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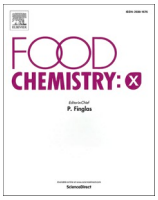

Polysaccharide Conjugates' contribution to mellow and thick taste of Pu-erh ripe tea, besides Theabrownin

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ABSTRACT

Mellow and thick taste (MTT) is considered to be a typical taste characteristic of high-quality Pu-erh ripe tea. However, the role of polysaccharide conjugates remains unclear. In this study, the infusion of different grades of Pu-erh ripe tea was isolated to fractions by sensory-guided ultrafiltration technology and the key taste substances of MTT in Pu-erh ripe tea were identified and confirmed in the sensory reconstruction experiment. Further separation, purification and structural identification of the polysaccharide conjugates were carried out. Involving in aggregation morphology, the ultrafiltration fraction exhibited obvious MTT than other fractions. The main MTT compound (PRTPS-5), mainly composed of the rhamnose, galactose, arabinose and mannose, had a molecular weight of 22.93 kDa. The main chain of PRTPS-5 comprised α-L-Ara*f*-(1→, →2,4)-α-L-Rha*p*-(1→, →2)-α-L-Rha*p*-(1→, α-D-Gal*p*-(1→, →4)-α-D-Gal*p*A-6-OMe-(1→, →4)-α-D-Man*p*-(1→, →3,6)-β-D-Gal*p*-(1 → and →5)-α-L-Ara*f*-(1 → and contained multiple pectic characteristic peaks. This result had scientific guiding significance for the quality enhancement of Pu-erh ripe tea.

1. Introduction

Pu-erh ripe tea is a long-established post-fermented tea in Yunnan Province of China, which has various pharmacological effects including anti-diabetes, anti-obesity, anti-hyperglycemia and anti-oxidation (Huang et al., 2015; Liu et al., 2022). As one of the most promising teas in China, the market quality of Pu-erh ripe tea requirement was gradually increasing, also the related research has become more attractive. Tea infusion is a mixed system composed of taste compounds extracted from tea, which usually has a unique taste decided by the content and interactions among biochemical components (Rahman et al., 2012). Mellow and thick taste (MTT) are the most remarkable character of Pu-erh ripe tea, however, the key flavor-presenting compounds that contribute to this are still unclear.

Our previous research found that theabrownin (TB) and crude polysaccharides had the effect of enhancing the strength of the MTT of Pu-erh ripe tea (Deng et al., 2022). TB is the principal bioactive ingredient of Pu-erh ripe tea with an average content of up to 12%. Compared with other teas, thearubigins and theaflavins in Pu-erh ripe tea are further oxidized and polymerized, and are more likely to bind to proteins, lipids and polysaccharides, and form TB, thereby improving the sensory quality of Pu-erh ripe tea (Hu, Li, et al., 2022; Liao et al., 2023). This is due to the fermentation of Pu-erh ripe tea, the enzymes (primarily polyphenol oxidase) still remain in cells, and the rolling and fermentation build an environment for the enzymatic incorporation of polysaccharide conjugates (Hou et al., 2020; Xu et al., 2021). The taste characteristics of polysaccharides in tea were sweet, sticky and inhibit bitterness (Yue et al., 2017). According to Yu et al. (2013), the polysaccharide in Pu-erh ripe tea played a role in neutralizing bitterness and irritation.

As aging time increased, the amount of total phenolic compounds decreased while the polysaccharides significantly increased (Xu et al., 2014). This might be attributed to the fact that longer aging time facilitates the oxidation of polyphenols and enhance the conjugation of polyphenols with proteins. The majority of current research on the usage of tea polysaccharides is on defining their biological properties, such as

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their immunomodulatory, hypoglycemic and antioxidation (Chen, Huang, et al., 2019; Hu, Wu, et al., 2022) However, little is understood about the function of polysaccharide conjugates (PC) in MMT of Pu-erh ripe tea. The purpose of this work was to clarify the relationship between polysaccharide conjugates and MTT of Pu-erh ripe tea. The MTT fractions were isolated by sensory-guided separation and purification technology. The purified PC were further identified, and the effect of PC on the MTT was verified by taste recombination experiment. These findings would contribute to a theoretical foundation for the quantitative evaluation of MTT, and have scientific guidance on the quality improvement of Pu-erh ripe tea.

2. Materials and methods

2.1. Materials

Pu-erh ripe tea samples with different grades were provided by the Yunnan Agriculture University. Anthrone, deuterium oxide, catechins, Caffeine (CAF), gallic acid (GA), monosaccharide standards, sodium acetate, potassium bromide, sodium boron deuteride, acetic anhydride, Folin-Ciocalteu phenol reagent, acetonitrile, n-butanol, ethyl acetate, sodium nitrate, galacturonic acid (GalA), glucose Cellulose acetate filter membrane (0.45 μm) and dialysis bag were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA); Methanol, dichloromethane, trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), ammonia and methyl iodide were purchased from ANPEL Laboratory Technologies (Shanghai) Inc. (Shanghai, China); Theabrownin (TB, 93%) was bought from Yunnan Tangren Biotechnology Co., Ltd. (Honghe, China); polysaccharide conjugates (pectin) was purchased from Beijing solarbio science&technology co., ltd. (Beijing, China); Bradford Protein Assay Kit was acquired from Beyotime Biotechnology Co., Ltd. (Shanghai, China). DEAE seplife FF was purchased from Sunresin (Xi'an, China); Sephacryl S-400HR was purchased from GE Healthcare (Massachusetts, BSN, USA); pellicon XL membrane (3 kDa) obtained from Millipore (Billerica, MA, USA).

2.2. Preparation of Pu-erh ripe tea infusion

Tea infusions were prepared as a literature description (Deng et al., 2022). Briefly, tea leaves were brewed in boiling water with the ratio 3:150 (*w/v*) for 5 min, then the dregs portions were quickly strained to obtain the initial tea infusion of Pu-erh ripe tea (PRT). The PRT were cooled down to 50 °C and maintained in water bath for subsequent experiments. The PRT were filtered through cellulose acetate membranes (0.45 μm) and then ultrafiltered at 50 °C through an ultrafiltration membrane (3 kDa). Rejection liquid (RL) made up the upper section of the membrane, and filtering liquid (FL) made up the lower portion. For further purification, part of RL was freeze-dried and the rest were used for sensory evaluation and physicochemical composition analysis.

