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Structural Identification of Theaflavin Trigallate and Tetragallate from Black Tea Using Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry

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ABSTRACT: Black tea contains two major pigments, theaflavins and thearubigins. These polyphenols have been associated with certain health benefits including prevention of heart disease and cancer. Elucidating and characterizing the structural aspects of thearubigins, the most abundant pigment in black tea, has been a challenge for many years. Therefore further studies of black tea polyphenols must be conducted in effort to solve this thearubigin dispute. In the present study, black tea extract was found to possess theaflavin trigallate and tetragallate by means of liquid chromatography/electrospray ionization mass spectrometry. These structures were confirmed by analysis of the MS^n (n = 1−4) spectra and comparison of the MS/MS spectra of the product ions to the MS/MS spectra of authentic (−)-epigallocatechin-3-gallate, (−)-epicatechin-3-gallate and theaflavin-3,3′-digallate. To our knowledge, this is the first report to confirm the presence of theaflavin trigallate and tetragallate in black tea.

KEYWORDS: *Black tea, LC-MS, theaflavin trigallate, theaflavin tetragallate, thearubigins*

INTRODUCTION

Camellia sinensis an evergreen shrub native to south China is renowned for the utilization of its shoots to produce tea, the world's second most consumed beverage.[1] The leaves can be manipulated to generate over 300 different kinds of tea. These are subdivided into three types: nonfermented green tea, semifermented oolong tea and fermented black tea. About 78% of the tea production worldwide is black tea, whereas green tea, mainly consumed in China and Japan, constitutes about 20%. Since the accidental discovery of tea in 2737BC by the Chinese Emperor Shen-Nun, tea production has become a massive industry.[2]

The consumption of tea is correlated to beneficial cardiovascular effects and inhibition or prevention of cancer.[3−6] These advantageous properties are due to polyphenolic compounds present in tea. Fermentation accounts for the differing polyphenols found in green, oolong, and black tea.[7] Flavan-3-ols, catechins are the major components found in green tea. Catechins possess a meta-5,7-dihydroxy substitution of the A ring and a di- or trihydroxyl group substitution of the B ring. Of the catechins present in green tea, (−)-eigallocatechin-3-gallate (EGCG, **1**) (Figure 1), (−)-epicatechin-3-gallate (ECG, **2**) (Figure 1), (−)-epigallocatechin (EGC) and (−)-epicatechin (EC) are the most prominent, with EGCG being the most abundant.[7] Black tea is characterized by two main pigments theaflavins (TFs) and thearubigins (TRs) which are formed during green tea fermentation. TFs have an orange-red pigmentation whereas TRs are red-brown in color. There are four main TFs present in black tea, these include: theaflavin (TF), theaflavin-3-monogallate (TF3G, **5**), theaflavine-3′-monogallate (TF3′G, **6**), and theaflavin-3,3′-digallate (TFDG, **7**) (Figure 1) (7). The conversion of catechins and their gallates to TFs involves co-oxidation of a *vic*-trihydroxyphenyl and *ortho*-dihydroxyphenyl catechin.[8] TRs are known to be heterogeneous polymers although their formation and characterization has yet to be elucidated.[7,9−12] Despite these drawbacks, Ozawa was able to partially reveal the structures of TRs by chemical degradation, which suggest they are heterogeneous polymers of flavan-3-ols and flavan-3-ol gallates and their bonds are presumably present at C-4, C-6, C-8, C-2′, C-5′, and C-6′ in the flavan-3-ol unit.[11] The MALDI-TOF results from a more recent study hint that TRs are oligomers that are not over 2100 Da, which coincided with their ESI-FTICR data that imply gallated-catechin oligomerization.[10]

Insight on black tea polyphenol bioactivity requires continuous investigation and has proven to be somewhat frustrating due to the limited knowledge of thearubigin chemistry. Despite the challenges posed by TRs, it is believed that black tea contributes to cardiovascular health benefits. In one study, the impact tea polyphenols have on nitric oxide production and vasodilation was researched to validate their influence on cardiovascular disease.[13] Both black tea and green tea were shown to have similar stimulation of eNOS activity and phosphorylation in bovine aortic endothelial cells and also proved to inflict vasorelaxation in rat aortic rings. Even though similar activities were reported, black tea polyphenols especially TRs were shown to have greater activity than catechins from green tea.[13] In addition to heart health association, black tea polyphenols can positively affect carcinogenesis.[6] It has been

