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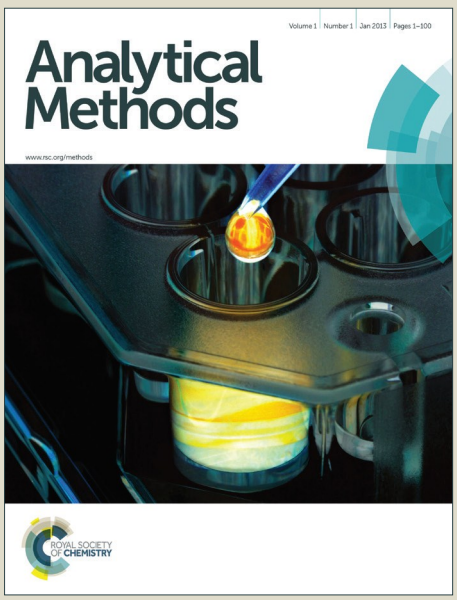

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Differentiation of two types of pu-erh teas by electronic nose and ultrasound-assisted extraction-dispersive liquid-Liquid microextraction-gas chromatography-mass spectrometry

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Abstract: It is a challenge task to discriminate raw pu-erh tea, notably aged raw tea, from ripened pu-erh tea, both of which are the two primary types of pu-erh teas, only based on taster's sensory evaluation. In current study, a workflow was proposed to differentiate those two clusters of pu-erh teas, as well as to point out and verify the markers being responsible for the discrimination. Initially, electronic nose was utilized for the rapid discrimination. Then, an efficient method based on ultrasound-assisted extraction-dispersive liquid-liquid microextraction-gas chromatography-mass spectrometry (UAE-DLLME-GC-MS) coupled with chemometrics methods was developed to disclose the metabolic profiles and pinpoint the markers for discrimination. Afterwards seven methoxyphenlic derivatives were simultaneously determined in both pu-erh teas. The role of volatile components for the classification of pu-erh teas was proved using the electronic nose (E-nose). Diverse parameters were optimized for UAE-DLLME-GC-MS, and a total of 84 volatile constituents were detected and identified. The methoxyphenlic derivatives as well as some alcohol derivatives were screened out as the primary markers by principle component analysis, and significant differences were revealed for the contents of methoxyphenlic compounds in these two types of pu-erh teas. Taken together, methoxyphenolic compounds as well as alcohol derivatives were found and verified as the markers for the differentiation between raw and ripened pu-erh teas, and either E-nose or UAE-DLLME-GC-MS could be applied as a reliable tool to achieve the discrimination.

Key words: pu-erh tea, differentiation, methoxyphenolic compounds, electronic nose,

ultrasound-assisted extraction-dispersive liquid-liquid microextraction coupled with gas chromatography-mass spectrometry (UAE-DLLME-GC-MS)

1. Introduction

Pu-erh tea, a famous and special post-fermentation tea originated from China about 3000 years ago, is made of the leaves of *Camellia sinensis* var. *assamica* (Mast.) Kitamura from Yunnan Province.[1] Nowadays, pu-erh tea has gained growing popularity among tea lovers all over the world because of its pleasant flavor properties as well as promising health benefits, such as antioxidation,[2,3] anti-obesity,[4,5] and antidiabetics.[6,7] Pu-erh teas are traditionally classified into two types according to the manufacturing processes, namely raw and ripen pu-erh teas. Raw pu-erh tea is made of the sun-dried green tea by autoclaving and compressing, and then being stored for several years at room temperature, whereas ripened pu-erh tea is "ripened" for several months using microbes under high temperature and humidity conditions prior to being pressed.[8] Today, pu-erh tea is not only a beverage, but also a collection for pu-erh tea enthusiasts. The prices of pu-erh teas with different storage years are varied. The longer the storage periods, the higher the prices, and the price difference can be up to tens to hundreds of times in the market.[9] In addition, it is worth noting that the compressed raw pu-erh tea can gradually be similar to ripened pu-erh tea in appearance and quality characteristics after long-term natural storage. Thus, the low-end ripened pu-erh teas sometimes were counterfeited as the high-end raw pu-erh teas by unscrupulous sellers for more profits. The tea consumers, who are confused by the two types of pu-erh teas in appearance and suffered from cheating, are thereby eager to learn the differences between them and how to distinguish. In particular, it is hard to discriminate the aged raw pu-erh tea from ripened pu-erh tea only according to

taster's sensory assessment, which is a conventional quality evaluation method in the tea industry. Therefore, it is of great importance to develop a simple and rapid discrimination method to act as an alternative of the sensory evaluation for the tea industry.

Aroma is one of the key indicators for the quality evaluation of teas and exhibits a great influence on its appreciation by consumers.[10] Tea's aroma is originated from the volatile components contained in *C. sinensis*, which can be affected by plant variety, harvest time, tea types, and processing techniques.[11] The characteristic volatile components would be important factors for the diagnosis of these two kinds of pu-erh teas.

The commonly used analytical techniques for aroma analysis in food industry include gas chromatography coupled with mass spectrometry (GC-MS) and electronic nose (E-nose). The E-nose, designed to mimic the mammalian olfactory system, is an analytical device with the ability to identify the mixture of volatile constituents as a whole.[12] The array of non-selective sensors interact with the volatile components presenting in the headspace and produce an electronic fingerprint or pattern characteristic to the odor or volatile compounds.[13] Nowadays, E-nose, with the advantages of non-invasive, fast-response, convenience, and low price, has been successfully applied in different food fields, such as identification of different types of teas,[14] discrimination of the propolis from different geographical and botanical origins,[15] and classification of cheeses.[16] However, the E-nose identifies volatile constituents as a whole rather than detects the individual chemical constituents

contained in the complex matrices.[17] When it is needed to correlate the sensor response pattern to the chemical pattern of the analyzed samples, E-nose technology is far from satisfactory.

On the other side, GC-MS is one of the most widely used techniques for the analysis of food volatile components because of its excellent performance in separating, identifying, and quantifying individual volatile compounds from complex systems. To date, GC-MS technique has been widely applied for the identification of the volatile components of foods and herbal drugs,[18,19] and determination of the content of pesticides in various fruit juices,[20] vegetables,[21] and honey products.[22]

In recent years, analytical techniques combining with multivariate analysis such as the similarity analysis (SA), hierarchical cluster analysis (HCA), principal component analysis (PCA), and partial least squares-discrimination analysis (PLS-DA) have been widely implemented in quality control, chemical classification, and chromatographic profile aligning of various foods.[23,24] PCA and HCA, severed as the most commonly used chemometric methods, are used to sort samples into groups by measuring similarity between samples.[25,26] Recently, PCA and HCA have been successfully used for discrimination of teas of different types,[25,27] different fermentation degrees,[28,29] and different grades.[30] Therefore, strategy integrating chemometrics with E-nose as well as GC-MS would have great application prospect for discriminating raw from ripened pu-erh teas, and also for the disclosure of discriminative contributors.

The aim of the current study is to differentiate those two clusters of pu-erh teas and to point out the markers being responsible for the discrimination, as well as to

simultaneously determine the distribution of seven primary methoxyphenolic compounds in the two types of pu-erh teas. To achieve this goal, a systematic strategy integrating E-nose, ultrasound-assisted extraction-dispersive liquid-liquid microextraction coupled with gas chromatography (UAE-DLLME-GC-MS), and chemometric methods was proposed. The workflow is elucidated in Fig. 1. Firstly, raw and ripened pu-erh teas were differentiated rapidly and objectively by the E-nose coupled with chemometrics. Then, UAE-DLLME-GC-MS-based metabolic profiling was introduced to reveal the chemical markers responsible for classification of these two types of pu-erh teas using chemometrics. The developed UAE-DLLME-GC-MS method was also employed for simultaneous determination of the methoxyphenolic compounds in pu-erh teas to validate those chemical markers.