2.3. Extraction and purification of polysaccharide conjugates from the Pu-erh ripe tea

Crude Pu-erh ripe tea polysaccharide conjugates (CPTPS) were prepared according to the procedure described in our previous study (Deng et al., 2022). The freeze-dried RL was redissolved in water (10 mg/mL), then ethanol was added to the concentration of 75% (*v*/v) at 4 °C. The mixture solution was centrifuged (4000 ×*g*, 15 min) after 24 h, the precipitate was allowed to dry naturally and then prepared as a 10 mg/mL solution and dialyzed in pure water for 24 h. The CPTPS was maintained at −20 °C after freeze-drying.

The Pu-erh ripe tea polysaccharide conjugates (PRTPS) were dissolved in pure water by the method of Du et al. (2019). The prepared solution was centrifuged (10 min, 10,000 ×*g*), and the supernatant was extracted to a DEAE seplife FF anion-exchange chromatography column (26 mm × 400 mm) which was sequentially eluted with distilled water at 4 mL/min, followed by different concentrations of NaCl (0.1 M, 0.2 M and 0.3 M). The carbohydrate content of each fraction (PRTPS-1,2,3,4) was measured using the phenol-sulfuric acid method (490 nm). The fraction, PRTPS-2 (eluted by 0.1 M NaCl) with the highest purity and yield, was selected dialyzed at 4 °C for 48 h, subsequently freeze-dried. After that, the PRTPS-2 was purified by a Sephacryl S-400HR gel chromatography column (26 mm × 1000 mm) on a AKTA exlorer system (GE Healthcare) to obtain PRTPS-5. It had a 92.5% concentration that was measured by means of the Phenol-sulfuric acid method after being eluted with distilled water flowing at a rate of 1 mL/min. The scheme of separation procedure was shown in Fig. 1.

2.4. Sensory evaluation

2.4.1. Taste dilution analysis

The taste dilution analysis (TDA) method was used with slight modifications to screen the MTT fractions in Pu-erh ripe tea. The three liquid fractions of PRT, RL and FL separated in 2.2 were heated to 50 °C, and 5 mL of each was mixed together in 5 mL of pure water at 50 °C. The samples were gradually diluted at a ratio of 1:1 and reviewed to characterize their infusion color and taste. We recruited 10 trained sensory evaluators of both sexes (four males and six females) with a mean age of 32.00 ± 11.72 years to complete all sensory experiments. PR1 was used as the reference sample of MMT, and the evaluators with strong preference and rejection of MMT taste were excluded. The evaluators were screened and trained to make them clear the definition of MMT and establish a good resolution of MMT, so as to meet the basic requirements of the evaluators who can carry out the experiment. The evaluator used the difference test until the taste was not tasted in the dilution, which was the taste dilution factor (TD-factor) of the fraction. The samples supplied to the sensory evaluators were obtained multiple times and then combined.

2.4.2. Reconstituted experimental for MTT

A descriptive evaluation method was used to investigate the effect of candidate components on the mellow and thick of Pu-erh ripe tea using PRT as the reference standard for MTT (Liu et al., 2014). The scoring table and sample concentrations were shown in Table 1, and 10 trained sensory evaluators mentioned in 2.4.1 performed the descriptive evaluation and scored the similarity of the MTT.

2.4.3. The taste attribute scale score of reconstituted samples

The 2.4.2 selected MTT standards were prepared at different concentrations for flavor strength analysis, keeping the ratios between flavor substances constant. The 10-point scale (0–1: weak; 2–3: slightly strong; 4–5: strong; 6–7: more strong; 8–9: extremely strong) used to establish a standard curve for the MTT.

The taste intensity of reconstituted samples was assessed using taste equivalence quantification using adjustments to the approach used by Liu et al. (2014). Caffeine, EGCG, glucose and TB + polysaccharide conjugates + citric acid were used as chemical standards for the four taste attributes of bitterness, astringency, sweetness and mellow and thick. Table S1 gave information on the recombinant samples and standards for sensory evaluation.

2.5. Determination of tea polyphenols (PP), soluble proteins (SP), polysaccharide conjugates (PC), theabrownin (TB) and galacturonic acid (GalA)

The PP in samples were measured using the Folin-Ciocalteu reagent and measured at 765 nm (Deng et al., 2022). The Bradford Protein Assay Kit was used to quantify the SP in tea samples (Beyotime Institute of Biotechnology, Shanghai, China). The anthrone-sulfuric acid colorimetric was used to analyze the PC in tea samples (Deng et al., 2022). The GalA was measured using the sulfate-carbazole method (Taylor, 1993). The TB was determined by the approach of Roberts and Smith (1961),