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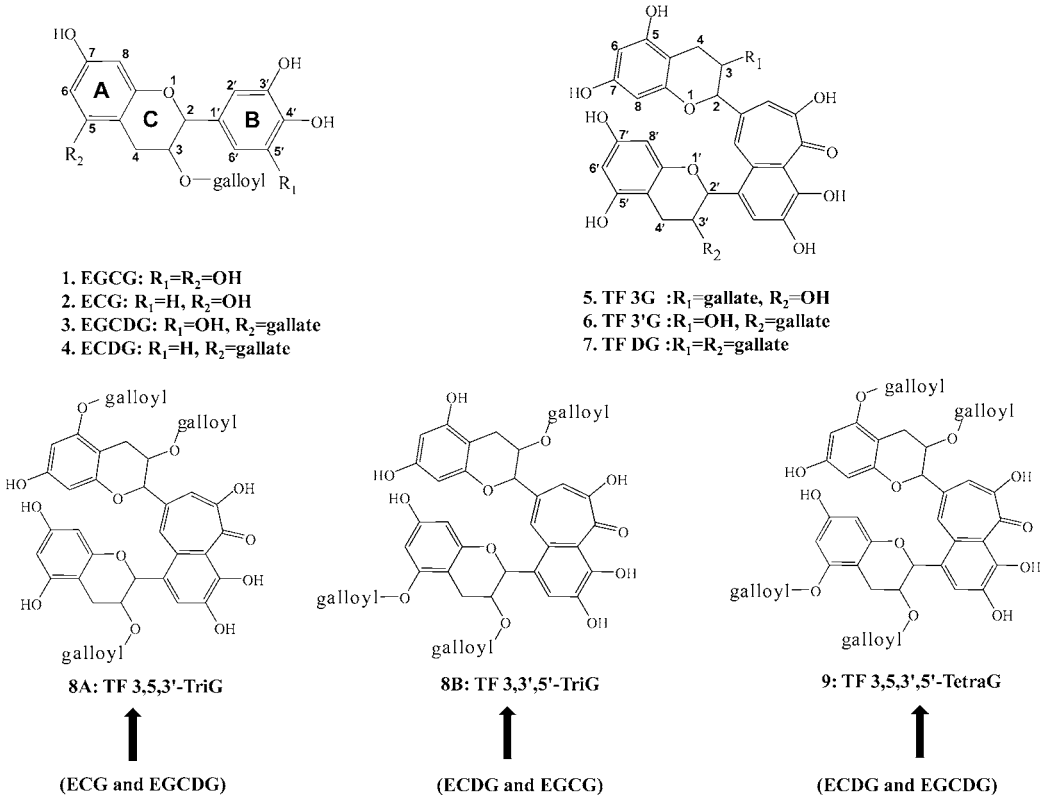

Figure 1. Chemical structures of the major tea catechins (**1**–**4**) and theaflavins (**5**–**7**) as well as theaflavin trigallate (TF-TriG, **8**) and theaflavin tetragallate (TF-TetraG, **9**).

reported that TFs (0.1% in drinking fluid) significantly reduced tumor multiplicity and volume by 23% and 34%, respectively, in 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorigenesis in A/J mice.[14] TRs were shown to decrease tumor volume and multiplicity in a 1,2-dimethylhydrazine (DMH)-induced colorectal carcinogenesis model in Sprague–Dawley rats.[15] Considering these reported anticarcinogenic effects, mechanisms for black tea bioactivity are of interest. Black tea polyphenols are thought to target the initiation and/or promotion stages of carcinogenesis by acting as suppressors and/or blockers.[16] From these studies, it is apparent that black tea polyphenols process anticarcinogenic properties and could be utilized in cancer prevention. Therefore chemical eludication of TRs is crucial to understanding black tea bioactivity.

In addition to the four most common catechins, flavanol digallates are also present in green tea, these being (−)-epigallocatechin-3,5-digallate (EGCDG, **3**) and (−)-epicatechin-3,5-digallate (ECDG, **4**) (Figure 1).[17,18] We hypothesize that EGCDG and ECDG can react with other catechins to produce TFs containing three or four galloyl groups. In the current study, the presence of theaflavin trigallate and theaflavin 3,3′,5,5′-tetragallate in black tea extract was investigated by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). The structures of theaflavin trigallate and tetragallate were confirmed by analyzing the MS^n (n = 1–4) spectra, along with comparing the MS/MS spectra of the product ions to the MS/MS spectra of authentic EGCG, ECG, and TFDG.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. EGCG, ECG, TF3G, TF3′G, and TFDG were prepared previously in our laboratory.[8] Lipton black tea bags and Yunnan black tea were purchased from local supermarkets. LC/MS-grade solvents and other reagents were obtained from Thermo Fisher Scientific (Pittsburgh, PA, USA).

Preparation of Black Tea Extract. One bag of Lipton black tea (1.9 g of black tea) was extracted with boiling water (120 mL) for five minutes. The extract was centrifuged at 17 × 1000 rpm for 10 min, the supernatants were transferred into vials for LC/MS analysis.

The extract of Yunnan black tea was prepared according to our previous method. In brief, Yunnan black tea (908 g) was extracted with 80% acetone (five times) at room temperature for two weeks. The extract was concentrated to dryness under reduced pressure, and the residue was dissolved in water and partitioned with chloroform, ethyl acetate, and *n*-butanol, respectively. The ethyl acetate fraction was subjected to Sephadex LH-20 eluted by acetone/water solvent system (30%-60%) to give 14 subfractions.

LC/ESI-MS Method. LC/MS analysis was carried out with a Thermo-Finnigan Spectra System which consisted of an Accela high-speed pump, an Accela refrigerated autosampler, and an LTQ Velos ion trap mass detector (Thermo Electron, San Jose, CA) incorporated with heated electrospray ionization (H-ESI) interfaces. A Luna C18 column (250 × 4.6 mm i.d., 5 μm; Phenomenex, Torrance, CA) was used at a flow rate of 0.8 mL/min. The column was eluted from 99% solvent A (H_2O with 0.1% formic acid), followed by linear increases in B (acetonitrile with 0.1% formic acid) to 100% in 35 min and then with 100% B from 35 to 40 min. The column was re-equilibrated with 100% A for 10 min. The LC eluent was introduced into the H-ESI interface. The negative ion polarity mode was set for the H-ESI source with the voltage on the H-ESI interface maintained at approximately 4 kV. Nitrogen gas was used as the sheath gas and auxiliary gas. To detect the components of black tea, optimized source parameters, including ESI capillary temperature (300 °C), capillary voltage (−50 V), ion spray voltage (3.6 kV), sheath gas flow rate (60 units), auxiliary

