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Jianrong Jiang

University of Science and Technology Beijing

Guoquan Lu

Shandong University

Qing Wang

Shandong University

Shuya Wei (✉ sywei66@hotmail.com)

University of Science and Technology Beijing

Research Article

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The analysis and identification of charred suspected tea remains unearthed from Warring State Period Tomb

Jianrong Jiang¹, Guoquan Lu^{2*}, Qing Wang², Shuya Wei^{1*}

1. Institute of Cultural Heritage and History of Science & Technology,
University of Science and Technology Beijing, Beijing 100083, China

2. School of History and Culture, Shandong University,
Jinan 250100, China

*Corresponding author: Email: Wei: sywei66@hotmail.com

Lu: luguoqan@126.com

Abstract

Recently, a tea bowl containing charred suspected tea remains unearthed from the early stage of Warring States period tomb in Zoucheng City, Shandong Province, China. To identify the remains is significant for understanding the origin of tea and tea drinking culture. Scientific investigations of the remains were carried out by using calcium phytoliths analysis, Fourier transform infrared spectroscopy (FTIR), Gas Chromatograph Mass Spectrometer (GC/MS) and thermally assisted hydrolysis - methylation Pyrolysis Gas Chromatography Mass Spectrometry (THM-Py-GC/MS) techniques. Modern tea and modern tea residue were used as reference samples. Through phytoliths analyses, calcium phytoliths identifiable from tea were determined in the archeological remains. The infrared spectra of the archaeological remains was found similar as modern tea residue reference sample. In addition, the biomarker compound of tea – caffeine was determined in the archaeological remains by THM-Py-GC/MS analysis. Furthermore, through GC/MS analysis, some compounds were found both in the archeological remains and the modern tea residue reference samples. Putting the information together, it can be concluded that the archaeological remains in the tea bowl are tea residue after boiling or brewing by the ancient.

Keywords: Tea; Charred suspected tea remains; Caffeine; Calcium phytoliths; Py-GC/MS; GC/MS

Introduction

China is the first country in the world to discover and cultivate tea. In Chinese legend, tea was first discovered as an antidote by Emperor Shen Nung in 2737 B.C, according to the first monograph on Chinese herbal medicine Shennong's Classic of Materia Medica(神农本草经)¹. The first mention of tea planting is believed to occur in the *Xiaxiaozheng*(夏小正), a Chinese earliest almanac recording traditional agricultural affairs, probably written in the Warring States Period(475-221BC). According to the literature, in the Spring and Autumn Period (770-476 BC), tea had been used as a sacrifice and vegetable, in the Warring States period and the early Western Han Dynasty, tea cultivation, tea making techniques and tea drinking custom in Sichuan province began to spread to other places².

The physical evidence of tea is very important to confirm the origin, development, function and culture of tea. As archaeological plant leaves remains have been buried for many years, most of them have rotted or charred, it is difficult to find archaeological plant leaves remains in archeological excavation. The first tea remains were found in Northern Song tomb of Lu'an, Anhui Province³. The oldest physical evidence of tea remains were from other two funerary sites: the Han Yangling Mausoleum in Xi'an, Sha'anxi Province, and the Gurgyam Cemetery in Ngari district, western Tibet, revealing that tea was used by Han Dynasty emperors as early as 2100 yr BP and had been introduced into the Tibetan Plateau by 1800 yr BP⁴, but whether the tea was used as beverage, food, medicine is unclear. Recently, some charred suspected tea remains (CST) were found in a tea bowl unearthed from tomb No.1 at Xigang in the Ancient Capital City Site of the Zhu Kingdom in Zoucheng City (The early stage of Warring States, approximately 2400 years ago), Shandong Province (Figure. 1)⁵. If the remains could be determined as tea, that would be the direct evidence for tea drinking in the ancient time.

Previously, the researches commonly used for the identification of plant remains were mainly based on the morphology of the plant, however, most of archaeological plant remains have rotted or charred due to the interference of various microorganisms, oxidation and other factors in the buried environment for many years, the morphology of the plants also changed dramatically, therefore, to identify the plant species by morphology is not applicable to the sample CST. Recently calcium phytoliths (calcium oxalate plant crystals), biomarkers (caffeine and theanine) were identified in

archaeological tea remains by using Gas Chromatography/Mass spectrometry (GC/MS) and Ultra-performance Liquid Chromatography and Mass Spectrometry (UPLC-MS) techniques⁴. For modern tea study, Fourier transform infrared spectroscopy (FTIR)^{6,7,8,9}, Spectrophotometry, Thermospray-LC-MS¹⁰, Head space solids-phase microextraction in combination with gas chromatography-mass spectrometry (HS-SPME/GC-MS)¹¹, Gas chromatography-mass spectrometry(GC-MS)^{12,13}are the mainly techniques applied.

In this study, methods of calcium phytoliths analysis, Fourier transform infrared spectroscopy (FTIR), Gas Chromatography/Mass spectrometry (GC/MS) and Thermally assisted hydrolysis - methylation Pyrolysis Gas Chromatography/Mass Spectrometry (THM-Py-GC/MS) were chosen for the identification of the sample CST found in the Warring State tomb. In the meantime, modern reference samples were studied by using the same analytical methods as the archaeological sample for comparison.

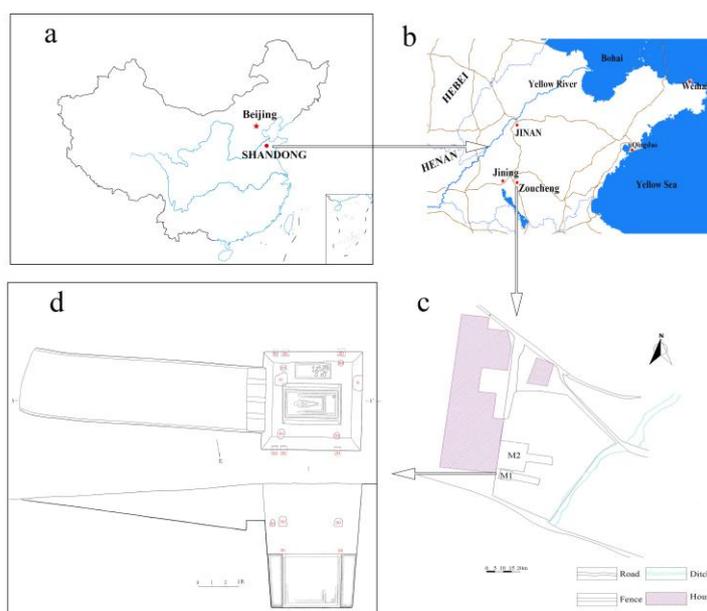


Figure. 1 The map shows a: Location of Shandong Province City in China; b: The Ancient Capital City Site of the Zhu Kingdom in Zoucheng City; c: The plan of the tomb; d: The plan and profile of tomb No.1 at Xigang

Materials and methods

Archaeological sample: the Archaeological sample is from a tea bowl unearthed from the Ancient Capital City Site of the Zhu Kingdom in Zoucheng (Figure. 2). In order to avoid the influence of contamination, the sample CST was taken from the

innermost part of Figure. 2 (d).

