Dynamic Chemical and Flavor Changes in Black Tea During Fermentation

A Nontarget Metabolomics Study

Application Note

Food Testing

Authors

Junfeng Tan, Weidong Dai, Haipeng Lv, Li Guo, Yue Zhang, Yin Zhu, Qunhua Peng, and Zhi Lin
Tea Research Institute of CAAS, Hangzhou, China

Meiling Lu and Shan Zhou
Agilent Technologies, Inc.
China

Abstract

Fermentation is one of the key steps to produce high-quality black tea, during which the chemical compositions change dramatically. However, the dynamic changes of this sophisticated process are far from clear, and are often characterized through identifying a few compounds. A nontargeted metabolomics approach using Agilent ultrahigh performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry and Agilent Mass Profiler Professional software was applied to map the variations of metabolites in tea samples during fermentation, which was partially based on the work published by Tan *et al.* recently[1]. Principal component analysis indicated obvious stepwise changes to the tea metabolome during the fermentation. Sixty-two compounds including catechins, dimeric catechins, flavonol glycosides, amino acids, phenolic acids, alkaloids, and nucleosides were identified or tentatively identified. Some of the compounds identified correlated excellently with the varying bitterness and astringency of the black tea. This study provides a comprehensive picture of the metabolite changes during the tea fermentation process. This enhanced understanding will be beneficial for improvement of tea quality in both taste and nutrition during black tea manufacturing.

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Introduction

Tea is a popular beverage worldwide, particularly in China, where tea drinking can be traced back at least 1,500 years. Over tea's long history, different processing methods have been developed. One of the primary classes of tea, black tea, is highly fermented, and its flavor quality is dependent upon the fermentation process. Recent studies have demonstrated that several enzymatic oxidation reactions take place during the fermentation process[2-3]. These enzymatic reactions inevitably change the chemical compositions as well as the flavor of the tea. The chemical characteristic of such changes in the tea metabolome during the fermentation are currently not clear, although some studies have been conducted[4-5]. This work aims to investigate how the chemical components change during the fermentation process in black tea.

The flavor of tea, mainly its bitterness and astringency, is derived from the aqueous solution, meaning that the less volatile components should primarily contribute to the flavor. An HPLC method would fit for the analysis of these less volatile compounds. Combined with accurate mass and tandem mass spectrometry, and chemometric analysis, HPLC would serve as a powerful metabolomics tool to profile the changes in tea components during fermentation. Therefore, we applied a UHPLC-Q-TOF/MS based metabolomics approach to investigate the dynamic changes of the chemical compositions in the black tea through the entire fermentation process.

Experimental

Materials and reagents

Reference compounds: epigallocatechin (EGC), catechin (C), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epicatechin (EC), kaempferol-3-O-glucoside (astragalin), kaempferol-3-O-rutinoside, isovitexin, isoquercitrin, tryptophan, glutamic acid, proline, glutamine, and adenine were obtained from Sigma-Aldrich (St. Louis, MO). Myricetin-3-galactoside and procyanidin B_2 were from Chemfaces (Wuhan, China). Theanine, rutin, quercetin-3-O-galactoside, theobromine, tyrosine, and phenylalanine were from J&K Scientific Ltd. (Beijing, China). Epiafzelechin from Yuanye Bio-Technology co., Ltd (Shanghai, China). Caffeine was from Enzo Life Sciences Inc. (Farmingdale, NY). Methanol was of LC/MS grade and obtained from Merck (Darmstadt, Germany). Ammonium acetate and formic acid were all HPLC grade, and were purchased from Sigma-Aldrich. Deionized water was produced in the lab using a Milli-Q water purification system.

Creation of a Personal Compound Database and Library for tea (Tea PCDL)

The reference compound solutions were prepared at a concentration of 100 ppb using pure methanol, and analyzed by UHPLC-Q-TOF/MS using the conditions shown in Table 1. The Q-TOF/MS was operated under both scan mode and target MS/MS mode to acquire the retention time, accurate *m/z*, and accurate MS/MS spectra. The information obtained was transferred to an Agilent MassHunter PCDL manager (B.07.00) for creation of the Tea PCDL.

Sample extraction and cleanup

Figure 1 shows the tea sample preparation process. Briefly, fresh tea leaves were plucked from the tea garden of the Tea Research Institute of CAAS with a moisture content of approximately 75 % (w.b.). They were then withered and rolled to a moisture content of approximately 65 % (w.b.). The tea leaves were equally divided into six portions. Each portion was rolled for 20 minutes, followed by fermentation at 30 °C with a relative humidity of 90 %. Equal tea samples were collected at fermentation times of 0, 1, 2, 4, 6, 8, 10, 12, and 14 hours, respectively. These samples were immediately dried at 120 °C to a moisture content of approximately 5 % (w.b.), stopping the fermentation.

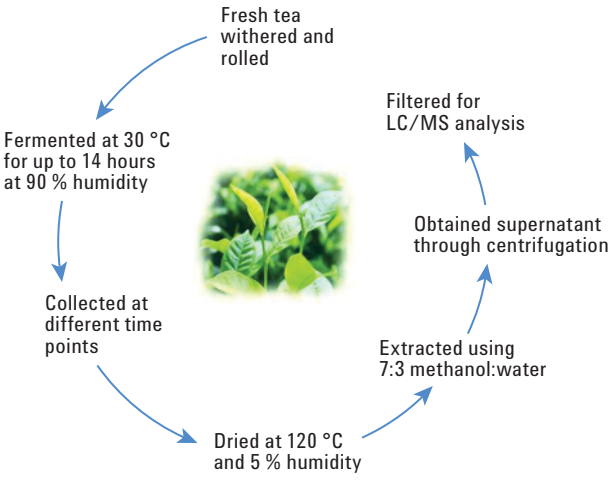

Figure 1. Schematic graph showing the tea sample preparation procedure for UHPLC-Q-TOF/MS analysis.

One portion of each tea sample collected was used for taste evaluation by a panel of five tea flavor evaluation experts based on bitterness and astringency. The other portion was extracted using solvents for UHPLC-Q-TOF/MS analysis. For the extraction, 10 mL of methanol/water solution (70/30, v/v) was added to 0.2 g of ground tea powder. The mixture was then sonicated to extract the tea metabolites, and 1.5 mL of the resulting solution was centrifuged at 10,000 rpm for 10 minutes. The supernatants were collected, and further filtered using a 0.22-µm nylon membrane for LC/MS analysis. A pooled sample, a mixture of a small portion from each tea sample, was used as quality control (QC), and analyzed once every 4 hours. The internal standards mixture contained sulfacetamide, sulfafurazole, and flumequine (0.2 µg/mL), which were added to each tea sample including the QC sample. Both QC samples and internal standards were used to evaluate stability and repeatability during the LC/MS analysis.