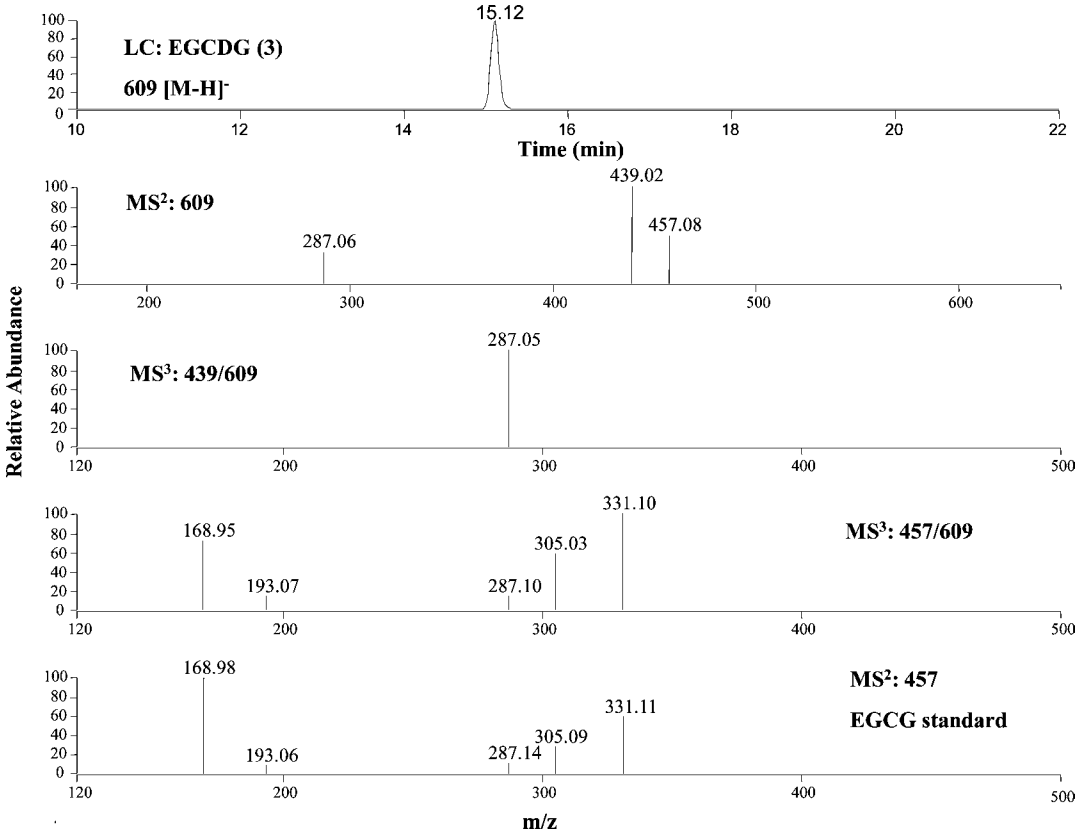

Figure 2. Extracted ion chromatogram (EIC) and ESI-MS^2 and MS^3 spectra of EGCDG and the ESI-MS^2 spectrum of authentic EGCG.

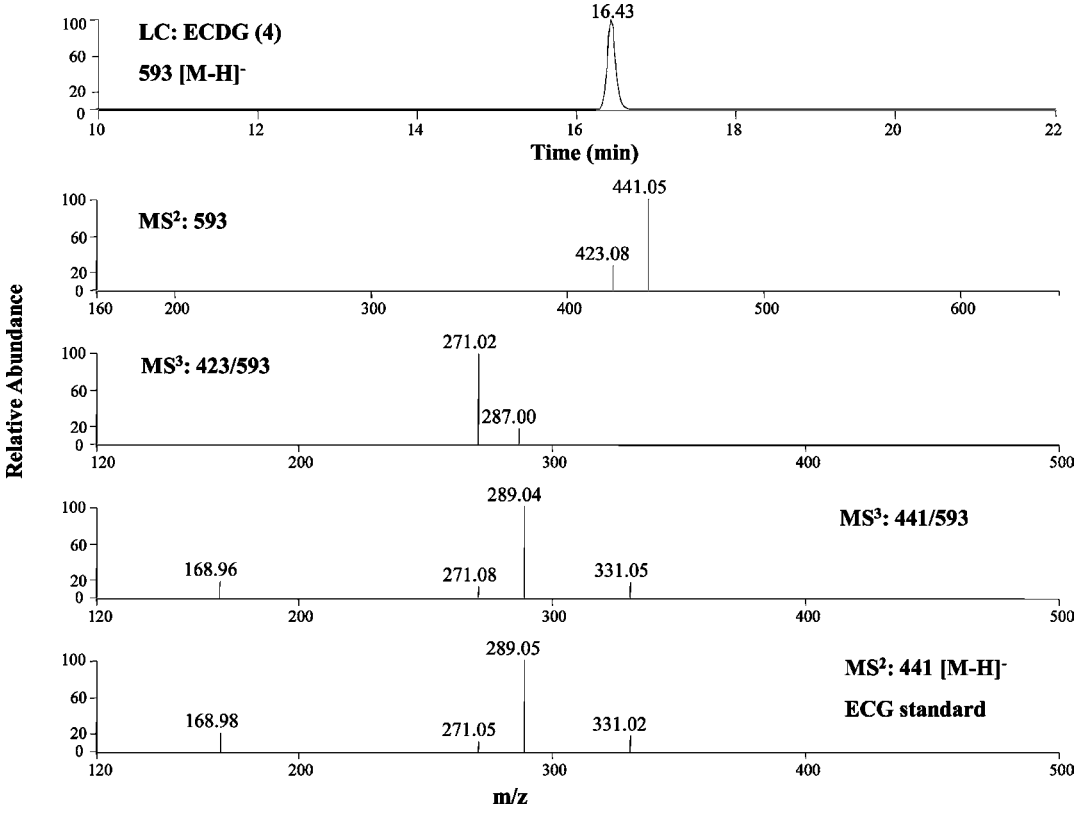

Figure 3. Extracted ion chromatogram (EIC) and ESI-MS^2 and MS^3 spectra of ECDG and the ESI-MS^2 spectrum of authentic ECG.