Reference samples: since the sample CST was unearthed in a tea bowl, therefore, it is not excluded that the sample is tea residue left after boiling or brewing by the ancient, so in this study, modern tea and modern tea residue were used as reference samples. Modern tea residue was produced from brewing tea with water for several times, then dry thoroughly and finally grinded into powders.



Figure. 2 The map shows a: Tomb No.1 at Xigang; b: Burial objects in the ware box; c: Tea bowl; d: The sample CST from the bottom of the tea bowl

Analysis of calcium phytoliths

Calcium phytoliths experiment was performed according to the procedure described in (Lu et al. 2016). Identification of calcium phytoliths was performed under a LEICA DM2700P microscope.

Fourier transform infrared spectroscopy (FTIR)

For FTIR analysis, Nicolet 6700 Advanced Fourier transform infrared spectrometer (America Thermo Fisher Scientific) was used. The spectra were collected over the 4000-500 cm^{-1} region, using attenuated total reflectance (ATR) for the

measurements, the spectral resolution is 4 cm^{-1} and the number of scans is 64. Each sample was scanned at 25°C and the data acquisition system used was OMNIC.

Gas Chromatograph-Mass Spectrometer (GC/MS)

GC-MS analysis was performed using Agilent GC-MS-QP2010Ultra (Shimadzu, Japan). A capillary column Ultra-5MS (5% diphenyl / 95% dimethyl siloxane), 0.25 mm internal diameter, 0.25 μm film thickness and 30 m length [Frontier lab, Japan] was used for the separation. Temperature programmed: initially keeping the column at 120°C for 2 min, followed by a gradient of $5^{\circ}\text{C}/\text{min}$ to 270°C and hold for 10 min. The injector temperature was set to 240°C . 30:1 split ratio. The carrier gas used was Helium (purity 99.999%). The electronic pressure control was set to a constant flow of 1 ml/min; Electron ionization (EI) temperature was at 280°C ; Transmission lines temperature was at 220°C ; Range of Scanning: 35~510 m/z.

Analysis procedure: A sample (20 mg) was weighed, transferred into a sampling vial with ultrapure water, boiled the sample for 10 min in a water bath, then extracted under sonication at 60°C for 30 min, centrifuged. Afterwards, the supernatant was transferred to a sample vial, then it was evaporated and dried in a stream of N_2 at 60°C . Finally, the dried tea extract was dissolved in solvent acetonitrile (ACN) and derivatized with N-(tert-Butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA, 1: 1 to ACN, v/v) at 110°C for 30 min, transfer the mixed fluid into an auto sampling vial for GC/MS analysis.

Pyrolysis Gas Chromatography Mass Spectrometry (Py-GC/MS)

For Py-GC/MS analysis, A Multi-Shot pyrolyzer, type EGA/PY-3030D, made by Frontier Lab, Japan, and a gas chromatograph mass spectrometer, GC-MS-QP2010 Ultra (Shimadzu, Japan). Shimadzu GC-MS real time analysis software was used for GC-MS control, peak integration and mass spectra evaluation.

The pyrolysis was performed at 550°C for 12 s. The pyrolyser interface was set to 290°C and the injector was set to 250°C . A capillary column SLB-5MS (5% diphenyl / 95% dimethyl siloxane), 0.25 mm internal diameter, 0.25 μm film thickness and 30 m length [Supelco] was used in order to provide an adequate separation of the components. The chromatographic conditions were as follows: The oven initial temperature was set

to 35 °C for 5 min, followed by a gradient of 60 °C /min to 100 °C, for 3 min, 14 °C /min to 240 °C , then 6 °C/min to 315 °C and hold for 1.5 min, The carrier gas was Helium (He, purity 99.999%). The electronic pressure control was set to a constant flow of 0.92 ml/min, in split mode at 1:20 ratios. Ions were generated by electron ionization (145.3 eV) in the ionization chamber of the mass spectrometer. The mass spectrometer was set from m/z 35 to 750. EI mass spectra were acquired by total ion monitoring mode. The temperatures of the interface and the source were 280 °C and 200 °C, respectively.

NIST14 and NIST14s Library of Mass Spectra were used for identifying the compounds.

Analysis procedure: About 50 µg sample was placed in a sample cup, 3µL of 25% aqueous TMAH (analytical pure, Sinopharm Chemical Reagent Co., Ltd) solution were injected into the sample cup, the cup was placed on top of the pyrolyzer at ambient temperature and then pyrolyzed immediately, afterwards the temperature program for the GC/MS analysis was started. During the process of analysis, blank tests were conducted before each sample.

Results and discussion

Analysis of calcium phytoliths

Calcium phytolith analyses were carried out according to the procedure described in⁴. The morphology observation of sample CST under microscope were depicted in (Figure. 3), which reveals that the sample contains abundant calcium phytoliths. By comparing with the literature⁴, the morphology of the calcium phytoliths found in the sample CST are similar as that from tea.



