Application Note

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Discovery of the Potential Marker Compounds for Stored White Tea by a Metabolomics Approach

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Abstract

This Application Note describes a metabolomic approach for profiling nonvolatile compounds in white tea, to search for possible chemical markers of white tea aging, which was modified from a recent work[1] using MPP as the primary chemometric software for statistics analysis. The established workflow uses an Agilent 1290 Infinity II LC together with an Agilent 6540/6545 Q-TOF LC/MS. Results showed that some compounds in white tea changed significantly over aging time, with 125 such differential compounds tentatively identified. Among these tentatively identified compounds, seven new compounds formed during storage, identified as 8-C N-ethyl-2-pyrrolidinone substituted flavan-3-ols (EPSFs). The content of the EPSFs increased over storage duration, accompanying by decreasing amount of their precursors, theanine and flavan-3-ols, suggesting that EPSFs can be promising marker compounds of white tea under long-term storage.

Introduction

Traditional Chinese white tea has shown potential beneficial health effects,[2] thus promoting the consumption of white tea in China.[3] White teas stored for long periods are considered to have high health-protective effect and high commercial value. Only some commonly known highly abundant substances in white tea have been investigated during storage, for example, catechins and amino acids were found to be decreasing in white teas under different storage times, while the content of gallic acid increased.[3] However, the decrease of the common metabolites could be accompanied with the formation of new compounds during storage that were rarely seen in previous reports. In this study, a metabolomics approach based on ultrahigh performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF/MS) was applied to comprehensively study the nonvolatile components in white tea during storage, and seek the potential markers related to tea quality change over storage time.

Experimental

Sample preparation

Two types of white tea samples, Bai Hao Yin Zhen (BHYZ) and Bai Mu Dan (BMD), produced from 2000 to 2015 were collected from two tea companies in Fujian, China. BHYZ was produced from only one bud, and BMD was produced from one bud with two leaves. The raw fresh leaves were processed into white tea according to a typical white tea manufacturing procedure, which includes withering and drying processes.[4] The white tea products were preserved in a storehouse maintained at 15 to 25 °C and 25 to 50% humidity. The stored white tea was ground into powder, and the tea metabolome was then extracted using methanol/water (v/v: 7/3). The extractant was centrifuged, filtered, and submitted to LC/MS analysis. To make the pooled quality control samples, an equal volume of each extracted sample was mixed thoroughly.

LC Conditions

Parameter	Value	
HPLC	• Agilent 1290 Infinity II LC with built-in degasser • Autosampler with temperature control • Column temperature control compartment	
Column	Agilent ZORBAX Eclipse Plus C18, 150 × 3.0 mm, 1.8 µm	
Column Temperature	40 °C	
Mobile Phase	**Solvent A:** 0.1 % formic acid in H_2O	
	Solvent B: methanol	
Flow Rate	0.4 mL/min	
Injection Volume	3.0 µL	
Needle Backflush	5 seconds with pure methanol	
Gradient Elution Profile	0 to 4 minutes	10 to 15 % B
	4 to 7 minutes	15 to 25 % B
	7 to 9 minutes	25 to 32 % B
	9 to 16 minutes	32 to 40 % B
	16 to 22 minutes	40 to 55 % B
	22 to 28 minutes	55 to 95 % B
	28 to 30 minutes	95 % B
	30 to 31 minutes	95 to 10 % B
	31 to 35 minutes	10 % B

ESI-Q-TOF MS conditions

Parameter	Value
MS	Agilent 6540/6545 Q-TOF LC/MS with dual Jet Stream ESI
Polarity	Positive ionization
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
MS Scan Range	*m/z* 100 to 1,100
MS/MS Scan Range	*m/z* 50 to 1,100
Reference Ions	*m/z* 121.0509/922.0098