gas flow rate (25 units), and tube lens (−120 V), were tuned using authentic TFDG. The collision-induced dissociation (CID) for H-ESI was conducted with an isolation width of 2 Da and normalized collision energy of 35 for MS^2, MS^3, and MS^4. Default automated gain

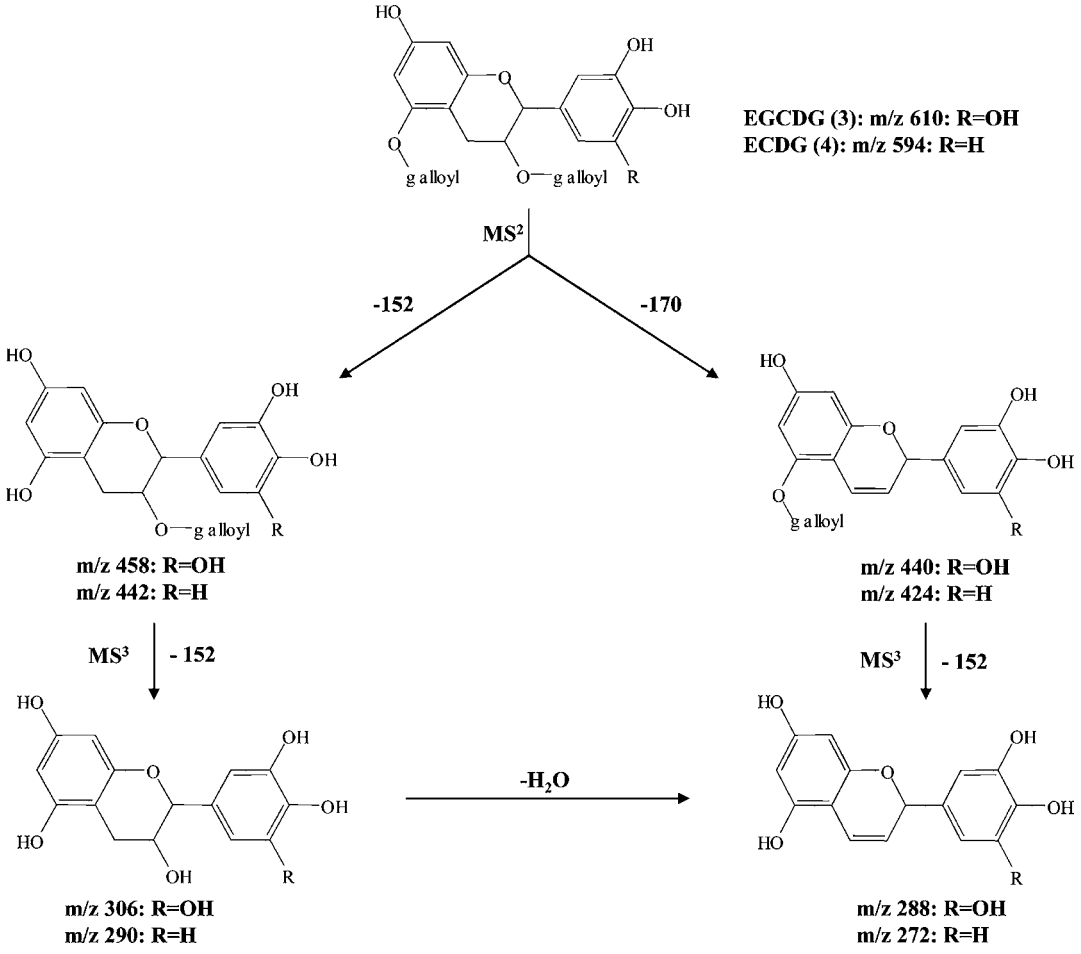

Figure 4. The potential fragment pathways of EGCDG (**3**) and ECDG (**4**).

control target ion values were used for MS, MS^2, MS^3, and MS^4 analyses. The mass range was from 50 to 1500 *m/z*. The mass resolution was 0.6 amu fwhm. Data acquisition was performed with Xcalibur version 2.1.0 (Thermo Electron, San Jose, CA).

RESULTS AND DISCUSSION

The fermentation of tea leaves induces enzymatic oxidation of flavan-3-ols and leads to the formation of two major pigments in black tea, TFs and TRs. The structures of TFs were formed by co-oxidation of selected pairs of catechins, one with a *vic*-trihydroxyphenyl moiety, and the other with an *ortho*-dihydroxyphenyl structure. For example, TF, TF3G (**5**), TF3′G (**6**), TFDG (**7**) (Figure 1) are formed by oxidative coupling of the EC and EGC, ECG and EGC, EC and EGCG, and ECG and EGCG, respectively. Since ECDG and EGCDG were found in green tea, which lead us to hypothesize that they can react with other catechins to produce higher molecular weight TFs containing three or four galloyl groups during the fermentation of tea leaves. Based on this hypothesis, we searched the existence of EGCDG (*m/z* 610), ECDG (*m/z* 594), TF 3,5,3′TriG (EGCDG and ECG, *m/z* 1020), TF 3,3′,5′TriG (EGCG and ECDG, *m/z* 1020), and TF 3,5,3′,5′TetraG (ECDG and EGCDG, *m/z* 1172) and identified EGCDG, ECDG, TF 3,5,3′TriG or TF 3,3′,5′TriG, and TF 3,5,3′,5′TetraG in two different black tea samples, the Lipton black tea and the Yunnan black tea (subfractions 13 and 14).