Figure. 3 Photographs under microscope of calcium phytoliths from sample CST

FTIR analysis

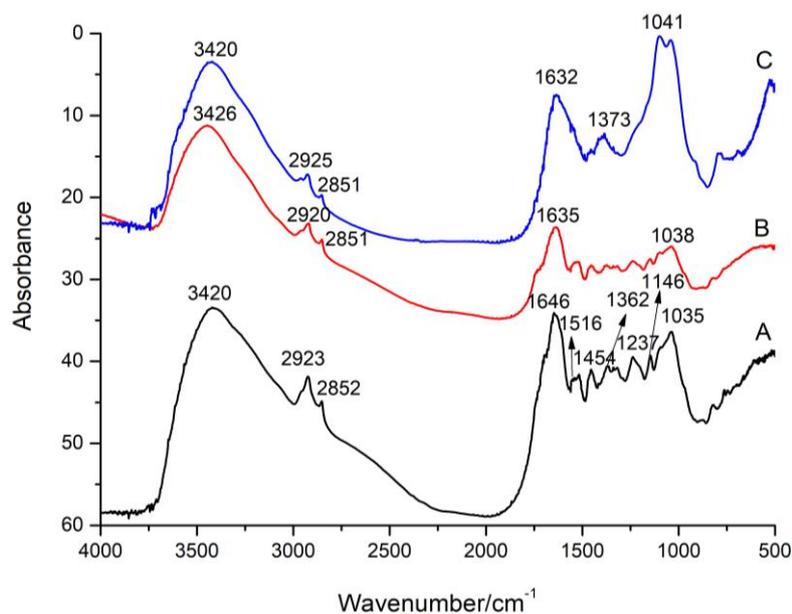


Figure. 4 Infrared spectra of A-modern tea; B-modern tea residue; C-sample CST

The infrared spectra of modern tea, modern tea residue and the sample CST are shown in Figure. 4. The vibrations of the functional groups of the compounds in tea and their corresponding infrared absorption characteristic peaks are consistent with the literatures^{6,7,8,2}. Taking infrared spectrum of modern tea as an example, the band assignment to chemical bonds for the vibrational FTIR spectra of modern tea is summarized in Table 1.

Table 1. Band assignments for the FTIR spectra obtained from modern tea

Wavenumber (cm ⁻¹)	Vibrational mode assignment	Absorption peak intensity: s (strong) /m (medium) /w (weak)
3420	-OH stretching vibration of tea-polyphenols and tea-polysaccharides	s
2923、2852	saturated C-H stretching vibration	m、w
1646	C=C stretching vibration peak of sugars and flavonoids	s
1516	-NO ₂ stretching vibration peak of aromatic compounds in tea	w

1454	saturated C-H deformation vibration	w
1362	-NO ₂ stretching vibration peaks of aliphatic compounds	w
1237	C-O stretching vibration peak in amides	w
1146	C-O-C antisymmetric stretching vibration	w
1035	O-H in-plane deformation vibration	s

All the samples, including both the archaeological sample CST and the reference samples (modern tea and modern tea residue), exhibit similar spectra, the peak shapes and peak positions of main absorption peaks (three strongest peaks: 3420、1632、1041 cm^{-1}) are also very close (Figure. 4), so it is speculated that the sample CST is most likely ancient tea.

The intensity of some absorption peaks in modern tea residue and sample CST have a tendency to decrease and even gradually disappeared compared with modern tea, for example, compared with modern tea, the infrared spectral peaks (3420, 2923, 1646, 1516, 1454, 1237 cm^{-1}) of theanine (the main amino acids in tea) gradually disappeared in the modern tea residue and sample CST, indicating most of water-soluble components in tea were leached out in the process of brewing tea¹⁴. The intensity of peaks about at 1373、1041 cm^{-1} of sample CST increased significantly, compared with modern tea and modern tea residue sample. Studies have shown that the intensity of peaks about at 1041 cm^{-1} of tea increases with the prolongation of tea aging time¹⁵. For peak 1373 cm^{-1} , it may be due to the nitration reaction of aliphatic hydrocarbons and aromatic hydrocarbons in tea under burial environment to generate a large amount of aliphatic nitro compounds^{16,7}. In addition, since sample CST is an archaeological sample, which has been buried for hundreds of years, microorganisms and other factors in the burial environment may cause chemical changes, resulting in the subtle differences in infrared spectra between the archaeological sample CST and reference samples. To confirm whether the sample CST is ancient tea or not, further study by other techniques were carried out as following.

Biomarker analysis

THM-Py-GC/MS analysis. The chromatograms of modern tea, modern tea residue and the sample CST obtained by THM-Py-GC/MS are shown in Figure. 5.

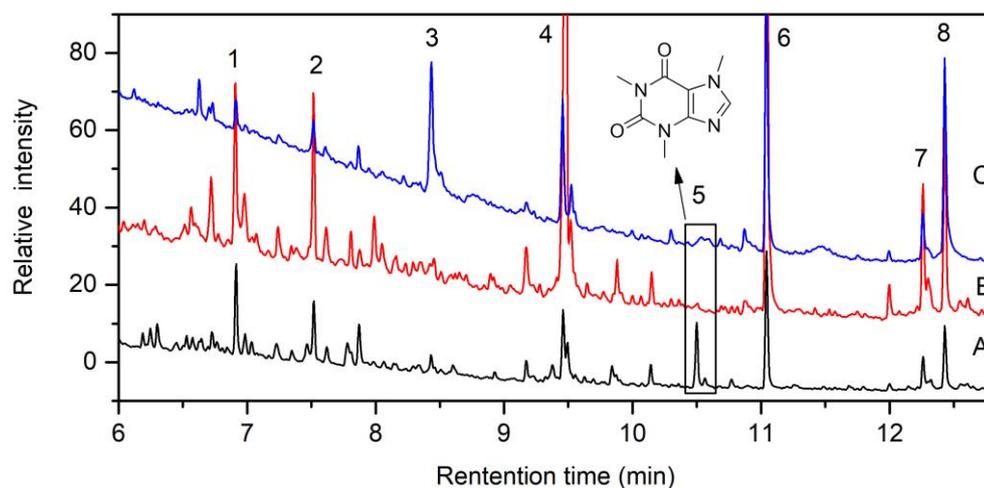


Figure. 5 TIC chromatogram obtained by THM-Py-GC/MS of A- modern tea;
B- modern tea residue; C-sample CST

1: 1,3,5-trimethoxy-benzene; 2: 2,4,6-trimethoxytoluene; 3: 3,4-dimethoxy-benzoic acid, methyl ester; 4: 3,4,5-trimethoxy-benzoic acid, methyl ester; 5: caffeine; 6: hexadecanoic acid, methyl ester; 7: 9-octadecenoic acid, methyl ester; 8: methyl stearate