Workflow for metabolomics analysis

The analysis process of tea extracts was slightly modified from a previous report,[5] which is presented in Figure 1. After acquiring the raw data for the tea extract with different quality and varying storing years, the data were then subjected to a data mining algorithm of molecular feature extraction to extract reliable compounds. The resulting compounds information were imported into Agilent Mass Profilier Professional software (MPP, version 14.9), in which compounds were first aligned across the samples within the allowed variation of retention time window and accurate mass window, then annotated uniformly using the retention time and accurate mass. A compound (peak) table was obtained through reasonable data filtration. A previous report used Simca-P and PASWstat software[1], however this study used MPP for PCA/PLSDA analysis, hierarchy cluster analysis, and correlation analysis. Feature identification and markers validation were conducted using Agilent MassHunter Acquisition and Qualitative Analysis software packages.

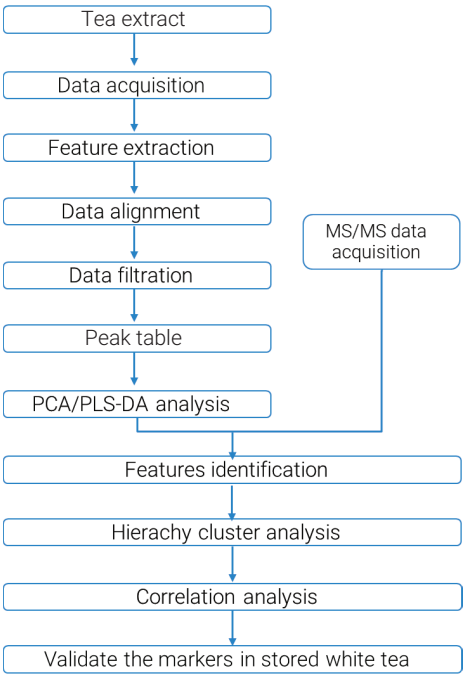

Figure 1. Schematic diagram showing the workflow for metabolomics analysis.

Results and discussion

Data quality validation

An optimized UHPLC gradient elution was applied for separation of thousands of compounds in the tea extract as described in a previous report.[4,5] Accurate Q-TOF MS in scanning mode was initially applied for detection of the compounds present in the tea metabolome following separation. Pooled quality control samples were analyzed through the sample set to evaluate the data quality every 10 samples. Using the molecular feature extraction algorithm provided in MassHunter Profinder software (v. 8.0), the extracted compound results from two types of white tea samples under different storage durations were imported into MPP software for data alignment and statistical analysis. Initial data alignment of the total data set resulted in 2,584 compound features for the dataset. The compound features with relative deviation below 35% for QC samples were then subjected to univariate and multivariate analysis.

Figure 2 shows that nonsupervised PCA analysis demonstrated that the QC samples are tightly clustered in the center of the score plot. This result demonstrates excellent reproducibility of the tea sample extraction and the UHPLC/Q-TOF MS analysis during the metabolomics investigation. The two types of white tea, BHYZ and BMD samples, are clearly separated in PCA score plot (Figure 2), suggesting that the quality type of white tea has a larger influence on the nonvolatile chemical constituents than the storage duration. Therefore, in the following analysis, the storage duration for both types of white tea were investigated separately.

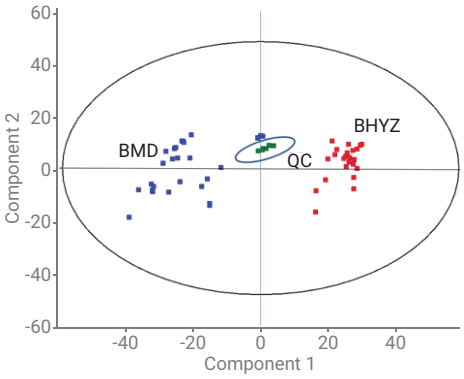

Figure 2. PCA score plot showing the tight clustering of QC samples and a clear separation of both types of white tea (BHYZ and BMD) under different storage duration.