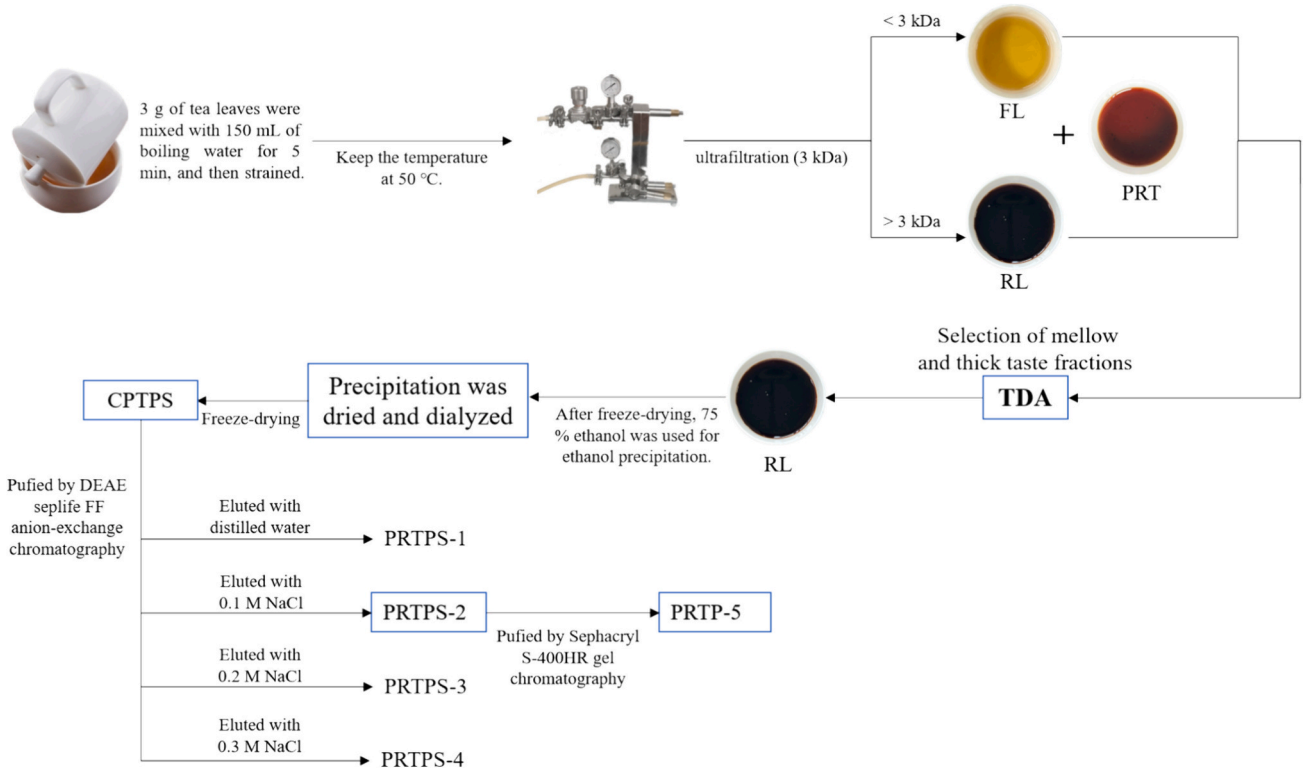

Fig. 1. Preparation of tea infusion and scheme for separation procedure of PRTPS-5.
FL: filtration liquid; RL: rejection liquid; PRT: initial tea infusion. TDA: taste dilution analysis.

Table 1
Sensory recombination solutions and description of mellow and thick taste.

Number	Samples	Formulation	Taste description	Similarity of mellow and thick taste[a]
b1	TB	1.5 g/L	mellow and lack of thick	3
b2	CPTPS	3 g/L	mellow and approach thick	3
b3	PC	30 mg/L	thick, smooth and lack of thick	3
b4	TB+ CPTPS	1.5 g/L + 3 g/L	slightly astringent, mellow and thick, approach smooth	4
b5	TB + PC	1.5 g/L + 30 mg/L	mellow and thick, smooth, brisk and mould taste	4.3
b6	TB + PC + citric acid	1.5 g/L + 30 mg/L + 150 mg/L	mellow and thick, smooth and brisk	5

Notes:a represents the similarity with the MMT of PR1, 5 points system. And the higher the score, the higher the similarity. Citric acid was only used to adjust the pH of the solution. The concentration of each formulation was determined by pre-experiments.

with alterations (Deng et al., 2022). It was extracted with n-butanol and then detected at 380 nm.

2.6. Analysis of catechins, gallic acid (GA), and caffeine

According to Deng et al. (2022), the caffeine, GA, and catechins were measured using Shimadzu High-Performance Liquid Chromatography (Tokyo, Japan) with Dikma Technologies Inc. DiamonsiTM C18 chromatographic column (4.6 mm × 250 mm; 5 μm, Lake Forest, CA, USA), using acetonitrile as the mobile phase B and 2 % acetic acid in aqueous solution as the mobile phase A at a flow rate of one milliliter per minute, and the column temperature was set at 40 °C, with a detection wavelength of 280 nm. The gradient elution of mobile phase B transitioned from 6.5% to 25% in 16 min, and then returned to the initial state in 25 min, and equilibrated for 10 min.

2.7. Structure characterization of PRTPS-5 fraction

2.7.1. Ultraviolet analysis

The ultraviolet-vis spectrum of PRTPS-5 (5 mg/mL) was measured on the Multiskan GO Multifunctional microplate reader (Thermo Fisher Scientific, USA) with wavelengths between 200 and 1000 nm. Pure water was used as blank.

2.7.2. Molecular weight determination

The PRTPS-5 was diluted in DMSO solution with 0.5% *w*/w lithium bromide (DMSO/LiBr) to attain a concentration of 1 mg/mL, whereafter filtered through 0.45 μm filter. The SEC-MALLS-RI was utilized to investigate the homogeneity and molecular weight of each fraction (Julakanti et al., 2023). The polydispersity index and weight and number-average molecular weights of the PRTPS-5 were measured using the laser photometer (DAWN HELEOS-II, Wyatt Technology Co., USA). The Shodex OH-pak SB-803, 804, and 805 chromatographic column was (300 × 8 mm, Showa Denko K.K., Tokyo, Japan), and the column temperature was sustained at 60 °C. 0.3 mL/min of fluid was flowing.

2.7.3. Monosaccharide composition

The monosaccharide composition of PRTPS-5 was identified by Pereira et al. (2024) with a few modifications. Add 5 mg of PRTPS-5 to sealed tubes and hydrolyze with 2 mol/L trifluoroacetic acid for 2 h (121 °C). Blow dry with nitrogen. It was washed with methanol, blown dry, repeated 2–3 times. The residue was filtered across microporous membranes (0.22 μm) after being redissolved in deionized water and measured.

The PRTPS-5 extracts were examined using Dionex ICS 5000+

pulsed amperometric detector with high-performance anion-exchange chromatography on an anion-exchange column (3.0 × 150 mm, 10 μm, CarboPac PA-20, Dionex). The flow rate was 0.5 mL/min, and the volume of injection was 5 μL. Mobile phase (A: ddH_2O, B: 0.1 M NaOH, C: 0.1 M NaOH and 0.2 M sodium acetate). The gradient program, at 0 min, the volume ratio of solutions A, B and C was 95:5:0; at 26 min, 85:5:10; at 42 min, 85:5:10; at 42.1 min, 60:0:40; at 52 min, 60:40:0; at 52.1 min, 95:5:0; at 60 min, 95:5:0.

2.7.4. Fourier transform infrared (FT-IR) analysis

A Nicolet iZ-10 spectrometer (Thermo Nicolet, USA) was employed to obtain the FT-IR spectra of PRTPS-5. For FT-IR measurements between the 4000 and 400 cm^{-1} range, the PRTPS-5 was combined with KBr powder before being pressed into 1 mm pellets by the medium of Pereira et al. (2024).

2.7.5. Methylation analysis

Acid methylation analysis: The reduction of uronic acids of the PRTPS-5 were performed using methylation. The PRTPS-5 was reduced with $NaBH_4$ and $NaBD_4$, dialyzed and lyophilized to acquire the reduzates were methylated in NaOH/DMSO with CH_3I. After complete methylation, the permethylated products were reduced with $NaBD_4$ and acetylated with acetic anhydride for 2.5 h at 100 °C after being hydrolyzed with 2 M TFA for 1.5 h at 121 °C (Zhu et al., 2021).

Neutral methylation analysis: The PRTPS-5 (in DMSO) were methylated using DMSO/NaOH with CH_3I. The remaining steps are consistent with acid methylation analysis (Yang et al., 2021).

