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Short communication

Combination of HSCCC and Sephadex LH-20 methods An approach to isolation and purification of the main individual theaflavins from black tea

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Abstract

In order to separate the main individual theaflavin monomers from black tea, high-speed countercurrent chromatography (HSCCC) and Sephadex LH-20 column chromatography were applied. The results showed that theaflavin (TF1), theaflavin-3-gallate (TF2A), theaflavin-3′-gallate (TF2B) and theaflavin-3,3′-digallate (TF3) can be obtained by HSCCC using a solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:3:1:6, v/v/v/v), but the TF1 was containing epicatechin-3-gallate (ECG). Similarly, Sephadex LH-20 can also effectively separate TF2A(B) and TF3, but epigallocatechin-3-gallate (EGCG) contaminated TF1, too. Combination of HSCCC and Sephadex LH-20, the preferably purified TF1, TF2A(B) and TF3 were obtained than single separation technique. In addition, ECG and EGCG were also suggested to be able to be comprehensively separated by combination of the two techniques.

Keywords: High-speed countercurrent chromatography; Sephadex LH-20; Theaflavin; Theaflavin-3-gallate; Theaflavin-3′-gallate; Theaflavin-3,3′-digallate; Black tea

1. Introduction

Tea is one of the most widely consumed beverages in the world, just second to water. According to different manufacturing processes, it was mainly divided into three types: green tea (non-fermented), oolong tea (semi-fermented) and black tea (fermented). Green tea and oolong tea are more popular in China and Japan, whereas black tea is preferred in India and the most western countries. Epidemiological and *in vitro* studies have shown that consumption of tea is linked to the reduction of cardiovascular diseases and cancer [1,2]. Theaflavins (TFs), one of the main constituents of black tea, which account for 2–6% of the dry weight and contribute greatly to the quality of black tea in terms of color, 'mouthfeel' and the extent of tea cream formation. They are formed from the enzymatic coupled oxidation of flavanols (catechins) catalyzed by the endogenous enzyme polyphenols oxidase (PPO) in tea leaves during the "fermentation" process. Theaflavin (TF1), theaflavin-3-gallate (TF2A), theaflavin-3′-gallate (TF2B) and theaflavin-3,3′-digallate (TF3) were well identified and characterized, which were generally considered to compose the main theaflavins [3]. Recently, considerable studies increasingly showed that theaflavins have attracted more attention on their bioactivities and pharmacological effects than green tea catechins. They also possess antioxidative properties, for example, TF3 has been proved to show higher antioxidative activity than epigallocatechin-3-gallate (EGCG), which is the strongest antioxidant among all catechins and a precursor of TF3 [4]. In addition, theaflavins can exert anti-mutagenicity [5], anti-inflammatory action [6] and suppression of extracellular signals to inhibit cell proliferation [7] as well as repair DNA oxidative damage [6]. Due to their low abundance and challenging purification procedure, previous studies have mostly been focused on using theaflavins mixture. Alternatively, the four main individual theaflavins are not commercially available. Therefore, in order to further investigate the molecular mechanisms of these theaflavins induced different biological activities or pharmacological function, it is necessary to prepare the main individual theaflavin monomers. Isolation

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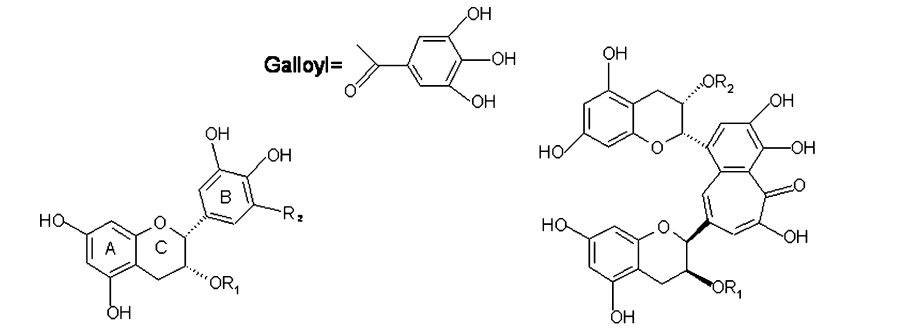