Influence of storage on the white tea compounds

Both principle component analysis (PCA) and partial least square differential analysis (PLS-DA) were applied to investigate the influence of storage duration on the white tea samples based on the abundance of the compounds. As shown in Figure 3, with PCA analysis, the score plot in the top shows slight separation of the tea samples with different storage duration. In comparison, with PLS-DA analysis, it is clear that for either type of white tea (BHYZ and BMD), tea samples with similar duration time cluster in the same region of the score plot (Figure 3). Such patterns demonstrate that the nonvolatile white tea compounds change significantly over the extended storage time.

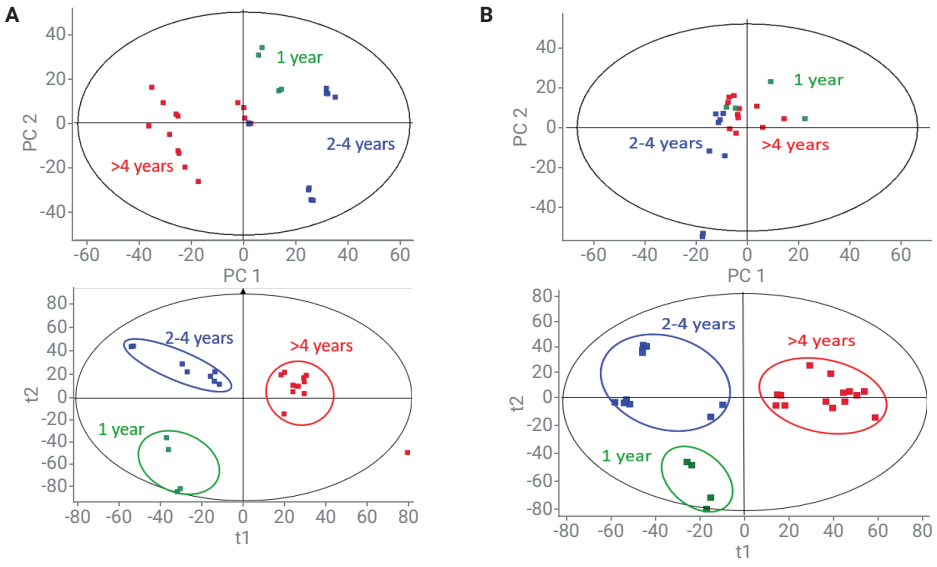

Figure 3. PCA (top) and PLS-DA (bottom, with 50-time permutation test) score plots demonstrating the change of the two subclasses of white tea over storage time. A. BMD; B. BHYZ.

Identified metabolites change during storage

There were 125 differential metabolite features tentatively identified by searching against HMDB and MetLin database and library, and by interpreting the accurate MS/MS spectra using Molecular Structure Correlator software. The following were confirmed using the authentic standards:

- Flavan-3-ols
- Dimeric flavan-3-ols (theaflavins, theasinensins, and procyanidins)
- Alkaloids
- Flavonol/flavone glycosides
- Amino acids
- Phenolic acids
- Nucleosides
- Organic acids
- Lipids
- Carbohydrates

The hierarchy cluster analysis diagram in Figure 4 demonstrates the obvious abundance change of the identified compounds in the subclasses of white tea. Due to space limitations, only the BMD subclass is shown as an example. As shown by the color scale of the abundance for each compound, these compounds can be classified into five major groups:

- **G1:** Very low intensity for one and two to four years of storage, and significantly high intensity for the tea stored for eight years or longer.
- **G2:** Medium or relatively high intensity for tea stored for one year, low intensity for the tea stored two to four years, but increased intensity when stored much longer.
- **G3:** Medium to high intensity when stored for one or two to four years, but much lower intensity when stored for eight years or longer.
- **G4:** High intensity when stored within one year, and slight decrease when stored longer.
- **G5:** Low intensity when stored for one year, and high intensity when stored for two to four years, and a slight decrease when stored much longer.