GC–MS was accomplished with an Agilent 6890 A-5977B connected with a chromatographic column (30 m × 0.25 mm × 0.25 μm, Agilent BPX70, SGE, Australia). The high-purity helium was utilized as the carrier gas with a 10:1 split ratio and a 1 μL injection volume. The initial temperature was 140 °C and maintained for 2 min. At a rate of 3 °C per minute, the temperature was pushed up to 230 °C and maintained there for 3 min.

2.7.6. NMR analysis

In 0.5 mL of D_2O, the PRTPS-5 was dissolved to a final concentration of 40 mg/mL. A spectrometer system (AVANCE NEO 500 M, Bruker, Rheinstetten, Germany) running at 500 MHz was used to record the ^1H NMR, ^{13}C NMR, COSY, NOESY, HMBC and HSQC at 25 °C (Yang et al., 2021).

2.8. Statistical analysis

The data were reckoned as mean ± standard deviation, with each experiment being performed three times. One-way analysis of variance through SPSS 23.0 (IBM Corporation, Armonk, NY) followed by Duncan's multiple range test to exemplify significant differences between means ($p < 0.05$). Partial least squares-discriminant analysis (PLS-DA) was carried out employing the Simca-P 13.0 software (Umetric, Umea, Sweden), while the graphs were generated utilizing Origin 2021 software (OriginLab, Northampton, MA). The data of homogeneity and molecular weight were acquired and processed using ASTRA6.1 (Wyatt Technology). The data of monosaccharide composition were obtained using the ICS5000+ (Thermo Scientific) and edited using chromeleon 7.2 CDS (Thermo Scientific). The data of NMR were processed using MestReNova software (Bruker, Germany).

3. Results and discussion

3.1. Isolation and selection of MTT fractions in Pu-erh ripe tea

Based on our previous studies (Deng et al., 2022), it was discovered that the key components affecting the MTT of Pu-erh ripe tea infusion were macromolecular compounds. In order to further clarify its influence, a representative Pu − erh ripe tea samples of mellow and thick taste (1:50 *w/v*, PR1–3) were consequently fractionated by ultrafiltration (3 kDa).

The sensory evaluation revealed that the MTT and color had a significant impact after the ultrafiltration process (Table 2). A TDA was conducted to further determine the intensity of the taste intensity and TD-factor in the fractions. The fraction with a higher TD-factor had a stronger mellow and thick intensity. The RL had a TD-factor of 8 and was mellower and stickier than the PRT. On the contrary, the TD factor of FL was 3, and the taste experienced a substantial decline, showing bitterness and astringency. The results indicated that RL might be crucial to the MTT of Pu-erh ripe tea, and ultrafiltration could separate the components of tea infusion, which had a significant effect on the taste of tea infusion, same as the research of Lin et al. (2016). The existence and proportion of colloid phase in tea infusion that can't pass through the ultrafiltration membrane affect the taste change.

3.2. Effects of main characteristic taste components on MTT of different fractions in Pu-erh ripe tea

PLS-DA was utilized to offer an overview of the intricate correlations among the multivariate statistical variables in the tea infusion of Pu-erh ripe tea (Fig. 2). Typical HPLC chromatogram were shown in Fig. S1. The PLS-DA model was divided into three groups, R^2X (cum), R^2Y (cum), and Q^2 (cum) were 0.946, 0.976, and 0.950 respectively (> 0.5), which signified that the model was a good fit. This meant that the ultrafiltration treatment had a good separation effect on the taste components in tea infusion. The concentration of PC and TB, mainly enriched in RL, were 635.08 mg/L and 8.86% respectively. PC and TB were the most strongly correlated with MTT, with significant positive correlations (TS: $r = 0.975$, $p < 0.0001$; TB: $r = 0.975$, $p < 0.0001$). So, we supposed that these components were the key components affecting the MTT of Pu-erh ripe tea infusion.

Simultaneously, the distribution rate of the above flavor components in FL decreased significantly ($p < 0.05$). PC, as a sweet substance in tea infusion, can alleviate the astringency and bitterness (Deng et al., 2022). The content of TB proved to be a crucial gauge in assessing the standards of Pu-erh tea, and it exhibited a positive correlation with the quality. The enzymatic action of hydrolase and polyphenol oxidase released by microorganisms was the primary factor in the development of TB (Peng et al., 2013). In summary, we speculate that MTT of Pu-erh ripe tea was related to both PC and TB, which was in line with prior findings (Deng et al., 2022).

3.3. Verification of the effects of screened MTT compounds

An analysis of the reconstituted solution's descriptive sensory properties was done so as to confirm the effectiveness of the above-identified MTT compounds. PC and TB were macromolecular compounds in tea infusion, which were difficult to accurately quantify.

Table 2
Sensory evaluation of different components of Pu-erh ripe tea.

Samples	Infusion color	Taste profile	TD
PR1	bright and brownish red	mellow and thick and sweet after taste	6
RL1	vandyke brown	bitter and mellow and thick	8
FL1	orange and bright	bitter, astringent and neutral	4
PR2	bright and brownish red	more mellow and thick	5
RL2	vandyke brown	bitter and mellow and thick	7
FL2	orange and approach bright	more bitter, astringent and neutral	3
PR3	light brownish red and bright	more mellow and thick	5
FL3	orange and approach bright	more bitter, astringent and neutral	3

Notes: Different letters denote significant differences between different columns as tested by ANOVA followed by Duncan's test ($p < 0.05$).

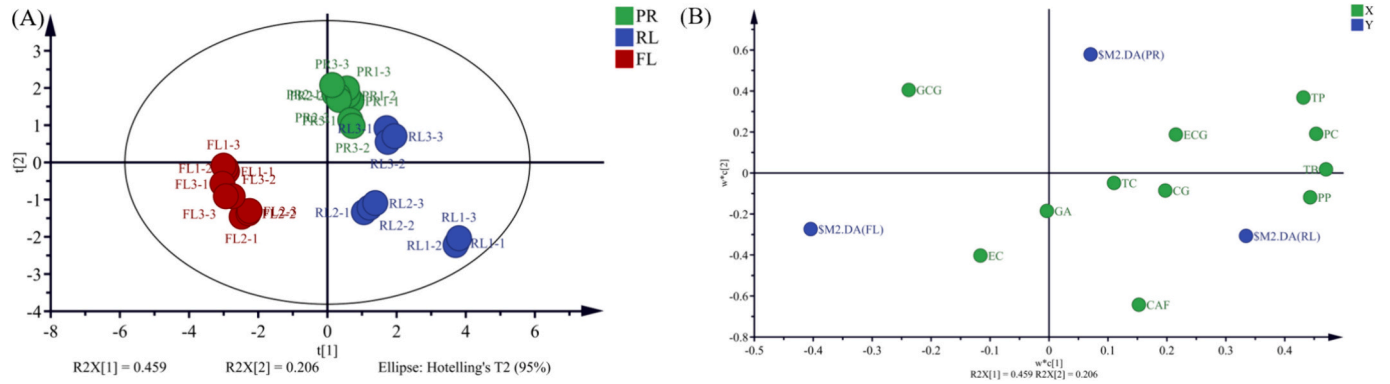

Fig. 2. The results of PLS-DA based on the chemical components of different fractions in Pu-erh ripe tea.
A: The score scatter plots; B: The loading scatter plots. FL: filtration liquid; RL: rejection liquid; PR: initial tea infusion. TP: total soluble protein; PC: polysaccharide conjugates; TB: theabrownin; PP: Total tea polyphenols; TC: total catechins; CAF: caffeine.