Fig. 1. Structure of tea catechins and theaflavins.

of theaflavins has been, so far, based on the lipophilic Sephadex LH-20 column chromatography and semi-preparative HPLC [8]. The all-liquid chromatographic technique of high-speed countercurrent chromatography (HSCCC) was also recently applied to the isolation of tea polyphones [9] or tea pigments from black tea [10,11]. However, the separation procedure of these techniques is not efficient to obtain monomeric theaflavin because they were contaminated by other components such as tea catechins or polymerized thearubigins (TRs). Based on the respective data obtained from HSCCC and Sephadex LH-20, we combined them to obtain the TF1, TF2A(B) and TF3 from black tea, especially the epicatechin-3-gallate (ECG) and EGCG, two main abundant catechins, can also be suggested to be efficiently isolated from TF1 through the combined procedures, which will be chosen as an effective approach to purify TF1.

2. Experimental

2.1. Isolation of crude theaflavins mixture from black tea

Twenty grams of a commercial black broken tea was extracted for 40 min with 70% aqueous ethanol (400 ml 2×) in sonication water bath (60 °C, 40 Hz), concentrated on a rotavapor (40 °C) to about 500 ml and partitioned with an equal volume of chloroform for twice to remove caffeine. The aqueous layer was partitioned with equal volume of ethyl acetate (EtOAc) for twice again and combined the EtOAc layer. The solvent was evaporated in vacuo and lyophilized. The yield was 1.86 g of a brown–red powder. The lyophilysate was dissolved in 95% ethanol and analyzed by HPLC, peak assignments using HPLC are based on the retention time and characteristics spectra previously described through comparison with authentic reference compounds [12]. The crude theaflavins mixture prepared above was used for the following experiments.

2.2. High-speed countercurrent chromatography (HSCCC)

Preparative HSCCC was carried out using a Model GS10A with a multilayer coil of 1.6 mm I.D. and 110 m in length with a total capacity of 230 ml. The β values of this preparative column range from 0.5 at internal to 0.8 at the external ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R ($R = 8$ cm) is the revolution radius or the distances between the holder axis and central axis of

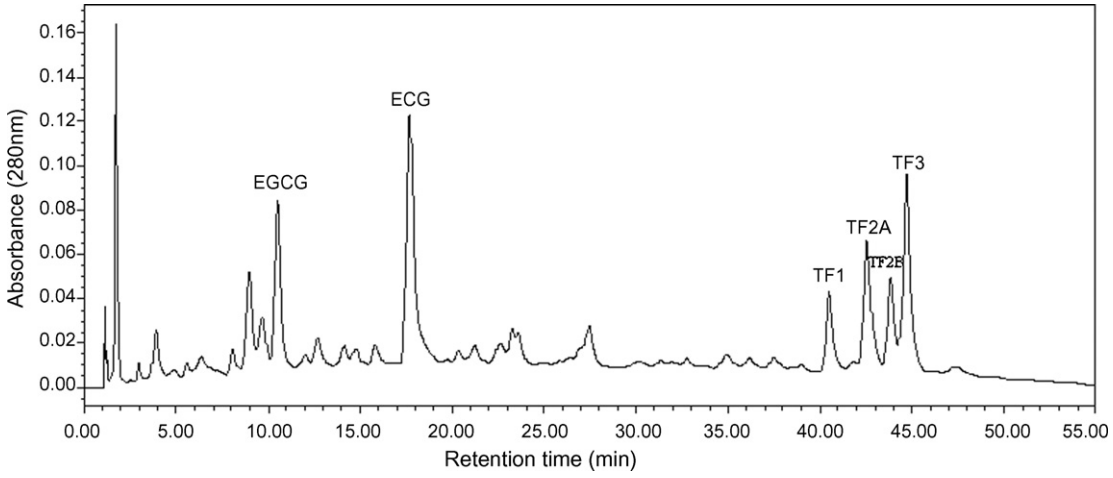

Fig. 2. Analysis of the crude theaflavins mixture extract from black tea by HPLC. Experimental conditions: Nucleosil RP 18 column (3 μm, 100 mm × 4.6 mm I.D.); mobile phase: solvent A (2% aqueous acetic acid) and solvent B (acetonitrile) using a linear gradient from 100 to 92% A in 2 min, 92 to 69% A in 50 min, back to initial conditions in 3 min; flow rate: 1.2 ml/min; detection: 280 nm and 380 nm; injection volume: 5 μl.