Agilent workflow for metabolomic profiling

Raw data were acquired by UHPLC-Q-TOF/MS with the conditions shown in Table 1. The acquired data were then processed following the procedures shown in Figure 2. Briefly, the data were extracted using Find by Molecular Feature (MFE) in Agilent MassHunter Qual. (B.07.00), and the results were exported as cef files. Alternatively, the total sets of data could be loaded into the Agilent MassHunter Profinder software (B.06.00) for recursive MFE extraction, and exported as cef files. The cef files were then imported into Agilent Mass Profiler Professional 13.1.1 for retention time/mass calibration, peak alignment, data filtration, multivariate and univariate statistics, principal component analysis (PCA), and so forth, to find the metabolites with significant changes during the fermentation procedure. Pearson correlations were performed to seek the correlation between the tea bitterness/astringency and the abundance of the differential metabolites.

LC/MS Conditions

Table 1. Instrument conditions.

Parameter	Value
LC conditions	
Instrument	Agilent 1290 Infinity LC System with built-in degasser
Autosampler	Agilent 1290 Infinity Autosampler with temperature control
Column compartment	Agilent 1290 Infinity Thermostatted Column Compartment
Column	Agilent ZORBAX Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm
Column temperature	40 °C
Mobile phase	A) aqueous solution containing 5 mmol/L ammonium acetate and 0.1 % formic acid B) methanol containing 5 mmol/L ammonium acetate and 0.1% formic acid
Flow rate	0.4 mL/min
Injection volume	1.0 µL
Post time	4 minutes
Gradient elution profile	0–4 minutes: 10–15 %B; 4–7 minutes: 15–25 %B; 7–9 minutes: 25–32 %B; 9–16 minutes: 32–40 %B; 16–22 minutes: 40–55 %B; 22–28 minutes: 55–95 %; 28–30 minutes: 95 %
ESI-MS/MS conditions	
Instrument	Agilent 6540 Q-TOF LC/MS system with Agilent Dual Jet Stream electrospray ionization source
Ionization mode	Positive
Drying gas temperature	300 °C
Drying gas flow rate	8 L/min
Nebulizer gas pressure	35 psi
Sheath gas temperature	300 °C
Sheath gas flow rate	11 L/min
Capillary voltage	3,500 V
Nozzle voltage	500 V
Scanning mode	TOF scan and target MS/MS scan
Scan range	Scan range: 100–1,100 (MS)/ 50–1,100 (MS2)
Reference ions	121.0509/922.0098

Results and Discussion

Optimized UHPLC separation and accurate Q-TOF/MS detection

Tea is a complex matrix, containing thousands of compounds. Hence, an appropriate separation is required to obtain as many compounds as possible within an acceptable time frame. By carefully adjusting the gradient elution with the selected C18 column and the mobile phase, a 30-minute gradient elution can separate the major components in the tea extracts. Figure 3A shows the separation in the total ion chromatogram. Using the MFE extraction algorithm, more than 1,000 compounds could be extracted from the tea samples (Figure 3B). Thus, such a gradient elution was selected for further Tea PCDL creation, and for the tea samples profiling analysis.

Recursive molecular feature extraction for compound alignment across sample groups

For any omics-related studies, reliable extraction of unknown compounds and annotation of the compounds across all sample groups are required. The recursive MFE algorithm in MassHunter Profinder was developed for such data analysis. It allows the user to extract the unknown compounds from multiple groups of data, and to examine the same compounds across the sample groups. With careful selection of the parameters for extraction, all the internal standard compounds showed excellent retention time repeatability within and across groups, with a maximum retention time deviation of 0.10 minutes (Figure 4). The mass accuracies for the internal standards in all samples were within 3.0 ppm. In addition, the internal standards, spiked in the QC samples, were analyzed in every eight samples (~4 hours) through the entire data set. These internal standards also showed good reproducibility in retention time, mass accuracy, and abundance (bottom traces in Figure 4). This reproducibility indicates that the data acquired are reliable and could be used for metabolomics analysis.

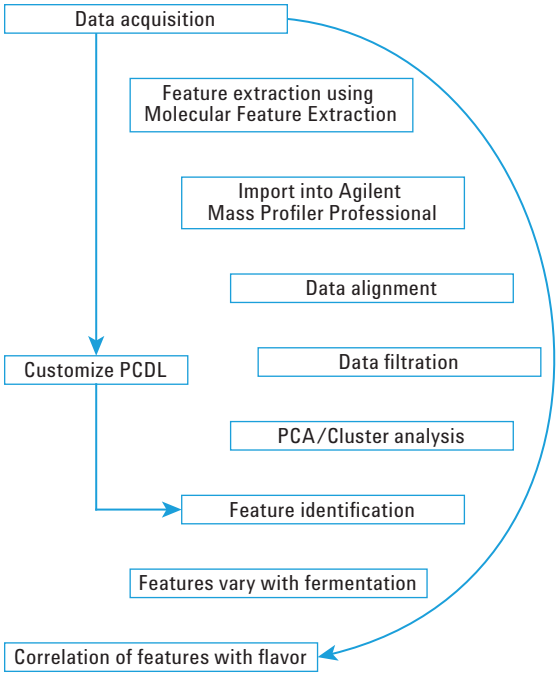

Figure 2. Schematic graph showing the workflow for data analysis.