2. Experimental

2.1. Reagents and standards

Methanol, ethanol, sodium chloride, chloroform, carbon tetrachloride, dichloromethane, tetrachloroethylene, ethyl acetate, acetone, and acetonitrile were purchased from Beijing Chemical Works (Beijing, China), and redistilled twice before use. Homologous series of C8-C40 *n*-alkanes were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultra-high purity helium (99.9999%) was supplied by Qianxi Gas Company (Beijing, China) and used as the carrier gas for GC-MS. 1,2-Dimethoxybenzene (> 99%), 3,4-dimethoxybenzene (> 99%), 1,2,3-trimethoxybenzene (> 99%), 1,2,4-trimethoxybenzene (> 97%), 3,4,5-trimethoxytoluene (> 98%), and 1,2,3-trimethoxy-5-methylbenzene (> 98%)

were purchased from TCI (Shanghai) Development Co., Ltd.; ethyl decanoate (internal standard, ≧99%) and 1-methoxy-4-(1-propenyl)-benzene (≧99%) were the products of Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water (Millipore, Bedford, MA, USA) of 18.2 MΩ/cm was used for the preparation of all aqueous solutions.

2.2. Apparatus

A KQ2200E (Jiangsu, China) ultrasonic water bath was used to facilitate the extraction. For centrifugation, an Anke TGL-16G-A centrifuge (Shanghai, China) was used. The injections into GC-MS were carried out using a 1 μL Hamilton microsyringe (Bonaduz, Switzerland). A 100 μL Hamilton syringe was used to inject organic solvents into sample solutions. An Agilent 6890N/5973N GC-MS (Agilent, CA, USA) was used for separation, identification, and quantification. Electronic nose A FOX-3000 (Alpha MOS, Toulouse, France), consisting of a sampling apparatus, an array of sensors, an HS-100 autosampler, an air generator equipment, and a pattern recognition software (SOFT V11.0) for data recording and analyzing, was used to analyze the odors of pu-erh teas. The sensor array of electronic nose was composed of 12 metal oxide semiconductors: LY2/LG, LY2/G, LY2/AA, LY2/GH, LY2/gCTL, LY2/gCT, T30/1, P10/1, P10/2, P40/1, T70/2, and PA/2.

2.3. Pu-erh tea sample collection

Thirteen batches of commercial raw pu-erh teas and thirteen batches of commercial ripened pu-erh teas with different post-fermentation years from different producing areas and companies were collected from Yunnan Province, China (Table S1). The samples were stored at -70 °C. All samples were grinded to pass through a 60 mesh

sieve before analysis.

2.4. Preparation of standard and sample solutions

The stock standard solution for GC-MS analysis was prepared by dissolving the standards of methoxyphenolic compounds in chloroform, which contained 0.972 g/L 1,2-dimethoxybenzene, 1.008 g/L 3,4-dimethoxybenzene, 0.944 g/L 1,2,3-trimethoxybenzene, 1.064 g/L 1,2,4-trimethoxybenzene, 0.968 g/L 1,2,3-trimethoxy-5-methylbenzene, 1.024 g/L 1-methoxy-4-(1-propenyl)-benzene, and 0.992 g/L 3,4,5-trimethoxytoluene. The stock solution of the internal standard (ethyl decanoate) was diluted with chloroform to yield a working standard of 201.0 ng/mL. A series of working standard solutions were prepared from the stock solutions by dilution with chloroform. All of these solutions were stored at 4 °C before use.

Sample solutions for GC-MS analysis were prepared by UAE-DLLME.

2.5. Electronic nose experimental procedure

The grated pu-erh teas were accurately weighed for 0.50 g, and placed in 10 mL headspace vials, sealed, and loaded into the autosampler tray. The heating time and temperature of the headspace vials were 600 s and 80 °C, respectively. Afterwards, 1000 μL of the aroma in the headspace vial was introduced into the testing chamber by a syringe on a flow rate of 150 mL/min and an injection rate of 1000 μL/s. The temperature of injector was set at 80 °C. The acquisition time and the time between injections were 120 s and 600 s, respectively. The response values of the 12 sensors for each sample were recorded. All samples were analyzed in triplicate.

2.6. UAE-DLLME procedure

A portion (100 mg) of accurately weighed pu-erh tea was transferred into a 15 mL screw cap glass test tube to which 1.5 mL of methanol was added. The sample was placed in the ultrasonic water bath for 45 min at 44 ℃ followed by centrifugation at 4500 rpm for 5 min. Then, 1.0 mL of the supernatant was transferred into another centrifuge tube. Afterward, 25 μL of chloroform (preconcentration solvent) was slowly injected into it with a 100 μL Hamilton syringe and the solution was vortex-mixed for 30 s. Then, 1.0 mL of the methanol-chloroform mixture was injected immediately into a conical tube containing 3.0 ml of 7.5% (w/v) NaCl solution, which served as the immiscible aqueous phase to initiate dispersive extraction. And a cloudy solution was formed in this step. The mixture was then centrifuged for 5 min at 4500 rpm to separate the organic and water phases. Finally, the preconcentrated, sedimented phase was transferred to a sample vial for GC-MS analysis.

2.7. Optimization of DLLME conditions

The single-factor experiment was utilized to select extraction and preconcentration solvents of DLLME. The Plackett-Burman (PB) design was performed to find out the significant factors for the UAE-DLLME. Then, response surface methodology (RSM) based on central composite design (CCD) was performed to optimize the extraction process.

2.8. GC-MS analytical method

An HP-5MS capillary column (5% phenyl methyl siloxane, 30 m × 0.25 mm i.d. × 0.25 μm film thickness) was used for analysis of the volatile components. High purity helium was used as carrier gas with a linear velocity of 1.0 mL/min. The injection

port was held at 260 °C in split mode at a ratio of 15:1. The initial oven temperature was 50 °C (2 min), which was ramped up at 3 °C/min to 200 °C and held for 5 min. Then it was ramped at a rate of 10 °C/min to 250 °C and held for 5 min. The detector temperature was 280 °C. The mass spectrometer was operated in electron impact (EI) mode with ionization energy of 70 eV and the transfer line temperature was set at 270 °C. Full scan mode was applied to screen for 40–550 amu.

2.9. Calculation of enrichment factors and retention indices

The enrichment factor (EF) was defined as the ratio between the analyte concentration in the sedimented phase (C_{sed}) and the initial concentration in the sample (C_0):

$$EF = \frac{C_{sed}}{C_0} \quad (1)$$

The retention indices (RIs) of all the components were calculated from gas chromatogram by linear interpolation of the related peaks located between two successive *n*-alkanes.

$$RI = 100[\frac{t_{R(i)} - t_{R(z)}}{t_{R(z+1)} - t_{R(z)}} + Z] \quad (2)$$

where z is the number of carbon atoms in the smaller *n*-alkane, $t_{R(i)}$, $t_{R(z)}$, and $t_{R(z+1)}$ are the retention times of the desired compound, the smaller *n*-alkane, and the larger *n*-alkane, respectively.