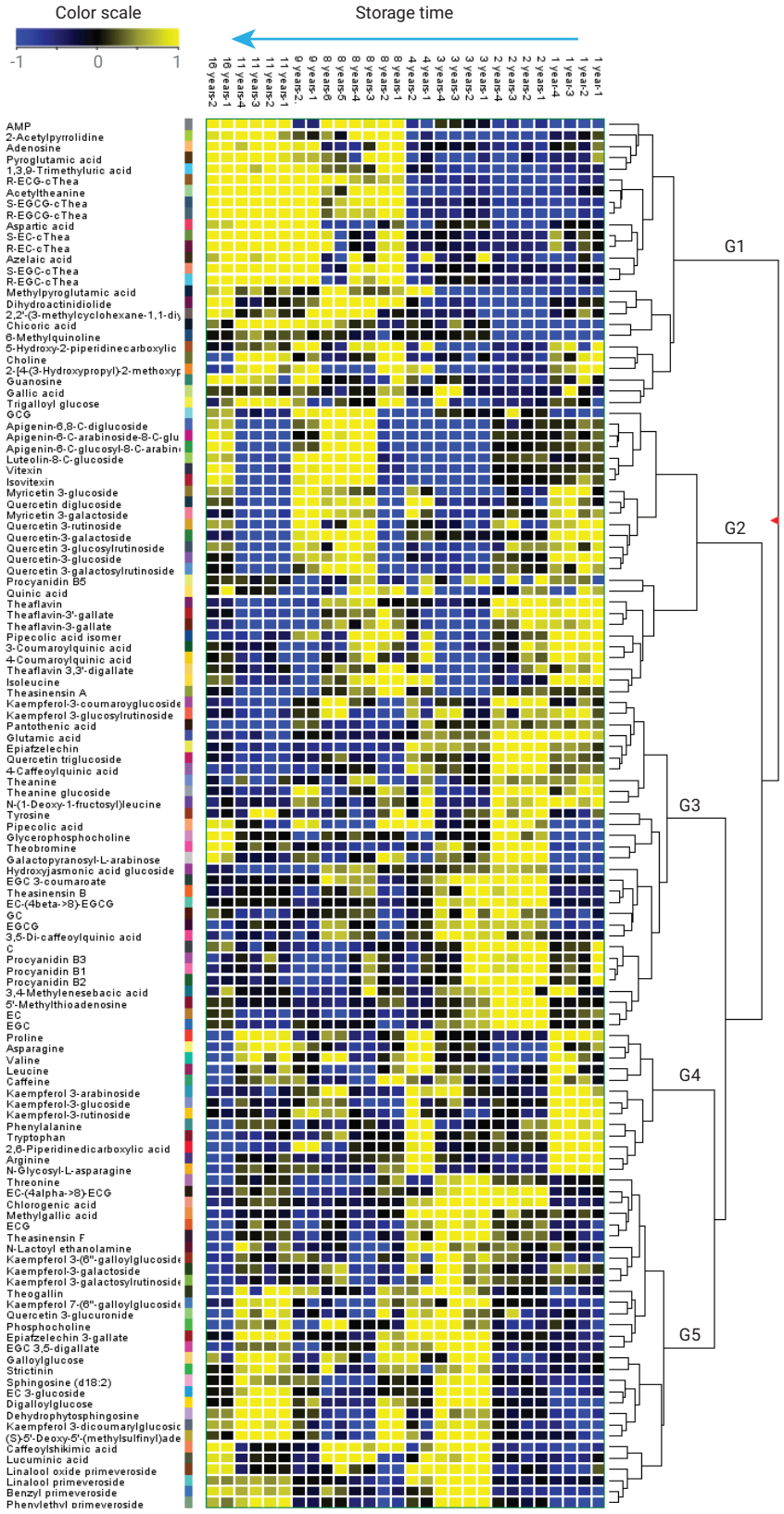

Figure 4. Hierarchy cluster analysis demonstrating the identified compounds abundance change over the storage time for BMD, one subclass of white tea.