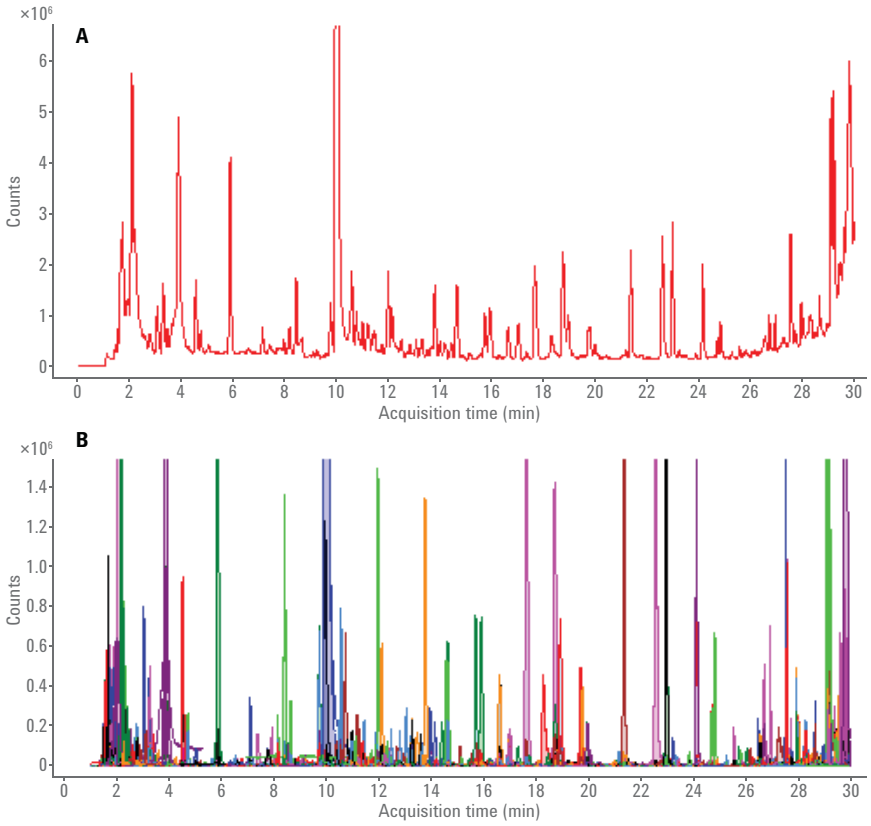

Figure 3. The typical total ion chromatograms (A) and the overlapped extracted compound chromatograms through MFE (B) demonstrating the high separation efficiency of the optimized LC/MS method. Note: the fermentation time for this sample was 6 hours.

With the selected parameters, all the data from each group of samples were subjected to recursive MFE. The resultant peaks were exported as cef files and imported into MPP for further chemometric analysis.

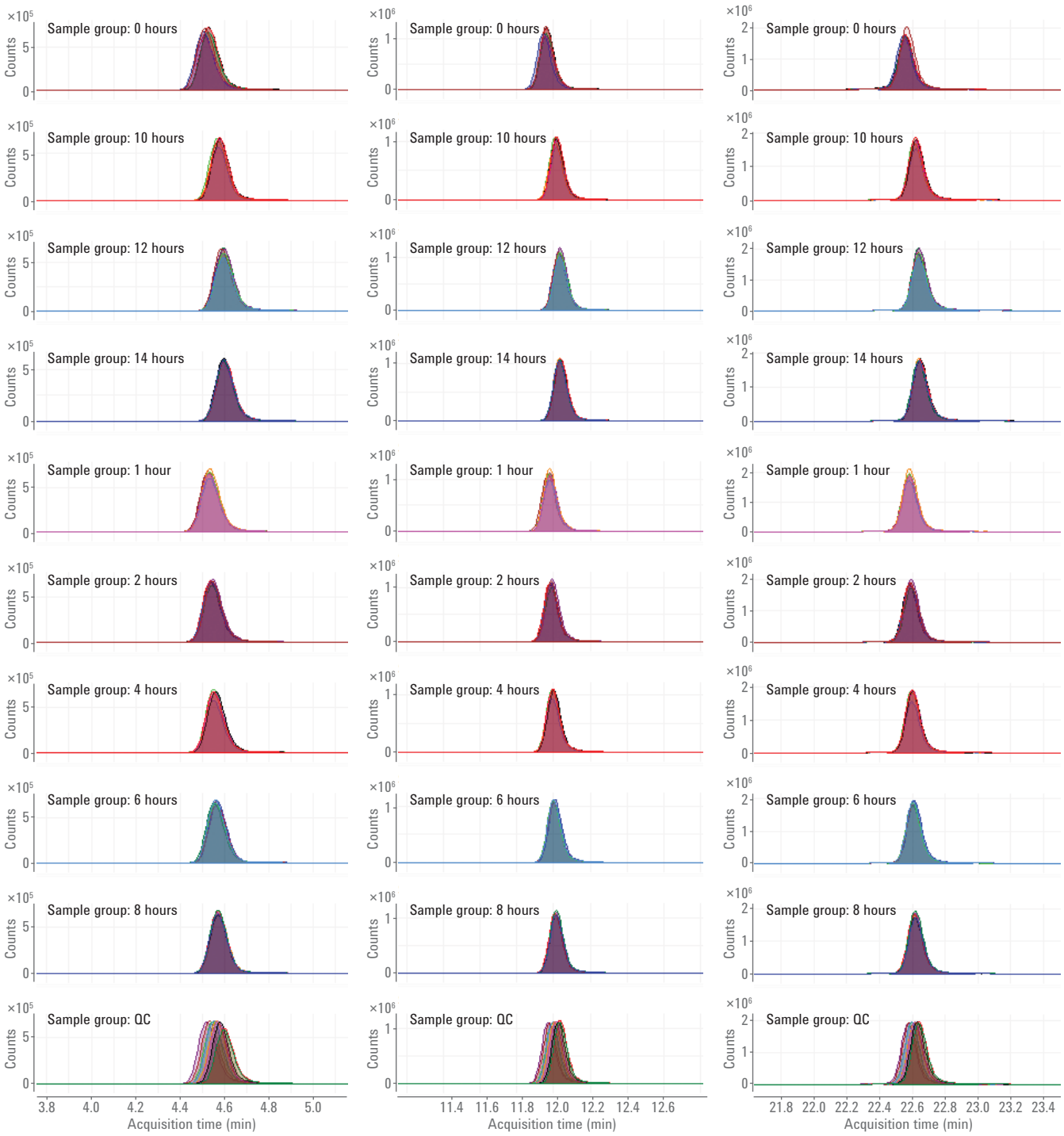

Figure 4. Comparing the internal standards within and across groups. Note: QC samples are the mixture of all samples at equal volume, and were analyzed in every eight samples through the entire sample sets.

Chemometric analysis of the chemical profile change during fermentation

To investigate how the metabolites change during the fermentation process, the previously mentioned processed data were imported into MPP for chemometric analysis. The retention times (RTs) and mass values for the compounds extracted through recursive MFE from all the collected tea samples were aligned in MPP. This alignment used the same specification of the RT window and the mass deviation window as those in MassHunter Profinder. In addition, each aligned compound was treated as an individual entity in MPP, and annotated using both the RT and accurate mass.

Using this alignment, 3,601 entities from all sample groups were obtained. These entities were then subjected to filtration by excluding those entities with a presence frequency less than 65 % and a RSD ≥30 % within QC samples to remove chemical/instrumental noise. Principle component analysis of the resulting 2,470 entities across all sample groups, including QC, demonstrated a crowd clustering of QC samples (Figure 5A). This clustering indicated that the data acquired were reliable and could be used for metabolomics profiling, consistent with Figure 4, in which the internal standards show good reproducibility in RT, mass accuracy, and abundance across the groups.

The entities were further filtered by abundance, and the remaining 1,141 entities were subjected to statistical analysis (ANOVA) using fresh tea (not fermented) as control. With a P-value cutoff of 0.01, 1,061 entities showed significant differences between control samples and the fermented samples. Up to 850 entities were 1.5 times higher, in at least two of the fermented groups, than the control group, with an absolute abundance difference greater than 2.0×10^4.