2.10. Data analysis

The volatile compounds were identified by comparing retention indexes and retention times with those obtained for authentic standards, or those of literature data, or with mass spectra in the Wiley and NIST11 libraries. The RIs were determined via sample

injection with a homologous series of alkanes (C8−C40) (Sigma-Aldrich, St. Louis, MO, USA).[31-34] The odor description of the volatile compounds were from literatures.[31, 33-35]

HCA was performed using SPSS statistical package (version 17.0 for Windows, SPSS, Inc., Chicago, IL, USA), and PCA was carried out using SIMCA-P 12.0 software (Umetrics, Umea, Sweden).The PB design matrix was generated, and the results were evaluated using Minitab16.0 software (Minitab, Inc., State College, PA). Design Expert 8.0.6 software (Stat Ease, Inc., Minneapolis, MN) was used to generate the CCD matrix and quadratic models that fit the experimental data as well as to draw the response surface plots.

3. Results and discussion

3.1. Classification of pu-erh teas by E-nose

The representative E-nose sensing signals of 12 sensors for raw and ripened pu-erh teas were shown in Fig. S1. The radar chart and response value map for the 26 batches of pu-erh teas were shown in Fig. S2 and Fig. S3, respectively. Each curve represents one sensor's conductivity induced by electro-valve action when volatile gas reaches the measurement chamber. As shown in Fig. S1-S3, the odor intensities of raw pu-erh teas differed from those of ripened pu-erh teas, indicating that the sensor responses of the E-nose varied with the manufacturing processes. PCA was further applied to the 26 batches of pu-erh teas. PCA is an unsupervised method and is used to reduce the dimensionality of dataset in order to obtain the maximum variation among samples. Fig. 2 showed the score plot for the two principal components (PC1 and PC2),

representing 98.87% of the total variability. The results demonstrated that pu-erh teas with different manufacturing processes could be distinguished according to their odors using the E-nose combined with PCA (Fig. 2). Hence, the E-nose could be accepted as a quick and useful analysis tool to distinguish raw pu-erh tea from ripened pu-erh tea.

3.2 Differentiation of pu-erh teas by UAE-DLLME-GC-MS

Although E-nose analysis based on PCA could realize the classification of pu-erh teas of different manufacturing processes, but it is not clear that what constituents contained in the teas, and what make the difference. In order to clarify the chemical constitution and to point out the markers of pu-erh teas with different manufacturing processes, an UAE-DLLME-GC-MS analysis was established carried out.

3.2.1. Selection of the extraction solvent

The solvent for UAE should exhibit high extraction capability of the targeted compounds and miscibility with both organic and aqueous phases in DLLME. Therefore, methanol, acetonitrile, ethanol, acetone, methanol-water (1:1, v/v), methanol-acetonitrile (1:1, v/v), acetonitrile-water (1:1, v/v), and ethanol-acetonitrile (1:1, v/v) were assessed. Among them, methanol displayed the highest extraction efficiency (Fig. 3), thus was selected as the extraction solvent for the subsequent experiments.

3.2.2. Selection of preconcentration solvent

The suitable preconcentration solvents which are vital for the success of DLLME should have higher density than water and good gas chromatography behavior. In our

study, four commonly used halogenated solvents including dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), tetrachloromethane (CCl_4), and chlorobenzene (C_6H_5Cl) were selected for extraction. The results showed that $CHCl_3$ was the most efficient (data not shown), and was therefore used in the subsequent experiments.

3.2.3. PB design for screening significant variables

An experiment based on the Plackett-Burman (PB) design was adopted to screen the significant factors for the extraction of the volatile components of pu-erh teas. Eight variables including the ultrasound temperature (°C), centrifuge rate (rpm), concentration of salt (%, w/v), volume of extraction solvent (mL), volume of preconcentration solvent (μL), sonication power (W), ultrasonic time (min), and centrifuge time (min) were analyzed according to their effects on the relative peak areas of the extracted components. Two levels (high and low, represented by +1 and −1) were chosen for each factor. A total of 12 experimental runs were performed. The investigated factors with their names and their levels are presented in Table S2.

Analysis of variation (ANOVA) was used to evaluate the data. The results were visualized using the Pareto chart (Fig. 4). The bar beyond the line corresponds to the effects that were statistically significant at the 95% confidence level. As shown in Fig. 4, the significant factors included ultrasound temperature, sonication time, concentration of NaCl, and volume of enrichment solvent. Among them, ultrasound temperature and sonication time displayed positive effects on the extraction efficiency, whereas the latter two factors resulted in negative effects.

3.2.4. Optimization of the extraction parameters using CCD

In this step, CCD was employed to optimize the significant extraction factors screened by PB design to obtain the best responses. The experiments were randomized in order to minimize the effect of uncontrolled factors. The main factors, their symbols and levels are shown in Table 1. The design matrix including the experiments, level of factors in each experiment, and the related responses is given in Table S3.

Based on the results of the performed experiments, the second order polynomial equation was obtained as following:

$$Y = 27.93 + 1.84\,X_1 + 0.54\,X_2 - 3.43\,X_3 - 2.93\,X_4 - 0.88\,X_1X_2 - 0.44\,X_1X_2 - 0.44\,X_1X_4 - 1.51\,X_1X\,4 - 1.68\,X_2X_3 - 0.67\,X_2X_4 - 0.48\,X_3X_4 - 1.98\,X_1^2 + 0.88\,X_2^2 - 0.68\,X_3^2 - 0.32\,X_4^2 \quad (3)$$

where Y is the response (relative peak area). ANOVA analysis indicated that the obtained model could be used to predict the response (Table S4).

With the RSM analysis, the optimum conditions that obtained from Design-Expert software were extraction temperature of 43.68 °C, extraction time of 45 min, chloroform volume of 25 μL, and sodium chloride concentration of 7.5 % (W/V). Under the optimized conditions, the predicted relative peak area was 37.6. In order to evaluate the accuracy of the results obtained by the response surface model, three experiments were performed under the optimized conditions with slightly modification of extraction temperature to 44 °C and the mean value (n = 3) was 36.5, which was well in agreement with the predicted value.

3.2.5. Analysis of volatile components in the commercial pu-erh teas

The volatile components of 26 batches of pu-erh teas were extracted under the optimal program and analyzed by GC-MS. The representative GC-MS chromatogram

of raw and ripened pu-erh teas are mapped in Fig. 5. The identified volatile components of pu-erh teas, their odor note and relative contents in percentage are shown in Table 2. Totally, 84 volatile components, including 18 hydrocarbons, 16 methoxyphenolic compounds, 14 alcohols, 14 aldehydes, 10 keones, four esters, three nitrogenous compounds, two phenolic compounds, two lactones, and one acid were identified in the 26 batches of pu-erh teas.