Reconstruction by simulating the concentration of characteristic taste components in tea infusion was difficult to achieve in this experiment (Dai et al., 2015). CPTPS was obtained by freeze-dried RL following a series of purification procedures including ethanol sedimentation, freeze-dried and dialysis. Meanwhile, the content of GalA in CPTPS was 61.44 ± 1.37%, it was preliminarily inferred that CPTPS was a PC with GalA as backbone (Seggiani et al., 2009). Moreover, Soultani et al. (2014) showed that pectin added to tea infusion can thickening and it does not mask the capacity of physicochemical components in tea infusion. Using food-grade TB and PC (pectin) as alternative materials, a reconstituted solution model close to the MTT intensity of Pu-erh ripe tea infusion was prepared by adjusting the different combinations and concentration of taste compounds, so as to verify the effect of PC on MTT. For the review of panelists, six samples were created, the PR1 served as the control sample. The reconstituted solution was described, and the similarity of MTT between the reconstituted solution and PR1 was scored (Table 1). A single flavor compound was difficult to form a complete MTT. When the concentration of TB + PC reached 1.5 g/L + 30 mg/L, it showed MTT with aroma by pile fermentation. After adding citric acid to the solution to bring its pH value to that of PR1, b6 showed MTT, which was almost consistent with the MTT intensity of PR1, and the similarity score was 5 points. The traditional evaluation of MTT is that the entrance is comfortable, and the aftertaste is sticky and greasy (Zhao et al., 2017), but in this study, we believed that the definition of MTT of Pu-erh ripe tea is: refreshing and comfortable in the mouth, rich in inclusions, and harmonious flavor with viscous taste. At the same time, the MTT intensity score of b6 was consistent with PR1. Based on the formula of b6, TB, PC and citric acid were selected as the chemical standard material of MTT property, and the concentration ratio of the three compounds was maintained at 50:1:5.

Based on the constructed taste score attribute standard curve, to verify the effect of PC on the MTT of Pu-erh ripe tea, CPTPS, PC and TB were added to the model tea solutions with the ratio 1:150 (*w/v*) in different combinations (Fig. 3). Firstly, the addition of CPTPS (TG2), PC (TG3) and TB (TG4) to TG1 resulted in different enhancements to the MTT compared to TG1, and the most significant enhancement of MTT was TG4, from 3.33 ± 0.29 to 4.33 ± 0.58. The MTT substances slightly reduced the scores of bitterness, astringency and overall taste compared to TG1. Next, the three MTT substances were blended in the TG1 and their taste intensity was evaluated. In TG5 (CPTPS and TB) and TG6 (PC and TB), the MTT intensity was significantly increased to 6.26 ± 0.23 and 6.16 ± 0.29 ($p < 0.05$), which was close to CG (6.33 ± 0.29). In addition, except the overall taste, the scores of other taste attributes were not significantly different from CG ($p > 0.05$).

In summary, the taste interaction produced by the mixture of CPTPS, PC and TB not only significantly improved the MTT intensity of tea infusion, but also had no significant effect on the taste of tea infusion itself. It can be seen that the MTT was a compound taste, which was mainly formed by the interaction of PC and TB. However, the specific content and structure of CPTPS need further purification and structural identification.

3.4. Isolation and purification of PRTPS-5 and their structural characterization

3.4.1. Isolation and purification of polysaccharide conjugates from RL

CPTPS was purified with a DEAE eplife FF exchange chromatography column and a total of 82 tubes of eluate (A1-A82) were collected, yielding three main polysaccharide elution peak fractions (Fig. S2A). PRTPS-2 (A31-A40), with the most MMT taste，also with highest purity and yield, was selected for further separated by gel filtration chromatography. After separation on a Sephacryl S-400HR gel chromatography column (Fig. S2B), a total of 63 tubes of eluate (B1-B63) were collected to give a single elution peak, collecting B31-B41 combined as PRTPS-5 with a purity of 92.49%.

3.4.2. Determination of molecular weight and monosaccharide composition

From Fig. 4A, compared with the blank control, PRTPS-5 had no significant absorption in the 200–400 nm wavelength, indicating that there were few residual impurities such as pigments, proteins and nucleic acids in the sample. The homogeneity and molecular weight of PRTPS-5 were determined using SEC-MALLS-RI analysis (Julakanti et al., 2023) (Fig. 4B). The chromatographic peaks of PRTPS-5 were single and close to normal distributions in the molar mass range, demonstrating good homogeneity (Yan et al., 2014). The number average molecular weight, the values of the weight and the molecular weight distribution of the PRTPS-5 were 20.77 kDa, 22.93 kDa and 1.10, respectively. The molecular weight distribution was approached 1, revealing they were highly homogenous and monodispersed. This result was similar to the yellow tea polysaccharide extracted by Wang et al. (2021), and the tea polysaccharide with a smaller molecular weight and superior antioxidant activity (Chen et al., 2004).

The gas chromatogram (Fig. 4C) showed that nine monosaccharides were detected in PRTPS-5, which mainly consisted of Rha (81.67 ± 3.14 μg/mg), Gal (75.47 ± 1.40 μg/mg), Ara (61.11 ± 2.01 μg/mg) and Man (40.70 ± 1.28 μg/mg), with a molar ratio of 2.01:1.85:1.50:1. The composition of monosaccharides was similar to previous reports (Deng et al., 2015)., Xu et al. (2014) also found that the content of Rha and Gal in Pu-erh tea polysaccharides increased with the increment with the years. It is generally recognized that the taste of Pu-erh tea increased with the increase of storage years (Duan et al., 2012). Therefore, the high content of Rha and Gal in PRTPS-5 also further verified that it played an essential role in improving the MMT of Pu-erh ripe tea.