Principle component analysis of the fermentation conditions based on the 850 entities showed a clear stepwise change of profile with the increase of the fermentation time. In addition, the sum of three components (PC1+PC2+PC3) can explain up to 84.67 % of the total covariances among the control samples and the fermented samples. Even the highest two components can explain 77.29 % of the total covariances, suggesting that these entities contribute the primary changes during the fermentation of tea (Figure 5B). Further principle component analysis of these 850 entities based on the fermentation conditions demonstrated that some differential metabolites can define the major trends more prominently than the others (Figure 5C).

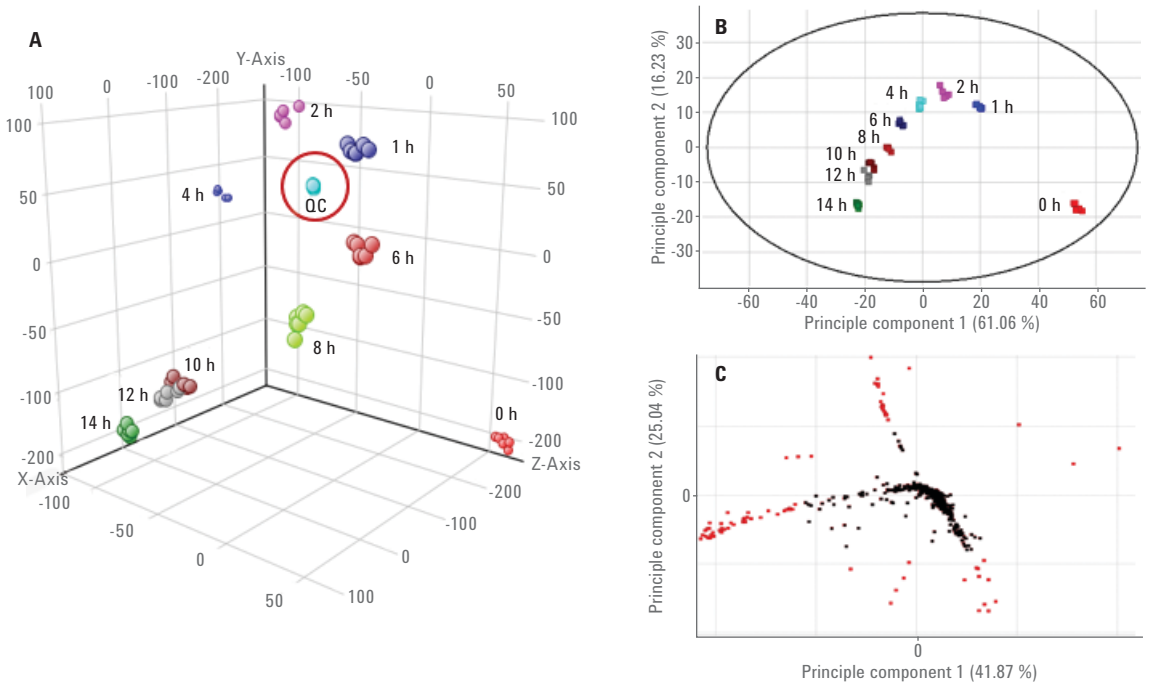

Figure 5. Principle component analysis of conditions and entities demonstrated the variation of the samples and the relative contribution of differential metabolites during the fermentation process. A) 3D PCA score plot on conditions including QCs after preliminary filtration shows a crowd clustering of QC samples, indicating the reliability of data for profiling studies. B) A 2D PCA score plot of conditions using 850 entities demonstrates a stepwise change of tea with increasing fermentation time. C) A 2D PCA score plot of the 850 entities based on the fermentation conditions shows that some differential metabolites can define the major trends more prominently (red square) than others (black square).

Identification of chemical entities with significant differences between fresh tea and fermented tea

The entities with significant differences between the control tea samples and the fermented tea samples were exported for target MS/MS analysis in MassHunter acquisition software. These entities were identified by comparison with the authentic standards (the customized tea PCDL in the lab), elucidation of MS2 spectra, or matching by accurate mass and MS2 spectra against metabolomics databases such as Metlin and HMDB.

In total, more than 60 compounds were identified, including catechins, flavones glycosides, theaflavins, amino acids, alkaloids, nucleosides, phenolic acids, theasinensins, and procyanidins. Figure 6 shows the intensity change for (–)-epigallocatechin-3-gallate (EGCG) over the fermentation duration and its MS2 spectrum/retention time search against the customized tea PCDL, suggesting that it is a common tea metabolite. To date, most compounds in tea have not been identified, and the corresponding authentic standard compounds are not commercially available. Hence, the accurate MS2 spectra were applied to illustrate the possible structure of the metabolites. Taking epigallocatechin methyl gallate as an example. Figure 7 shows the obtained accurate MS2 spectrum for the compound at 11.40 minutes with an *m/z* of 473.1081. The primary fragment ions can reasonably be illustrated with the aid of the Agilent molecular structure correlation (MSC) software (B.05.00). The possible fragmentation pathway could then be deduced, as displayed at the top of Figure 7. Table 2 lists all identified compounds with authentic compound confirmation, and tentatively identified compounds.