The results indicated that methoxyphenolic compounds with a stale scent, accounting for 38.83% of the total compounds, were the primary components in ripened pu-erh teas. In addition, the total content of methoxyphenolic compounds in raw pu-erh teas was only 10.11%, suggesting that the content of this type of compounds was increased with post-fermentation process. Therefore, the methoxyphenolic compounds could be considered to be the special characteristic odor for pu-erh teas. Among the identified methoxyphenolic compounds, 1,2,3-trimethoxybenzene occupied the highest percentage, followed by 1,2,4-trimeoxybenzene, 4-ethyl-1,2-dimethoxybenzene, 1,2-dimethoxybenzene, and 3,4-dimethoxybenzene, successively, in ripened pu-erh teas. The similar results were found in raw pu-erh teas, 1,2,3-trimethoxybenzene, 1,2-dimethoxybenzene, and 1,2,4-trimethoxybenzene were the primary methoxyphenolic compounds. These findings were consistent with the previous reports that methoxyphenolic compounds accounted for 33.58% of the total aroma constituents in ripened pu-erh teas,[31] and 1,2,3-trimethoxybenzene was the most abundant methoxyphenolic compound.[36]

Among the identified alcohols, linalool with a floral and sweet scent, being rich in

various green teas,[37] was 24.53% in raw pu-erh teas, while decreased to 3.36% in ripened pu-erh teas. Meanwhile, linalool oxides, such as linalool oxides I–IV, increased in ripened pu-erh teas. It could be deduced that linalool had undergone obvious oxygenation during the fermentation process. In addition, *α*-terpineol, with a floral and sweet scent as a major aroma components in Lapsang Souchong and smoked Lapsang Souchong,[38] increased in ripened pu-erh teas, agreed with the previous studies that linalool oxides and *α*-terpineol were the major alcohols in pu-erh tea,[31,39] and that the formation of *α*-terpineol was due to the result of the microbial activity during post-fermentation.[40]

As far as aldehydes were concerned, *β*-cyclocitral and safranal were the major compounds in raw pu-erh teas, which decreased to almost the half in ripened pu-erh teas during post-fermentation. While, (*E*,*E*)2,4-heptadienal and *β*-cyclocitral were dominant in ripened pu-erh teas. These results were different from the previous reports that citral was the most abundant aldehyde in pu-erh tea.[31,41] This difference could be attributed to the difference of the extraction method used in this analysis.

A total of ten saturated and eight unsaturated hydrocarbons were identified in 26 batches of pu-erh teas. Saturated hydrocarbons were considered to have no contribution to the tea flavor, while, unsaturated hydrocarbons played an important role in the flavor of tea.[42] Naphthalene with mint odor and *β*-guaiene with wood odor were present at relatively high levels in both raw and ripened pu-erh teas, which were consistent with the previous report.[31]

Among the 10 ketones, *β*-ionone with a low human odor perception threshold

which significantly contribute to the flavor of tea,[11] was found to be the highest ketone in the raw and ripened pu-erh teas followed by geranyl acetone and α-ionone which endowed pu-erh tea with the scent of flora and woody.

With respect to the esters identified in the volatiles, methyl linolenate was the major ester in raw and ripened pu-erh teas, and caffeine and dihydroactinidiolide were found at high levels in all the pu-erh teas. Although these compounds were also reported in other teas, there has been no report about their contribution to tea flavor.[18]

3.2.6. Multivariate statistical analysis

To highlight the chemical markers for discrimination of raw and ripened pu-erh teas, the relative contents in percentage of all the 84 volatile compounds of the raw and ripened pu-erh teas obtained by GC-MS were analyzed by PCA. Fig. 6A shows the score plot on the two principal components (PC1 and PC2), representing 75.94% of the total variation. As shown in Fig. 6A, 13 batches of raw pu-erh teas were clearly distinguished from 13 batches of ripened pu-erh teas in the PCA model. These findings were in good agreement with the results obtained by E-nose as they both separated the pu-erh teas into the two groups with different manufacturing processes.

Fig. 6B shows the loading scatter plot which displayed the relative importance of each variable. The variables giving higher loading values were considered to be important for the separation of raw and ripened pu-erh teas. As shown in Fig. 6B, linalool (C6), linalool oxide III (C8), linalool oxide IV (C9), eucarvone (C32), 1,2-dimethoxybenzene (C65), 3,4-dimethoxybenzene (C66), 1-methoxy-4-(1-propenyl)-benzene (C69), 3,4,5-trimethoxybenzene (C70), 1,2,3-trimethoxybenzene

(C71), 4-ethyl-1,2-dimethoxybenzene (C72), 1,2,4-trimethoxybenzene (C74), 1,2,3-trimethoxy-5-methylbenzene (C75), 1,2,3,4-tetramethoxybenzene (C79), α-terpilenol (C10), α-cedrene (C50), and caffeine (C63) are the most important volatile compounds for the differentiation of raw and ripened pu-erh teas, implying that these compounds may be the potential chemical markers. These potential markers include methoxyphenolic compounds, alcohols, hydrocarbons, and nitrogenous compounds. Among them, methoxyphenolic and alcohol compounds are the major ones, which contributed a lot to the classification of pu-erh teas with different manufacturing processes.

3.3 Simultaneous determination of methoxyphenolic compounds in pu-erh teas

3.3.1. Method validation

The PCA of the volatile compounds of commercial pu-erh teas identified by GC-MS indicated that mthoxypheonlic compounds were the major chemical markers for the discrimination of raw pu-erh tea from ripened pu-erh tea. In order to comprehensively understand the distribution of the methoxyphenolic compounds in raw and ripened pu-erh teas, a quantitative method for determination of the major methoxyphenolic compounds in pu-erh teas using the above established UAE-DLLME-GC-MS approach was validated. The calibration curve of each methoxyphenolic compound was constructed on the basis of the peak area ratio of the analyte to the internal standard versus the concentration of analyte. The sensitivity of the method was presented as the limit of detection (LOD) and the limit of quantification (LOQ) which were determined on the basis of signal to noise ratio (S/N) of 3 and 10, respectively.

The results of calibration curves, the correlation coefficients, the linear ranges, and the LOD and LOQ values were summarized in Table 3. Good linearity ranges were obtained for the calibration curves, with R^2 higher than 0.9986. The LODs ranged from 6.30 to 8.20 ng/mL and the LOQs ranged from 24.20 to 26.60 ng/mL. The EFs for the seven methoxyphenolic compounds ranged from 34 to 43.

Precision of the method was determined by analyzing the quality control samples containing approximately 200 ng/mL of the analytes. The RSDs located in the ranges of 2.43–5.32% and 3.67–7.12% for intra-day and inter-day determinations, respectively (Table 4).The seven investigated methoxyphenolic compounds were stable at room temperature for at least 24 h with RSD values less than 4.32%.

Recovery experiments were performed to evaluate the accuracy of the optimized method. Known amount of methoxyphenolic compounds at three concentration levels were added to pu-erh tea samples. The average recoveries of seven investigated methoxyphenolic compounds ranged from 89.7 to 111.5% with RSD values less than 10.43% (Table 5).

3.4.2. Determination of seven methoxyphenolic compounds in pu-erh teas

The developed method was applied for the determination of the contents of seven methoxyphenolic compounds in the 26 batches of pu-erh teas. Representative chromatogram from UAE-DLLME-GC-MS analysis of a pu-erh tea sample is shown in Fig. 5. The results are shown in Table 6. It was observed that 1,2-dimethoxybenzene, 3,4-methoxybenzene, 1,2,3-trimethoxybenzene, and 1,2,4-trimethoxybenzene were widely distributed in the pu-erh teas, whereas

1,2,3-trimethoxy-5-methylbenzene, 1-methoxy-4-(1-propenyl)-benzene, and 3,4,5-trimethoxytoluene were not detected in some batches of pu-erh teas. This might attribute to the difference of the collection places of tea leaves in Yunnan Province and the difference of microbes in pu-erh teas. The total amount of methoxyphenolic compounds in pu-erh teas was associated with the post-fermentation year (with few exceptions) and the preservation time, the longer the higher. The contents of methoxyphenolic compounds in ripened pu-erh teas ranged from 275.03 to 627.41 μg/g, much higher than those in raw pu-erh teas from 69.76 to 235.76 μg/g. This may be due to the continuing fermentation during the ageing of pu-erh tea. These results could partly explain why the pu-erh tea aged for a longer period was supposed to have a better odor, a better taste, and a better quality.

