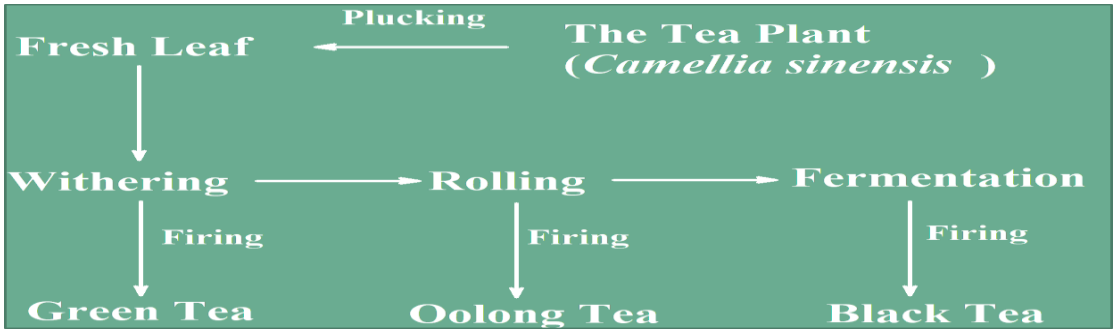


Figure 1.1 The manufacture of various types of tea

Huangshan Maofeng green tea is a type of tea where the production locates in the south eastern area of Anhui province, China. As one of the most well-known tea types, its name is never missed on the China Famous Tea list. The typical feature possessed by Huangshan Maofeng green tea is the leaves' color. The color of the tea liquor is jade green and a light flowery fragrance is presented. The area that grow Huangshan Maofeng green tea is the mountain districts of Huangshan (Yellow Mountain), where many other famous types of green tea was grown as well. The English translation of Huangshan Mao Feng tea is "A tiny sharp peak of Huangshan Mountain" due to the tiny white parts at the top and a similar general shape of mountain peak of the leaves. Before China's Qingming Festival in the early spring, is said to be the optimum time to pick Huangshan Maofeng green tea. New leaves and buds are selectively picked during the tea picking process. The best or high grade of HSMF tea is usually labelled as "tè" (T), which means the special high grade of tea. The HSMF tea could be rated as six grades, "T1, T2, T3, 1, 2, 3" ordered from the high to low grade. The price of HSMF tea is highly correlated to its grade, which is mainly depended on the sensory evaluation by tea specialists.

1.2 Green Tea Extracts:

Common green tea fusion consists of over 99% of water, with a lack of significant

nutrient content (Table1.1) but includes polyphenols, such as catechins and other compounds such as caffeine.

Nutritional value per 100 g (3.5 oz)	
Energy	4 kJ (0.96 kcal)
Carbohydrates	0 g
Fat	0 g
Protein	0.2 g
Vitamins	**Quantity %DV**†
Thiamine (B1)	1%
	0.007 mg
Riboflavin (B2)	5%
	0.06 mg
Niacin (B3)	0%
	0.03 mg
Vitamin B6	0%
	0.005 mg
Vitamin C	0%
	0.3 mg
Minerals	**Quantity %DV**†
Calcium	0%

	0 mg
Iron	0%
	0.02 mg
Magnesium	0%
	1 mg
Manganese	9%
	0.18 mg
Potassium	0%
	8 mg
Sodium	0%
	1 mg
Other constituents	**Quantity**
Water	99.9 g
Caffeine	12 mg

%DV: Percentage of daily value

Table 1.1 Nutrition value table for brewed, regular green tea linked to USDA report

Green tea polyphenols take up about 30% of the solid extract and the major constituent are classified as catechins, including epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG) (Khan & Mukhtar, 2013). The potential chemical as well as biological effects of catechins have been studied and

analyzed in vivo (Johnson & Williamson, 2003). Compounds of flavan-3-ols refer to a main category of polyphenols in green tea. Other components including flavonoids, which primarily known as kaempferol, quercetin, and myricetin, and also caffeine, theanine and phenolic acids are also presented. Table 1.2 below gives the main polyphenolic compounds in green tea.

Polyphenolic compounds	**Green tea**
Catechins	31–40
Flavonols	6–10
Other flavonoids	2–5
Theogallin	1–3
Gallic acid	0.5
Quinic acid	2
Theanine	4–7
Methylxanthines	6–9
Theaflavins	–
Thearubigens	–

Table 1.2 Polyphenolic compounds in green tea (% w/w of extract solids)

The major components of green tea catechins consist of epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) (Rains et al., 2011Ahmad and Mukhtar, 1999). Figure 1.2 below shows the chemical structures of these primary catechins.

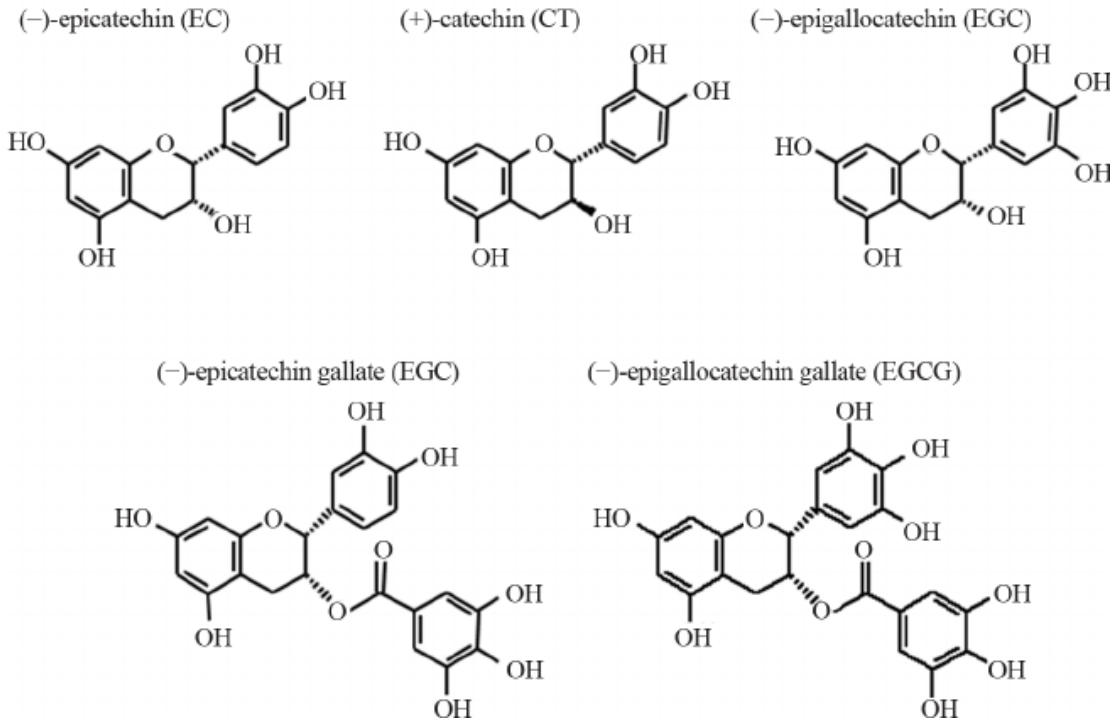


Fig. 1.2 Chemical structure of major green tea catechins. EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin gallate

Comparing to the other types of tea, for example oolong tea and black tea, higher levels

of catechins are presented in green tea (Khokhar & Magnusdottir, 2002). Except for the catechins, other polyphenolic compound groups are also identified in green tea, such as flavonoids (Figure 1.3) and phenolic acids.

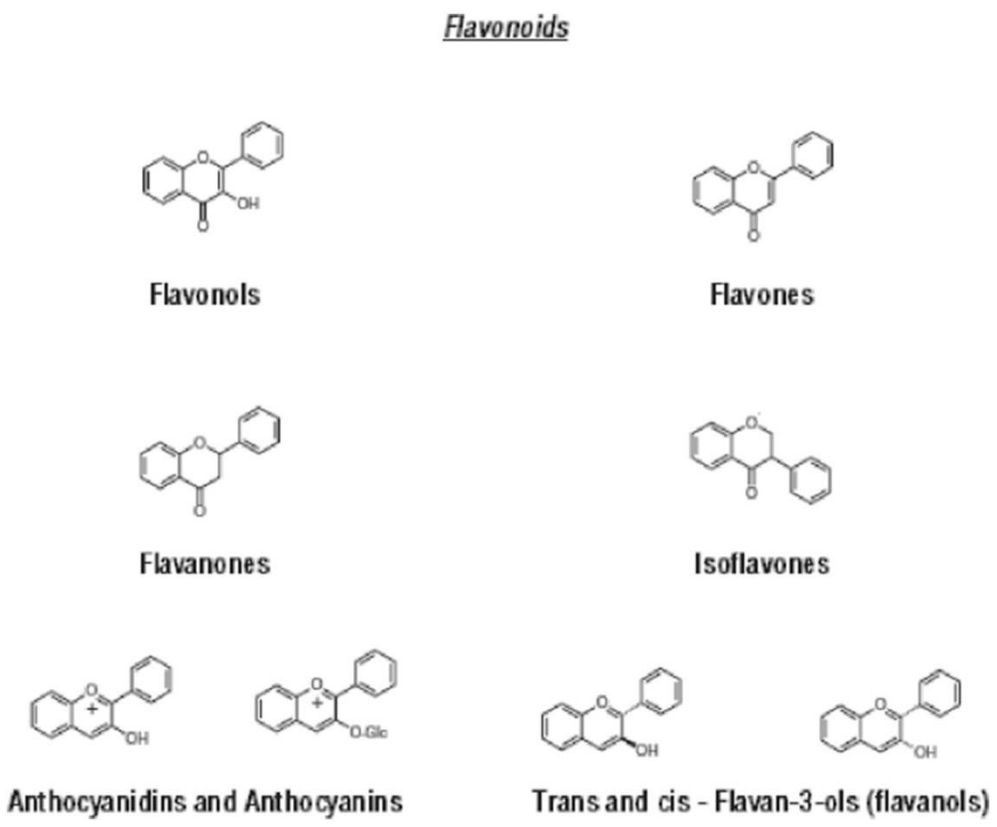


Figure 1.3 Chemical structure of flavonoids

Gallocatechin, epicatechin, epigallocatechin, catechin gallate, epicatechin gallate, gallocatechin gallate, and epigallocatechin gallate together composed flavonols. The differences within each flavonol compound are indicated by their extent of alkylation

and glycosylation as well as their own hydroxyl groups in different numbers and special arrangements. The unsaturated heterocyclic ring, a 3-positioned hydroxyl group, and an C4-postioned oxygen atom are the characteristic features of flavonol compounds. Major components of flavonols include quercetin, kaempferol, and myricetin (Figure 1.4).

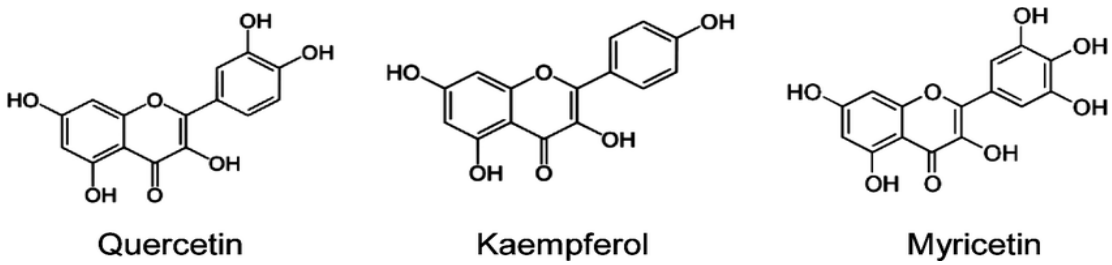


Figure 1.4 Structure of quercetin, kaempferol and myricetin

The major components of phenolic compounds are generally categorized into hydroxybenzoic and hydroxycinnamic acids. In addition, there are also phenolic acids which are phenols attached by one carboxylic acid group within the structure. The expression level of these phenolic compounds in green tea are very low.

1.3 Health benefits:

The health promotion effects of green tea have been broadly studied within recent years. There have been numerous positive impacts on health reported in various types of studies. Within decades, many interest has been gained in accordance to researches that conclude the beneficial impacts on health through consumption of green tea polyphenols (Chen & Chan, 1996). Similar researches indicated that polyphenols are the major contributors for the health promotion effects of green tea (Graham, 1992).

The antioxidant property of green tea polyphenols is the well-known property that are widely reported. Several studies provides the conclusion that very high level of antioxidant activities can be observed from isolated green tea flavanols (Gramza et al., 2005, Zhang et al., 2004). The phenolic hydroxy groups that are presented in the B- and D-rings of the galloylated catechins (ECG and EGCG) and in the B-ring in ungalloylated catechins (EC and EGC) are major contributors to green tea's antioxidant properties (Salah et al., 1995). In addition, the metal-chelating properties of green tea catechins as well as the 3,4,5-trihydroxy B-ring structure were also demonstrated to be responsible for the antioxidant properties of green tea polyphenols (Valcic, Muders, Jacobsen, Liebler, & Timmermann, 1999). It is also reported in the literature that the defense against multiple types of oxidative damage as well as overall antioxidative status within human body can be promoted through the intake of a balanced diet added green tea polyphenols (Erba et al., 2005). In addition, a two to fourfold in antioxidant activity level than that of a typical antioxidant compound, α-tocopherol at the same

concentration can be observed for green tea catechins. The inhibition effects on oxidation process in various types of meat and muscles were generally presented (Tang, Sheehan, Buckley, Morrissey, & Kerry, 2001). Researches were also done on marine oils, where higher antioxidant activity can be observed from a green tea polyphenol extract over typical antioxidants such as α-tocopherol, BHT and BHA at 200, 500, and 500 ppm (Wanasundara & Shahidi, 1998).