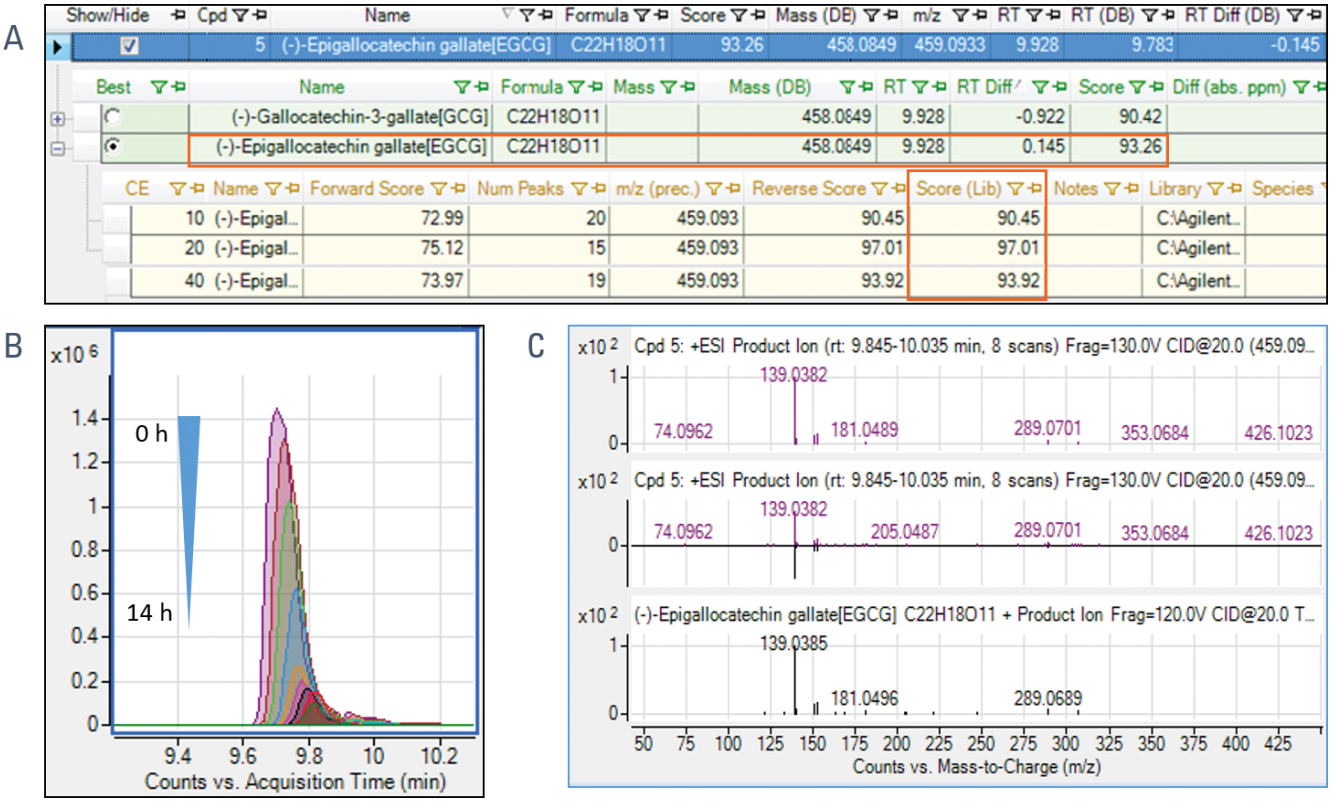

Figure 6. Identification of a common tea metabolite (–)-epigallocatechin gallate (EGCG) by matching the accurate MS/MS spectrum and retention time against the customized tea PCDL in the lab. A) The MS2 spectrum/retention time matching result. B) The differential metabolite changes over the fermentation duration. C) The mirror display of the acquired MS2 spectrum and the reference spectrum in the tea PCDL.

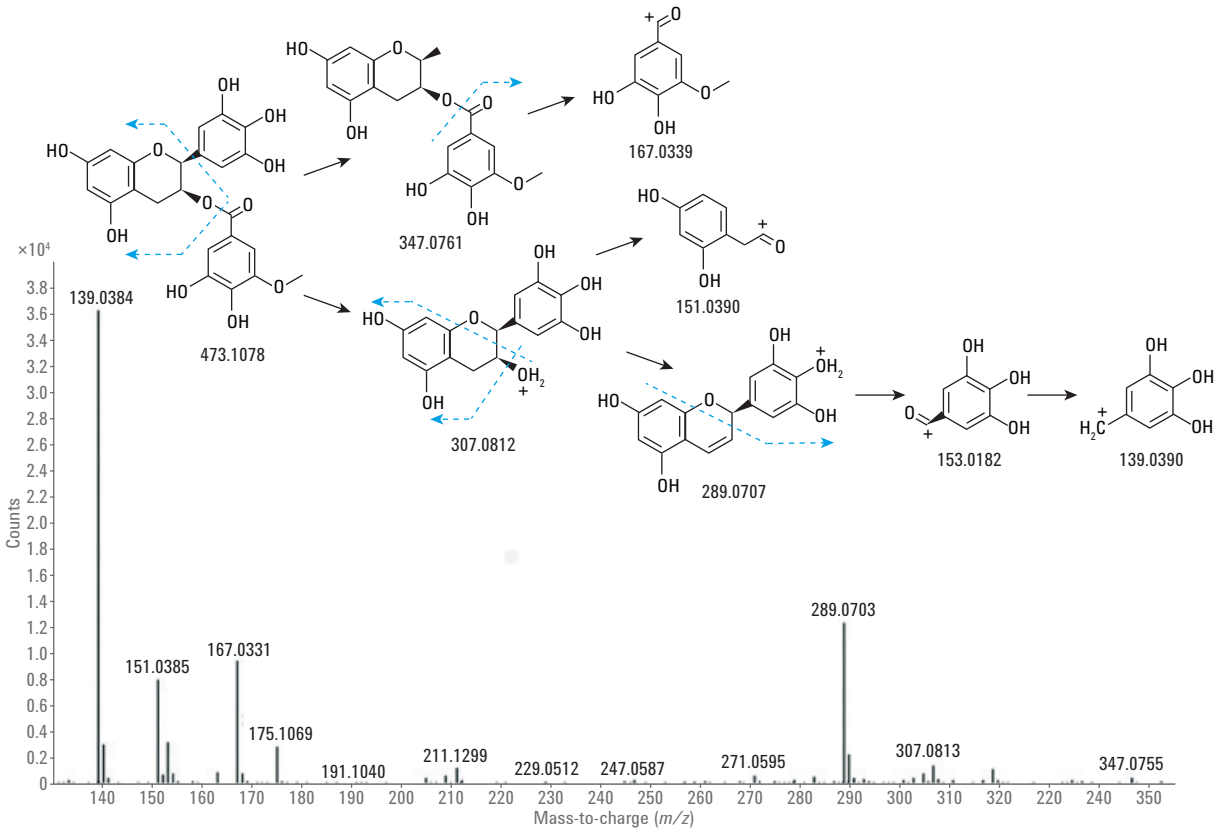

Figure 7. A typical metabolite with an *m/z* of 473.1081 at 11.4 minutes was tentatively identified as epigallocatechin methyl gallate. This was based on its accurate MS2 spectrum, and the possible fragmentation pathway due to collision-induced dissociation in the Q-TOF mass analyzer, shown in the inset.

Table 2. A list of the major identified and tentatively identified compounds in black tea during fermentation processing (A and B in the note column represents the compound confirmed using authentic standard and identified tentatively, respectively).