3.4.3. Quality assessment by HCA

The content data of the seven methoxyphenolic compounds from all the pu-erh tea samples were subjected for HCA. As shown in Fig. 7, the dendrogram of HCA demonstrated clearly that the 26 batches of pu-erh tea could be classified into two main groups, corresponding to the different manufacturing processes. These findings were in perfect accordance with those obtained by E-nose and GC-MS. All of them distinguished raw pu-erh teas from ripened pu-erh teas, indicating methoxyphenolic compounds to be the pivotal compounds for the differentiation of pu-erh teas.

4. Conclusion

In conclusion, a systemic strategy integrating electronic nose, an improved GC-MS method with a new UAE-DLLME-based sample treatment, and chemometrics

methods was applied to distinguish between raw and ripened pu-erh teas, and to point out and validate the discriminative markers. Meanwhile, the optimized UAE-DLLME-GC-MS method was also employed for the quantitative analysis of the methoxyphenolic compounds in raw and ripened pu-erh teas. The results obtained from E-nose coupled with PCA have shown that this method can differentiate raw pu-erh tea from ripened pu-erh tea by their odors with the advantages of being rapid and easy to use. The further metabolic profile of the volatile constituents of pu-erh teas by UAE-DLLME-GC-MS combined with PCA revealed that alcohols and methoxyphenolic compounds could be the chemical markers for the classification of raw pu-erh tea and ripened pu-erh tea. The contents of methoxyphenolic compounds in pu-erh teas increased along with the storage years. In summary, methoxyphenolic compounds as well as alcohol derivatives were found and verified as the markers for the differentiation between raw and ripened pu-erh teas, and either E-nose or UAE-DLLME-GC-MS could be applied as a reliable tool to achieve the discrimination.

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Table 1 Independent factors, their symbols and levels for the central composite design.

Factor	Symbol	Level				
		$-\alpha$	-1	0	1	α
Sonication temperature (°C)	X_1	30	35	40	45	50
Sonication time (min)	X_2	15	25	35	45	55
Volume of preconcentration solvent (μL)	X_3	15	25	35	45	55
Salt concentration (%, w/v)	X_4	5	7.5	10	12.5	15

Table 2 Volatile Components and their Relative Contents in Percentage (%) in the Pu-erh Teas Fermented by Fungi and Commercial Pu-erh Teas

No.	Components	Odor note	RIs[c]	ID[d]	Raw pu-erh tea	Ripened pu-erh tea	*p* value[f]
					Average content (content range)	Average content (content range)	
	Alcohol						
1	Hexyl alcohol	—[b]	861	MS, RI	0.01 (0-0.08)	0.20 (0-0.41)	0.001
2	2-Ethyl-1-hexanol	—[b]	1030	MS, RI	0.24 (0-0.38)	0.46 (0.24-0.67)	0.000
3	Benzylalcohol	—[b]	1034	MS, RI	0.79 (0.54-1.00)	0.80 (0.45-1.18)	0.424
4	Linalool oxide I	Flower, wood	1072	MS, RI	1.90 (1.08-2.46)	2.72 (1.32-4.01)	0.005
5	Linalool oxide II	Flower, wood	1088	MS, RI	2.64 (1.55-4.15)	3.51 (1.44-5.89)	0.019
6	**Linalool**	**Flower, lavender, Wood**	**1096**	**MS, RI**	**24.53(14.98-34.21)[e]**	**3.36 (1.38-4.93)[e]**	**0.000**
7	Phenethyl alcohol	Rose	1110	MS, RI	0.75 (0.34-1.12)	0.67 (0.14-0.99)	0.225
8	**Linalool oxide III**	**Flower, wood**	**1169**	**MS, RI**	**0.75 (0.25-1.32)[e]**	**1.82 (0.54-3.12)[e]**	**0.001**
9	**Linalool oxide IV**	**Flower, wood**	**1175**	**MS, RI**	**1.09 (0-0.201)[e]**	**3.08 (1.04-4.55)[e]**	**0.000**
10	***α*-Terpilenol**	**Mint**	**1188**	**MS, RI**	**2.94 (1.09-4.76)[e]**	**3.79(1.65-6.01)[e]**	**0.048**
11	Nerol	Sweet	1228	MS, RI	0.72 (0.34-1.06)	0.44 (0-0.89)	0.007
12	Geraniol	Rose, geranium	1256	MS, RI	1.90 (1.53-2.35)	0.42 (0-0.92)	0.000

13	Nerolidol	Wood, flower, wax	1554	MS, RI	0.52 (0.34-0.75)	0.54 (0.23-0.94)	0.384
14	Cedrol	Wood	1598	MS, RI	0.75 (0.29-1.04)	0.15 (0-0.43)	0.000
	Aldehydes						
15	(*E*)-2-Hexenal	Grass, tallow, fat	814	MS, RI	0	0	
16	Benzaldehyde	Almond, burnt sugar	958	MS, RI	0.33 (0-0.65)	0.46 (0-0.68)	0.068
17	2-Pyrrolecarbaldehyde	—[b]	1005	MS, RI	0	0.35 (0-0.55)	0.000
18	(*E,E*)2,4-Heptadienal	Nut, fat	1007	MS, RI	1.10 (0.65-1.46)	1.30 (0.89-1.68)	0.036
19	Hyacinthin	—[b]	1042	MS, RI	0	0	
20	(*E*)-2-Nonenal	—[b]	1046	MS, RI	0.15 (0-0.32)	0.14 (0-0.29)	0.463
21	1-Ethyl-1H-pyrrole-2-carbaldehyde	—[b]	1050	MS, RI	0.35 (0-0.76)	0.47 (0-0.76)	0.140
22	Nonanal	Fat, citrus, green	1094	MS, RI	0.37 (0.26-0.45)	0.35 (0.21-0.44)	0.256
23	Safranal	Herb, sweet	1195	MS, RI	1.28 (0.96-1.66)	0.67 (0.26-1.11)	0.000
24	Decanal	Soap, orange peel, tallow	1199	MS, RI	0.23 (0.18-0.28)	0.26 (0-0.36)	0.234
25	*β*-Cyclocitral	Mint	1218	MS, RI	1.81 (1.22-2.19)	0.73 (0.45-0.98)	0.000
26	2-Phenyl-2-butenal	—[b]	1270	MS, RI	0.21 (0.15-0.22)	0.48 (0.32-0.74)	0.000
27	2-Butyl-2-octenealdehyde	—[b]	1371	MS, RI	0	0.41 (0.12-0.62)	0.000
28	5-Methyl-2-phenyl-2-hexenal	—[b]	1488	MS, RI	0	0.37 (0-0.87)	0.000