Identification of EGCDG and ECDG in Black Tea. In the extracted chromatogram of *m/z* 609 $[M\text{-}H]^-$ (molecular ion of EGCDG under ESI negative mode), one peak (**3**, RT: 15.12 min) was found in the extract of black tea. **3** showed 152 mass units higher than that of EGCG, indicating that there is one more galloyl group in **3** than in EGCG. This peak showed tandem mass spectrum with *m/z* 457 (molecular ion of EGCG under negative mode) and 439 (molecular ion of dehydrated EGCG under negative mode) as the major product ions (Figure 2). The tandem mass spectrum of product ion 457 (MS^3: *m/z* 457/609) of **3** was almost identical to the MS^2 spectrum of authentic EGCG (MS^2: *m/z* 457) (Figure 2), suggesting that **3** was the monogallated EGCG. The structure of **3** was then shown in Figure 1.

Similarly, one peak (**4**, RT: 16.43 min) was observed at *m/z* 593 (molecular ion of ECDG under negative mode), which was 152 mass units higher than that of ECG, indicating that there is one more galloyl group in **4** than in ECG. We then compared the tandem mass spectrum of its product ion *m/z* 441 (MS^3: *m/z* 441/593) with that of authentic ECG (MS^2: *m/z* 441). Our results indicated that they had almost identical mass fragments (Figures 3). Hence, we established **4** as ECDG (Figure 1). The major fragment pathways of **3** and **4** were proposed in Figure 4.

Identification of TF Trigallate. In the extracted chromatogram of *m/z* 1019 $[M\text{-}H]^-$ (molecular ion of TF trigallate under ESI negative mode), one peak (**8**, RT: 18.09 min) was found in black tea extract. This peak showed tandem mass

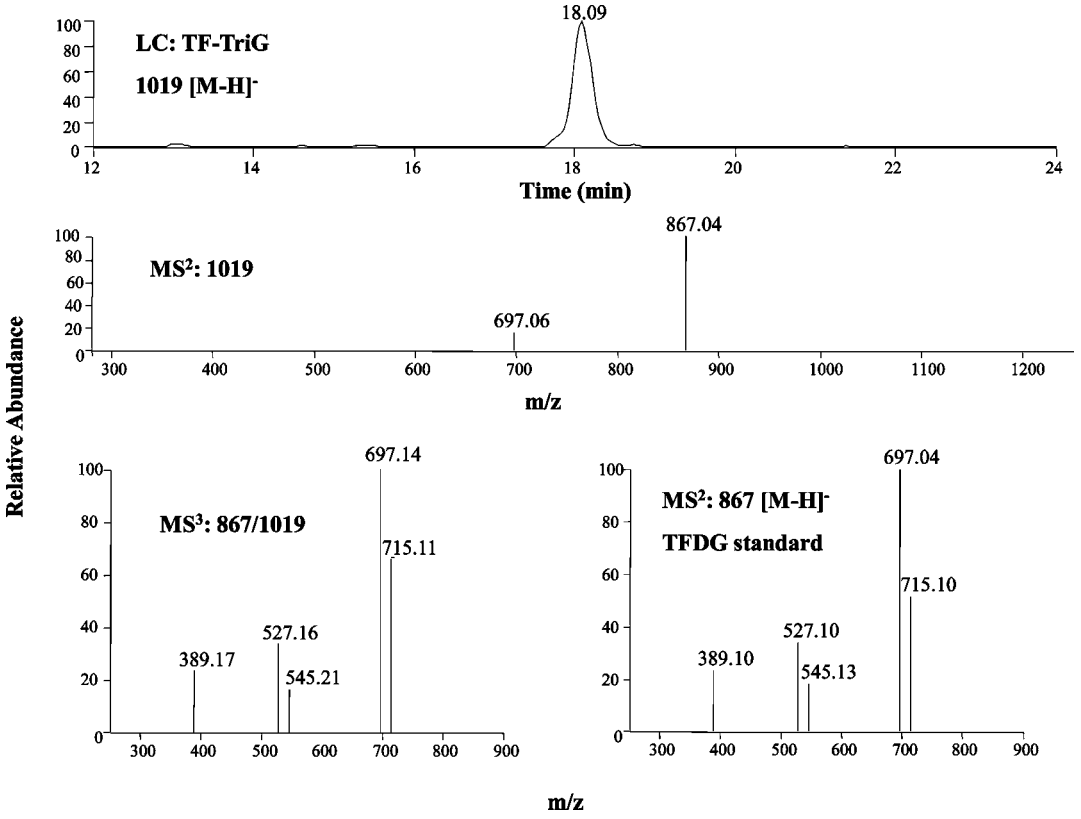

Figure 5. Extracted ion chromatogram (EIC) and ESI-MS^2 and MS^3 spectra of theaflavin trigallate (TF-TriG) and the ESI-MS^2 spectrum of authentic theaflavin digallate (TFDG).