Other than the antioxidant effects, green tea polyphenols still show positive effects on cardiovascular diseases. It is demonstrated by epidemiological studies that the antioxidant effect presented by green tea are important contributors to the beneficial effect on cardiovascular diseases. In addition, it is demonstrated by Higdon and Frei (2003) that the antioxidant activity of blood serum could be increased by the consumption of green tea. Furthermore, there were numerous studies indicating that the level of blood cholesterol can be reduced by consumption of green tea linked with a low-fat diet.

Green tea might also present benefits in the prevention and control over diabetes.

Previous study indicated that, green tea extract contributes to inhibit the developing process of hyperglycemia, insulin resistance, and other defects in metabolism when administered to rats with a fructose induced diet (Wu et al., 2004b).

Green tea polyphenols are also displaying anti-carcinogenic features which are gaining

rising attention of researchers. There were numerous studies over the discovery of the potential relationships between the consumption of green tea and cancer. The anticarcinogenic properties of green tea were majorly determined by the catechins, of which EGCG presented as the major contributor (Mukhtar & Ahmad, 1999). In addition, researchers conducted an observation on over 100 patients suffering from confirmed prostate cancer and 250 ordinary patients. The result surprisingly indicated a reduced risk of getting prostate cancer with a diet associated with continuous consumption of green tea on a daily basis (Jian, Xie, Lee, & Binns, 2004). In addition, researchers in Japan found that by drinking green tea of 750mL or more on a daily basis is likely to produce an inhibition effect over the development of early-stage tumors (Inoue et al., 2001).

Green tea also possesses anti-viral and antibacterial activities. According to relevant researches, green tea catechins are contributors of vital importance in inhibiting the activity of several vital enzymes associated with microbes (Okamoto, Leung, Ansai, Sugimoto, & Maeda, 2003). It is also suggested that one of the representative catechins, EGCG, showed protein inhibition effects over the activity of tyrosine phosphatase (PTPase) activity in *Proveta intermedia* at 0.5 μmol, and related species at 5 μmol.

1.4 Overall Sensory Study of Green Tea

In the tea market, it is by the tea specialists and analysts who set the quality for green tea products based on general evaluation and determination. The main aspects that are taken into consideration by the tea specialists and analysts include the appearance of tea leaves and buds, and also the aroma, taste and color of the tea infusion. Among these aspects, the taste of tea infusion can be mainly characterized into four categories, which are bitterness, astringency, sweetness, and umami.

Based on the information above, it seems that only tea specialists and analysts who are well-trained for years with sensory evaluation of green tea are the ones who can depend on. In addition, although when describing the taste of green tea, tea specialists and analysts will be using normative words and expressions with standards, it is still likely that many consumers finding it hard to comprehend. In order to overcome these disadvantages, plenty of works have been done to discover the relationships between the sensory quality and chemical components of different types of tea. The targets were to find objective approaches to assess or determine the quality attributes in sensory of tea that can be used to replace the temporary evaluation methods (Fraser et al., 2014, Chen et al., 2008, Daglia et al., 2014, Sliwinska et al., 2014).

Based on previous studies conducted by researchers, it is the chemical components including polyphenols, alkaloids, organic acids, amino acids, pigments, and also volatile compounds that contribute to the quality aspects of tea (Ho et al., 2015, Stodt and Engelhardt, 2013, Zhang and Ruan, 2016). Take chlorophylls as examples, it is well known that chlorophylls are the main reason that leaves including tea leaves getting their color. However, the chlorophylls obtained from green tea associated with quercetin have a large influence on the color of tea brew (Wang, Park, Chung, Baik, & Park, 2004). Flavonoids, which include catechins and flavonoid glycosides were analyzed to be responsible for the astringency taste of tea (Scharbert and Hofmann, 2005, Masataka et al., 2010 and Dai et al., 2015). In addition, proteins that are presenting in tea, of which is characteristic in tea, theanine, is suggested to be the main contributor to the umami taste of tea infusion (Kaneko et al., 2006, Feng et al., 2014). While the quality attributes of tea are evaluated from aspects of appearance, color, aroma and taste, a systematic identification on the potential link between chemical compositions and sensory quality values of tea is required (Daglia et al., 2014, Fraser et al., 2014, Ho et al., 2015, Stodt and Engelhardt, 2013, Zhang and Ruan, 2016).

1.5 Astringency and Bitterness Study of Green Tea

Among the quality attributes of green tea, the green tea infusion’s taste is no doubt the

most important factor associated with consumption. There are three levels of taste interactions that can be observed: chemical level, oral physiological level and cognitive level (Keast & Breslin, 2002), with the first two levels being commonly observed in green tea (Yu et al., 2014, Yin et al., 2014). Bitterness, astringency, umami and a unique type of sweet aftertaste together build up the general taste of green tea infusion (Yu et al., 2014, Zhang et al., 2016). Astringency is defined as a tactile sensation felt on the tongue caused by the interaction between polyphenols and salivary proteins (Yu et al., 2014), which has been proved to have significant impact on the tea brew taste (Yin et al., 2014). Combined with bitterness are the vital sensory features of green tea brew (Zhang et al., 2016). There have been studies reporting the quantitative evaluation for astringency and bitterness of oolong and black tea infusions (Hayashi, Ujihara, Chen, Irie, & Ikezaki, 2013). Nevertheless, researchers are still working hard to develop scientific approaches for quantitative determination of bitterness and astringency of green tea infusions.

Previous researches suggested that catechins are mainly responsible for the astringency and bitterness of green tea (Zhang et al., 2016, Narukawa et al., 2010). Epicatechins, which include (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG) are a major group of components of green tea polyphenols (Wu, Xu, Heritier, & Andlauer, 2012), among which EGCG suggested as the strongest one (Jin, Ma, Ma, Yao, & Chen, 2014). Though

presenting as isomers, the properties of epicatechins and non-epicatechins are quite differ to some extent and they lead to different levels of sensory attributes (Scharbert & Hofmann, 2005). For example, at the same concentration, the solution of EC gives a higher level of bitterness and astringency compared to that of a C solution (Thorngate & Noble, 1995). In addition, astringency and bitterness of epicatechins with variations with special arrangements and molecular structures presents markable difference. For instance, gallated catechins produce a significantly higher level of astringency and bitterness than non-gallated types (Zhang et al., 2016), among which ECG presenting the highest level of bitterness and astringency (Narukawa et al., 2010). It is still required for the determination of astringency and bitterness levels of green tea quantitatively.

Besides catechins, other compounds are also contributing to the astringency and bitterness of green tea, which are caffeine as reported in previous researches and flavonol glycosides (Zhang et al., 2015). One type of astringency is formed in front of the upper teeth and at the tip of the tongue is defined as velvety astringency. Another type of astringency taste leads to a reflex reaction of tongue and cheeks against a dry and tactile sense felt in the oral cavity is described as puckering astringency (Gonzalodiago, Dizy, & Fernándezzurbano, 2013).Velvety astringency in green tea is suggested to form from flavonol glycosides (Zhang et al., 2015), while puckering astringency might present with catechins as the major contributor (Scharbert & Hofmann, 2005). There are other components in green tea which also have effects on

the astringency and bitterness of green tea infusion. For instance, Rutin displays a promoting effect on the caffeine-produced bitterness, while caffeine contributes to EGCG's astringency attribute (Xu, Li, Zhang, Tang, & Wan, 2010). Also the presence of Ca^{2+} is suggested to increase the astringency level while decline the bitterness level of EGCG solution (Yin et al., 2014). Since the interaction within the taste contributors are complicated, further studying is necessarily required.

The relationship within the sensory of tea and contained chemical components is really complex and the relationship can never be scientifically and supportively proved by targeted determination of particular components while lacking data of non-targeted compounds (Daglia et al., 2014, Fraser et al., 2014). Recently, we just studied the metabolomics of black tea and the chemical contributors of glucose-producing enzymes inhibition effect. Similarly, the metabolomics can also be used in exploring the marker compounds, especially the minor or trace composition of tea under different treatments, or from different geographical locations. Combining with the chemical standards, tea plant chemistry database, and mass fragment regulations of typical types of secondary metabolites of tea plant, many marker compounds have been identified from tea. To find the grade-related compounds of tea, metabolomics could also be a potent tool.

1.6 Metabolomics Study

The definition of metabolomics is referred as the systematic analysis of the identification and quantification of metabolites occurring in biological systems, such as a cell, tissue, organ and etc. A metabolic profile is obtained during metabolomics analysis with the application of analytical tools and data processing software to observe the state of metabolites of selected samples. Just like we focus on DNA and genetic information in case of genomics study, RNA and information of mRNA expression in transcriptomics study, we focus on the metabolites and their state, with which particular factors and variables involved, in metabolomics study (Dayalan et al., 2019).

The application of metabolomics study has spread to multiple fields and agriculture is one representative. With the increase of population in today's world, the demand for higher amount of farm production becomes more and more necessary. By applying metabolomics approaches, we can obtain important information indicating the biochemical status at specific time points of genetic modified crop strains, thus enabling us to set up critical control over the growth and harvest process for higher quality and quantity in production. Moreover, since numerous of primary and secondary metabolites are presenting in plants, and with traditional approaches it is only possible to identify up to 400 metabolites at a time, the application of metabolomics in plant is

of vital importance by enabling us to identify and determine thousands of plant metabolites at specific time points.

Metabolomics is also successfully applied in the discovery of biomarkers, which are compounds responsible for the separation of set of samples with certain level of similarity. For instance, if there are two groups of samples with one suffering from particular stress and the other being the control group, by discovering the biomarkers that distinguish samples within the two groups with metabolomics approaches, it is easy and clear to identify the compounds that mainly contribute due to stress or occurring without stress control.

Personalized medicine is a new scientific field which is drawing increasing attention of the public. In the diagnosis process it would be essential if the characteristic signal of certain decease could be detected in time, leading to a higher efficiency in offering a cure or solution to the situation. Metabolomics study provides with a perfect tool which can contribute to identifying and determining of over thousands of compounds at specific time points so that the decease state to some extent could be observed rapidly if important markers in significant level could be observed.

The two technology platforms that are used most widely in metabolomics study are mass spectrometry (MS) and nuclear magnetic resonance (NMR). MS-based metabolomics is highly sensitive when applied to the analysis of massive quantities of metabolites thus provides essential benefits and convenience. When there is a lack of related standards for quantification, liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) is available to present required information in adequate quantities over the change in patterns of metabolites in the whole metabolic system (Burgess, Rankin & Weidt, 2014). Although the sensitivity of NMR metabolomics is in a lower level comparing to MS-based metabolomics, the outstanding capability of identifying structures of metabolic compounds makes NMR metabolomics another useful tool for effective analysis. (Burgess, Rankin & Weidt, 2014).

Statistical analysis is a very important part in metabolomics study. There are generally two types of methods on stat analysis which are categorized into univariate statistical analysis and multivariate statistical analysis. The main target of multivariate statistical analysis is dealing with data which contain various variables and through analyze providing the correlations between all variables. The difference between univariate statistical analysis and multivariate statistical analysis is that the former only focuses on one variable at a time. In MS-based metabolomics study, the essential variables that are taken into consideration are the m/z ratio and signal intensity. Integrated signals that

meets the requirement of research in nuclear magnetic resonance-based metabolomics are selected. Statistical tools were applied to analyze the intrinsic differences of data obtained from each sample groups, common analyzing models used are hierarchical cluster analysis (HCA), principal component analysis (PCA) and partial least squares projection discriminant analysis (PLS-DA). With the assistance of these statistical models in capturing information from sample data, metabolomics study is capable of demonstrating a prediction and categorization of samples with particular properties.