	Ketones						
29	2-Heptanone	—[b]	884	MS, RI	0.28 (0.17-0.55)	0.23 (0.14-0.29)	0.054
30	6-Methyl-5-heptene-2-ketone	—[b]	958	MS, RI	0	0	
31	Isophorone	—[d]	1112	MS, RI	0.12 (0-0.27)	0.27 (0-0.42)	0.003
32	**Eucarvone**	**Herb**	**1210**	**MS, RI**	**0**	**0.18 (0.1-0.32)** [e]	**0.000**
33	Menthone	**Mint**	1231	MS, RI	0.34 (0.18-0.48)	0.46 (0.21-0.66)	0.013
34	*β*-Damascenone	Apple, rose, honey	1382	MS, RI	0.76 (0.12-1.12)	0.77 (0.42-1.14)	0.460
35	(*E*)-*α*-Ionone	Wood, violet	1428	MS, RI	0.90 (0.61-1.12)	0.85 (0.59-1.16)	0.258
36	Geranyl acetone	Magnolia, green	1452	MS, RI	0.43 (0.15-0.68)	0.58 (0.21-0.89)	0.056
37	*β*-Ionone	Seaweed, violet, flower, raspberry	1486	MS, RI	2.36 (1.89-2.98)	2.24 (1.49-2.89)	0.186
38	Phytone	—[b]	1846	MS, RI	0	0.40 (0.12-0.59)	0.000
	Esters						
39	Methyl salicylate	Peppermint	1190	MS, RI	0.34 (0-0.52)	0.41 (0-0.65)	0.185
40	Methyl linoleate	—[b]	2093	MS, RI	0.43 (0-0.66)	0.36 (0-0.49)	0.184
41	Methyl linolenate	—[b]	2096	MS, RI	0.51 (0.23-0.68)	0.78 (0.55-1.02)	0.000
42	Dimethyl itaconate	—[b]	2124	MS, RI	0.34 (0.12-0.45)	0.53 (0.22-0.76)	0.000

	Hydrocarbons						
43	1-Octen-3-ol	—[b]	978	MS, RI	0.42 (0.27-0.62)	0.49 (0.21-0.64)	0.092
44	Naphthalene	Tar, mint	1177	MS, RI	1.23 (0.67-1.65)	1.49 (0.78-2.12)	0.070
45	Dodecane	—[b]	1200	MS, RI	0.32 (0.13-0.43)	0.50 (0.23-0.65)	0.000
46	2-Methylnaphthalene	Grass	1287	MS, RI	0.63 (0.41-0.79)	0.60 (0.32-0.8)	0.346
47	1-Methylnaphthalene	Grass	1302	MS, RI	0.67 (0.26-1.14)	0.43 (0.29-0.55)	0.008
48	*β*-Guaiene	Wood, balsamic	1387	MS, RI	1.17 (0.65-1.54)	0.86 (0.32-1.22)	0.003
49	Tetradecane	—[b]	1400	MS, RI	0.34 (0.18-0.46)	0.58 (0.32-0.87)	0.000
50	***α*-Cedrene**	**Wood**	**1408**	**MS, RI**	**3.03 (0.87-4.12)** [e]	**2.16 (1.02-2.98)** [e]	**0.009**
51	*β*-Caryophyllene	Wood, spice	1417	MS, RI	0.86 (0-1.28)	0.59 (0-0.97)	0.038
52	Cumarin	—[b]	1435	MS, RI	0.53 (0.18-0.76)	0.44 (0.12-0.69)	0.125
53	Dibenzofuran	—[b]	1502	MS, RI	0.29 (0.13-0.42)	0.44 (0.29-0.68)	0.000
54	*α*-Farnesene	Wood, sweet	1508	MS, RI	0	0.58 (0.39-0.89)	0.000
55	Fluorene	—[b]	1572	MS, RI	1.68 (1.32-2.01)	1.58 (0.89-2.01)	0.225
56	Hexadecane	—[b]	1600	MS, RI	1.55 (1.09-1.91)	1.20 (0.67-1.78)	0.007
57	Heptadecane	—[b]	1700	MS, RI	0.98 (0-1.43)	0.58 (0.32-0.87)	0.005
58	Anthracene	—[b]	1765	MS, RI	0.48 (0-0.71)	0.34 (0-0.54)	0.060

59	Octadecane	—[b]	1800	MS, RI	0.25 (0.01-0.49)	0.29 (0.07-0.55)	0.236
60	Nonadecane	—[b]	1900	MS, RI	0.71 (0.45-0.99)	0.77 (0.21-1.12)	0.254
	Nitrogenous compounds						
61	Acetophenone	—[b]	1064	MS, RI	0.34 (0.19-0.55)	0.24 (0-0.4)	0.023
62	N-Ethyl succinimide	—[b]	1137	MS, RI	0.36 (0.16-0.46)	0.33 (0.21-0.44)	0.171
63	**Caffeine**	—[b]	**1840**	**MS, RI**	**3.35 (0.32-4.22)** [e]	**2.51 (0.98-3.89)** [e]	**0.033**
	Acid						
64	Hexadecanoic acid	—[b]	1975	MS, RI	0.28 (0-0.47)	0.53 (0.32-0.69)	0.000
	methoxyphenolic compounds						
65	**1,2-Dimethoxybenzene**	**stale**	**1148**	MS, RI, Std	**1.68 (0.98-2.44)** [e]	**3.83 (3.12-4.87)** [e]	**0.000**
66	**3,4-Dimethoxybenzene**	**stale**	**1242**	MS, RI, Std	**0.94 (0.36-2.01)** [e]	**3.26 (1.16-4.78)** [e]	**0.000**
67	1,2-Dimethoxy-3-toluene	stale	1252	MS, RI	0.32 (0.22-0.44)	0.25 (0-0.5)	0.068
68	3,5-Dimethoxytoluene	stale	1266	MS, RI	0.18 (0.12-0.23)	0.35 (0.21-0.43)	0.000
69	**1-Methoxy-4-(1-propenyl)-benzene**	**stale**	**1281**	MS, RI, Std	**0.21 (0-0.34)** [e]	**1.89 (1.59-2.54)** [e]	**0.000**
70	**3,4,5-Trimethoxytoluene**	—[b]	**1308**	MS, RI, Std	**0.16 (0-0.26)** [e]	**1.68 (0.96-2.84)** [e]	**0.000**
71	**1,2,3-Trimethoxybenzene**	**Stale**	**1326**	MS, RI, Std	**3.55 (1.67-4.68)** [e]	**15.84(11.56-18.97)** [e]	**0.000**

72	**4-Ethyl-1,2-dimethoxybenzene**	**Stale**	**1335**	MS, RI	**0.61 (0.14-1.21)** [e]	**3.89 (2.23-6.01)** [e]	**0.000**
73	1,3,5-Trimethoxybenzene	Stale	1350	MS, RI	0.23 (0.11-0.34)	0.34 (0.13-0.54)	0.133
74	**1,2,4-Trimethoxybenzene**	**Stale**	**1375**	MS, RI, Std	**1.20** [e] **(0.96-1.46)**	**4.20** [e] **(2.01-6.98)**	**0.000**
75	**1,2,3-Trimethoxy-5- methylbenzene**	**Stale**	**1404**	MS, RI, Std	**0.38 (0-0.76)** [e]	**2.31 (1.43-3.23)** [e]	**0.000**
76	1,3-Dimethoxybenzene	Stale	1414	MS, RI	0.15 (0-0.25)	0.25 (0-0.41)	0.189
77	Naphthalene, 1-methoxy	—[b]	1442	MS, RI	0	0	
78	Naphthalene, 2-methoxy	—[b]	1447	MS, RI	0.49 (0.29-0.74)	0.22 (0-0.62)	0.002
79	**1,2,3,4-Tetramethoxybenzene**	**Stale**	**1449**	**MS, RI**	**0.02 (0-0.14)**	**0.72 (0.39-1.12)**	**0.000**
80	3,4,5-Trimethoxybenzaldehyde	—[b]	1516	MS, RI	1.34 (0.56-1.92)	1.88 (0.19-2.48)	0.006
	Phenolic compounds						
81	2,6-Dimethoxy- phenol	—[b]	1351	MS, RI	0.33 (0-0.76)	0.54 (0.26-0.87)	0.008
82	Isoeugenol	Flower	1459	MS, RI	0.27 (0-0.55)	0.41 (0.26-0.66)	0.027
	Lactones						
83	Dihydroactinidiolide	Musk	1528	MS, RI	1.81 (1.28-2.42)	1.57 (0.96-2.64)	0.107
84	Tetrahydroactinidiolide	—[b]	1583	MS, RI	0.54 (0-0.74)	0.46 (0-0.66)	0.157

[a]The description of the odor from references 30, 32-34.