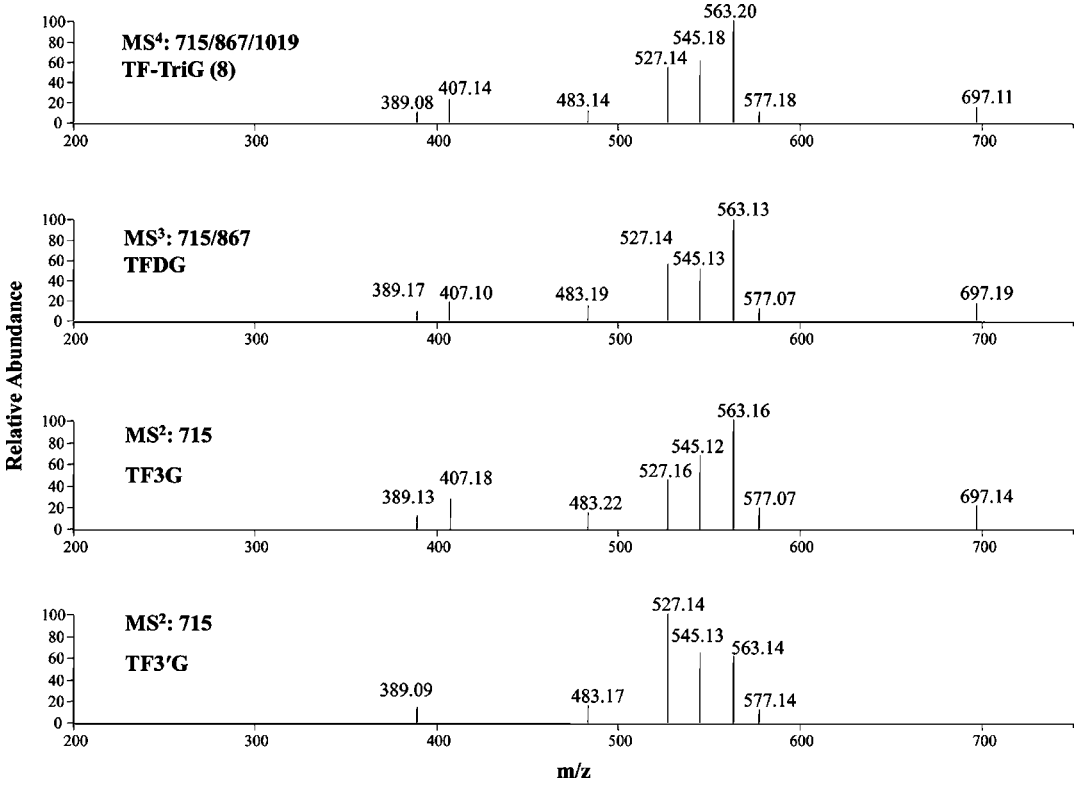

Figure 6. The MS^4 spectrum of the product ion m/z 715 of TF-TriG, the MS^3 spectrum of the product ion m/z 715 of authentic TFDG, and the MS^2 spectra of authentic TF3G and TF3′G.

spectrum with m/z 867 (molecular ion of TFDG under negative mode) and 697 as the major product ions, indicating that **8** has one more galloyl group than TFDG (molecular weight: 868) (Figure 5). The tandem mass spectrum of product

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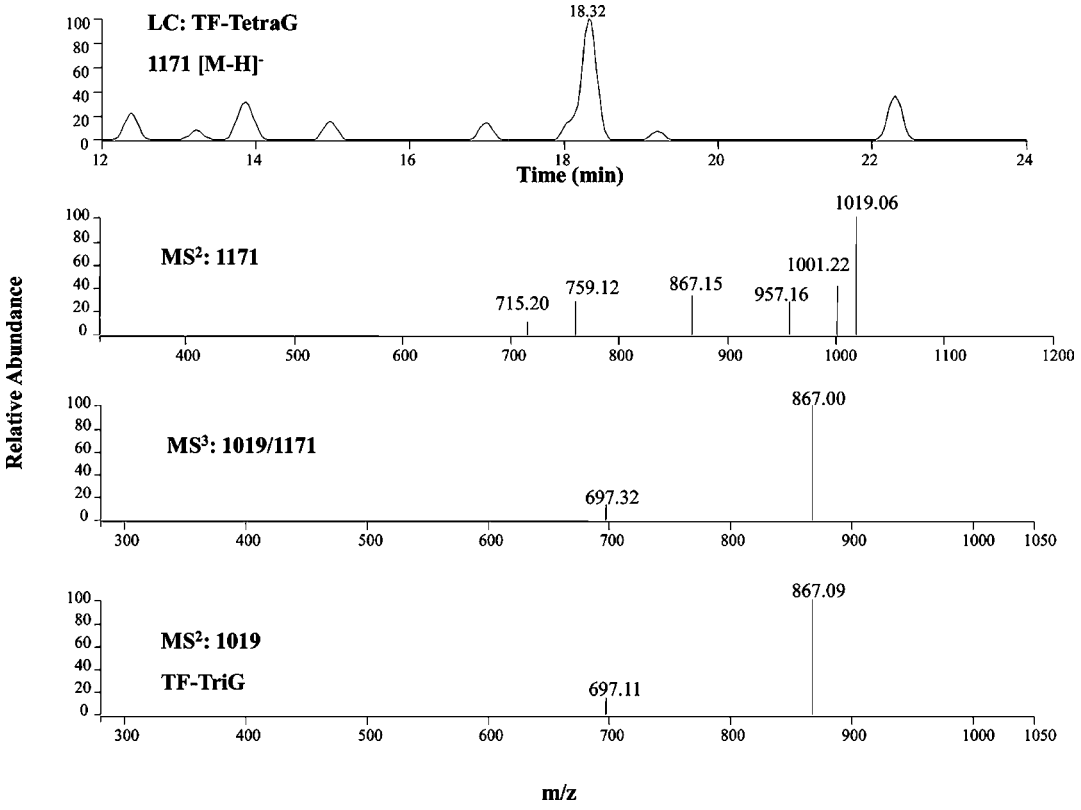

Figure 7. Extracted ion chromatogram (EIC) and ESI-MS^2 and MS^3 spectra of theaflavin tetragallate (TF-TetraG) and the ESI-MS^2 spectrum of theaflavin trigallate (TF-TriG).