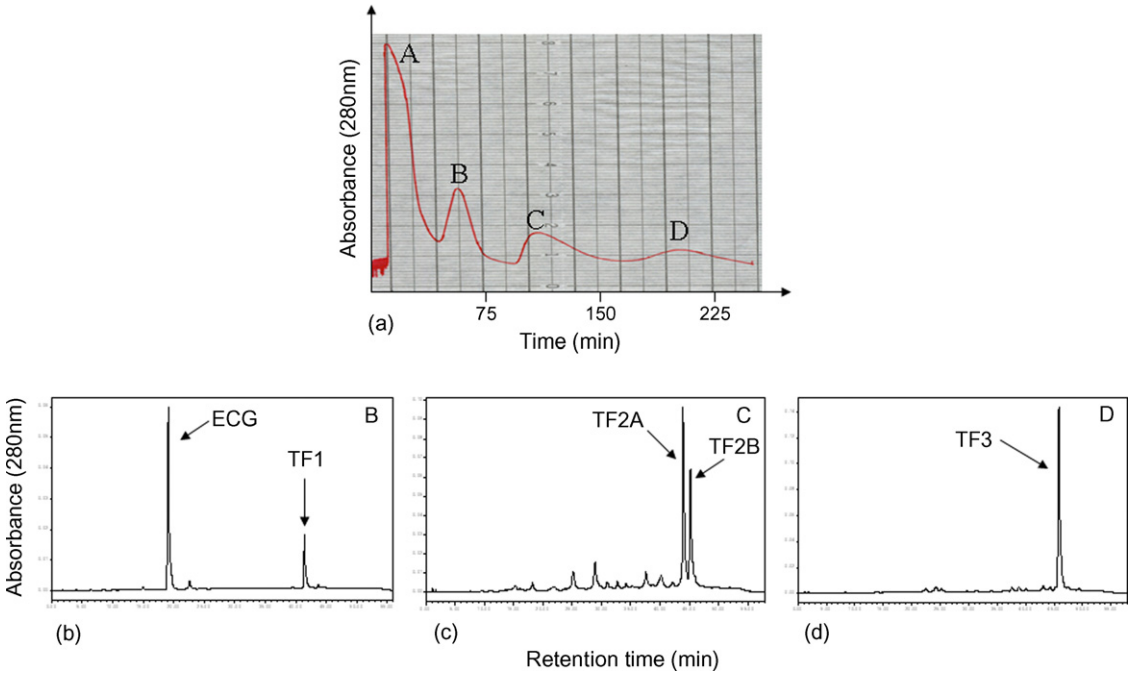

Fig. 3. Separation of the main theaflavin monomers from black tea preparation by HSCCC. Experimental conditions: revolution speed: 800 rpm; solvent system: hexane–ethyl acetate–methanol–water (1:3:1:6, v/v/v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 1.5 ml/min; sample size: 400 mg; retention of the stationary phase: 76%. (a) Profile for HSCCC separation of crude black tea extracts; peak A: a mixture of EGCG and un-identified complex compounds; peak B: a mixture of TF1 and ECG; peak C: isomer of TF2A and TF2B; peak D: TF3. (b, c and d) HPLC profiles for peaks B, C and D, respectively.

the centrifuge) (Beijing Institute of New Technology Application, Beijing, China). A mixture of two-phase solvent system composed of hexane–ethyl acetate–methanol–water (1:3:1:6, v/v/v/v) was chosen as previously reported [13]. The two-phase solvent was shaken vigorously in a funnel and equilibrated, two phases were separated shortly before use. The upper organic phase served as the stationary phase and pumped into the coils until the coils were filled up. The lower aqueous phase used as