[b]Means not found.

[c]Retention index of compounds on HP-5 Column.

[d]Method of identification: MS, mass spectrum comparison using Wiley and NIST11 library; RI, retention index in agreement with literature value; Std, confirmed by authentic standards.

[e]Sixteen volatile compounds with significant difference ($P < 0.05$) in pu-erh tea were indicated in bold.

[f]Two-sample t-test significant values at a level of 0.05.

Table 3 Method validation for the quantitation of methoxyphenolic compounds.

Analyte	Calibration curve	Linearity (ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)	EF
1,2-Dimethoxybenzene	y = 0.0057x + 0.0924	24.3-972.0	0.9994	6.8	24.3	38
3,4-Dimethoxytoluene	y = 0.0068x + 0.0594	25.2-1008.0	0.9992	6.5	25.2	40
1,2,3-Trimethoxybenzene	y = 0.0054x + 0.0031	23.6-944.0	0.9998	6.3	23.6	36
1,2,4-Trimethoxybenzene	y = 0.0079x + 0.0703	26.6-1064.0	0.9988	8.2	26.6	34
1,2,3-Trimethoxy-5-methylbenzene	y = 0.0072x + 0.0306	24.2-968.0	0.9982	7.5	24.2	43
1-Methoxy-4-(1-propenyl)-benzene	y = 0.0095x + 0.0132	25.6-1024.0	0.9988	7.8	25.6	42
3,4,5-Trimethoxytoluene	y = 0.0109x + 0.0462	24.8-992.0	0.9986	8.2	24.8	42

Table 4 Results of precision and stability (RSD%, $n = 6$)

Analyte	Intra-day	Inter-day	Stability
1,2-Dimethoxybenzene	2.43	7.12	2.66
3,4-Dimethoxytoluene	3.21	3.67	3.04
1,2,3-Trimethoxybenzene	3.56	4.88	3.32
1,2,4-Trimethoxybenzene	5.32	4.65	4.32
1,2,3-Trimethoxy-5-methylbenzene	3.56	4.54	3.77
1-Methoxy-4-(1-propenyl)-benzene	4.01	6.65	3.54
3,4,5-Trimethoxytoluene	3.66	3.99	3.76

Table 5 Recoveries of the seven methoxyphenolic compounds (n = 3)

Analyte	Low level		Middle level		High level	
	Spiked content (μg/g)	recovery (%)	Spiked content (μg/g)	Recovery (%)	Spiked Content (μg/g)	Recovery (%)
1,2-Dimethoxybenzene	60.10	95.6	120.20	107.5	240.40	104.3
3,4-Dimethoxytoluene	40.05	105.4	80.10	102.1	160.20	109.8
1,2,3-Trimethoxybenzene	60.65	100.5	121.30	89.7	242.60	107.3
1,2,4-Trimethoxybenzene	32.75	98.9	65.50	101.2	131.00	108.3
1,2,3-Trimethoxy-5-methylbenzene	30.13	106.1	60.25	102.3	120.50	105.5
1-Methoxy-4-(1-propenyl)-benzene	8.15	107.4	16.30	95.1	32.60	103.2
3,4,5-Trimethoxytoluene	6.30	92.4	12.60	98.5	25.20	111.5

Table 6 Contents of methoxyphenolic compounds in pu-erh teas (μg/g).

Sample [a]	1,2-Dimethoxybenzene	3,4-Dimethoxytoluene	1,2,3-Trimethoxybenzene	1,2,4-Trimethoxybenzene	1,2,3-Trimethoxy-5-methylbenzene	1-Methoxy-4-(1-propenyl)-benzene	3,4,5-Trimethoxytoluene
S01	47.01	44.11	66.11	33.21	27.22	16.04	2.06
S02	48.12	36.21	56.88	30.35	N.D.	0.88	2.26
S03	32.32	42.66	52.56	24.88	N.D.	12.06	N.D.
S04	37.66	26.45	49.32	18.56	17.92	11.82	2.66
S05	34.98	24.64	49.89	16.98	16.34	9.84	0.54
S06	22.43	32.66	41.78	15.43	12.98	N.D.	5.12
S07	27.22	20.17	40.98	15.11	N.D.	6.76	N.D.
S08	26.44	19.31	40.22	15.09	11.65	7.87	N.D.
S09	25.23	15.01	41.42	13.88	4.91	N.D.	3.01
S10	18.22	14.92	37.85	12.98	N.D.	N.D.	N.D.
S11	14.66	15.19	32.31	12.44	N.D.	N.D.	0.77
S12	14.92	15.03	31.43	11.98	N.D.	N.D.	N.D.
S13	14.02	14.98	28.54	12.22	N.D.	N.D.	N.D.
R01	143.22	115.44	160.99	87.66	77.33	26.55	16.22
R02	122.88	103.22	150.11	74.88	69.01	24.22	12.83
R03	133.55	97.66	145.78	75.44	1.22	21.54	N.D.
R04	123.87	89.44	127.07	67.01	61.22	14.93	N.D.
R05	99.56	105.11	121.54	62.02	57.21	N.D.	12.78

R06	126.22	81.02	111.13	64.55	53.88	15.01	10.76
R07	112.88	73.12	110.87	56.01	51.22	0.55	N.D.
R08	89.89	63.44	110.01	45.66	44.4	6.27	8.42
R09	94.22	58.1	99.44	43.99	13.22	4.54	4.9
R10	89.75	56.82	90.44	42.99	11.23		N.D.
R11	80.43	56.35	90.44	38.44	10.01	N.D.	N.D.
R12	80.99	65.12	92.98	43.43	N.D.	8.01	N.D.
R13	76.34	61.22	92.12	40.12	5.23	N.D.	N.D.

[a]: S01 – S13 are raw pu-erh teas; R01 – R13 are ripened pu-erh teas.

N.D.: not detected

Figures caption

Fig. 1 Strategy for characterization of raw and ripened pu-erh teas.

Fig. 2 PCA score plot of the 26 batches of pu-erh teas obtained by electronic nose measurement

Boxes represented for raw pu-erh teas, whereas triangles for ripened pu-erh teas.

Fig. 3 Effect of extraction solvents on the extraction efficiency (n = 3)

methanol (M), acetonitrile (ACN), aceton (A), ethanol (E), water (W)

Fig. 4 Standardized main effect Pareto chart for the PB design

The vertical line in the chart defines the 95% confidence level.

Fig. 5 Total ion chromatograms of UAE-DLLME/GC-MS analysis of pu-erh tea samples

raw pu-erh tea sample (A), ripened pu-erh tea sample (B);

3,4,5-trimethoxytoluene (1), 1,2-dimethoxybenzene (2), 3,4-dimethoxybenzene (3), 1-methoxy-4-(1-propenyl)-benzene (4), 1,2,3-trimethoxybenzene (5), ethyl decanoate (6), 1,2,4-trimethoxybenzene (7), 1,2,3-trimethoxy-5- methylbenzene (8).

Fig. 6 PCA score (A) and loading (B) plot derived from 84 volatile compounds of the 26 batches of pu-erh teas

(A) boxes represented for raw pu-erh teas, whereas triangles for ripened pu-erh teas;

(B) red color represented 16 volatiles with significant difference (p < 0.01) in the pu-erh teas on the basis of the results showed in Table 2.