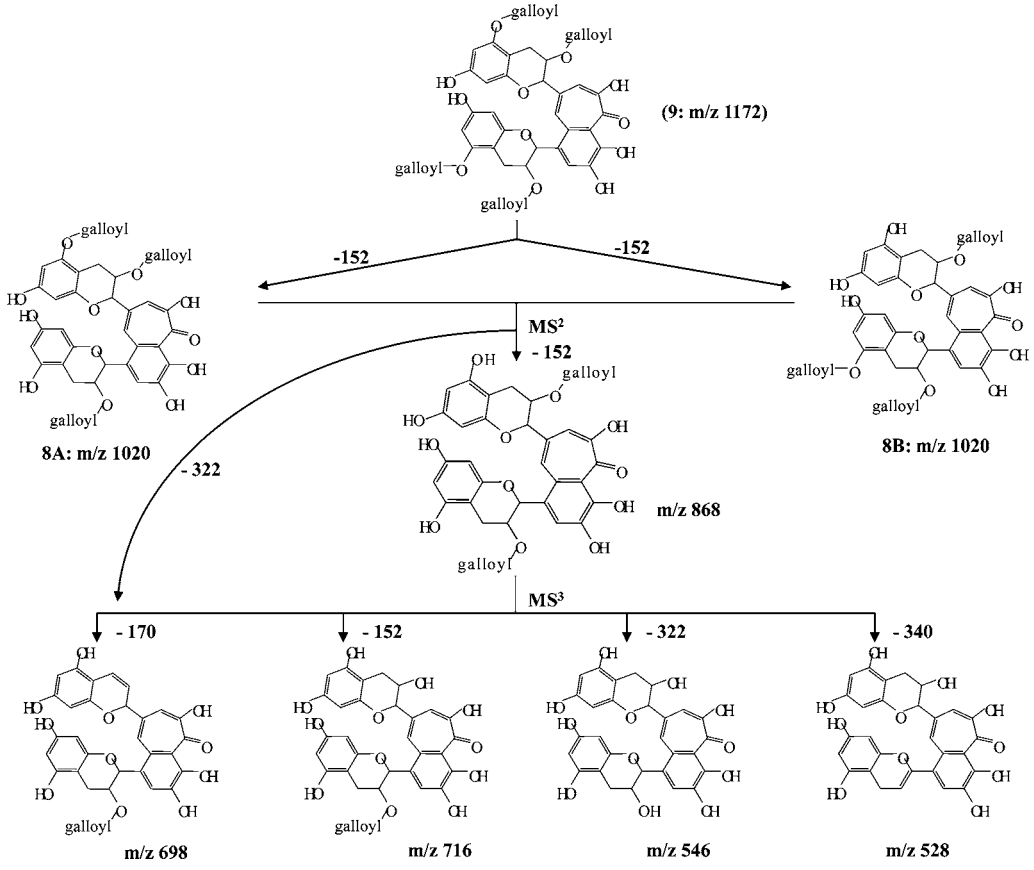

Figure 8. The potential fragment pathways of theaflavin tetragallate (TF-TetraG, **9**) and theaflavin trigallate (TF-TriG, **8**).

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ion 867 (MS^3: m/z 867/1019) of **8** was almost identical to the MS^2 spectrum of authentic TFDG (MS^2: m/z 867) (Figure 5), suggesting that **8** was the monogallated TFDG. The MS^4 spectrum of the product ion m/z 715 (MS^4: 715/867/1019) of **8** showed major product ions at m/z 527, 545, and 563 which was almost identical to the MS^3 spectrum of the product ion m/z 715 (MS^3: 715/867) of TFDG and the MS^2 spectrum of TF3G or TF3′G (MS^2: 715) (Figure 6), further confirming our elucidation of **8**. Based on the formation mechanism of the benzotropolone structure of TFs, we should be able to detect two theaflavin trigallates, TF 3,5,3′-TriG (**8A**) and TF 3,3′,5′-TriG (**8B**), which are generated by the oxidative coupling of ECG and EGCDG and of ECDG and EGCG, respectively. However, in the current study we only detected one peak in response to the molecular ion of TF-TriG. It is possible that this peak is a mixture of the two TF-TriGs that we are unable to separate them or one of them is the minor component that we cannot detect it. To further confirm the structure of **8**, authentic standards of TF 3,5,3′-TriG and TF 3,3′,5′-TriG are required. Therefore, we tentatively identify **8** as TF 3,5,3′-TriG or TF 3,3′,5′-TriG.

Identification of TF 3,5,3′,5′-TetraG. We observed one peak (peaks **9**, 18.32 min) in the LC chromatogram obtained from negative ESI-MS detection with the molecular ion m/z 1171 $[M - H]^-$ (1019 + 152), indicating that this peak had one more galloyl group than **8**. Peak **9** showed tandem mass spectrum (Figure 7) with m/z 1019 ($[M\text{-}152\text{-}H]^-$, molecular ion of **8** under negative mode) and m/z 867 ($[M\text{-}152\text{−}152\text{-}H]^-$, molecular ion of TFDG under negative mode) as the major product ions, indicating that **9** was theaflavin tetragallate. In order to corroborate this proposition, we compared the tandem mass spectrum of the product ion m/z 1019 (MS^3: m/z 1019/1171) of **9** with the MS^2 spectrum of **8** (MS^2: m/z 1019) (Figure 7). Our results clearly indicated that they had almost identical mass fragments, suggesting that **9** was the theaflavin tetragallate. In theory, TF tetragallate can only be generated by the oxidative coupling of the two catechin digallate, ECDG and EGCDG. Therefore, peak **9** was identified as TF 3,5,3′,5′-TetraG as shown in Figure 1. According to the tandem mass spectra of **8** and **9**, a detailed fragmentation pathway was proposed in Figure 8.

Catechin digallates, EGCDG and ECDG, have been reported in green tea by different research groups. However, this is the first time to report them in black tea. More importantly, we identified two novel black tea polyphenols, theaflavin trigallate (**8**) and tetragallate (**9**), by using liquid chromatography/electrospray ionization tandem mass spectrometry. Theaflavin trigallate (**8**) was formed through oxidative coupling of EGCDG (**3**) and ECG (**2**) or EGCG (**1**) and ECDG (**4**). Theaflavin tetragallate (**9**) was formed by oxidative coupling of EGCDG (**3**) and ECDG (**4**). All four compounds were identified in two different black tea samples, Lipton black tea and Yunnan black tea.