EPSFs identification and their presence in aged white tea

Among the identified differential compounds, seven compounds were newly identified in the stored white tea. Two compounds have the *m/z* of 570.1606, two have the *m/z* of 418.1496, two have the *m/z* of 402.1542, and one has the *m/z* of 554.1657. Figure 5 shows the corresponding extraction ion chromatograms. Using peak 1 in Figure 5 as an example, the precursor ion can generate a number of fragment ions as shown in Figure 6A. Further interpretation of MS/MS spectra through assigning each fragment leads to identification of peak 1 as S- EGCG-cThea, one type of 8-C N-ethyl-2-pyrrolidinone substituted flavan-3-ols (EPSFs), and the possible fragmentation pathway of this compound is shown is Figure 6B. The other six peaks were also identified as the EPSFs group of compounds with difference in the substrate of flavan-3-ols (Table 1). Three out of these seven compounds were reported for the first time, and all seven compounds were first observed in white tea.

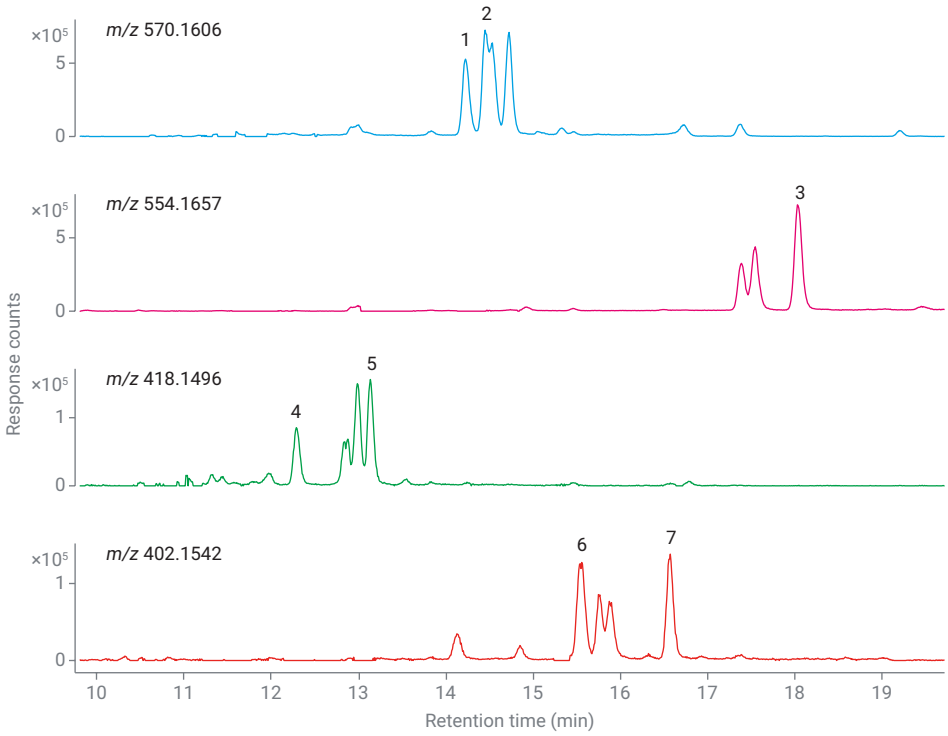

Figure 5. Typical extracted ion chromatograms (EICs) of seven EPSFs in stored white teas. Note: the white tea sample shown BHYZ subtype with storage time over six years.