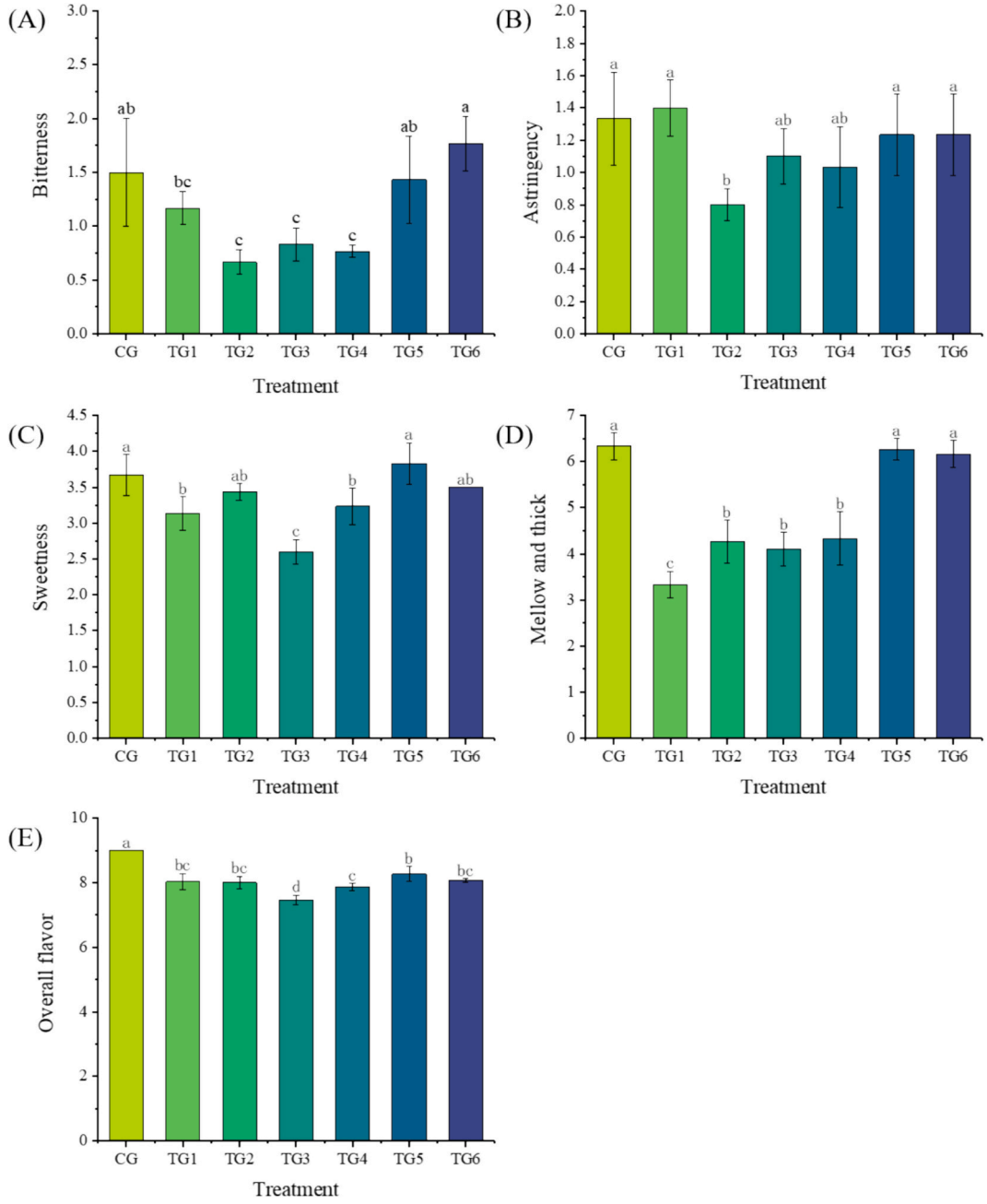

Fig. 3. Taste attributes for different combinations.
A: Bitterness; B: Astringency; C: Sweetness; D: Mellow and thick; E: Overall flavor. CG: Control Group (the initial tea infusion of PR1); TG: Treatment Group. Different letters denote significant differences between different columns as tested by ANOVA followed by Duncan's test ($p < 0.05$).

3.4.3. FT-IR analysis

Fig. 4D revealed the preliminary structural characterizations of PRTPS-5 that showed typical absorption peaks of pectin functional groups in the range of 4000–500 cm^{-1}. At 3410.95 cm^{-1}, a prominent absorption peak was produced by the tensile vibration of O-H; it was a recognizable polysaccharide absorption band (Zhou et al., 2021). The C—H tensile vibration was responsible for the signal at 2935.04 cm^{-1} (Chen, Xie, et al., 2019). The typical pectic peak was the absorption peak at 1735.22 cm^{-1}, which was formed by the C═O stretching vibration formed by the carboxyl group of galacturonic acid (Liang et al., 2022). Notably, the band at 1246.73 cm^{-1} indicated the presence of -*O*-CH_3 group in PRTPS-5, which was generally considered to be the fingerprint region of different species of pectic (Zhao et al., 2017). The glycosidic bond produced an absorption peak at 1048.03 cm^{-1}, which was attributed to the stretching vibration of C—O to form the characteristic absorption peak of pectic polysaccharide (Chen, Xie, et al., 2019).

3.4.4. Methylation and NMR analysis

The exact linkage patterns, glycosidic bond types, contents and configuration of PRTPS-5 were determined via methylation analysis and NMR. The total ion chromatograms were shown in Fig. S3., detailed information on the sugar residues were shown in Table S2. In this study, initial acid methylation was conducted, revealing the presence of an acid glycosidic bond, 4-Gal(p)-UA. However, due to the low molecular weight of PRTPS-5, the outcomes of acid methylation analysis significantly differed from those of monosaccharide composition analysis. Therefore, we additionally performed the neutral methylation analysis, integrating both the results of the acid and neutral methylation analysis