1.7 Previous Study on Tea Metabolomics

The chemical component analysis in quality and quantity are recommendable methods to determine tea types and tea grades (Zuo, Chen, & Deng, 2002; Del Rio et al., 2004; Sereshti, Samadi, & Jalali-Heravi, 2013; Memic, Mahic, Zero, & Muhic-Sarac, 2014). In accordance to the level of non-volatile compounds, such as purine alkaloids (Zuo et al., 2002; Del Rio et al., 2004), free amino acids (Ananingsih et al., 2013) and metal (Memic et al., 2014), as well as the volatile compounds (Sereshti et al., 2013), it is not hard to distinguish non-fermented types of teas, for example, green tea and white tea from the fermented types, for instance, black tea. The majority of related researches have targeted on teas with significant distinction in appearance and extent of fermentation. Nevertheless, for the tea types that show no obvious difference there is

limited study and research, especially for the non-fermented types of tea. The presence of multiple high-tech analytical platforms, such as high performance liquid chromatography (HPLC) (Ananingsih et al., 2013), gas chromatography–mass spectrometry/flame ionization detection (GC–MS/FID) (Sereshti et al., 2013), ultra-performance liquid chromatography (UPLC-MS) (Dai et al., 2017; Del Rio et al., 2004), FT-IR spectrophotometry (Ren et al., 2013), electrospray ionization ion mobility spectrometry (ESI-IMS) (Memic et al., 2014), and the electronic tongue technique (Scharbert, Jezussek, & Hofmann, 2004), has made it viable to categorize various types of tea according to their chemical component content. The broad application of these analytical approaches contribute a lot to metabolomics analysis (Lee et al., 2015; Dai et al., 2017). It is very difficult to scientifically identify and determine all of metabolites, including both primary and secondary from tea due to the huge amount in numbers and variety in properties. Therefore, conducting metabolomic analysis on different types of tea by applying multiple high-tech analyzing tools brings a lot convenience. In 2008, These metabolomics approaches were conducted on 96 samples of 3 non-fermented teas, which are white tea, yellow tea and green tea from China (Zhang et al., 2018). The identification and quantification of approximately six hundred metabolites were provided in the results. In addition, the samples of non-fermented types of tea were classified into 3 groups, in accordance to the analyzing result conducted through a partial least squares discriminant analysis (PLS-DA) model. Moreover, it was also suggested that the health promotion effects via the level of antioxidants of these three non-fermented types of tea is different mainly due to variation in their metabolic

components, such as ascorbate and vitexin.

One of the most commonly used analytical method (Dettmer, Aronov, & Hammock, 2007), liquid chromatography with mass spectrometry (LC-MS) is capable of identifying chemical compounds with large differences in molecular weights (Snyder et al., 2013, Kumazoe et al., 2015, Yang et al., 2016). The application of this effective tool has been conducted on studies of the relationship between harvesting conditions and sensory attributes of different types of tea (Dai et al., 2015), green tea catechins and related metabolite contents (Matabilbao et al., 2007) and shift in metabolite status during microbial fermentation process of Fu brick tea (Xu, Hu, Wang, Wan, & Bao, 2015). In 2018, this effective metabolomics tool was used to identify and determine metabolites from Pu-erh teas with various periods of storaging and manufacturing locations (Wang et al., 2018). 38 marker compounds were identified to differentiate the storaging time of Pu-erh teas due to partial least squares analysis with a desirable recognition rate. 19 characteristic compounds identified were capable of distinguishing ripened teas from the raw. Significant correlation between manufacturing locations and local water compositions were shown as a result of cluster analysis. This study offers instruction for distinguishing Pu-erh teas and contributes to set a mature and scientific tea market.

According to recent researches, some metabolomics studies on the classification of white tea with different types were conducted. In 2016, Ning et al. identified 29 main marker compounds quantitatively for four types of white tea by using metabolomics method. The conclusion being that it is not likely to distinguish white of different types though only one particular marker compound. In 2017, Tan, Engelhardt, Lin, Kaiser, and Maiwald not only identified 29 chemical compounds in quantity from white tea of three types, but also within four categorized groups of white tea demonstrated declined levels of catechins, phenolic acids, theanine, hydrolysable tannins and caffeine; on the contrary, increasing levels of flavonol glycosides and theaflavins were observed. Moreover, a significant correlation between white tea types and their producing seasons was indicated according to the results. In 2019, non-targeted metabolomics analysis was conducted to reveal distinguished metabolite composition among Bai Mudan white tea of four different grades (Yue et al., 2019). Liquid chromatography combined with mass spectrometry was applied in the research. The results indicated a high similarity in chemical composition for the two highest grades of white tea, while the results obtained from partial least square-discriminant analysis model was capable of providing supportive explanations. An overall of 93 compounds were determined with structure, among which 21 compounds presented significant difference within different grades of samples, thus indicating themselves as the marker compounds of Bai Mudan white tea

Research Objectives

2.1 Research Objectives

1. To reveal the critical compounds responsible for the grade classification of Huangshan-Maofeng green tea through LC-MS based metabolomics study.
2. To explore the potential relationship between sensory evaluation and critical compounds responsible for the grade classification of Huangshan-Maofeng green tea.

Experimental

3.1 Samples

All of the green tea samples were produced in the Xieyuda Tea Factory in the spring of 2018, at Huangshan city, China. All green tea samples were rated by tea masters according to the taste, flavor, color and appearance of tea infusion. The grade of green tea could be classified as T1, T2, T3, 1, 2, and 3. To produce the green tea from fresh tea leaves, briefly, the fresh tea leaves undergo three steps including fixation, rolling and drying.

3.2 Chemicals

Gallic acid (GA,>98%), caffeine (> 98%), theobromine (> 98%), (+)-catechin (C,>98%), (−)-epicatechin (EC,>98%), (−)-gallocatechin (GC,>98%), (−)-epigallocatechin (EGC,>98%), (−)-gallocatechin gallate (GCG,>98%), (−)-epigallocatechin gallate (EGCG,>98%) and (−)-epicatechin gallate (ECG,>98%) standards were purchased from Shanghai Tongtian Biotechnology company (Tongtian,

Shanghai, China) and Shanghai Yuanye Biotechnology Company (Yuanye, Shanghai, China) with their structure and purity confirmed by our laboratory. Rutin (> 98%) standard, β-glucogalin standard (> 98%), tannase, α-amylase and α-glucosidase were purchased from Shanghai Yuanye Biotechnology Company (Yuanye, Shanghai, China). HPLC-grade acetonitrile was purchased from TEDIA Company Inc. (Fairfield, OH, USA), and distilled water was used as mobile phase of HPLC. Other reagents were of analytical grade.

3.3 Method

3.3.1 Sample Preparation

HSMF green tea sample of each grade was milled and weighed for 0.1 g accurately, then transferred into 10 mL conical flasks with cover separately. 3mL of methanol was added into the conical flask and extracted twice by ultrasonic extraction at room temperature for 10 min followed by a 10-min centrifuging at 8000 rpm. The extracts were combined and accurately diluted with methanol to 10 mL. Each extract was filtered through a 0.22 μm Millipore filter.

3.3.2 Astringent scoring

A sensory panel constituting of nine panelists (4 males, 5 females) who do not smoke or addict to alcohol were recruited in the astringent evaluation for different grades of green tea. Before test, all panelists received specific training to recognize and score the intensity of astringency with various concentrations of EGCG solution (0.025, 0.25, 0.5, 1.0, 2.0, 4.0 g/L). A 12-point scale ranging from 0 (not detected) to 12 (very intense) was used in the sensory analyses. Panelists were avoided of taste-odor interactions by using nose clips, and then rate the taste intensities of different grades of HSMF tea infusion, which was previously brewed with 150mL of deionized water for 3 g of tea samples.

3.3.3 The determination of total flavone and polyphenols

The determination of total flavone and polyphenols were conducted as the published methods (Rusak et al., 2008), and all samples were detected in triplicate.

3.3.4 The determination of tea polyphenols and purine alkaloids

The Agilent HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisted of Infinity binary pump, integrated vacuum degasser, autosampler, thermo-stated column compartment and diode array detector was used to determine tea polyphenols and purine alkaloids. The analytical column used was Agilent SB-Aq C18 reversed phase column (250×4.6mm i.d., 5μm) protected to a Phenomenex C18 guard column (10mm×4.6 mm, 5μm; Phenomenex, Torrance, CA, USA). The mobile phase was composed of methanol (A) and 0.2% (v/v) formic acid–water (B). The flow rate was 1.0 mL/min and detection wavelength was set at 278 nm. The gradient elution was as below: 0–5 min, 5–20%B; 5–18 min, 20–25%B; 18–25 min, 25–42%B; 25–32 min, 42%B; 32–40 min, 42–100%B; 40–42 min, 100%-5%B and 42–55 min, 5%B. The injection volume was 5 μL for each sample extract. The temperature of the column was kept constant at 30 °C. The contents of main compound of tea were determined according to the published method with minor changes (Zhang et al., 2017).

3.3.5 LC-Q-TOF-MS

Chromatographic separation of HSMF green tea extract was conducted using a Acquity

UPLC shield RP-18 column (50mm×2.1 mm, 1.7μm) equipped with an Acquity UPLC C18 guard column (Waters, Milford, MA, USA) at a flow rate of 0.3 mL/min, and the column was thermostated at 30 °C, while the detection wavelength was selected at 278 nm. The mobile phase consisted of 0.1% formic acid in water (v/v) (A) and acetonitrile (B), and with the gradient elution at 0–5 min: 5–15% B; 5–8 min: 15–30% B; 8–13 min: 30%B; 13–23 min: 30–88%B; 23–28 min: 88–95%B; 28–30 min: 95%B; 30–33 min: 95–5%B; 33-35min: 5%B. The injection volume was 2.0μL and gas temperature is set as 320℃, with gas flow set as 8 L/min, nebulizer set as 35psig, sheath gas temperature set as 350℃, sheath gas flow set as 11 L/min. Metabolites were detected by full scan mass analysis from m/z 100–2000 at a scan rate of 1.00 spectra/sec.

3.3.6 Metabolomics analysis

Principal Component Analysis (PCA) was used to analyze the overall data using Simca-p software (Umetrics AB, Umea, Sweden). Experiment data obtained from LC-MS analysis were initially summarized and anomalous data were found. The data can be subsequently analyzed with Hierarchical Cluster Analysis (HCA) through the results of the PCA. The supervised orthonormal partial least squares discriminant analysis (OPLS-DA) was carried out while taking tea grade as the solely Y variable. This model can observe the influence of variable importance (VIP) and S-plot, to find a contribution

to the classification of the main marker compounds.

3.3.7 The quantitative analysis of marker compounds

The determination of procyanidins was conducted by using published method. The determination of flavonoid glycosides refereed the method developed by Guo et al. Briefly, the quantitative analysis was proceeding on Agilent 1260 series UHPLC system (Agilent Technologies, Palo Alto, CA, USA) containing an auto-injector, a quaternary solvent delivery system. The chromatographic separation was carried out by Acquity UPLC shield RP-18 column (50mm×2.1mm, 1.7μm) guarded with an Acquity C_{18} guard column (Waters, Milford, MA, USA) at a flow rate of 0.3 mL/min at 40 °C of column temperature. The mobile phase and mass parameters are same as published method of LC-QQQ-MS (Guo et al., 2018).

Multiple reaction monitoring (MRM) mode was used to determine the compounds by product ions from the parent ions (procyanidin dimer EC-EC, 577→407; procyanidin dimer EC-EGC, 593→407; procyanidin dimer EC-ECG, 729→407; kaempferol-rhamnose-glucose-glucoside, 755→285; quercetin-rhamnose-glucose-glucoside, 771→300), through the MRM mode. The contents of procyanidins, flavonoids

glycosides and hydrolysable tannins were calculated as the calibration curve of procyanidin B2, rutin and glucogallin respectively.

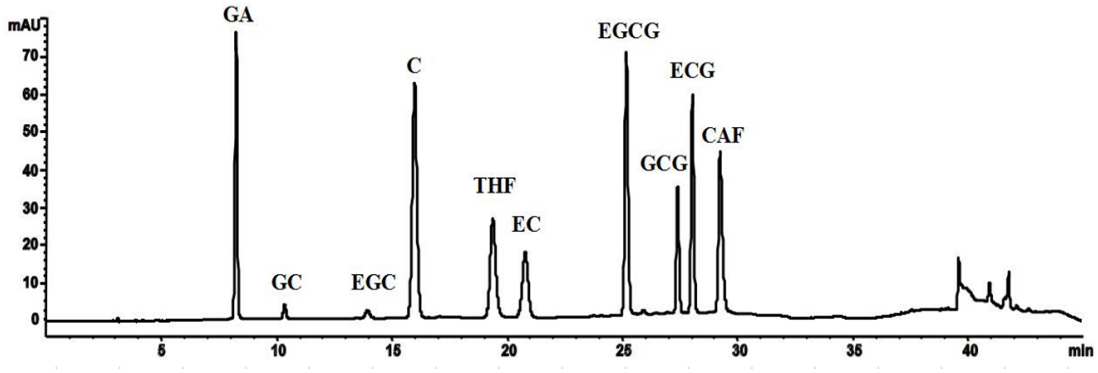
3.3.8 Statistical Analysis

Results were expressed as mean ± SD, with the number of determinations (n=3) representing separate experiments. Data were evaluated at a 0.05 level of significance with one-way ANOVA with post hoc testing by Fisher's protected least significant differences procedure.