Fig. 7 HCA dendrogram of pu-erh tea samples targeted analysis of the contents of methoxyphenolic compounds

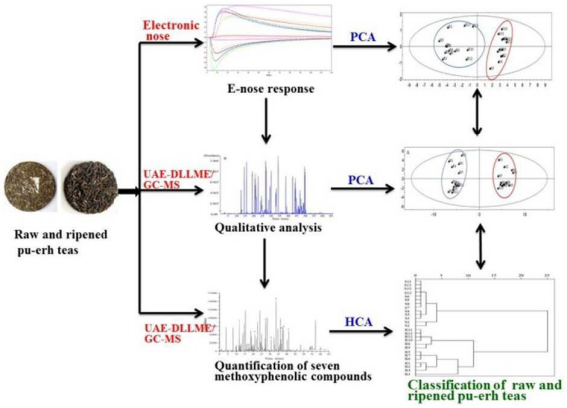

Fig. 1

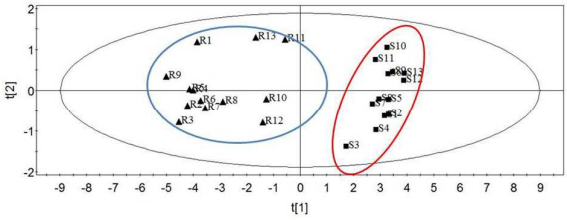

Fig. 2

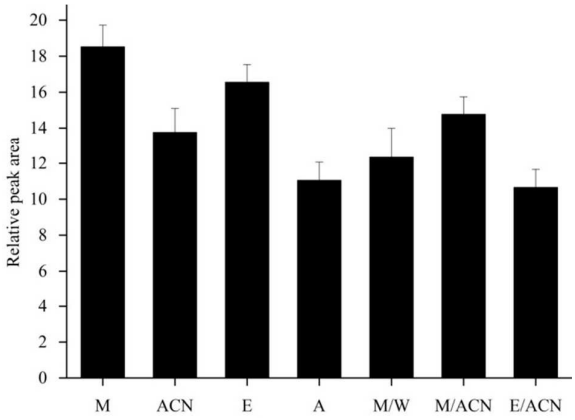

Fig. 3

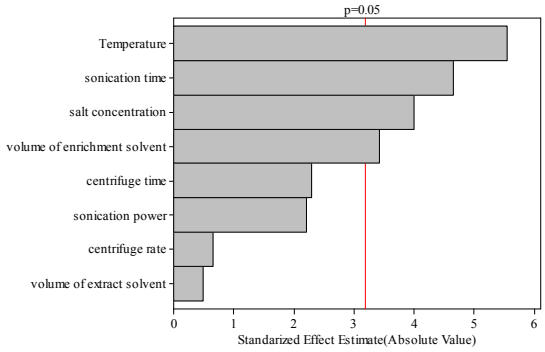

Fig. 4

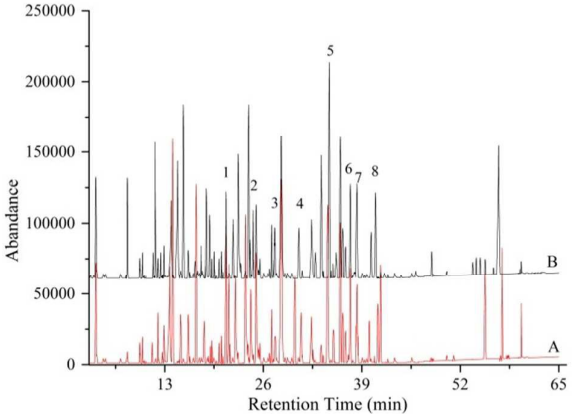

Fig. 5

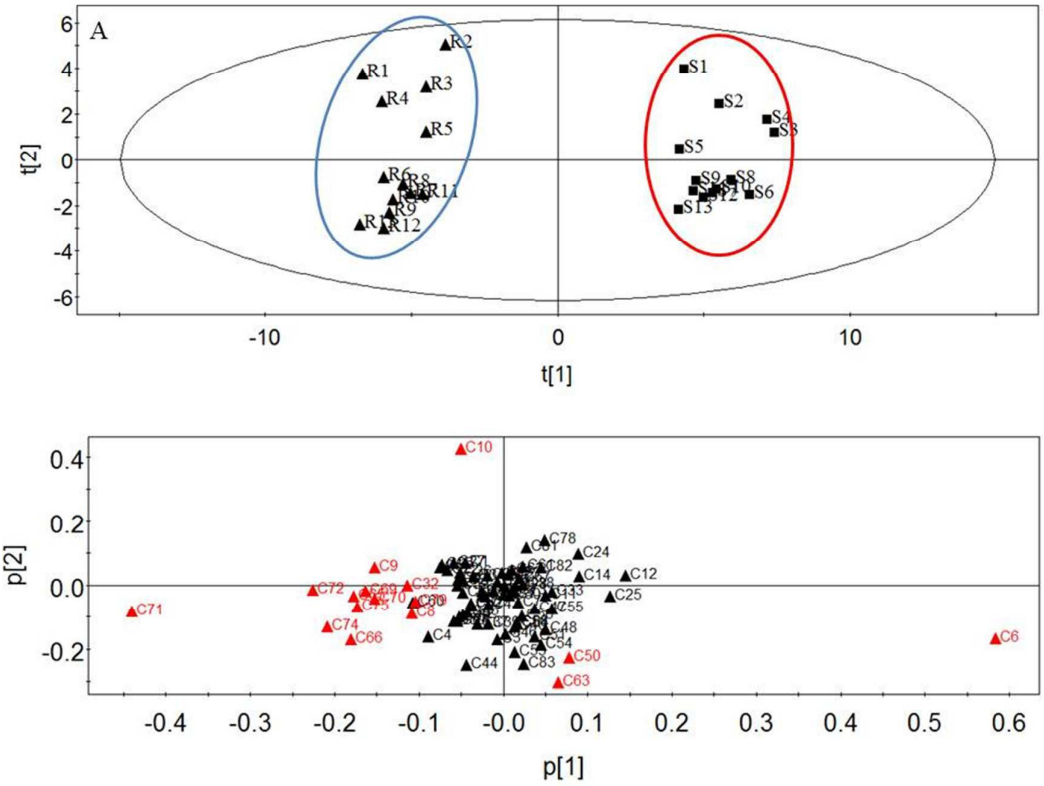

Fig. 6

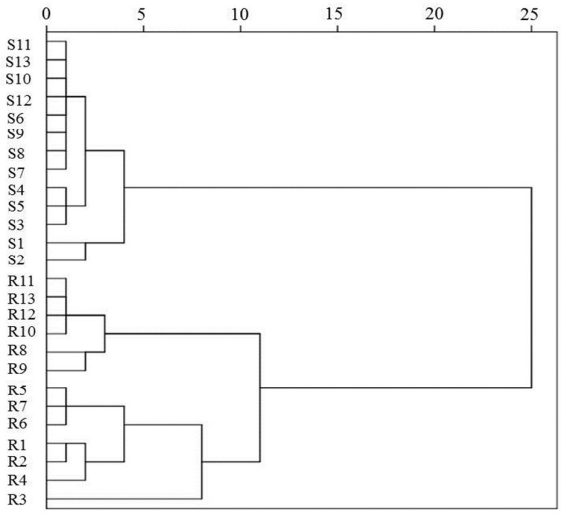

Fig. 7

Graphical Abstract