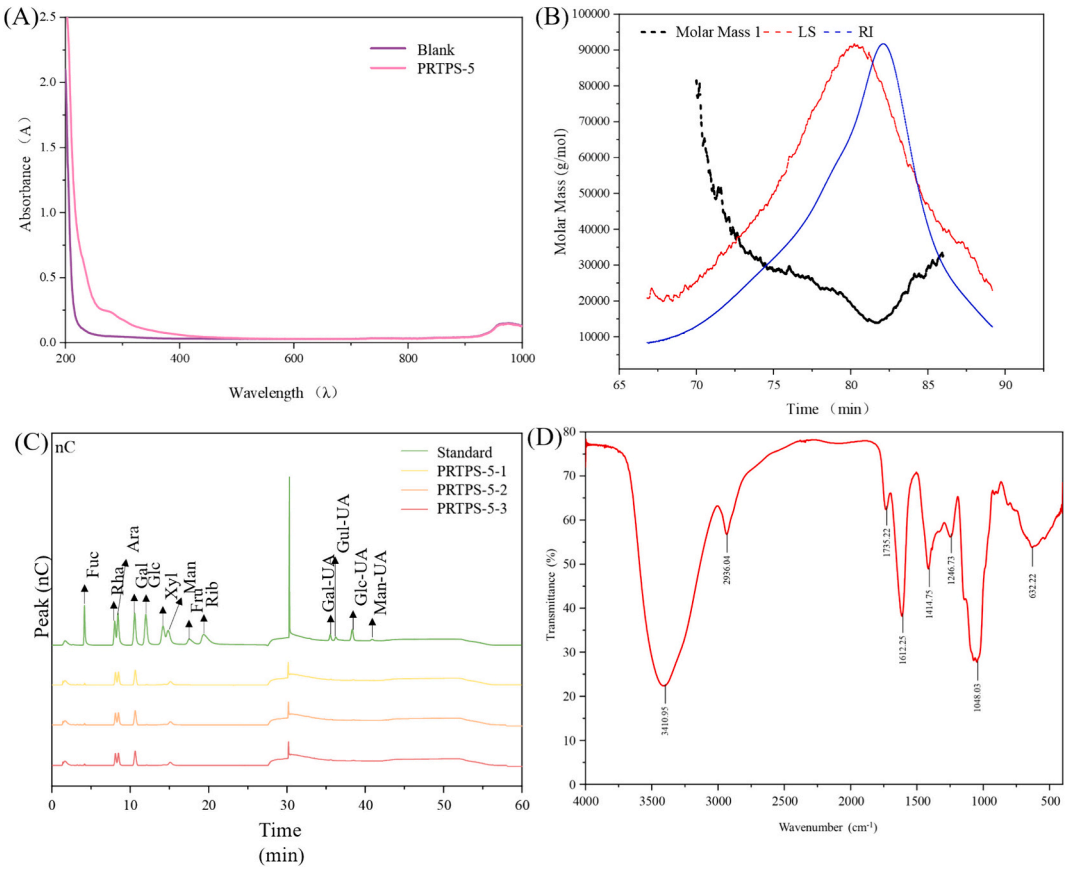

Fig. 4. Structure characterization of PRTPS-5.
A: UV spectra; B: Molecular weight determination; C: Monosaccharide composition; D: FT-IR spectrum.

to obtain more accurate and comprehensive reaction sample data. The outcomes revealed that PRTPS-5 had 14 derivatives: t-Ara(f), t-Ara(p), 2-Rha(p), t-Gal(p), 5-Ara(f), 4-Xyl(p), 2,4-Rha(p), 3-Glc(p), 4-Man(p), 4-Gal(p)-UA, 6-Man(p), 3,4-Gal(p), 2,6-Man(p), 3,6-Gal(p). Among the various sugar residues, t-Ara(f) accounts for the largest ratio, indicating that it may be the main backbone of PRTPS-5. And the ratio was approximately consistent with the results of monosaccharide composition.

The structure of PRTPS-5 was elucidated using NMR analysis, including 1D and 2D NMR spectrum. The ^{1}H NMR (500 MHz, Fig. 5A) showed the signal of PRTPS-5 was mainly concentrated between δ 3.0–5.4 ppm. The signal at δ 1.1–1.3 ppm was often considered to be the hydrogen signal of 6-deoxyglucose (Wang et al., 2021). The presence of many coupling signal peaks in the δ 4.3–5.4 ppm signal area suggests that the sample contains varying sugar residues with overlapped peaks. The signal peak near δ 3.43 ppm was the signal of hydrogen in -*O*-CH_3 (Liang et al., 2022). Combined ^{13}C NMR spectrum and HSQC spectrum identified anomeric signals of different residues and eight anomeric carbon/proton correlations were assigned as shown in Fig. 5B&C. The 8 anomeric signals were designated as A-H and the corresponding chemical shifts of carbon/proton were determined to be δ 5.16/109.65 (A)、5.17/98.59 (B)、5.15/98.71 (C)、5.03/99.32 (D)、4.95/97.84 (E)、4.83/99.63 (F)、4.42/103.06 (G) and 5.12/107.06 (H) ppm, respectively. The chemical shifts of non-anomeric protons of different residues were fully assigned according to COSY (Fig. 5D) and methylation analysis (Table S2), and the corresponding non-anomeric carbons were assigned with the assistance of previous reports (Guo et al., 2019; Zhang et al., 2020). The results of the full assignments of different residues were listed in Table 3.

Through the observation of HMBC and NOESY spectrum, the structure and linkage of the polysaccharide could be further analyzed. According to the HMBC (Fig. 5E) spectrum, there was a cross-peak at δ 5.16/76.81 ppm between H1 of sugar residue A and C4 of sugar residue B, and a cross-peak at δ 5.17/77.72 ppm between H1 of sugar residue B and C4 of sugar residue E. According to the NOESY spectrum (Fig. 5F), there was a cross-peak at δ 5.16/3.89 ppm between H1 of sugar residue A and H4 of sugar residue B, a cross-peak at δ 5.17/4.36 ppm between H1 of sugar residue B and H4 of sugar residue E, a cross-peak at δ 5.17/3.63 ppm between H1 of sugar residue B and H6 of sugar residue G, a cross-peak at δ 5.15/4.05 ppm between H1 of sugar residue C and H2 of sugar residue B, a cross-peak at δ 5.03/3.79 ppm between H1 of sugar residue D and H3 of sugar residue G, a cross-peak at δ 4.95/4.05 ppm between H1 of sugar residue E and H2 of sugar residue C, and a cross-peak at δ 4.55/3.84 ppm between H1 of sugar residue G and H4 of sugar residue F.

In summary, the structure of PRTPS-5 was proposed as shown in Fig. 5G by combining the results of monosaccharide composition, methylation and GC–MS analysis, and 1D & 2D NMR results.