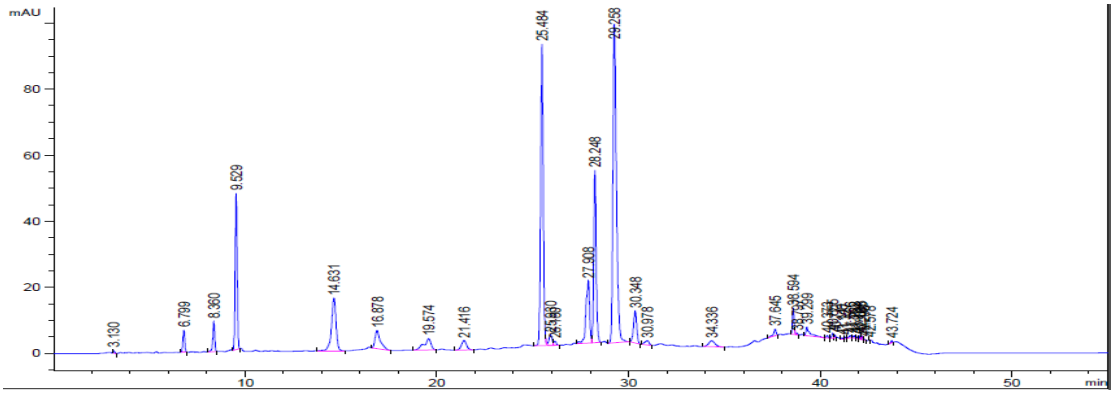
Results

4.1 HPLC Analysis of main compounds of Huangshan Maofeng Green Tea

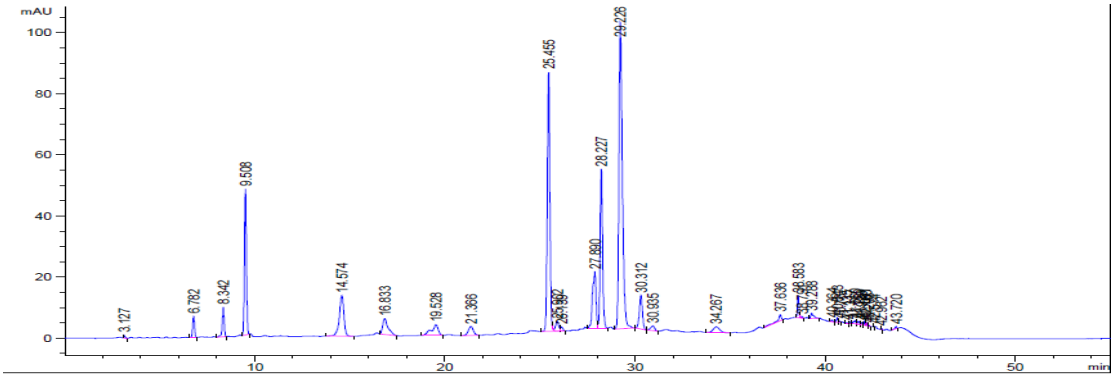
The HPLC chromatograph of 10 main compounds, gallic acid (GA), GC, EGC, C, theobromine (THB), EC, EGCG, GCG, ECG, caffeine (CAF) of our Huangshan Maofeng green tea samples is shown below in figure 4.1 (b), (c), (d), (e), (f) and (g). Major peaks were observed between 5 – 30 min and figure 4.1 (a) helps to confirm the retention time of each compound.



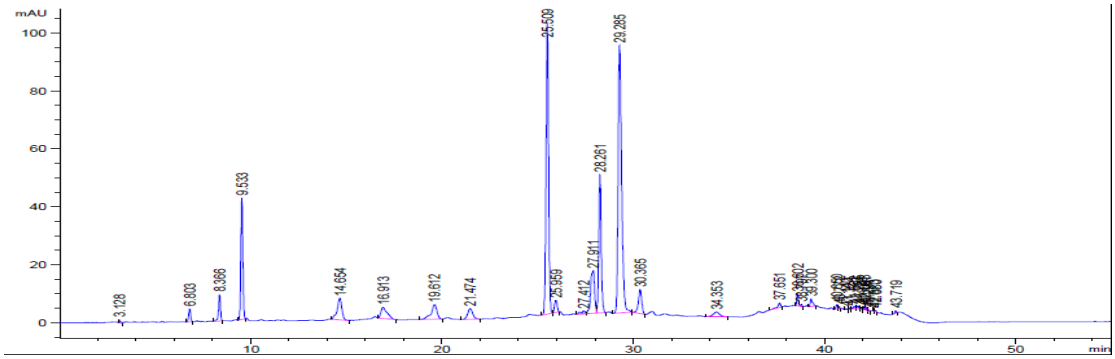
(a)



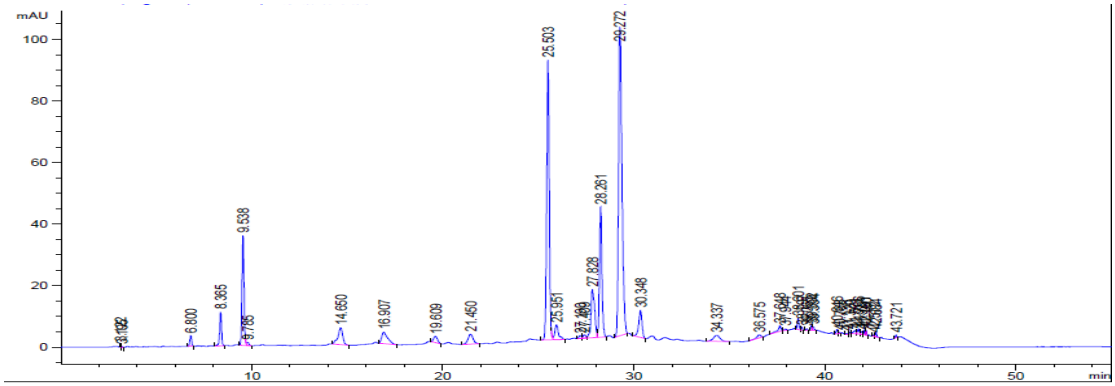
(b)



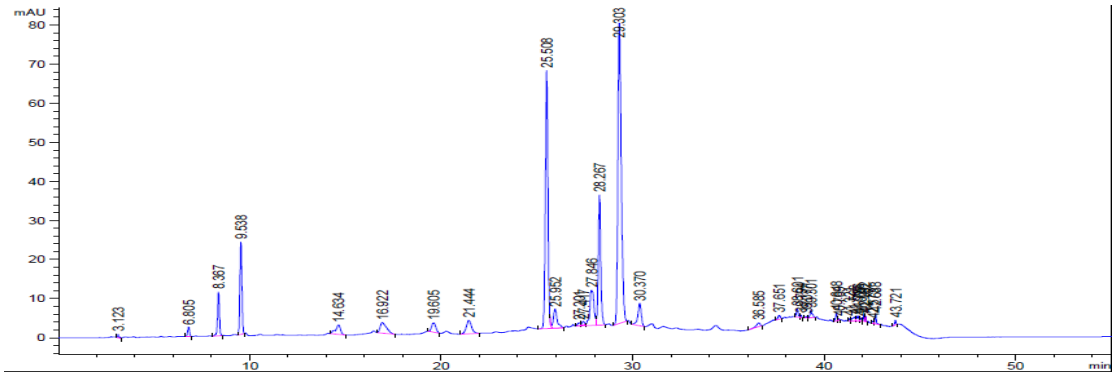
(c)


mAU
100
80
60
40
20
0
3.128
6.803
8.366
9.533
14.654
16.913
19.612
21.474
25.509
25.959
27.412
27.911
28.261
29.285
30.365
34.353
37.651
43.719
10
20
30
40
50
min

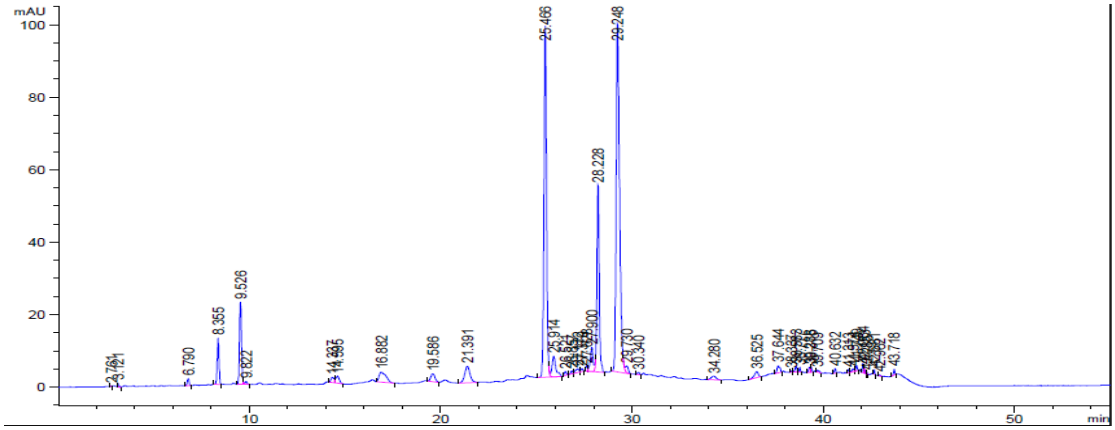
(d)


mAU
100
80
60
40
20
0
6.800
8.365
9.538
14.650
16.907
19.609
21.450
25.503
25.951
27.828
28.281
29.272
30.348
34.337
36.575
43.721
10
20
30
40
50
min

(e)


mAU
80
70
60
50
40
30
20
10
0
3.123
6.805
8.367
9.538
14.634
16.922
19.605
21.444
25.508
25.952
27.846
28.267
29.303
30.370
36.585
37.651
43.721
10
20
30
40
50
min

(f)



(g)

Figure 4.1 HPLC chromatograph of 10 main compounds of Huangshan Maofeng Green Tea samples. (a) The chromatograph showing retention time of 10 main compounds. (b) The chromatograph of grade T1 sample product. (c) The chromatograph of grade T2 sample product. (d) The chromatograph of grade T3 sample product. (e) The chromatograph of grade 1 sample product. (f) The chromatograph of grade 2 sample product. (g) The chromatograph of grade 3 sample product.

4.2 LC-Q-TOF-MS Result for Samples of Different Grades

Figure 4.2 shows the LC-QTOF-MS result. For each grade from six grades of Huangshan Maofeng green tea samples, 6 samples were taken into LC-Q-TOF-MS analysis. Experiment data obtained from LC-MS analysis were initially summarized and put into Simca-p software for further metabolomics analysis.

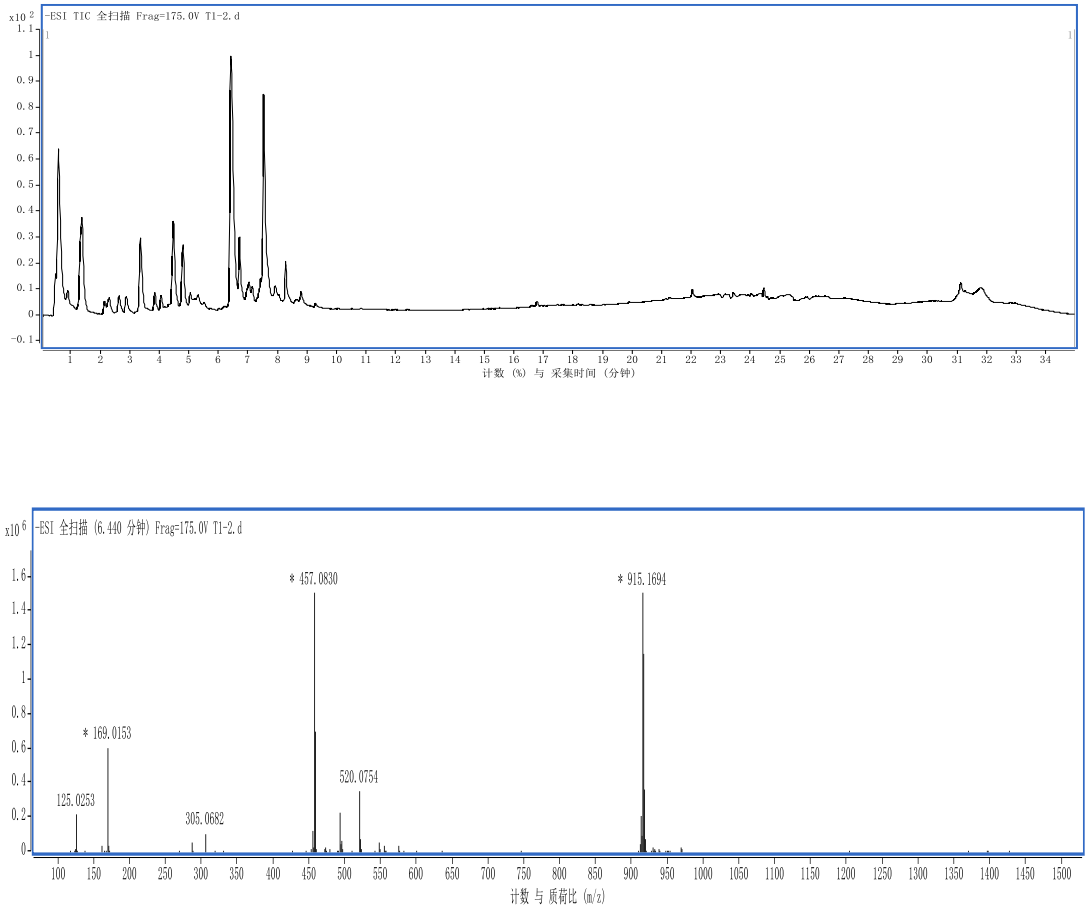
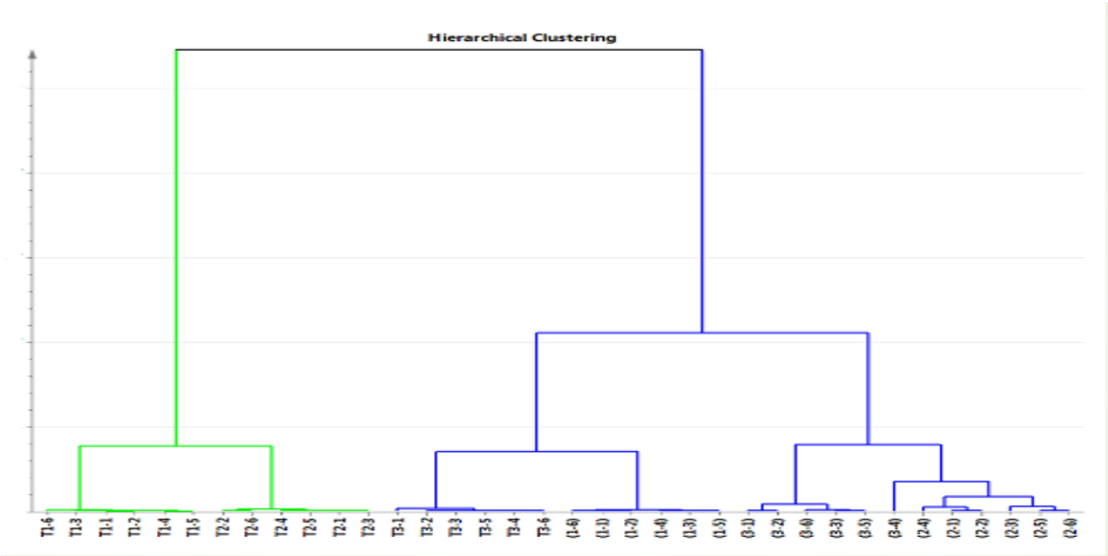