RT (min)	ΔtR (min)	Exp. *m/z*	Theo. *m/z*	Mass error (ppm)	Formula	Metabolite identification	Note
Catechins (10)							
4.60	–0.02	307.0815	307.0820	1.6	$C_{15}H_{14}O_7$	(+)-Gallocatechin [GC]	A
8.22	–0.01	291.0864	291.0863	0.3	$C_{15}H_{14}O_6$	(+/–)-Catechin [C]	A
9.77	–0.01	459.0923	459.0922	0.2	$C_{22}H_{18}O_{11}$	(–)-Epigallocatechin gallate [EGCG]	A
7.97	0.00	307.0811	307.0812	–0.3	$C_{15}H_{14}O_7$	(–)-Epigallocatechin [EGC]	A
10.57	0.01	291.0864	291.0863	0.3	$C_{15}H_{14}O_6$	(–)-Epicatechin [EC]	A
10.80	–0.05	459.0921	459.0922	–0.2	$C_{22}H_{18}O_{11}$	(–)-Gallocatechin–3–gallate [GCG]	A
11.40	–	473.1081	473.1078	0.6	$C_{23}H_{20}O_{11}$	Epigallocatechin methylgallate	B
12.16	–0.06	443.0983	443.0973	2.3	$C_{22}H_{18}O_{10}$	(–)-Epicatechin gallate [ECG]	A
12.39	–0.10	275.0914	275.0914	0.0	$C_{15}H_{14}O_5$	Epiafzelecin (2R,3R) (–)	A
14.24	–	457.1129	457.1129	0.0	$C_{23}H_{20}O_{10}$	Epicatechin-3-O-methlgallate	B

RT (min)	ΔtR (min)	Exp. *m/z*	Theo. *m/z*	Mass error (ppm)	Formula	Metabolite identification	Note
Dimers of catechins (9)							
5.08	–	763.1508	763.1505	0.4	$C_{37}H_{30}O_{18}$	Theasinensin B	B
7.45	–	915.1623	915.1614	1.0	$C_{44}H_{34}O_{22}$	Theasinensin A/D	B
7.81	–	579.1501	579.1497	0.7	$C_{30}H_{26}O_{12}$	Procyanidin B1	B
8.87	–0.13	579.1497	579.1497	0.0	$C_{30}H_{26}O_{12}$	Procyanidin B2	A
9.21	–	899.1658	899.1665	–0.8	$C_{44}H_{34}O_{21}$	Theasinensin F/G	B
9.63	–	731.1601	731.1607	–0.8	$C_{37}H_{30}O_{16}$	3-Galloylprocyanidin B1	B
22.95	–	717.1447	717.1450	–0.4	$C_{36}H_{28}O_{16}$	Theaflavin-3-gallate	B
23.26	–	869.1564	869.1560	0.5	$C_{43}H_{32}O_{20}$	Theaflavin 3, 3′-digallate	B
23.39	–	717.1447	717.1450	–0.4	$C_{36}H_{28}O_{16}$	Theaflavin 3′-gallate	B
Flavonol glycosides and flavone glycosides (20)							
11.77	–	595.1660	595.1658	0.3	$C_{27}H_{30}O_{15}$	Apigenin-6,8-C-diglucoside	A
13.32	–	565.1561	565.1552	1.6	$C_{26}H_{28}O_{14}$	Apigenin-6-C-glucoside-8-C-arabinoside	B
13.65	–	565.1558	565.1552	1.1	$C_{26}H_{28}O_{14}$	Apigenin-6-C-arabinoside-8-C-glucoside	B
14.01	–	481.0979	481.0977	0.4	$C_{21}H_{20}O_{13}$	Myricetin-3-galactoside	A
15.75	–0.06	465.1021	465.1028	–1.5	$C_{21}H_{20}O_{12}$	Myricitrin	A
15.94	–	773.2151	773.2135	2.1	$C_{33}H_{40}O_{21}$	Quercetin-3-O-glucosyl rutinoside	B
16.25	0.03	433.1138	433.1129	2.1	$C_{21}H_{20}O_{10}$	Isovitexin (saponaretin)	A
16.66	–0.03	465.1031	465.1028	0.6	$C_{21}H_{20}O_{12}$	quercetin-3-O-*beta*-D-galactopyranoside	A
16.81	–	465.1028	465.1028	0.6	$C_{21}H_{20}O_{12}$	Quercetin-3-O-galactoside	
17.01	–0.04	611.1602	611.1607	–0.8	$C_{27}H_{30}O_{16}$	rutin (quercetin-3-O-rutinoside)	A
17.07	0.00	465.1028	465.1028	0.0	$C_{21}H_{20}O_{12}$	Isoquercitrin (quercetin-3-O-glucoside)	A
17.68	–	757.2208	757.2186	2.9	$C_{33}H_{40}O_{20}$	Kaempferol-3-O-galactosyl rutinoside	B
18.76	–	757.2208	757.2186	2.9	$C_{33}H_{40}O_{20}$	Kaempferol-3-O-glucosyl rutinoside	B
18.93	–	449.1084	449.1078	1.3	$C_{21}H_{20}O_{11}$	Kaempferol-3-O-galactoside	B
19.15	–	449.1084	449.1078	1.3	$C_{21}H_{20}O_{11}$	Kaempferol-3-*beta*-D-glucopyranoside	B
19.75	–0.01	449.1081	449.1078	0.7	$C_{21}H_{20}O_{11}$	Astragalin (kaempferol-3-O-glucoside)	A
19.83	0.00	595.1661	595.1657	0.7	$C_{27}H_{30}O_{15}$	Kaempferol-3-O-[*alpha*-L-rhamnopyranosyl-(1-6)-O-*beta*-D-glucopyranoside]	A
23.63	–0.01	287.0549	287.0550	–0.3	$C_{15}H_{10}O_{6}$	Luteolin	A
24.96	–0.06	287.055	287.0550	0.0	$C_{15}H_{10}O_{6}$	Kaempferol	A
25.27	–0.05	271.0598	271.0601	–1.1	$C_{15}H_{10}O_{5}$	Apigenin	A
Organic acids (6)							
9.25	–	265.1441	265.1434	2.6	$C_{15}H_{20}O_{4}$	Abscisic acid	B
3.29	–	345.0827	345.0816	–3.2	$C_{14}H_{16}O_{10}$	Theogallin	B
8.19	–	339.1077	339.1074	0.9	$C_{16}H_{18}O_{8}$	4-O-*p*-coumaroylquinic acid	B
9.83	–	165.0544	165.0546	–1.2	$C_{9}H_{8}O_{3}$	*p*-Coumaric acid	B
10.95	–	339.1077	339.1074	0.9	$C_{16}H_{18}O_{8}$	3-O-*p*-coumaroylquinic acid	B
11.91	–	337.0908	337.0918	–3.0	$C_{16}H_{16}O_{8}$	Caffeoylshikimic acid	B