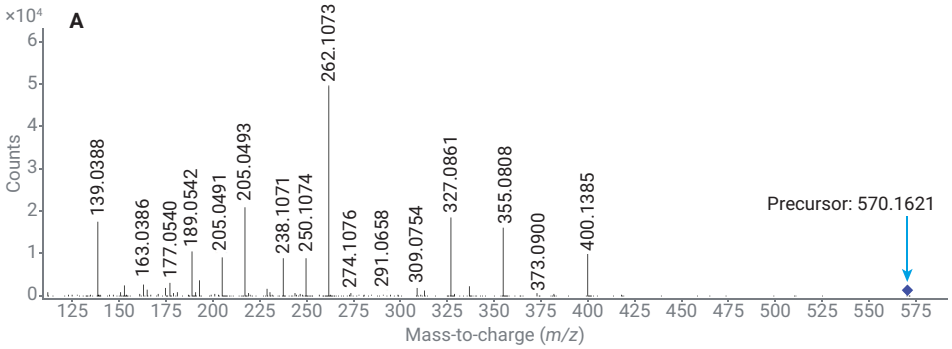

Figure 6A. MS/MS spectra of peak 1 in Figure 5.

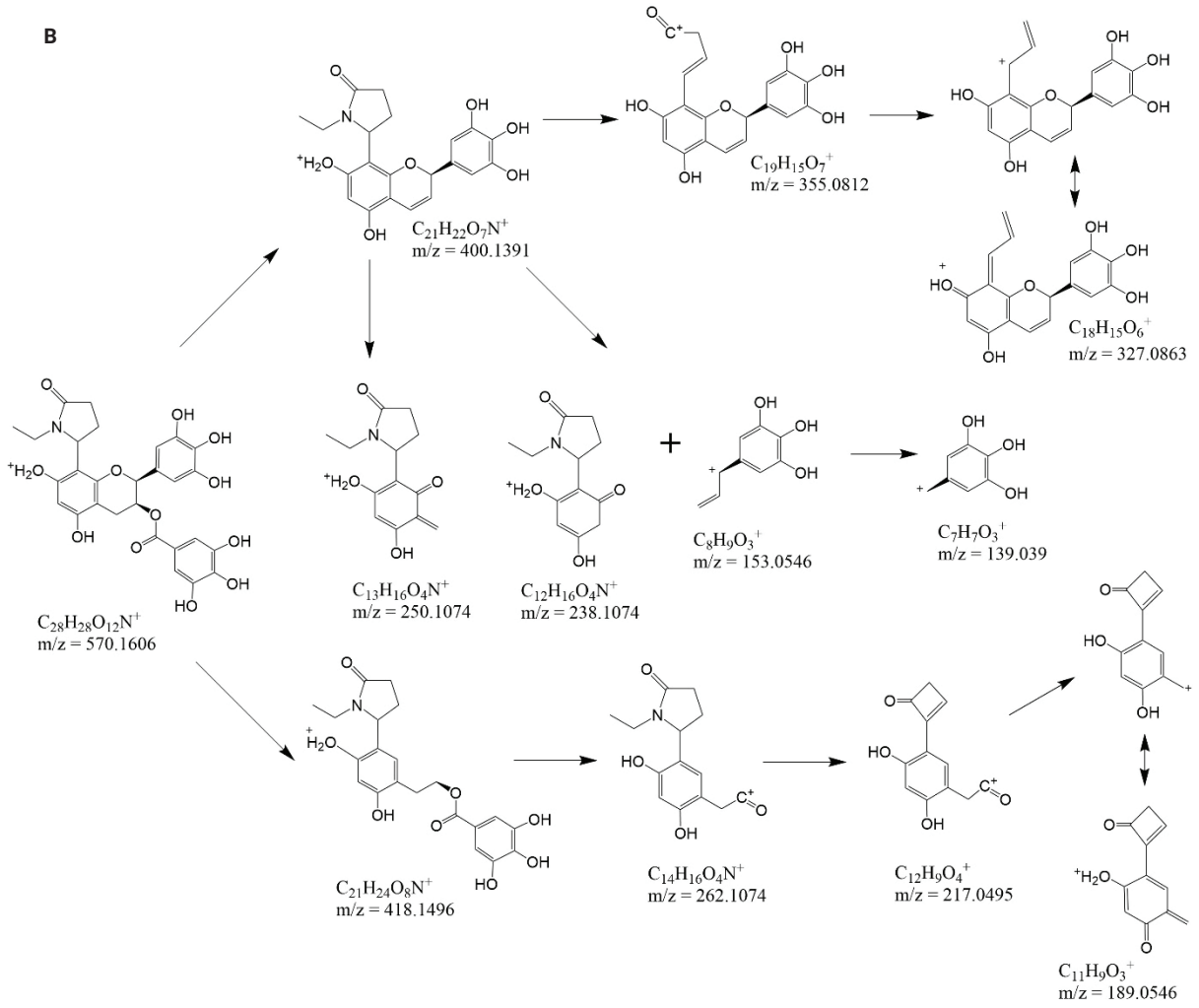

Figure 6B. Interpretation of each fragment results in the tentative identification of the compound and its possible fragment pathway.

Table 1. List of the identified ESPF compounds.

No.	Identification	RT (minutes)	Formula	$[M+H]^+$ $(m/z)_{exp}$	Error (ppm)	MS/MS Fragments (*m/z*)
1	S- EGCG-cThea	14.24	$C_{28}H_{28}O_{12}N$	570.1617	1.93	400.1391,355.0812, 327.0863, 262.1074, 250.1074, 238.1074, 217.0495, 139.0390
2	R- EGCG-cThea	14.45	$C_{28}H_{28}O_{12}N$	570.1613	1.23	
3	R-ECG-cThea	18.06	$C_{28}H_{28}O_{11}N$	554.1657	1.80	384.1229,339.0863, 311.0914, 262.1065, 250.0490, 177.0514, 123.0441
4	S-EGC-cThea	12.30	$C_{21}H_{23}O_8N$	418.1506	2.39	400.1391, 355.0812,327.0863, 262.1074,250.0490, 177.0546, 139.0390,
5	R-EGC-cThea	13.15	$C_{21}H_{23}O_8N$	418.1505	2.15	
6	S-EC-cThea	15.55	$C_{21}H_{23}O_7N$	402.1551	2.24	384.1447, 311.0914, 262.1074, 250.1074, 205.0490, 177.0546, 123.0411
7	R-EC-cThea	16.58	$C_{21}H_{23}O_7N$	402.1553	2.74	

Variation of ESPFs during storage, and their correlation with the precursor compounds in white tea

The amounts of the seven EPSFs increases with increasing storage duration for both subclasses of white tea (Figure 7A and B). More significant difference was observed between white tea over four years than those ≤ 4 years. It suggests that EPSFs could be a useful marker for aged white tea. The increasing abundance of these EPSFs is correlated with the decreasing abundance of theanine and flavan-3-ols (Figure 7C) over the storage time, indicating that both compounds are the potential precursor compounds of EPSFs in white tea. It was confirmed through *in vitro* reaction of theanine and flavan-3-ols in solution and the laboratory controlled speedy aging white tea analysis.[1]