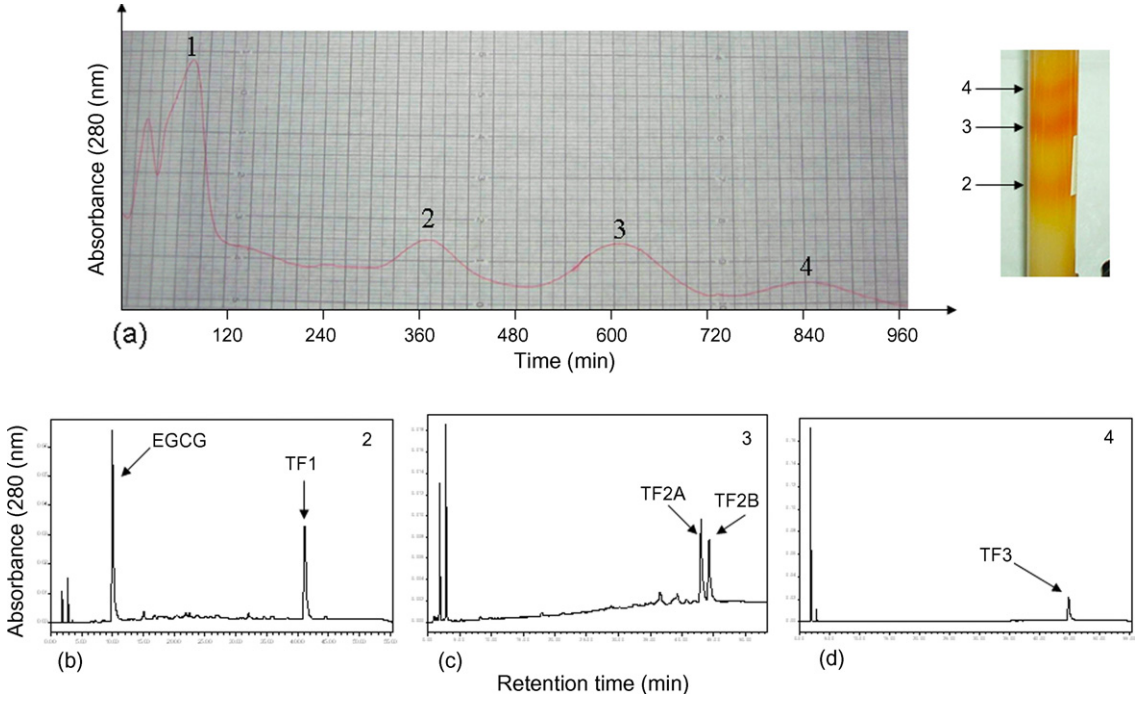

Fig. 4. Separation of the main theaflavin monomers from black tea preparation by Sephadex LH-20. Experimental conditions: column: glass column (30 cm × 2.5 cm I.D.); sample size: 80 mg of crude theaflavins mixture; mobile phase: 45% acetone; flow rate: 0.8 ml. (a) Profile for Sephadex LH-20 separation of crude black tea extracts; peak 1: a mixture of ECG and un-identified complex compounds; peak 2: a mixture of TF1 and EGCG; peak 3: isomer of TF2A and TF2B; peak 4: TF3. (b, c and d) HPLC profiles for peaks 2, 3 and 4, respectively.