RT (min)	ΔtR (min)	Exp. *m/z*	Theo. *m/z*	Mass error (ppm)	Formula	Metabolite identification	Note
Alkaloids (4)							
1.55	–	104.1067	105.1170	–2.9	$C_5H_{14}NO^+$	Choline	B
1.61	–	258.1108	258.1101	2.7	$C_8H_{21}NO_6P^+$	Glycerophosphocholine	A
6.00	0.12	181.0724	181.0720	2.2	$C_7H_8N_4O_2$	Theobromine	A
9.88	–0.01	195.0904	195.0877	13.8	$C_8H_{10}N_4O_2$	Caffeine	A
Amino acids (9)							
1.60	0.00	134.0450	134.0448	1.5	$C_4H_7NO_4$	L-Aspartic acid	A
1.62	0.02	148.0605	148.0604	0.7	$C_5H_9NO_4$	Glutamic acid	A
1.70	0.00	116.0710	116.0706	3.4	$C_5H_9NO_2$	Proline	A
1.94	–0.06	118.0865	118.0863	1.7	$C_5H_{11}NO_2$	Valine	A
2.08	–0.02	175.1082	175.1077	2.9	$C_7H_{14}N_2O_3$	L-Theanine	A
2.20	–	337.1615	337.1605	3.0	$C_{13}H_{24}N_2O_8$	Theanine glucoside	B
2.88	–0.02	132.1019	132.1019	0.0	$C_6H_{13}NO_2$	Leucine	A
3.09	–0.01	132.1020	132.1019	0.8	$C_6H_{13}NO_2$	Isoleucine	A
4.85	–0.11	166.0865	166.0863	1.2	$C_9H_{11}NO_2$	Phenylalanine	A
Nucleosides (4)							
2.84	–	314.0923	314.0918	1.6	$C_{11}H_{15}N_5O_4S$	(S)–5′-Deoxy–5′() adenosine	B
2.96	0.04	284.0990	284.0989	0.4	$C_{10}H_{13}N_5O_5$	Guanosine	A
2.99	–	269.0881	269.0880	0.4	$C_{10}H_{12}N_4O_5$	Inosine	B
8.78	–	298.0969	298.0968	0.3	$C_{11}H_{15}N_5O_3S$	5′-Deoxy-5′-(methylthio) adenosine	B

Metabolite change in tea during the fermentation process

To better illustrate the changes of the identified differential metabolites during the fermentation process, a heat map of tea samples versus these metabolites was plotted using hierarchy cluster analysis. Figure 8 shows this heat map; the red and blue colors in the figure represent respectively a higher and a lower content than the mean value, in yellow. The intensity of the color indicates the fold change compared to the mean value. By comparing the variation in color intensity for the samples, it was found that compounds belonging to the same subgroup exhibited similar dynamic change patterns during the entire fermentation process. Furthermore, the dynamic changes of these phenolic metabolites could be explained through the phenylpropanoid/flavonoid pathway, suggesting multichannel transformations of phenols during the fermentation process[1].

It is widely known that flavanols (catechins) are one group of the characteristic and abundant metabolites in fresh tea leaves. Flavanols are considered to be the major contributors to the puckering astringency and bitter taste[6]. They can be oxidized to dimeric, oligomeric, and polymeric compounds during tea fermentation processing[5,7].

In this study, the typical catechin-related compounds, C, EC, EGC, EGCG, ECG, epicatechin 3-O-(methyl gallate), and epigallocatechin methyl gallate all exhibited significant decrease within the initial 6 hours of fermentation before reaching a plateau after 8 hours (top part of Figure 8). The remaining contents of these catechins could be below 8.3 % of initial contents (0 hours) after 14 hours of fermentation. Almost all of the catechin (99.9 %) and epigallocatechin (99.1 %) were depleted. In comparison, the depleting rate of epiafzelechin, which is also a type of catechin, was much slower (64 % was depleted) than the previously described catechins.

The levels of dimerized catechins, theaflavins (TFs), were very low in the initial fresh tea leaves, but increased rapidly within the first 4 hours of the fermentation by enzymatic oxidation of the catechins. However, TF levels decreased between 6–14 hours, perhaps due to further transformation into oligomeric catechins. Other dimers of flavanols, theasinensins and procyanidins, were also found to be transformed dramatically during the fermentation. Theasinensin A and theasinensin B increased sharply within the first 4 hours, then slowly decreased in the following period. The levels of procyanidins were very high in the control sample and the first 2 hours of fermentation, then decreased rapidly within 4–6 hours, before remaining constant throughout the rest of the fermentation process.

Most compounds from other groups, including flavonol glycosides and flavone glycosides, organic acids, alkaloids, and amino acids also exhibited intensity differences during fermentation. However, these intensity differences were not as obvious as those of the catechins and their dimerization products. Some exceptions were mericitin-3-gallactoside, *p*-coumaric acid, and theogallin, which showed very high abundance in the fresh tea and during the initial 2 hours of fermentation. In addition, caffeoylshikimic acid clearly exhibited high abundance during 1–2 hours of fermentation, and then decreased in the following fermentation process.