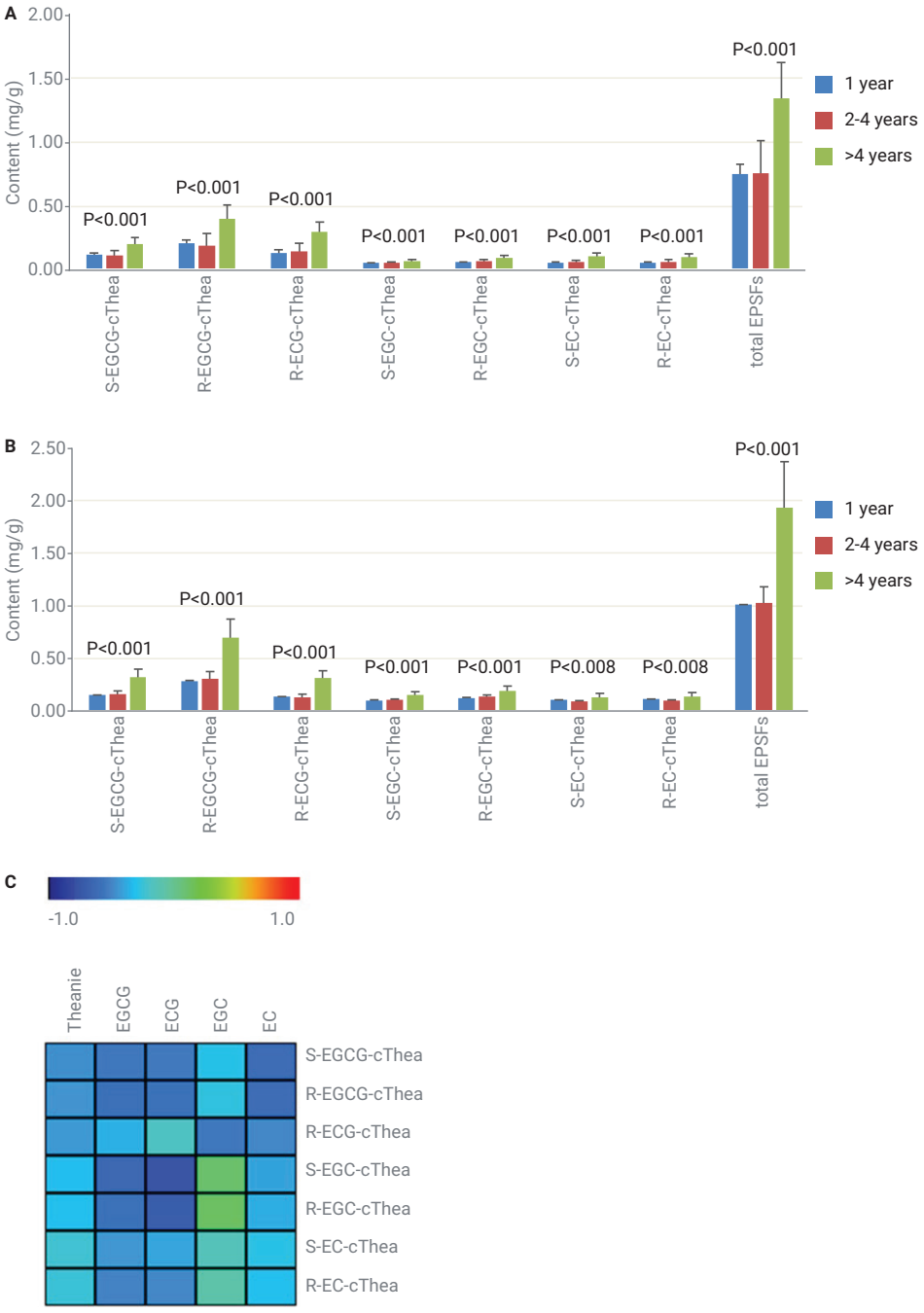

Figure 7. The contents of EPSFs in subclass of white tea, BHYZ (A) and BMD (B) (note: the significance of the compound amount difference among groups was tested using ANOVA). (C) Correlation analysis of the abundances between EPSFs and theanine and between EPSFs and flavan-3-ols in white teas.

Conclusion

Nontargeted metabolomic analysis was successfully performed for the study of white tea during extended storage. Up to 125 differential metabolites among the white tea samples were identified using accurate mass, MS/MS spectra, and the authentic/synthetic standards. EPSFs (8-C N-ethyl-2-pyrrolidinone substituted flavan-3-ols) are newly identified compounds in aged white tea samples, and their abundance is positively correlated with storage duration. This abundance is also correlated with the decrease in their precursor compounds, theanine and flavan-3-ols, suggesting that EPSFs can be promising aging markers of white tea under long-term storage.

References

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