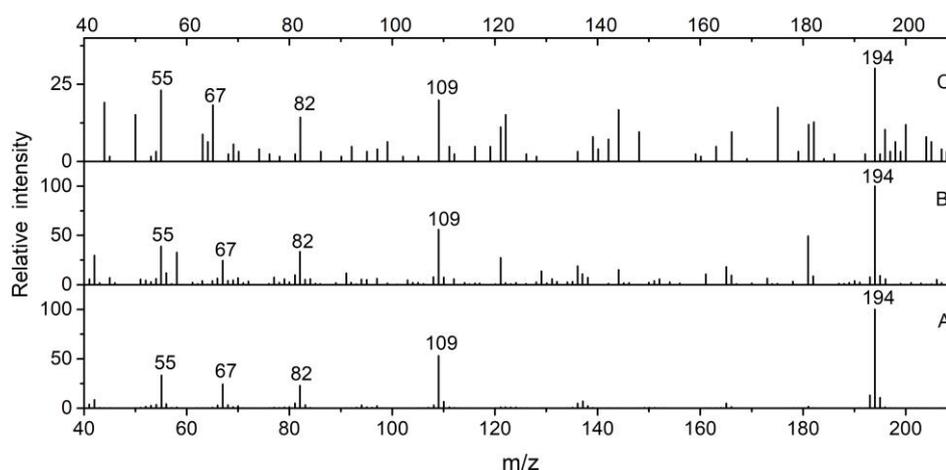


Figure. 6 The mass spectrum of caffeine (peak 5 in Figure .5) of A- modern tea;
B- modern tea residue; C-sample CST by THM Py-GC/MS analysis

Comparing retention time and mass spectrum of the main chromatographic peaks

in the sample CST and the reference samples (modern tea and modern tea residue), the main peaks found in the sample CST are also present in the reference samples (peak No.1-8), which are 1,3,5-trimethoxy-benzene, 2,4,6-trimethoxytoluene, 3,4-dimethoxy-benzoic acid, methyl ester, 3,4,5-trimethoxy-benzoic acid methyl ester, caffeine, hexadecanoic acid methyl ester, 9-octadecenoic acid methyl ester, methyl stearate, the peak 1, 2, 3, 4 belong to methoxybenzene compounds which are the characteristic components of tea aroma^{17,18}, and palmitic acid, 9-octadecenoic acid and methyl stearate are common fatty acids in tea¹⁹. Especially the biomarker compound of tea - caffeine was identified (peak No. 5, RT 10.5 min), the mass spectra are shown in Figure. 6. Caffeine is easily soluble in water, most of the caffeine in tea was leached out in the process of brewing tea, therefore, the content of caffeine in modern tea residue is significantly lower than that in modern tea (Figure. 5), which indicating that the sample CST is most likely tea residue after being boiled or brewed.

GC/MS analysis. The modern tea reference sample, modern tea residue reference sample and the sample CST were pretreated according to the procedure described in a previous section in this document. The chromatograms of them obtained by GC/MS analyses are shown in Figure. 7

The main amino acids contained in tea were detected in modern tea sample after derivatized by MTBSTFA, which is consistent with the literature^{20,21}. Especially the tea marker compound –theanine, two derivatized peaks of theanine were detected in modern tea reference sample (labeled as T1 and T2 in Figure. 7). In the modern tea residue sample, only a trace of theanine (T1) was found, but not found in the CST sample. Theanine is the main free amino acid in tea, its concentration is significantly decreased after tea was brewed due to its good solubility in water, therefore, the content of theanine in modern tea residue and sample CST is so low that even can not be detected, which is consistent with the results of infrared spectrum analysis. However, there are some other compounds were detected both in modern tea residue and sample CST, which are listed in table 2, most of these components are organic acids, which are the common contents in tea, and as a water-soluble substance, which can be also leached out in the process of brewing tea²². Although some of the compounds are not identified, but they are both present in modern tea residue and sample CST (peak No. 1, 4, 10,12, 13 in Figure. 7), which provide another evidence that the archaeological sample CST is most likely tea residue left after boiling or brewing.

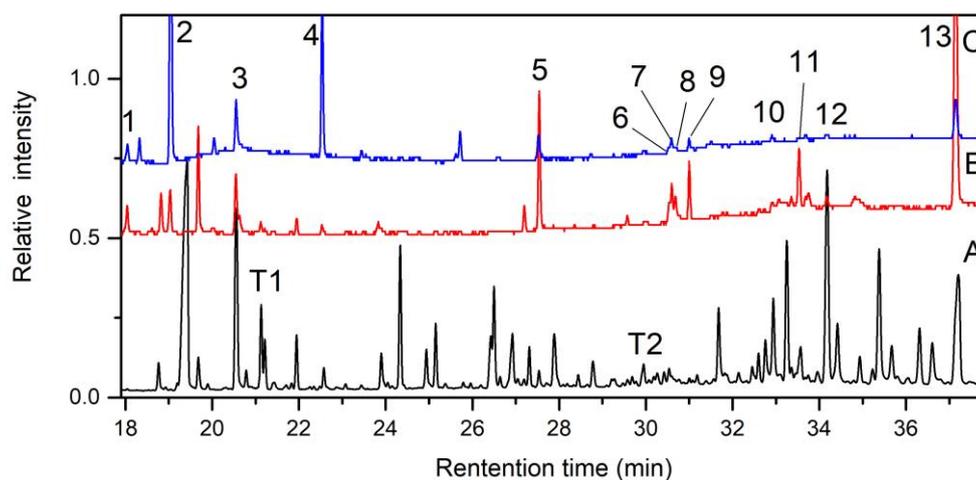


Figure . 7. TIC chromatogram obtained by GC/MS of A-modern tea; B- modern tea residue; C-sample CST; T1, T2: two derivatized peaks of theanine in sample modern tea(A)

Table 2. GC/MS analysis result of modern tea residue and sample CST

Peak No.	RT(min)	Main Ions (m/z)	Compounds identified
1	18.04	115,147,173, 259,386	unidentified
2	19.04	147,189,221, 263,355	Boric acid, 3TMS derivative
3	20.54	211,269,383,425	Phosphoric acid, tris(tert-butyldimethylsilyl) ester
4	22.56	147,221,263,337	unidentified
5	27.53	117,131,313	Palmitic Acid, TBDMS derivative
6	30.54	129,337	Linoelaidic acid, tert.-butyldimerthylsilyl ester
7	30.59	129,339,381	Petroselinic acid, TBDMS derivative
8	30.68	129,339	.alpha.-Linolenic acid, TBDMS derivative
9	31	117,129,341	Stearic acid, TBDMS derivative
10	32.92	117,143,237, 252,359	unidentified
11	33.54	223,339,455,469	2-Amino[1,3]thiazolo[4,5-d]pyrimidine-5,7-diol
12	34.15	185,241,256,359	unidentified
13	37.14	238,323,397, 439,495	unidentified

Conclusions

In this study, Calcium phytoliths analysis, Fourier transform infrared spectroscopy (FTIR), Gas Chromatograph Mass Spectrometer (GC/MS) and

Thermally assisted hydrolysis-methylation pyrolysis-gas chromatography/mass spectrometry (THM-Py-GC/MS) techniques were applied for the identification of archaeological remains—charred suspected tea (CST) excavated from the early stage of Warring State Period tomb in Shandong Province. The experimental results show that the sample CST contains abundant calcium phytoliths identifiable as tea, and it exhibits similar FTIR spectra with the modern tea residue. Moreover, caffeine, methoxybenzene compounds, organic acids, 2-Amino[1,3]thiazolo[4,5-d]pyrimidine-5,7-diol and several unidentified compounds were detected in both the sample CST and the reference sample (modern tea residue) by PY-GC/MS and GC-MS. Therefore, combined with experimental results and the residue container- the tea bowl, it can be concluded that the archaeological remains in the tea bowl are tea residue after boiling or brewing by the ancient.