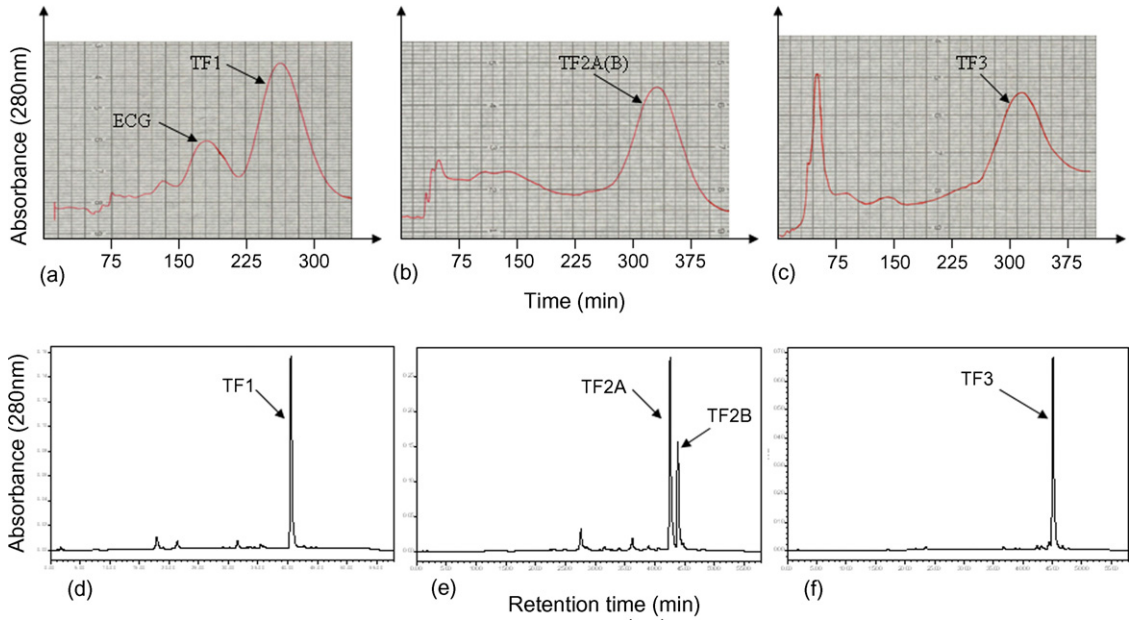

Fig. 5. Purification of the main individual theaflavin monomers through combination of HSCCC and Sephadex LH-20. Experimental conditions: column: glass column (30 cm × 2.5 cm I.D.); sample: peaks B–D obtained by HSCCC as depicted in Fig. 3; mobile phase: 45% acetone; flow rate: 0.8 ml. (a–c): Profiles for Sephadex LH-20 purification of the samples which were initially separated by HSCCC; (d–f): HPLC profiles for the relevant theaflavin monomers purified by Sephadex LH-20, respectively.

the mobile phase and eluted from head to tail of the multiplayer coil. When the stationary phase emerged from the tail end of the instrument, the flow was stopped and the coil planet was run at a revolution speed of 800 rpm. The flow rate of the mobile phase was set at 1.5 ml/min and delivered by a constant-flow pump. The 400 mg of crude theaflavins mixture (dissolved in 2 ml of mobile phase) was then injected into the coil through the injection loop. The effluent from the outlet of the column was continuously monitored with a UV detector at 280 nm and the fractions were collected with a fraction collector into one tube for every 6 min and the chromatograms were recorded simultaneously. According to the chromatograms recorded, combined those correlative tubes for each peak and then analyzed by HPLC.

2.3. Sephadex LH-20 column chromatography

Based on the previous report [8], we used column chromatography with Sephadex LH-20 gel (Pharmacia Fine Chemicals Inc., NJ) to isolate and purify different theaflavins. Briefly, weighed about 20 g of Sephadex LH-20 gel and saturated fully with 200 ml distilled water, and then transferred carefully to an eluting column (30 cm × 2.5 cm), eluted with 45% acetone for at least two fold column volume to equilibrate before exposing to sample.

2.4. High-performance liquid chromatography (HPLC)

Analysis of different fractions was carried out using HPLC on a Waters Model 600 analytical HPLC system consisting of a UV detector (Waters Model 2487) and a Millennium 32 data processor. Peak detection was monitored at 280 nm and 380 nm simultaneously. The chromatographic separation was performed on a Nucleosil RP 18 column (3 μm, 100 mm × 4.6 mm) at room temperature. The mobile phase was a linear gradient of 2% aqueous acetic acid (solvent A) and acetonitrile (solvent B). All solvents and sample solutions were filtered through a 0.45 μm filter prior to use. Conditions: 100–92% A in 2 min; 92–69% A in 50 min; back to initial conditions in 3 min; flow rate, 1.2 ml/min. The injection volume was 5 μl.