Our previous studies have found that the galloyl ester group of TF3G is as reactive as the B-ring (*vic*-trihydroxy) of EGCG or EGC and the galloyl ester group of ECG, and can further react with EC to form the new theaflavin type tea catechin trimer, theadibenzotropolone A.[8] In addition, we also observed that the two galloyl ester groups of TFDG can react with EC to generate the new theaflavin type tea catechin tetramer, theatribenzotropolone A.[19] The observation that the galloyl ester group of TFs can be oxidized to form di- or tribenzotropolone skeletons strongly implies that this type of oxidation is an important pathway to extend the molecular size of TRs. It is worthwhile to study whether the galloyl ester groups of theaflavin trigallate and tetragallate can be oxidized and react with EC to form tetra- and penta-benzotropolone skeletons, which will facilitate our understanding of the formation of TRs derived from the oxidation of TFs.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Ross, I. A. *Medicinal Plants of the World: Chemical Constituents, Traditional and Modern Medicinal Uses*; Humana Press Inc: Totowa, NJ, 2005; pp 1−27.

(2) Gallenkemper, G. About the effect of (black) tea compresses on the skin - Application study of inflammatory lesions on the legs in particular in connection with venous blood flow disturbance and literature review. *Phlebologie* **2009**, *38*, 276−294.

(3) Naito, Y.; Yoshikawa, T. Green tea and heart health. *J. Cardiovasc. Pharmacol.* **2009**, *54*, 385−90.

(4) Gardner, E. J.; Ruxton, C. H.; Leeds, A. R. Black tea—Helpful or harmful? A review of the evidence. *Eur J Clin Nutr* **2007**, *61*, 3−18.

(5) Yang, C. S.; Wang, H.; Li, G. X.; Yang, Z.; Guan, F.; Jin, H. Cancer prevention by tea: Evidence from laboratory studies. *Pharmacol. Res.* **2011**, *64*, 113−22.

(6) Kumar, G.; Pillare, S. P.; Maru, G. B. Black tea polyphenols-mediated in vivo cellular responses during carcinogenesis. *Mini-Rev. Med. Chem.* **2010**, *10*, 492−505.

(7) Sang, S.; Lambert, J. D.; Ho, C. T.; Yang, C. S., The chemistry and biotransformation of tea constituents. *Pharmacol. Res.* **2011**.

(8) Sang, S.; Lambert, J. D.; Tian, S.; Hong, J.; Hou, Z.; Ryu, J. H.; Stark, R. E.; Rosen, R. T.; Huang, M. T.; Yang, C. S.; Ho, C. T. Enzymatic synthesis of tea theaflavin derivatives and their anti-inflammatory and cytotoxic activities. *Bioorg. Med. Chem.* **2004**, *12*, 459−67.

(9) Haslam, E. Thoughts on thearubigins. *Phytochemistry* **2003**, *64*, 61−73.

(10) Kuhnert, N.; Drynan, J. W.; Obuchowicz, J.; Clifford, M. N.; Witt, M. Mass spectrometric characterization of black tea thearubigins leading to an oxidative cascade hypothesis for thearubigin formation. *Rapid Commun. Mass Spectrom.: RCM* **2010**, *24*, 3387−404.

(11) Ozawa, T.; Kataoka, M.; Morikawa, K.; Negishi, O. Elucidation of the partial structure of polymeric thearubigins from black tea by chemical degradation. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 2023−2027.

(12) Kuhnert, N. Unraveling the structure of the black tea thearubigins. *Arch. Biochem. Biophys.* **2010**, *501*, 37−51.

(13) Lorenz, M.; Urban, J.; Engelhardt, U.; Baumann, G.; Stangl, K.; Stangl, V. Green and black tea are equally potent stimuli of NO production and vasodilation: New insights into tea ingredients involved. *Basic Res Cardiol* **2009**, *104*, 100−110.

(14) Yang, G. Y.; Liu, Z.; Seril, D. N.; Liao, J.; Ding, W.; Kim, S.; Bondoc, F.; Yang, C. S. Black tea constituents, theaflavins, inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorigenesis in A/J mice. *Carcinogenesis* **1997**, *18*, 2361−5.

(15) Patel, R.; Ingle, A.; Maru, G. B. Polymeric black tea polyphenols inhibit 1,2-dimethylhydrazine induced colorectal carcinogenesis by inhibiting cell proliferation via Wnt/beta-catenin pathway. *Toxicol. Appl. Pharmacol.* **2008**, *227*, 136−46.

(16) Kumar, G.; Pillare, S. P.; Maru, G. B. Black tea polyphenols-mediated in vivo cellular responses during carcinogenesis. *Mini-Rev. Med. Chem.* **2010**, *10*, 492−505.
(17) Coxon, D. T.; Vora, V. C.; Tee, J. L.; Grant, M. S.; Ollis, W. D.; Holmes, A. Flavanol digallates in green tea leaf. *Tetrahedron* **1972**, *28*, 2819−2826.
(18) Lihu, Y.; Yueming, J.; Nivedita, D.; Riantong, S.; Xu, L.; Jun, D.; Katherine, R.; Alan, L.; Ying, X. HPLC analyses of flavanols and phenolic acids in the fresh young shoots of tea (*Camellia sinensis*) grown in Australia. *Food Chem.* **2004**, *84*, 253−263.
(19) Sang, S.; Tian, S.; Stark, R. E.; Yang, C. S.; Ho, C. T. New dibenzotropolone derivatives characterized from black tea using LC/MS/MS. *Bioorg. Med. Chem.* **2004**, *12*, 3009−17.