Tea drinking is one of the most representative traditional cultures in China, since ancient times, the Chinese people have always had the habit of drinking tea, but there is no physical evidence to prove when tea actually appeared, until the discovery of tea in the Han Yangling Mausoleum, which proved that Chinese tea has a history of at least 2150 years, which has earned recognition from Guinness World Records as the oldest tea in 2016 (Lu et al. 2016). The identification of the tea remains at the Ancient Capital Site of the Zhu Kingdom in Zoucheng (the early stage of Warring States, approximately 2400 years ago) has advanced the origin of tea by nearly 300 years. Furthermore, the tea was found in a tea bowl, providing additional evidence of the usage of tea. The results of this study indicate that tea drinking culture may start as early as in Warring State period.

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Author information

Affiliations

**Institute for Cultural Heritage and History of Science and Technology,
University of Science and Technology Beijing, Beijing, 100083, China**

Jianrong Jiang & Shuya Wei

School of History and Culture, Shandong University, Jinan, 250100, China

Guoquan Lu & Qing Wang

Contributions

J.J. performed all experiments, analyzed the data and produced figures. J.J. and S.W. drafted the manuscript. G.L. and Q.W. provided the archaeological data, materials and reviewed the manuscript.

Corresponding author

Correspondence to Guoquan Lu and Shuya Wei.

Ethics declarations

Competing interests

The authors declare no competing interests.

Figures

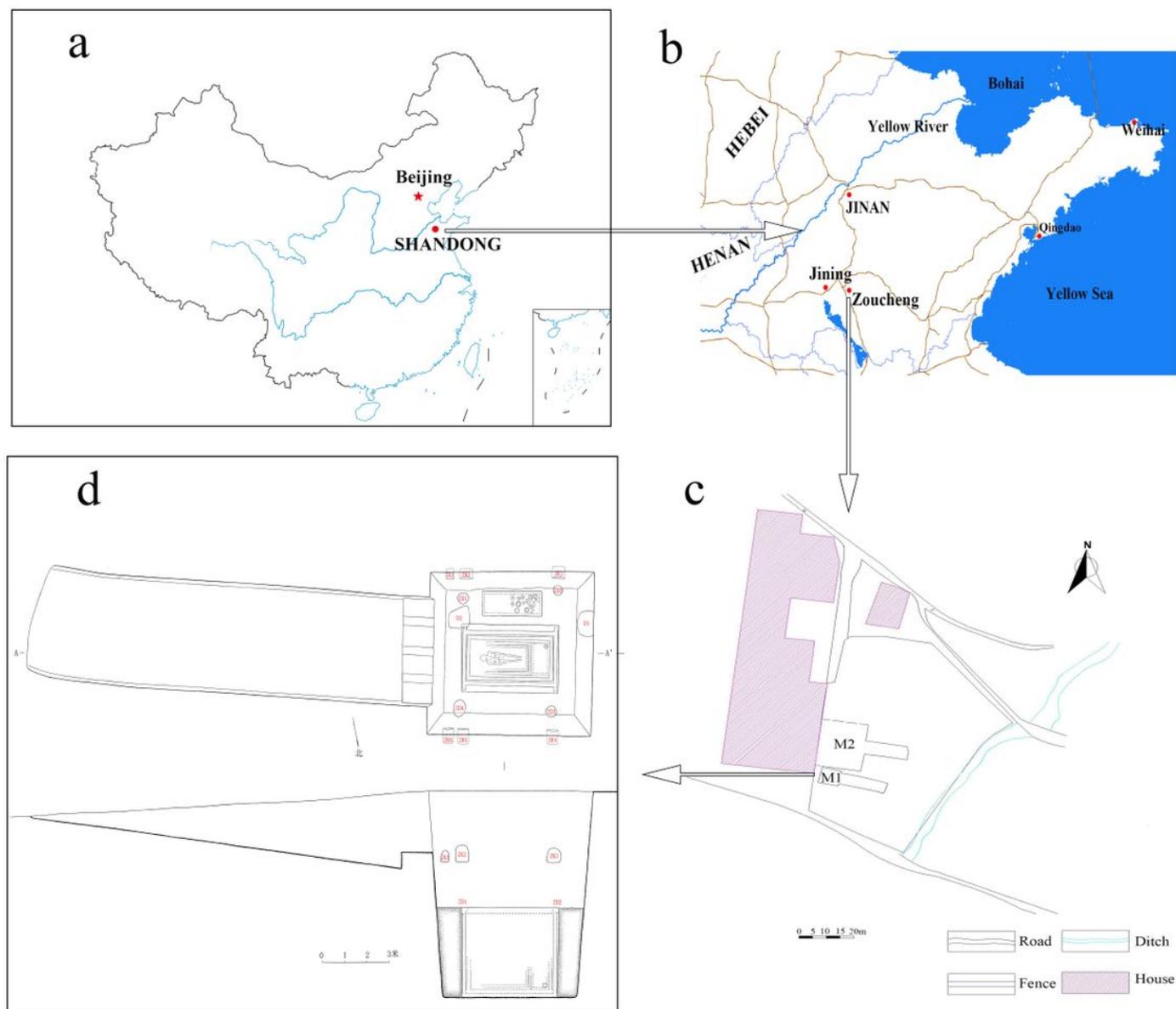


Figure 1

The map shows a: Location of Shandong Province City in China; b: The Ancient Capital City Site of the Zhu Kingdom in Zoucheng City; c: The plan of the tomb; d: The plan and profile of tomb No.1 at Xigang. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