3. Results and discussion

It is known that theaflavins account for 2–6% of the dry weight of solids in fermented black tea [14], which mainly consist of TF1, TF2A, TF2B and TF3. As depicted in Fig. 1, their structures as well as tea catechins were illustrated. Among black tea polyphenols, TF1, TF2A(B) and TF3 are generally considered to be the more effective components than tea catechins for the inhibition of carcinogenesis [15]. In order to fully develop the value of theaflavins, considerable studies were attracted to the isolation of the main individual theaflavins in recent years. We first prepared crude theaflavins mixture, which was also called tea pigments, from commercial black tea produced in Sri Lanka due to the large amount of theaflavins content compared with other black tea. As shown in Fig. 2, the crude theaflavins mixture isolated from black tea was detected by HPLC. Preliminary assignment of peak identities in sample infusion was based on the comparison of peak retention time and characteristics spectra as we previously reported [12]. Based on these observations, the following peak assignments were made as TF1, TF2A, TF2B and TF3, respectively, which was depicted in Fig. 2. However, the tea pigments preparation was still a theaflavins complex which was contaminated by catechins or polymerized polyphenols known as

thearubigins. Although the theaflavins were enriched in the tea pigments preparation, the further purification of them is required.

Recently, HSCCC was widely applied as a convenient and efficient technique for the separation of tea catechins or theaflavins. It was a support-free liquid–liquid partition chromatography and the theory and operation principle was described in details by Ito [16]. HSCCC separation of the main individual theaflavins was shown in Fig. 3, the separation was carried out with *n*-hexane/ethyl acetate/methanol/water (1:3:1:6, (v/v/v/v) less dense layer as stationary phase). The sample load was 400 mg of the black tea preparation, yielding 33.0 mg of B, 24.5 mg of C and 13.5 mg of D, respectively. Further analysis of these compounds by HPLC showed that the first peak A was a mixture of EGCG and unknown complex compounds which were not identified (data not shown). Peak B was determined to be mainly composed of TF1 and ECG. It was suggested that the polarity and partition coefficient between TF1 and ECG were close which caused difficulty in the separation of them. Peak C was a mixture of the two isomers of TF2A and TF2B, whereas it was still contaminated by little unknown compounds and further purification was necessary. Analysis of peak D by HPLC suggested that it was of good purity of TF3, which was able to use as an eligible therapeutic for biological research. It was noteworthy that a separation of theaflavins by HSCCC has been described by Jiang et al. [13], but the separation peaks were not identified and characterized. Furthermore, the ECG embedded in TF1 was not regarded as a potential compound for the comprehensive separation previously.

In order to compare the purification grade of the main theaflavin monomers with HSCCC, we employed Sephadex LH-20 to separate individual theaflavins from the same crude theaflavins mixture provided above. Although the method was widely used as a general approach for the separation of the main theaflavin monomers, the high purity of theaflavins cannot be obtained except for gradient elution with different reagents such as ethanol or acetone, which heavily burdened the separation procedure. In this study, we used 45% acetone, one constant concentration, to separate the main theaflavin monomers. As depicted in Fig. 4, the first shoulder peak 1 was analyzed by HPLC, which indicated it was a mixture of ECG and unidentified complex compounds (data not shown), just like what we obtained by HSCCC. Interestingly, peak 2 was a mixture and it mainly consisted of TF1 and EGCG, which suggested that the molecular weight and partition coefficient of TF1 and EGCG was relatively close than other compounds, therefore it increased the difficulty to separate them. As we expected, peak 3 was the isomer of TF2A and TF2B, and peak 4 was of good purity of TF3.

From the above data, it was found that the single separated technique was not enough to obtain preferably purified individual theaflavin monomers because TF1 was containing ECG or EGCG. Similarly, TF2A(B) was still contaminated by little other unknown polyphenols. Thus, in view of our work on the separation of theaflavins, we further used the Sephadex LH-20 column chromatography as a supplementary technique to purify the samples which were initially separated by HSCCC. As shown in Fig. 5, combination of HSCCC and Sephadex LH-20 effectively separated the main individual theaflavin monomers respectively, especially to the separation of TF1 and ECG. Further analysis of these compounds by HPLC indicated that TF1, TF2A(B) and TF3 were of better purity than those separated by single HSCCC or Sephadex LH-20 technique. Taken together, it was suggested that ECG, EGCG, TF1, TF2A(B) and TF3 may be comprehensively separated by combination of HSCCC and Sephadex LH-20 technique from black tea extract. Self-evidently, further improvement on the separation efficiency as well as time saving for the purification of theaflavins need to be investigated by future work.

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