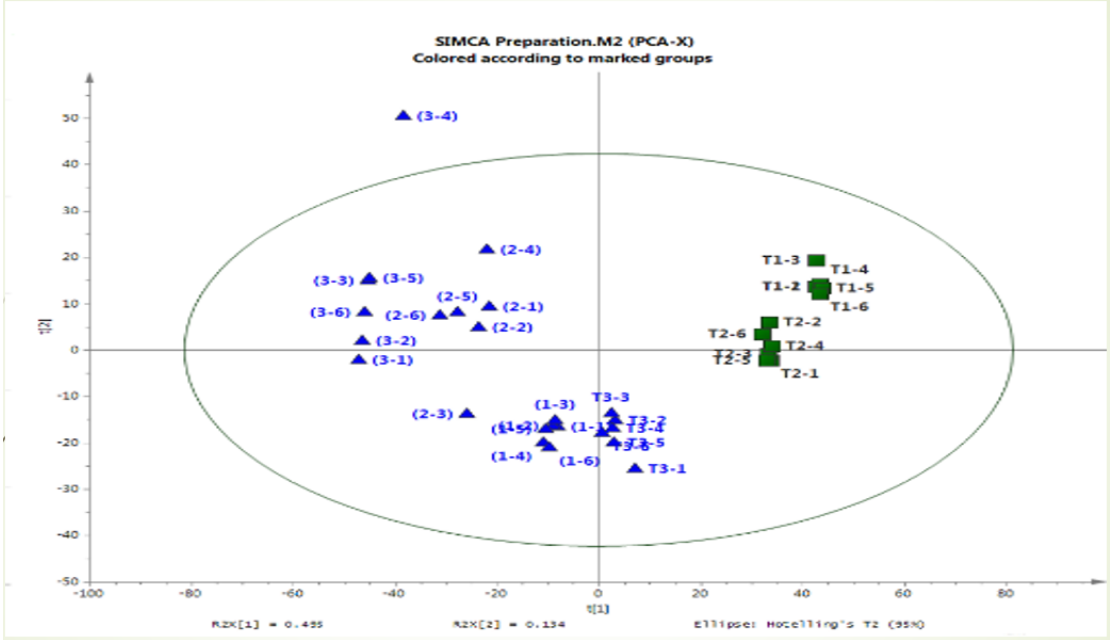
Figure 4.2 LC-Q-TOF-MS result for Huangshan Maofeng green tea sample

4.3 Metabolomics Analysis Result for Samples of Different Grades

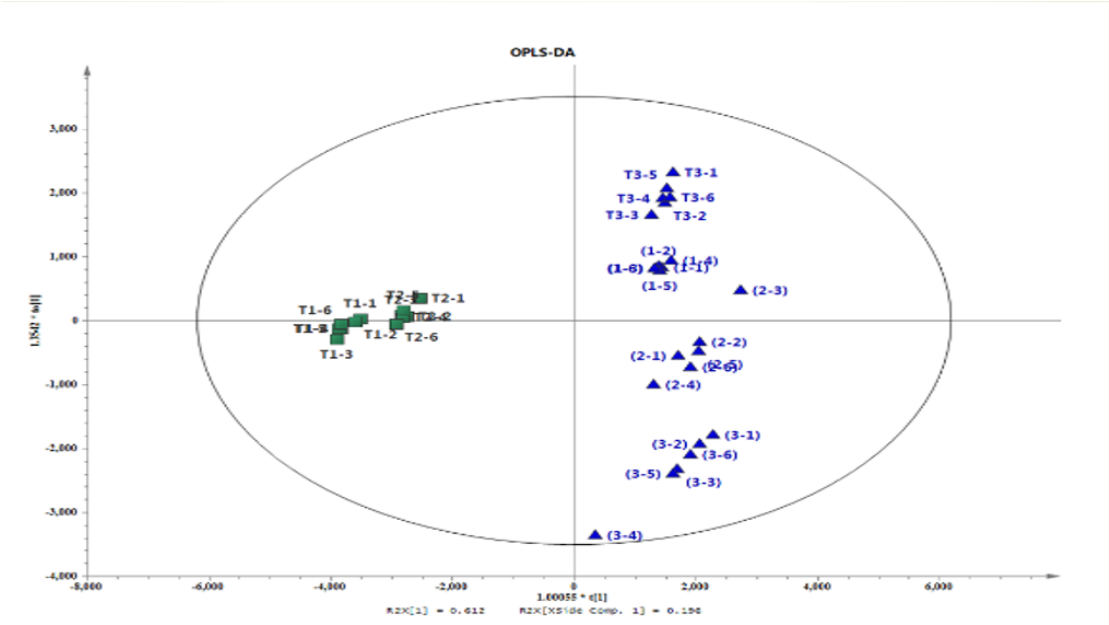
Experiment data obtained from LC-MS analysis were initially summarized and anomalous data were found in Simca-p software (Umetrics AB, Umea, Sweden). The data can be subsequently analyzed with Hierarchical Cluster Analysis (HCA) through the results of the PCA. The supervised orthonormal partial least squares discriminant analysis (OPLS-DA) was carried out while taking tea grade as the solely Y variable. This model can observe the influence of variable importance (VIP) and S-plot, to find a contribution to the classification of the main marker compounds. The results for clustering analysis through PCA, as well as score plots and S-plot from OPLS-DA model was given by Simca-p software and are shown in figure 4.3 below.



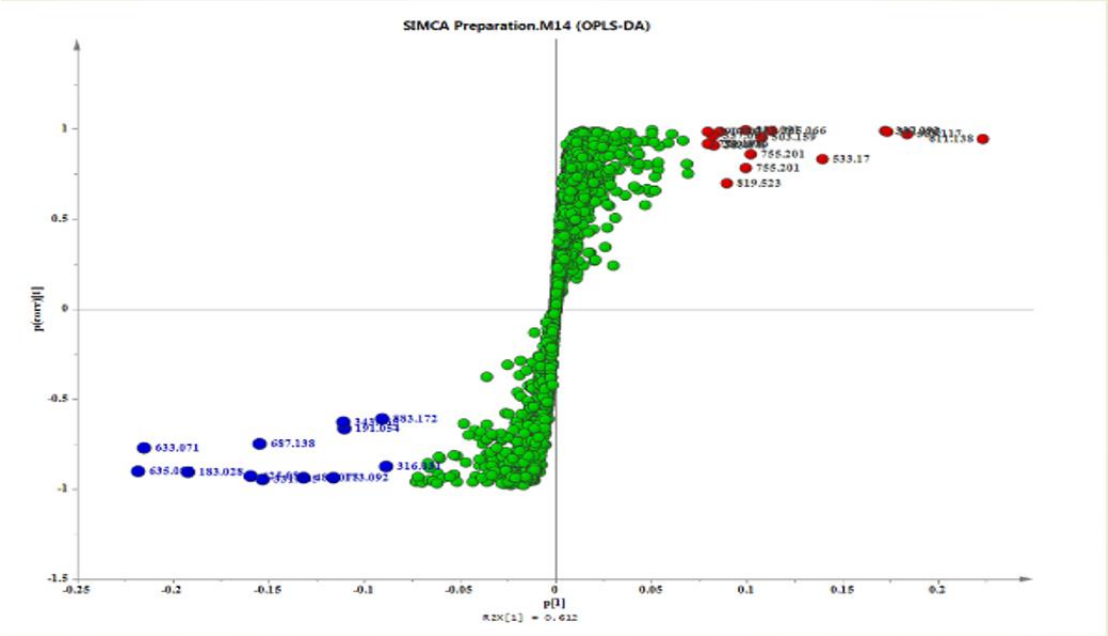
(a)


SIMCA Preparation.M2 (PCA-X)
Colored according to marked groups
t[1]
t[2]
R2X[1] = 0.495
R2X[2] = 0.134
Ellipse: Hotelling's T2 (95%)

(b)


OPLS-DA
1.00055 * t[1]
R2X[1] = 0.612
R2X[Xside Comp. 1] = 0.196

(c)

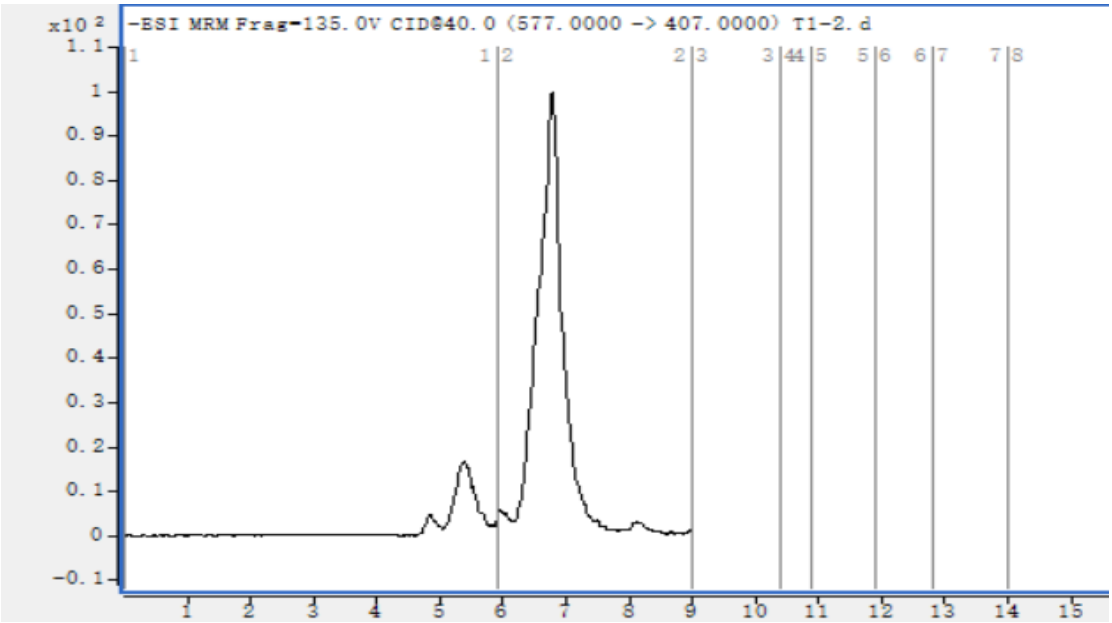


(d)

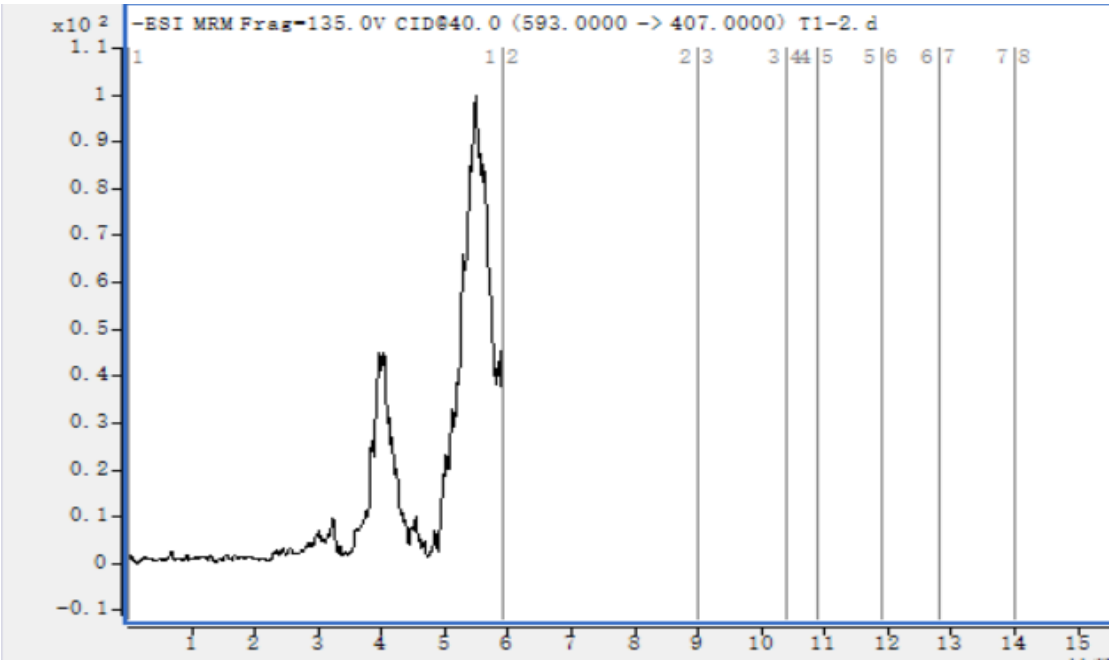
Figure 4.3 The classification of different grades of green tea (T1, T2, T3, 1, 2, 3) by LC-Q-TOF-MS based metabolomics and multivariate analysis. (a) The Clustering analysis based on LC-Q-TOF-MS metabolomics data. (b) Score plots of principle component analysis. (c) Score plots of orthogonal partial least squares discriminant analysis partial least squares analysis. (d) S-plot of high-grade and low-grade green tea (T1, T2 vs T3, 1, 2, 3).