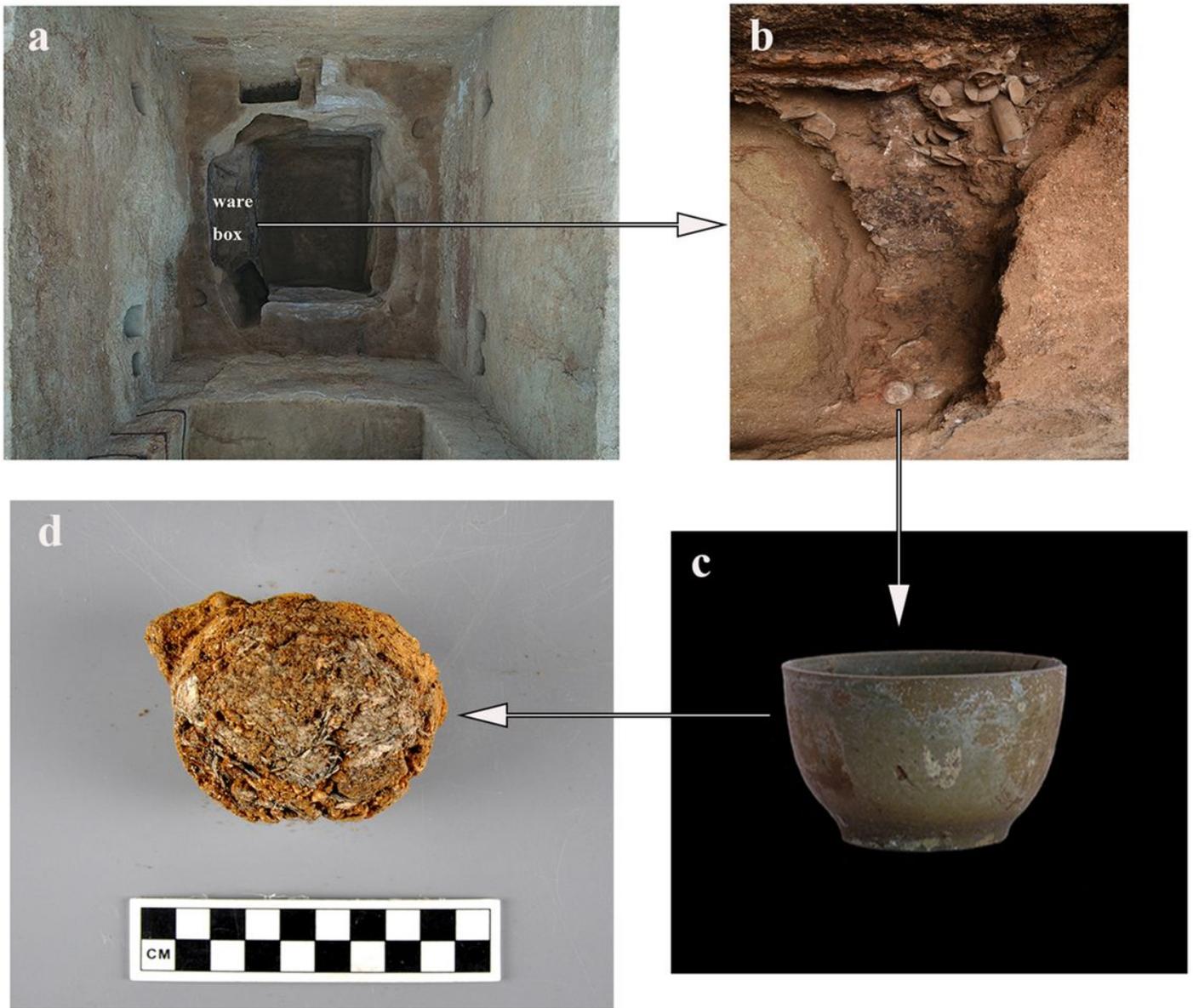


Figure 2

The map shows a: Tomb No.1 at Xigang; b: Burial objects in the ware box; c: Tea bowl; d: The sample CST from the bottom of the tea bowl