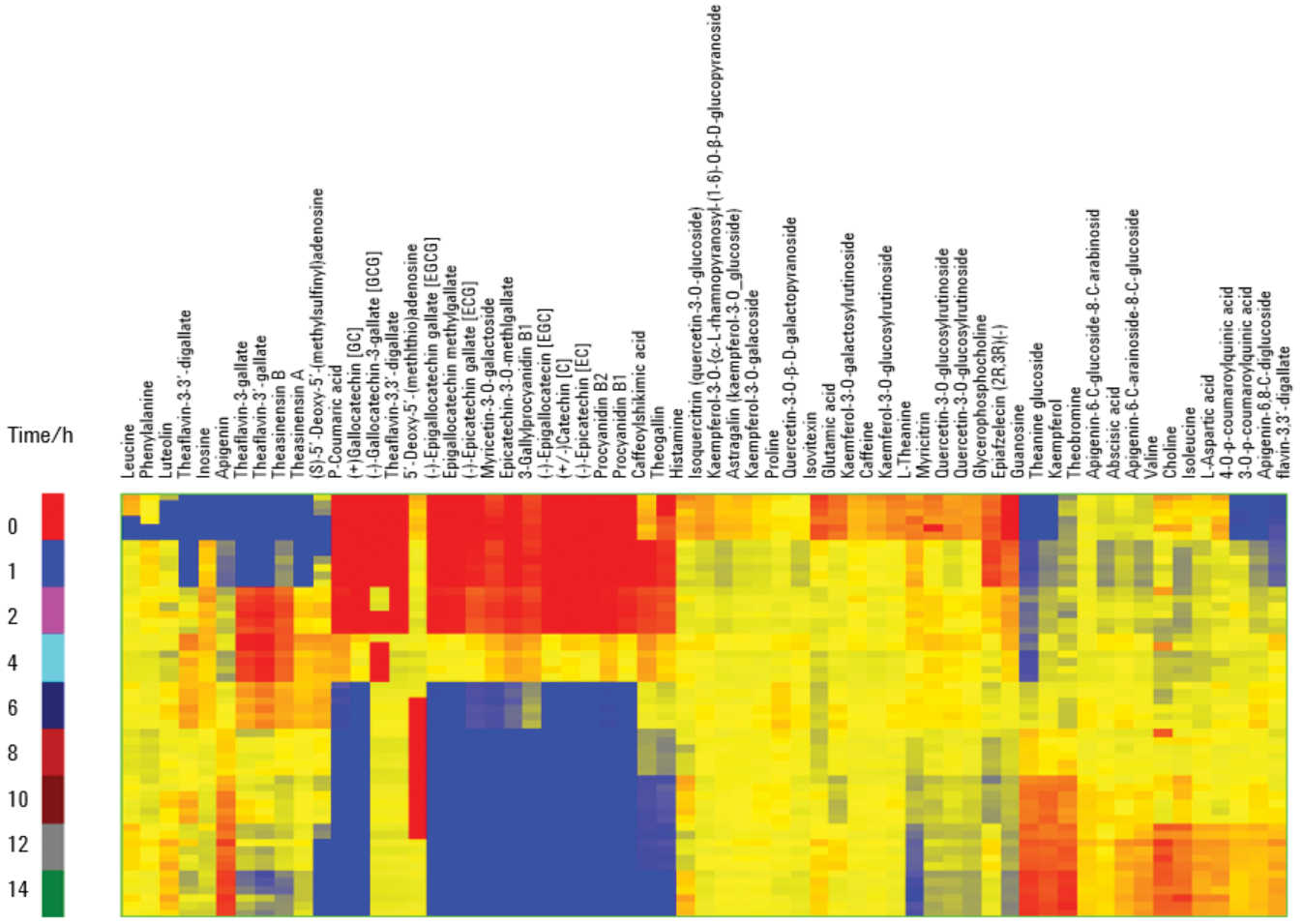

Figure 8. Heat map of the identified/tentatively identified differential metabolites during the tea fermentation process, showing an obvious and grouped change in intensity.

Correlation between tea flavor and the identified differential metabolites

The tea leaves were made into black tea, and tasted by an expert tea panel on the bitterness and astringency using a relevant score value from 1 to 10. This tasting was to further explore whether the changes in abundance of the identified compounds may contribute to the flavor changes over the fermentation time. The changes in score value of bitterness and astringency were correlated with the change in abundance of the identified differential compounds using the Pearson correlation analysis provided by MPP software. As shown in Figure 9A, most compounds are simultaneously either positively (red colored) or negatively (blue colored) correlated with both bitterness and astringency. Particularly for ECG, 3-galloyl procyanidin B1, myricetin-3-O-galactoside, L-theanine, and kaempferol, they exhibited good correlation with bitterness and astringency, with the correlation coefficients (R^2) of higher than 0.8 for at least one taste. Figure 9B shows the typical correlation curves for these compounds.

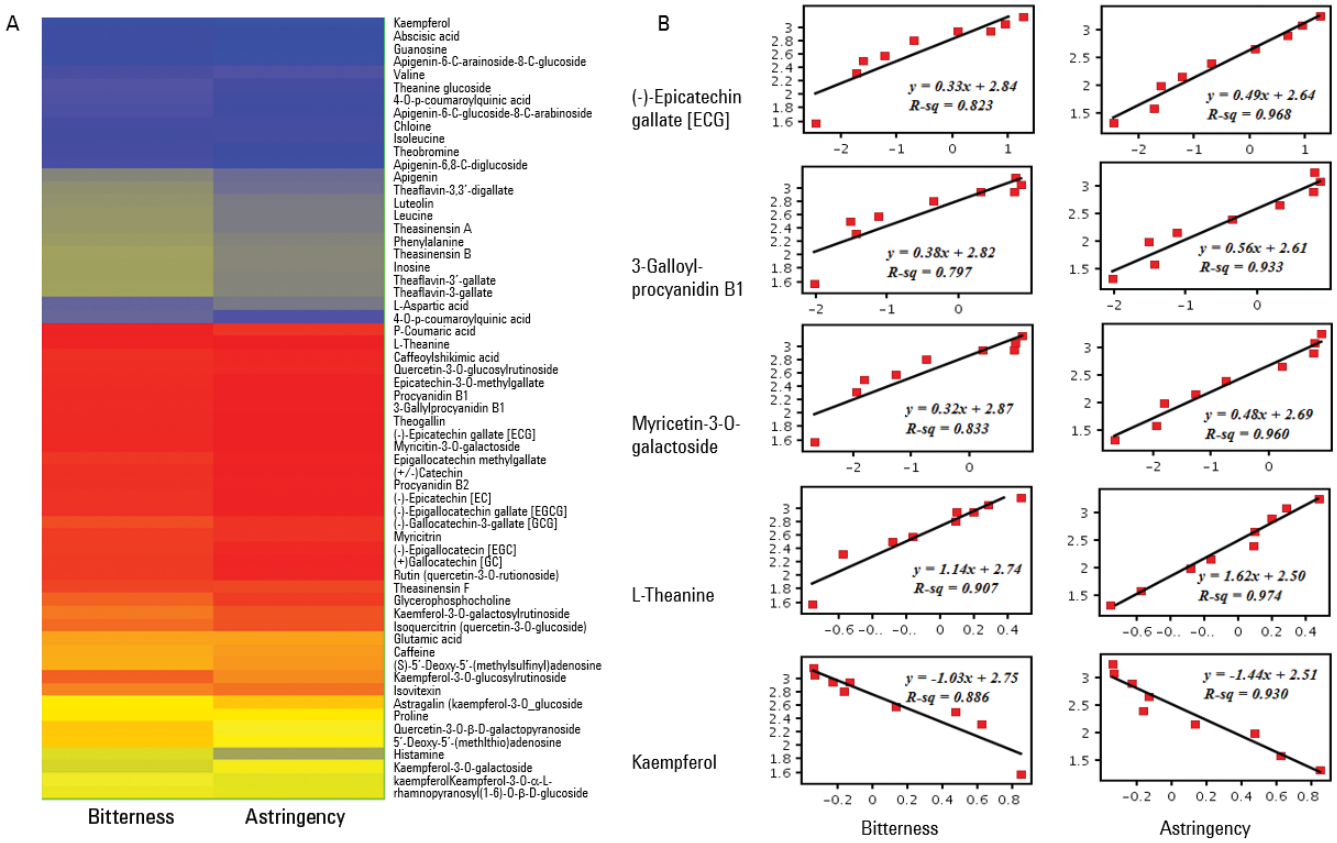

Figure 9. Pearson correlation analysis of the abundance change of the identified metabolites with the variation of the black tea taste (A), and the correlation curve for the typical differential metabolites (B).

Conclusions

Tea fermentation is a complex process in which various chemical reactions take place and induce distinct changes in the chemical constituents. Using a nontargeted metabolomics approach, more than 1,000 differential metabolite ion features were mapped with the fermentation time change, among which 62 differential compounds were structurally identified. The identified metabolites revealed distinct metabolomic patterns of teas with fermentation duration up to 14 hours, and most of them exhibited very good correlation with the bitterness and astringency of the black tea. This study offers a comprehensive picture for the variations of tea metabolites during fermentation. It can also be applied to control, and improve the sensory quality and nutritional value of the black tea during tea manufacturing.

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