4.4 LC-QQQ-MRM Results for the Determination of Procyanidins and Flavonoid Glycosides

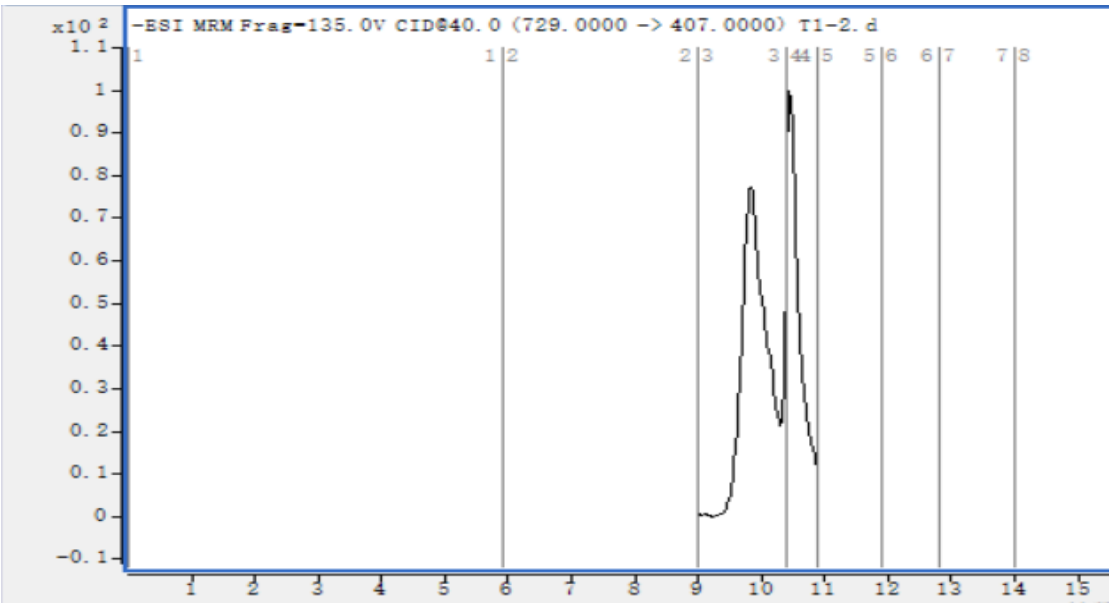
Multiple reaction monitoring (MRM) mode was used to determine the compounds by product ions from the parent ions (procyanidin dimer EC-EC, 577→407; procyanidin dimer EC-EGC, 593→407; procyanidin dimer EC-ECG, 729→407; kaempferol-rhamnose-glucose-glucoside, 755→285; quercetin-rhamnose-glucose-glucoside, 771→300), through the MRM mode. Figure 4.4 below shows the results for above compounds.



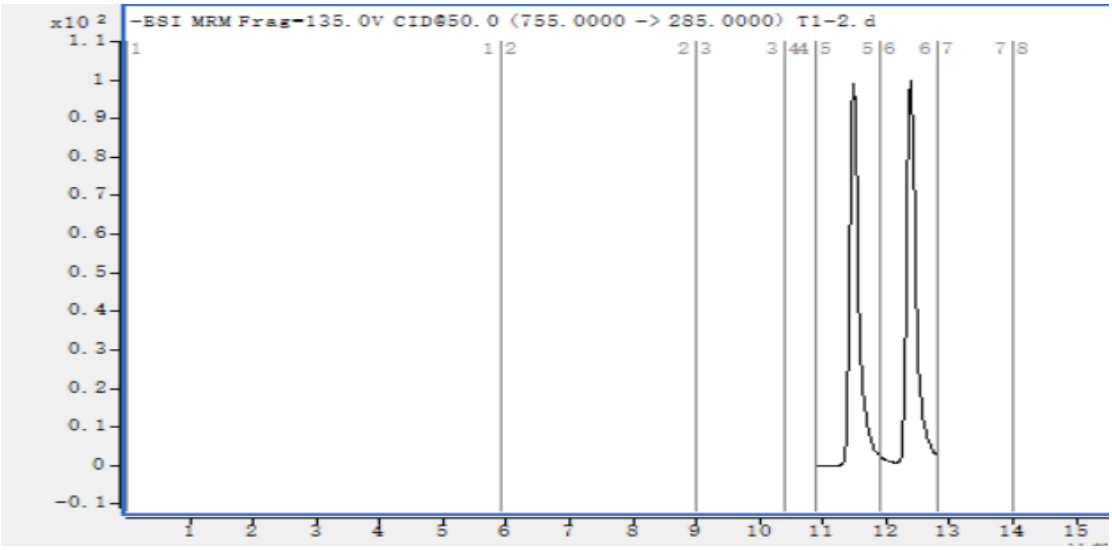
(a)


x10 2
-ESI MRM Frag=135.0V CID@40.0 (593.0000 -> 407.0000) T1-2.d

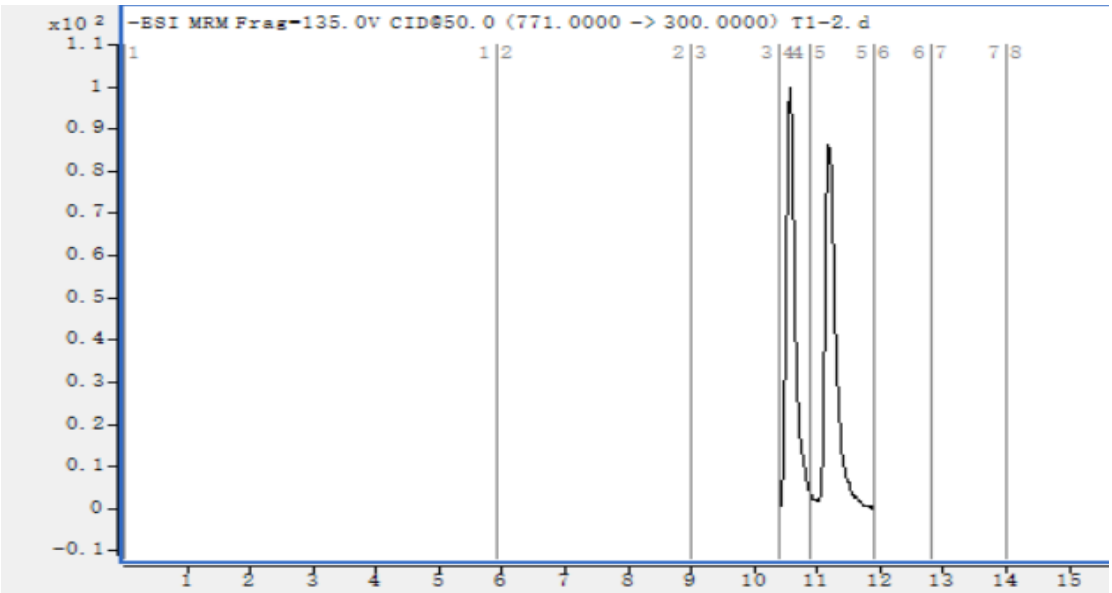
(b)


x10 2
-ESI MRM Frag=135.0V CID@40.0 (729.0000 -> 407.0000) T1-2.d

(c)



(d)



(e)

Figure 4.4 LC-QQQ-MRM results for procyanidins and flavonoid glycosides. (a) Procyanidin dimer EC-EC, 577→407. (b) procyanidin dimer EC-EGC, 593→407. (c) Procyanidin dimer EC-ECG, 729→407. (d) Kaempferol-rhamnose-glucose-glucoside, 755→285. (e) Quercetin-rhamnose-glucose-glucoside, 771→300.

4.5 Procyanidin B2 Standard Curve

Figure 4.5 shows the procyanidin B2 standard curve with concentration varied from 0.0001 mol/L, 0.001 mol/L, 0.01 mol/L to 0.02 mol/L. The R2 value is equal to 0.9993.

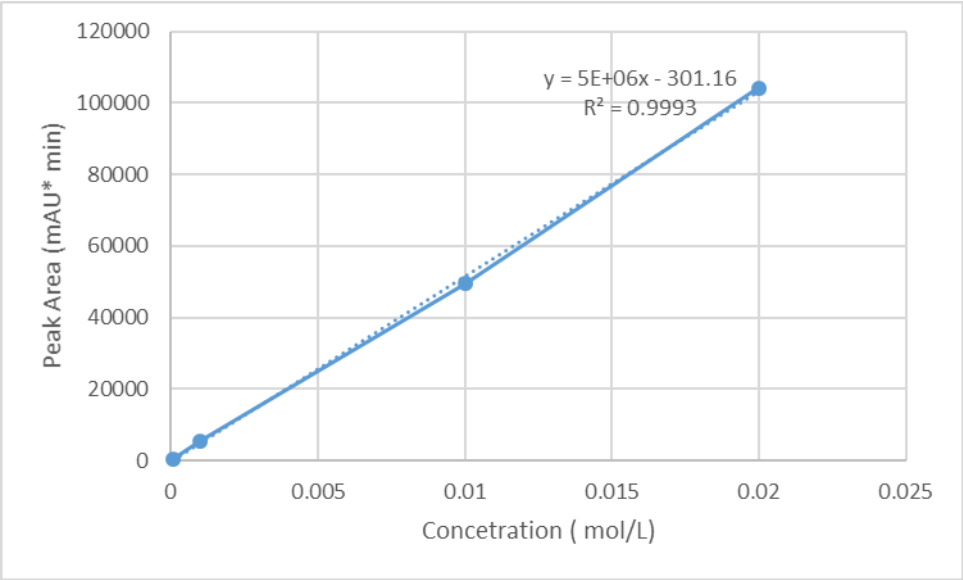


Figure 4.5 Procyanidin B2 standard curve

4.6 β-glucogallin Standard Curve

Figure 4.6 shows the β-glucogallin standard curve with concentration varied from 0.000064 mg/mL, 0.00032 mg/mL, 0.0016 mg/mL, 0.008 mg/mL to 0.04mg/mL. The R2 value is equal to 0.9978.

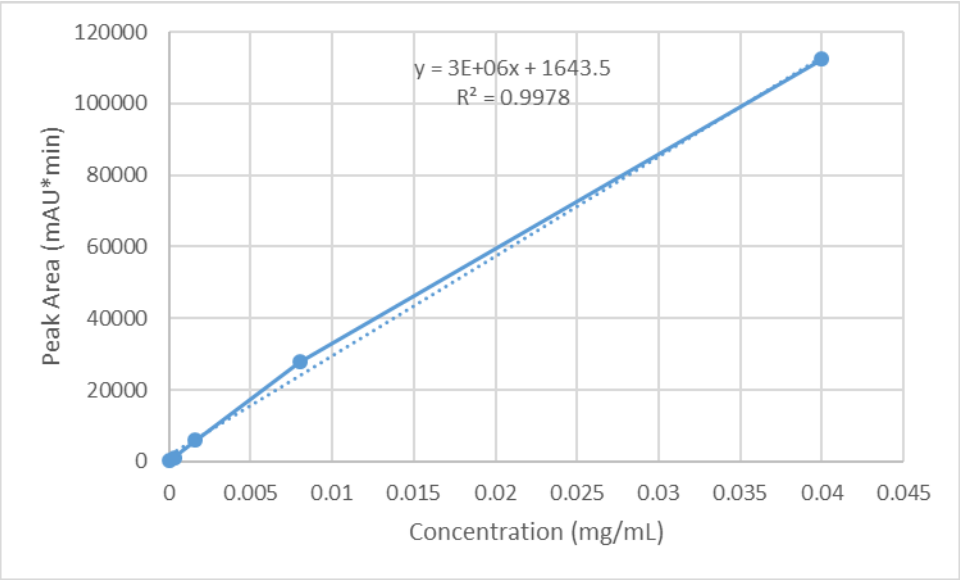


Figure 4.6 β-glucogallin standard curve

Discussion

5.1 The Contents of Main Compounds of Huangshan Maofeng Green Tea

The content of gallic acid (GA), GC, EGC, C, theobromine (THB), EC, EGCG, GCG, ECG, caffeine (CAF) of our Huangshan Maofeng green tea samples are calculated and shown below in table 5.1.