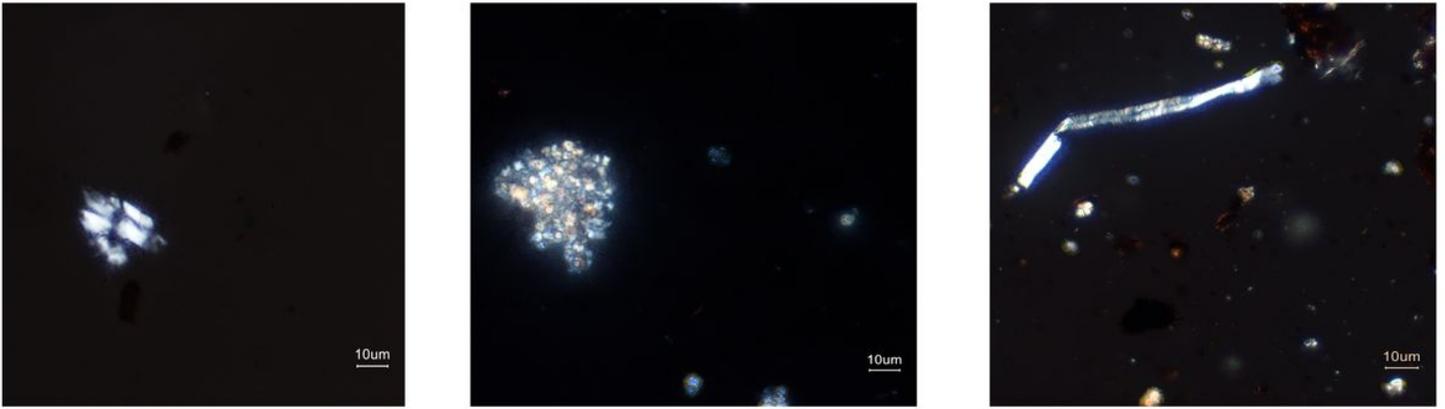


Figure 3

Photographs under microscope of calcium phytoliths from sample CST

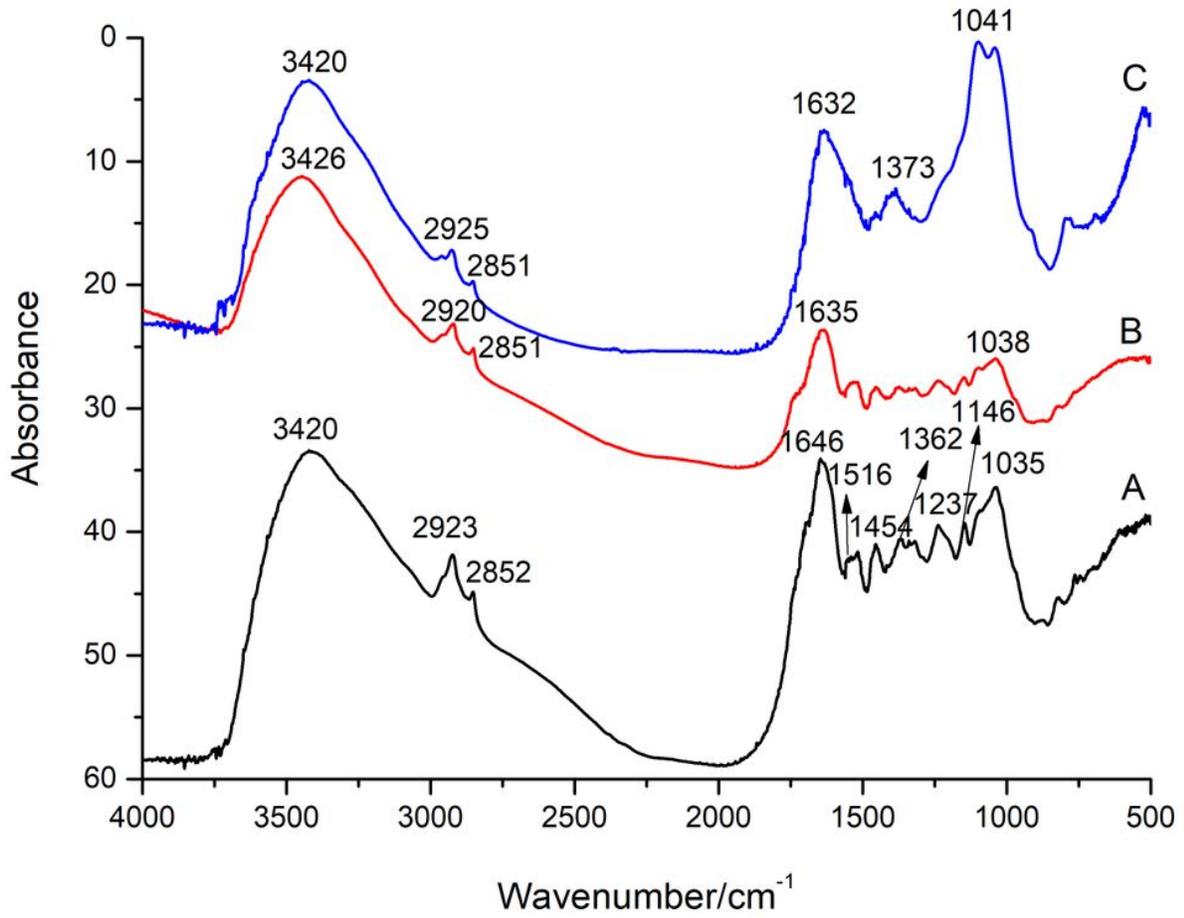


Figure 4

Infrared spectra of A-modern tea; B-modern tea residue; C-sample CST

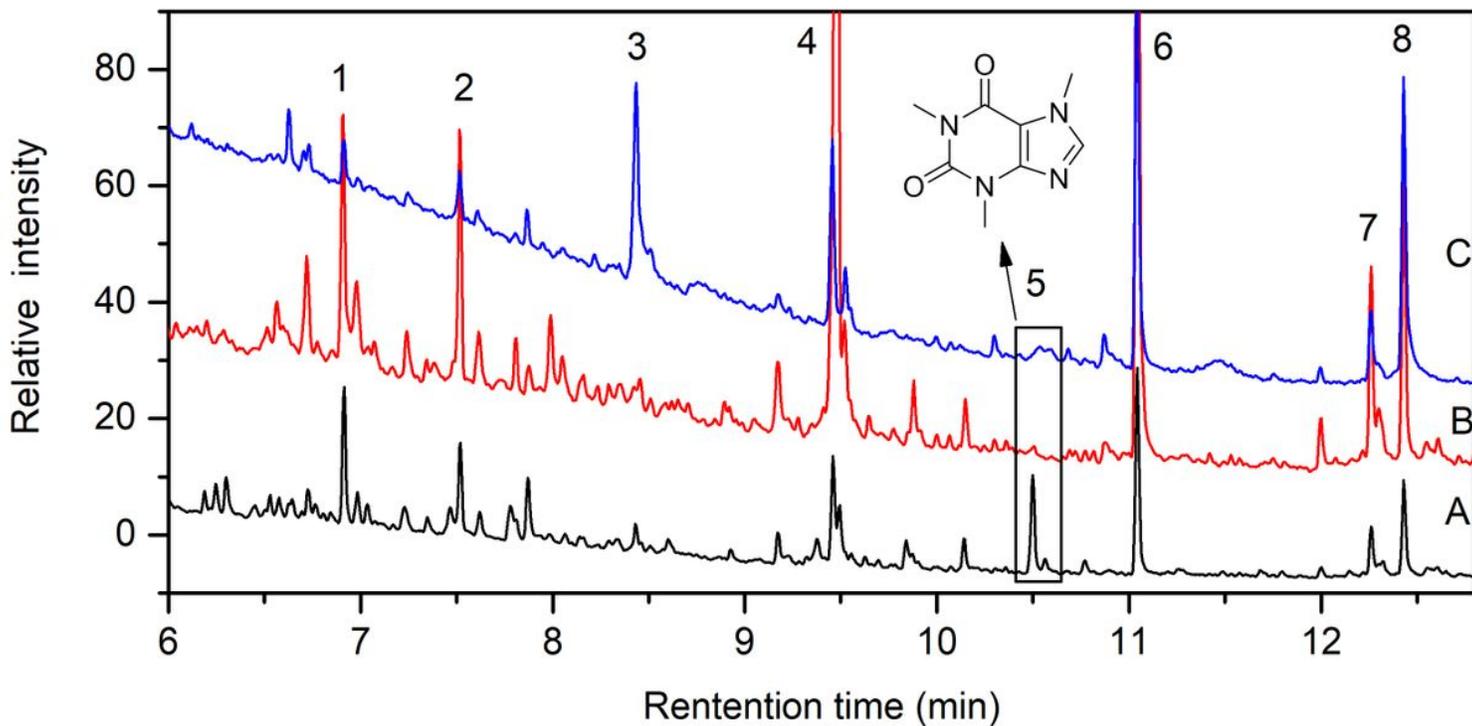


Figure 5

TIC chromatogram obtained by THM-Py-GC/MS of A- modern tea B- modern tea residue C- sample CST