4. Conclusion

In this research, the MTT compounds in Pu-erh ripe tea were investigated. By using microfiltration and ultrafiltration technology, the MTT components were locked into macromolecular compounds. Through physicochemical composition analysis and TDA, the key substances affecting the MTT of Pu-erh ripe tea were identified, which were mainly enriched in RL, namely PC and TB. This was confirmed in the taste recombination experiment. PC had a significant effect on improving the MTT of Pu-erh ripe tea, especially after interacting with TB, and did not affect other taste in the tea infusion. Further separation, purification and structural identification of PC in RL were found, PRTPS-5 is a distinctive highly branched polysaccharide with a backbone composed of α-L-Ara*f*-

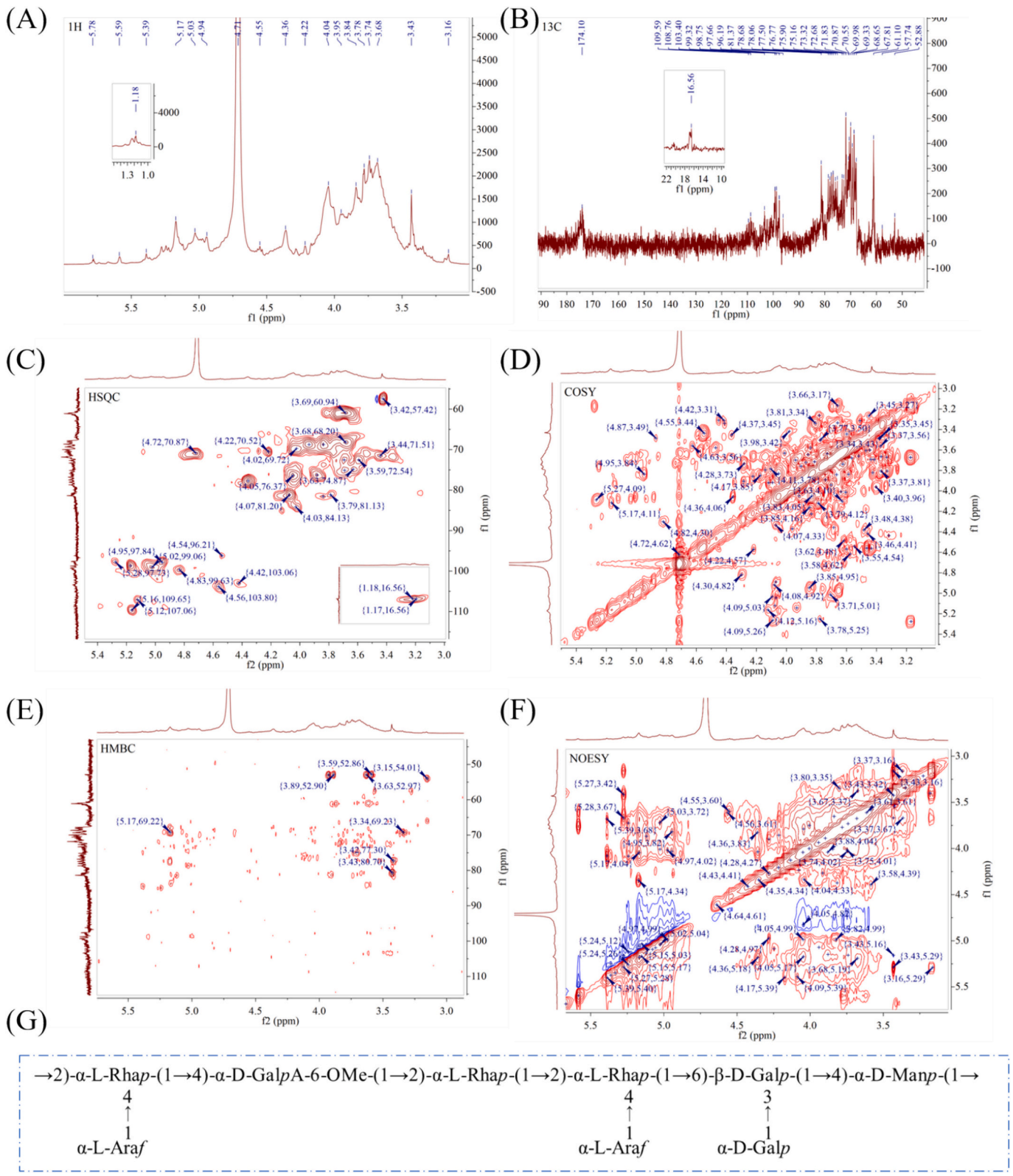

Fig. 5. NMR spectrum and the structural formula of PRTPS-5.
A: ^{1}H; B: ^{13}C; C: HSQC; D: COSY; E: HMBC; F: NOESY; G: the structural formula.

(1→, →2,4)-α-L-Rhap-(1→, →2)-α-L-Rhap-(1→, α-D-Galp-(1→, →4)-α-D-GalpA-6-OMe-(1→, →4)-α-D-Manp-(1→, →3,6)-β-D-Galp-(1 → and →5)-α-L-Araf-(1 → and contained multiple pectic characteristic peaks. These findings will improve the sensory evaluation method of Pu-erh ripe tea and supply a technical basis and scientific basis for the progression of PC as a taste modifying agents.

CRediT authorship contribution statement

Sihan Deng: Writing – original draft, Methodology, Data curation.

Table 3
Chemical shift assignments of the of PRTPS-5.

Code	Glycosyl residues	Chemical shifts (ppm)					
		H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
A	α-L-Ara*f*-(1→	5.16	4.12	4.01	4.03	3.743.68	n.d
		109.65	81.32	76.81	84.11	61.13	n.d
B	→2,4)-α-L-Rha*p*-(1→	5.17	4.05	3.82	3.89	3.66	1.18
		98.59	76.67	69.2	76.81	68.73	16.56
C	→2)-α-L-Rha*p*-(1→	5.15	4.05	3.83	3.45	3.68	1.17
		98.71	76.67	73.78	71.49	68.3	16.82
D	α-D-Gal*p*-(1→	5.03	3.68	3.94	3.83	3.63	3.82
		99.32	72.23	69.04	73.78	74.82	60.99
E	→4)-α-D-Gal*p*A-6-OMe-(1→	4.95	3.84	4.06	4.36	4.72	n.d
		97.84	68.73	69.81	77.72	70.96	174.1
F	→4)-α-D-Man*p*-(1→	4.83	4.29	3.73	3.84	n.d	n.d
		99.63	70.42	74.99	81.69	n.d	n.d
G	→3,6)-β-D-Gal*p*-(1→	4.42	3.34	3.79	n.d	n.d	3.633.76
		103.06	71.79	81.08	n.d	n.d	66.78
H	→5)-α-L-Ara*f*-(1→	5.12	4.08	n.d	n.d	n.d	n.d
		107.06	81.22	n.d	n.d	n.d	n.d

Notes: "n.d" means not detected.

Tianfang Zhang: Writing – review & editing, Conceptualization. **Suhang Fan:** Investigation. **Huahua Na:** Data curation. **Haiyu Dong:** Validation. **Baijuan Wang:** Resources. **Ying Gao:** Writing – review & editing, Conceptualization. **Yong-Quan Xu:** Writing – review & editing, Supervision, Project administration. **Xiaohui Liu:** Resources, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101726.

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