Compounds	T1	T2	T3	1	2	3
GA	1.68±0.16	2.40±0.16	2.14±0.27	2.57±0.20	2.86±0.15	3.08±0.06
GC	Nd	2.16±0.29	2.19±0.16	2.32±0.64	2.04±0.26	2.46±0.50
EGC	148.80±14.19	117.37±10.56	70.31±9.44	52.16±5.28	24.08±5.09	12.85±4.98
C	Nd	15.55±0.48	13.03±0.38	12.32±0.18	10.63±1.05	10.03±0.46
THB	1.43±0.05	2.73±0.06	3.18±0.09	0.98±0.03	1.48±0.31	0.92±0.13
EC	7.22±0.60	6.75±0.21	8.58±0.16	7.77±0.07	8.89±0.83	9.36±1.54
EGCG	85.87±2.06	69.59±3.75	78.78±2.55	76.62±5.78	58.16±12.79	54.35±19.10
GCG	2.55±0.38	2.49±0.02	2.60±0.05	2.34±0.03	2.58±0.41	2.14±0.06
ECG	11.30±0.10	21.43±1.08	13.91±5.65	18.23±0.33	16.86±3.22	17.75±3.18
CAF	33.44±1.66	36.50±0.96	33.01±1.33	36.21±0.81	29.36±2.19	29.22±5.48

Table 5.1 The content of 10 main compounds in Huangshan Maofeng green tea samples of different grades(mg/g). Nd, not detected.

Like most of the unfermented types of tea, green tea contained high contents of EGCG, ECG and EGC. In table 5.1, in the high grade of green tea, the EGC is the predominant polyphenol, with level about 110-150 mg/g, much higher than that of EGCG, but in the low grade of green tea, the highest content of polyphenol is EGCG. The levels of other compounds including caffeine, theobromine and theanine among various grades of green tea are similar.

Furthermore, the total flavone and polyphenols of different grade of tea were determined and shown in figure 5.1. Except for the T3 grade, the high grade of tea (T1, T2) has slightly higher levels of total flavone than low grade, while the contents of total polyphenols are similar. These results indicated that the basic compounds of various tea samples are similar, therefore it is necessary to look into the content of much more compounds in detail through metabolomics analysis.

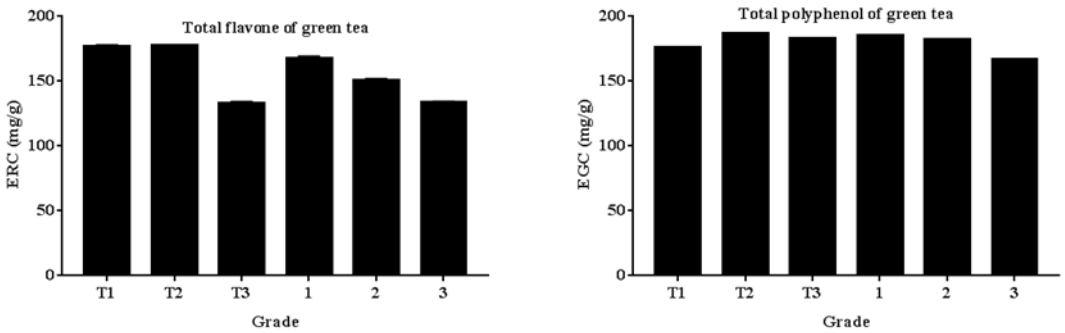


Figure 5.1 The total flavones and polyphenols of different grades of green tea (T1, T2, T3, 1, 2, 3).

5.2 Metabolomics Analysis of Different Grades of HSMF Green Tea

Usually, the chemical characteristics or chemotaxonomy was used in the classification of plant relationships or tea types' classification. The main compounds of tea were also used as contributors for the classification of different types of teas, such as green tea, black tea or oolong tea. However, in the present study, if classifying the different grades of green tea through the main compositions of tea, it was suggested that the re-classification results did not correspond to the tea grades based on sensory evaluation by tea masters. Therefore, to obtain more reasonable classification of different grades of green tea, non-targeted metabolomics may comprehensively reveal the differences of tea grades.

As shown in Figure 4.3, the clustering analysis showed that all grades of tea samples were not consistent to the tea rating by tea masters. Therefore, the non-targeted LC-MS based metabolomics was conducted on all tea samples. The results indicated that T1 and T2 grades were classified as one type, while other grades (T3, 1, 2, 3) belonged to other type. The classification was observed from the principal cluster analysis of LC-MS metabolomics data. To chemically depict the important compounds responsible for the classification of low and high grades of green tea, OPLS-DA analysis was conducted on the dataset. It gave some important marker compounds by S-plot.

5.3 The Grade-related Marker Compounds of HSMF Green Tea

In figure 4.3 (d), it clearly showed the critical compounds, which have been identified base on the high-resolution mass, and mass fragments of individual marker compound.

During the identification of marker compounds, three approaches were used to certify the compounds' structure. For example, some main compounds of tea could be matched by the chemical standards, like EC, EGC, GA, theanine, Galloyl-quinic acid. Otherwise, the compounds' molecule weight was retrieved in the TCDE database, and the fragments of targeted ion were also used to deduce the structure of compound. For example, the procyanidins have been widely identified from fresh tea leaves or green tea, they have typical fragments produced by disassociation of C4-C8 bond of procyanidins. Gallic tannins are also the variety of markers in distinguishing different grades of green tea. They could be deduced by the loss of galloyl moiety [-152 or -170]. As shown in Table 5.2, peak NO.9 had the quasi-molecular ion m/z at 483.075, which could yield the fragment ion m/z at 331.068 by losing 152Da, and continue to lose 152Da+H_2O to produce the fragment ion m/z at 160.838 [glucose-H_2O]. Similarly, peak No. 11, 12, were also tentatively identified as HHDP-galloyl-glucose and Tri-galloyl-glucose.

Peak No.	t_R (min)	m/z (-MS)	Corr.index	MS^n (-MS)	Identification	Fold (High/Low)	Identification
1	1.24	169.013	-0.96	124.883;	Gallic acid	2.22	s
2	0.57	173.092	-0.94	154.950, 128.011;	Theanine	1.38	s
3	2.87	183.028	-0.91	160.838;	Methyl gallate	2.49	d, MS^2
4	1.35	191.054	-0.66	169.0105;	Quinic acid	1.31	s
5	0.89	331.065	-0.95	191.052, 164.068;	β-Glucogallin	3.17	s
6	1.36	343.065	-0.63	191.052;	Galloyl-quinic acid	1.22	s
7	8.25	425.085	-0.93	273.072, 205.083, 169.010, 125.021;	Epi-afzelechin gallate	2.19	d, MS^2
8	6.68	441.081	-0.95	289.067, 169.010, 125.021;	(-)-epicatechin gallate (ECG)	5.31	s
9	4.05	483.075	-0.94	331.068, 197.804, 160.838, 116.926, 103.917;	Di-galloyl-glucoside	2.99	d, MS^2
10	0.56	485.197	-0.96	172.980, 154.969;		1.49	
11	4.46	633.071	-0.77	463.071, 316.0343, 300.960;	HHDP-galloyl-glucose (isostrictinin or strictinin)	1.94	d, MS^2
12	6.69	635.086	-0.90	483.105, 317.0422, 465.119, 313.157;	Tri-galloyl-glucose	2.30	d, MS^2
13	1.36	687.138	-0.75	343.143;	Galloyl-quinic acid	1.90	s
14	7.13	729.143	-0.83	577.052, 559.036, 451.214, 441.207, 407.229, 289.041;	Procyanidin dimer (EC-ECG)	1.62	d, MS^2
15	23.44	822.526	-0.82	716.429, 248.976, 200.035, 146.012;		1.52	
16	8.25	865.159	-0.93	549.947, 425.082, 273.072, 116.925;	Procyanidin dimer (ECG-afezelechin gallate)	2.66	d, MS^2
17	7.9	881.154	-0.86	739.201, 609.082, 441.077, 279.083, 197.804;	Procyanidin dimer (ECG-ECG)	2.02	d, MS^2
18	7.52	883.172	-0.61	441.077;	(-)-epicatechin gallate (ECG)	1.14	s
19	4.46	1267.15 [2M-H]	-0.97	633.071;	HHDP-galloyl-glucose (isostrictinin or strictinin)	7.55	d, MS^2
20	4.79	289.071	0.91	245.073, 204.999, 203.062, 178.855, 150.0942, 124.873;	(-)-epicatechin (EC)	0.94	s
21	3.35	305.066	0.99	287.054, 261.102, 220.976, 178.972, 164.821, 136.942, 124.919;	(-)-epigallocatechin (EGC)	0.90	s
22	5.04	337.091	0.99	191.099, 162.875, 118.815;	Quinic acid-rhanmnose	0.52	
23	0.57	503.159	0.95	190.999;	Quinic acid derivatives	0.66	
24	0.58	533.17	0.83	190.862;	Quinic acid derivatives	0.56	
25	3.35	611.138	0.94	305.061;	(-)-epigallocatechin (EGC)	0.39	
26	7.42	755.201	0.86	593.285, 447.147, 285.035;	Kaempferol-glucose-rhamnose-glucose	0.64	d, MS^2
27	7.12	771.196	0.92	609.177, 462.894, 301.105;	Quercetin-glucose-rhamnose-glucose	0.35	d, MS^2
28	24.43	819.523	0.70	747.482, 248.975, 146.011;		0.75	

Table 5.2 The marker compounds contributing to the re-classification of high and low grade of green tea (T1, T2 vs T3, 1, 2, 3). “s” stands for identification by chemical standards; “d” stands for identification by TCDB database (http://pcsb.ahau.edu.cn:8080/TCDB/f); “MS^2” stands for identification by mass fragments of parent ions.

As shown in table 5.3, in the high grade of green tea, the contents of gallic acid, theanine, methyl gallate, and some hydrolysable tannins were significantly higher than those of low grade. The fold of high vs low grade for each marker compound was also calculated. Except for the catechins, the glucogallin, di-galloyl-glucose, tri-galloyl-glucose and HHDP-gallyol-glucose were also observed as the main marker compounds of high grade of green tea. On the other hand, some flavonoid glycosides were also identified as the marker compounds in the low grade of green tea. It was suggested that flavonoids glycosides are important astringent compounds of black tea, especially with less threshold compared with common catechins, such as EGCG, GCG.

Peak No.	t_R (min)	m/z (-MS)	Corr. index	MS^n (-MS)	Identification	Fold (High/ Low)
277	1.24	169.013	-0.96	124.883;	Gallic acid*	2.22
292	0.57	173.092	-0.94	154.950, 128.011;	Theanine*	1.38
322	2.87	183.028	-0.91	160.838;	Methyl gallate	2.49
337	1.35	191.054	-0.66	169.0105;	Quinic acid*	1.31
724	0.89	331.065	-0.95	191.052, 164.068;	Glucogallin*	3.17
756	1.36	343.065	-0.63	191.052;	Galloyl-quinic acid	1.22
929	8.25	425.085	-0.93	273.072, 205.083, 169.010, 125.021;	Epi-afzelechin gallate	2.19
993	6.68	441.081	-0.95	289.067, 169.010, 125.021;	(-)-epicatechin gallate*	5.31
1148	4.05	483.075	-0.94	197.804, 160.838, 116.926, 103.917;	Di-galloyl-glucoside	2.99
1159	0.56	485.197	-0.96	172.980, 154.969;		1.49
1496	4.46	633.071	-0.77	463.071, 300.960;	HHDP-galloyl-glucose (isostrictinin or strictinin)	1.94
1502	6.69	635.086	-0.90	483.105, 465.119, 313.157;	Tri-galloyl-glucose	2.30
1560	1.36	687.138	-0.75	343.143;	Galloyl-quinic acid	1.90
1616	7.13	729.143	-0.83	577.052, 559.036, 451.214, 441.207, 407.229, 289.041;	Procyanidin dimer (EC-ECG)	1.62
1731	23.44	822.526	-0.82	716.429, 248.976, 200.035, 146.012;		1.52
1768	8.25	865.159	-0.93	549.947, 425.082, 273.072, 116.925;	Procyanidin dimer (ECG-afezelechin gallate)	2.66
1794	7.9	881.154	-0.86	739.201, 609.082, 441.077, 279.083, 197.804;	Procyanidin dimer (ECG-ECG)	2.02
1797	7.52	883.172	-0.61	441.077;	(-)-epicatechin gallate*	1.14
1930	4.46	1267.15	-0.97	633.071;	HHDP-galloyl-glucose (isostrictinin or strictinin)	7.55
591	4.79	289.071	0.91	245.073, 204.999, 203.062, 178.855, 150.0942, 124.873;	(-)-epicatechin*	0.94
644	3.35	305.066	0.99	287.054, 261.102, 220.976, 178.972, 164.821, 136.942, 124.919;	(-)-epigallocatechin*	0.90
736	5.04	337.091	0.99	191.099, 162.875, 118.815;	Quinic acid-rhanmnose	0.52
1205	0.57	503.159	0.95	190.999;	Quinic acid derivatives	0.66
1280	0.58	533.17	0.83	190.862;	Quinic acid derivatives	0.56
1453	3.35	611.138	0.94	305.061;	(-)-epigallocatechin*	0.39
1651	7.42	755.201	0.86	593.285, 447.147, 285.035;	Kaempferol-glucose-rhamnose-glucose	0.64
1671	7.12	771.196	0.92	609.177, 462.894, 301.105;	Quercetin-glucose-rhamnose-glucose	0.35
1728	24.43	819.523	0.70	747.482, 248.975, 146.011;		0.75

Table 5.3 The marker compounds contributing to the re-classification of high and low grade of green tea (T1, T2 vs T3, 1, 2, 3)

5.4 The Contents of Procyanidins and Flavonoid Glycoside of Different Grades of HSMF Green Tea

To quantitatively describe the chemical compositions of these minor marker compounds, which were hardly detected under the HPLC, there were three types of compounds determined by LC-QQQ-MRM with reference to chemical standard. As shown in Figure 5.2, the procyanidins dimers were determined by referring to the procyanidin B2. The total procyanidins' level of high grade of green tea (T1, T2) was significantly higher than those of low grade (T3, 1, 2, 3). These quantitative results were similar as the metabolomics analysis results. Although the condensed tannins are main astringent compounds in many herbs, in the present study, high grade tea may contain much more condensed tannins.