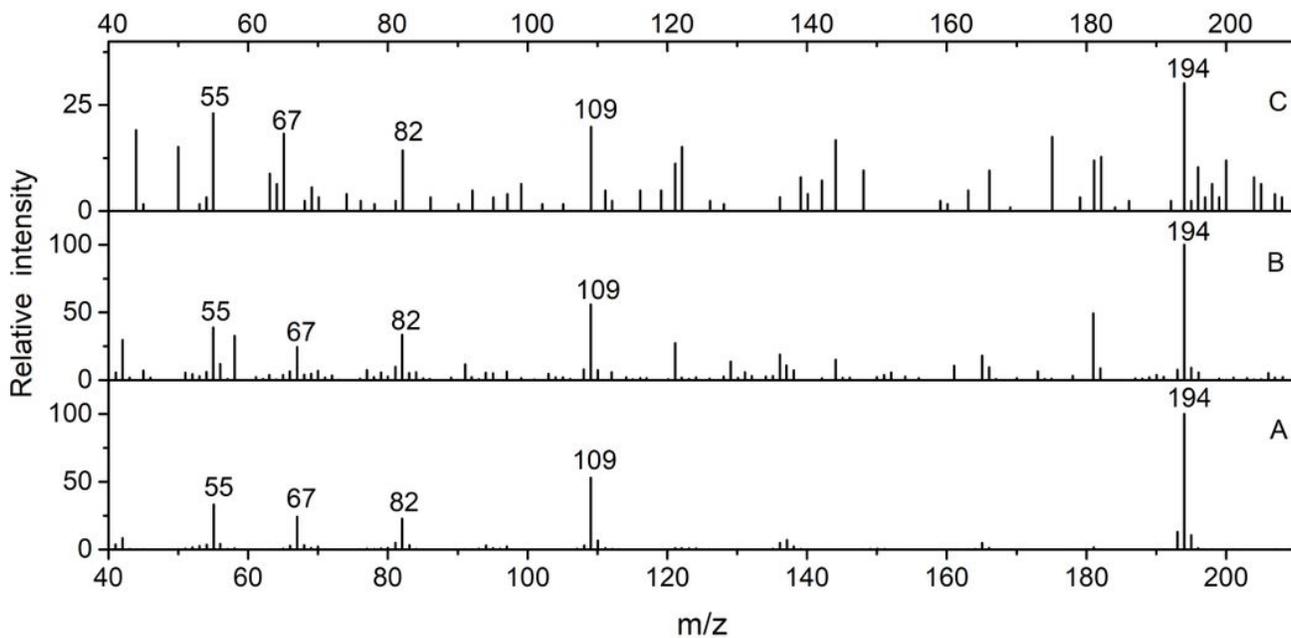


Figure 6

The mass spectrum of caffeine (peak 5 in Figure .5) of A- modern tea; B- modern tea residue; C- sample CST by THM Py-GC/MS analysis

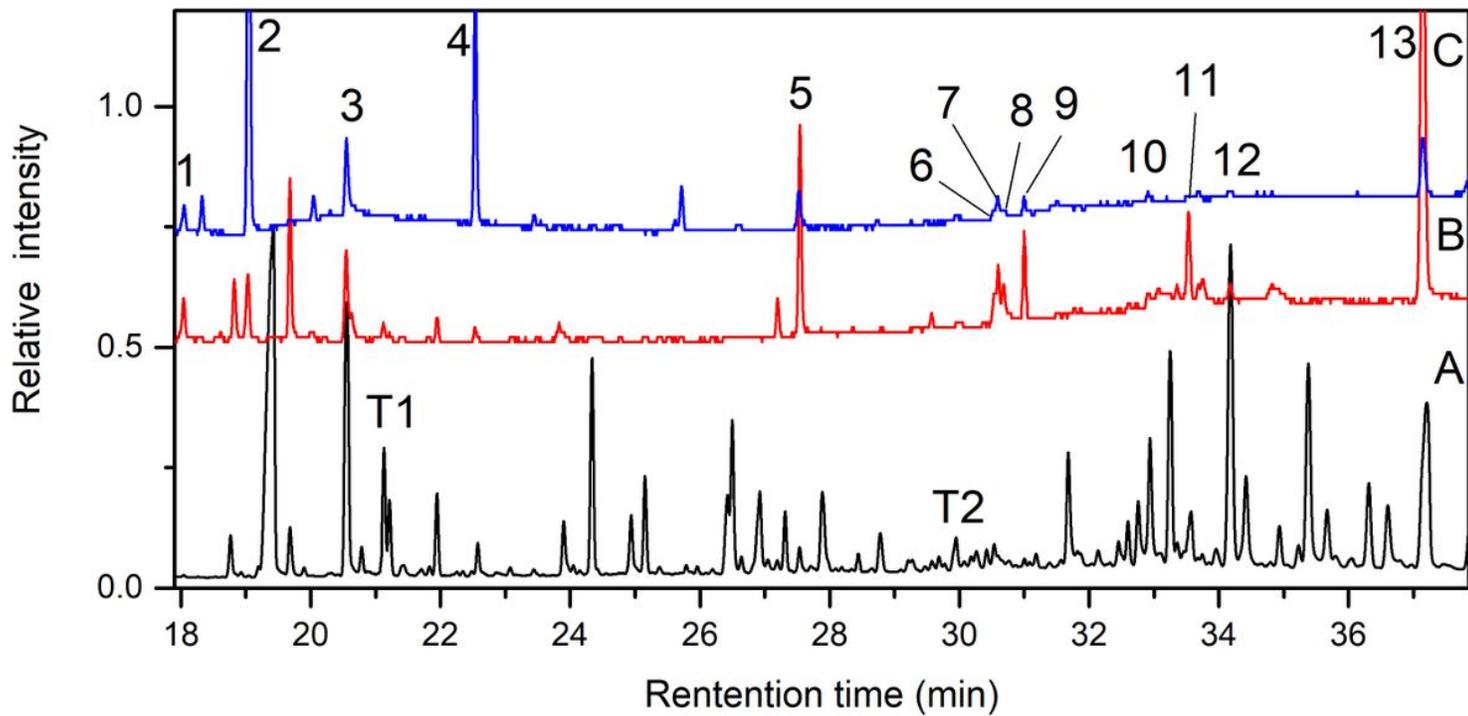


Figure 7

TIC chromatogram obtained by GC/MS of A-modern tea; B- modern tea residue; C-sample CST; T1, T2: two derivatized peaks of theanine in sample modern tea(A)