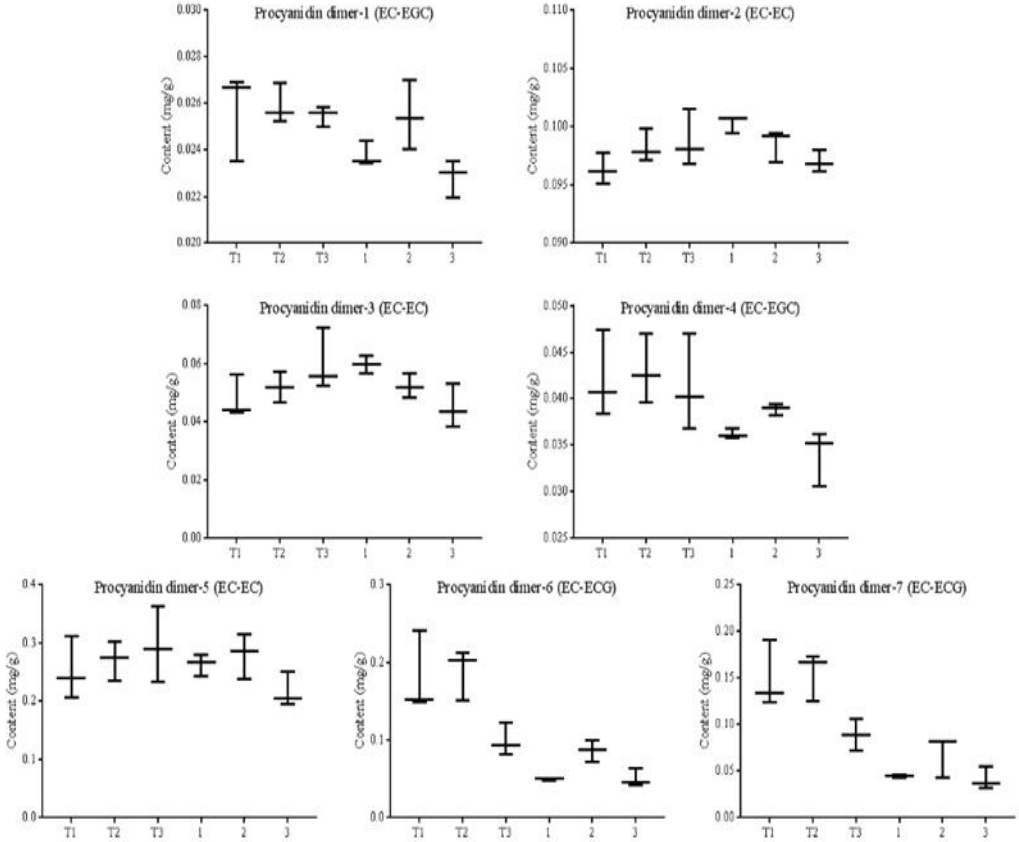


Figure 5.2 The contents of procyanidins of different grades of green tea by semi-quantitative determination of LC-QQQ-MRM (T1, T2, T3, 1, 2, 3)

However, the flavonoids glycosides' levels of high graded tea are highly less than those of low graded tea. Through the authentic compounds and referring to published articles, many flavonoids glycosides were determined and provided in supplementary material. In table 5.2, two marker compounds were identified, and therefore were also determined as shown in figure 5.3. In the low grade of green tea, in particular, the grade 2 and 3 tea contained much higher contents of Kaempferol-glucose-rhamnose-glucose and quercetin-glucose-rhamnose-glucose than high grade. These results may have

suggested that low grade tea had stronger astringency.

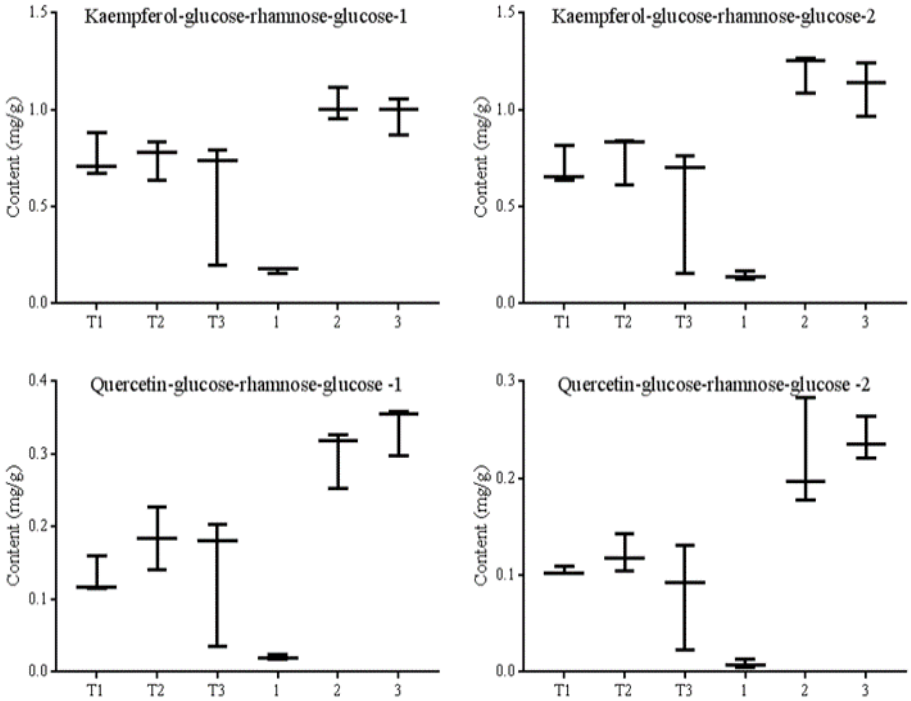


Figure 5.3 The contents of critical flavonoid glycosides in different grades of green tea by semi-quantitative determination of LC-QQQ-MRM (T1, T2, T3, 1, 2, 3)

5.5 The Astringency Test on Different Grades of HSMF Green Tea

Because all of the green tea was produced in same place and at the same time, so most of the contents of these teas were similar. Nine panelists were trained for four weeks

using EGCG and alum liquor as basic astringent taste. The astringent evaluation was given base on the 1-12 scores. As shown in figure 5.4, the astringency scores showed differences, though there was not statistical significance among groups. The high grade of green tea had less astringent taste than low grade teas. The basic theory of astringency may be caused by the galloylated catechins, condensed tannins, hydrolysable tannins and flavonoids glycosides. A comprehensive effect of different astringent compounds formed the astringent sensors during tea drinking.

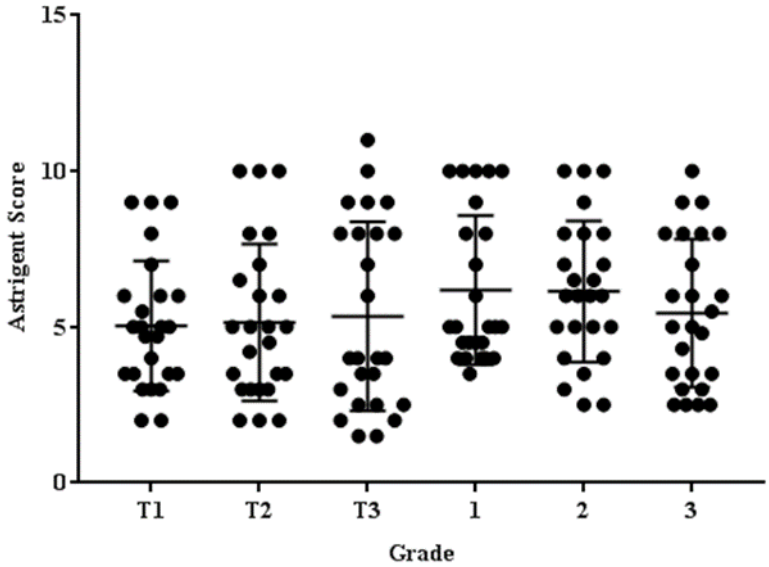


Figure 5.4 The astringent score of different grades of green tea (T1, T2, T3, 1, 2, 3)

Conclusion and Future Work

Huangshan Maofeng green tea, as one of the most famous types of green tea in China, has attracted a large number of customers for its unique sensory performance and potential health effect. The price of Huangshan Maofeng green tea in the market is determined by its grade corresponding to the evaluation by tea specialists. However, up to now. there hasn't been a systematic chemical standard for the grading for Huangshan Maofeng green tea based on its chemical composition. Therefore, a detailed and systematic chemical composition analysis is noticeable to try. It is also said by tea masters and specialists that lower grade green tea possess more astringency taste comparing to higher grade of green tea, thus the idea of discovering whether the content of astringency producing compounds varies in high and low grade of Huangshan Maofeng green tea came up to me.

The relationship between the sensory and quality of teas and chemical components is rather complicated. It is very likely that such relation is not sufficiently and reliably explained by targeted measurement of specific components while missing information of those not selected for analysis. Recently, researchers applied metabolomics study on

different types of tea products to discover chemical composition in detail and find marker compounds of various types of tea. This method has been proved efficient and reliable so I applied metabolomics analysis in my study of different grades of Huangshan Maofeng green tea.

In this study, Six grades of Huangshan Maofeng green tea were studied by LC-MS based metabolomics and then the dataset was processed by multivariate statistical analysis. The results showed (1) the main polyphenols' levels were not tightly related to tea grades, but non-targeted metabolomics revealed that all grades of teas could be classified into two types, one high grade (T1, T2) and low grade (T3, 1, 2, 3); (2) the main marker compounds causing the differences of high and low grades can be categorized into three groups, which are procyanidins, flavonoid glycosides and four types of hydrolysable tannins (mono-galloyl-glucose, di-galloyl-glucose, tri-galloyl-glucose and HHDP-galloyl-glucose).

There are still some work that are worthy to do in the future for discovering the relationship of marker compounds and sensory evaluation of different grades of Huangshan Maofeng green tea.

(1) More systematic sensory evaluation on Huangshan Maofeng green tea is required.

According to previous research, astringency and bitterness are two important quality attributes of green tea infusion. However, in this study only astringency is focused and sensory evaluation on bitterness is thus required. In addition, the number of sensory tests should be increased to obtain sufficient and reliable results.

(2) The mechanism of marker compounds on their influence of the taste of Huangshan Maofeng green tea.

Based on previous researches, catechins, procyanidins and flavonoid glycosides are contributors to the astringency taste of tea. However, in this study, the content of procyanidin dimers in higher grade (grade T1 and T2) of Huangshan Maofeng green tea is higher than lower grade (grade T3, 1, 2 and 3). Although the astringency test didn’t give much difference for the 6 grades of our samples, the difference in amount of procyanidins is in contrary to the conclusion of former researches. Therefore, it is necessary to discover in detail the mechanism of procyanidins and taste formation in Huangshan Maofeng green tea.

In addition, the third type of marker compounds obtained in this study, the hydrolysable tannins, have noticeable difference in content from higher grade and lower grade of Huangshan Maofeng green tea. The connection of hydrolysable tannins and tea taste hasn't been mentioned before, thus it is advisable to discover in detail the mechanism of hydrolysable tannins and taste formation. One thought on this direction is the hydrolysable tannins might undergo complexation reaction with the flavonoid glycosides in Huangshan Maofeng green tea, while the latter has been proved as a major astringency contributor of tea, to reduce astringency taste.

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