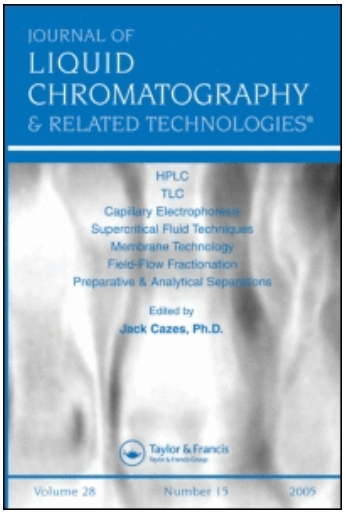

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

ISOLATION AND PURIFICATION OF FOUR INDIVIDUAL THEAFLAVINS USING SEMI-PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Yi Xu[a]; Yuxia Jin[a]; Yuanyuan Wu[a]; Youying Tu[ab]
[a] Department of Tea Science, The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Zhejiang University, Hangzhou, China [b] Easily Biotechnology Limited Company, Hangzhou, China

Online publication date: 04 December 2010

To cite this Article Xu, Yi , Jin, Yuxia , Wu, Yuanyuan and Tu, Youying(2010) 'ISOLATION AND PURIFICATION OF FOUR INDIVIDUAL THEAFLAVINS USING SEMI-PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY', Journal of Liquid Chromatography & Related Technologies, 33: 20, 1791 — 1801
To link to this Article: DOI: 10.1080/10826076.2010.526865
URL: http://dx.doi.org/10.1080/10826076.2010.526865

Journal of Liquid Chromatography & Related Technologies, 33:1791–1801, 2010

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826076.2010.526865

Taylor & Francis
Taylor & Francis Group

ISOLATION AND PURIFICATION OF FOUR INDIVIDUAL THEAFLAVINS USING SEMI-PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Yi Xu,[1] Yuxia Jin,[1] Yuanyuan Wu,[1] and Youying Tu[1,2]

[1]*Department of Tea Science, The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Zhejiang University, Hangzhou, China*
[2]*Easily Biotechnology Limited Company, Hangzhou, China*

□ *Theaflavin (TF_1), theaflavin-3-gallate (TF_2A), theaflavin-3′-gallate (TF_2B), and theaflavin-3,3′-digallate (TF_3) are the major theaflavins, which mostly contribute to the quality and bioactivity of black tea. In this study, a rapid isolation and purification of the four major individual theaflavins from crude theaflavins mixture was established. The crude theaflavins mixture was prepared by enzymatic oxidation of tea polyphenols using immobilized polyphenol oxidase and then fractionated using a Mitsubishi SP-207 resin chromatography with an elution gradient of 20%, 30%, 40%, 50%, and 70% aqueous ethanol to obtain a mixture of theaflavins with 80% purity (TF80). The TF80 was further purified using a semi-preparative high performance liquid chromatography (HPLC) equipped with a C18 column using isocratic elution with water/acetonitrile/glacial acetic acid (73.5:26:0.5, v/v/v) at a flow rate of 5 mL/min as optimized operating conditions. The purity of the isolated individual theaflavins were 92.48% for TF_2A, 90.05% for TF_2B, 92.40% for TF_3, and 73.02% for TF_1, respectively.*

Keywords macroporous resin, preparative chromatography, theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, theaflavin-3,3′-digallate

INTRODUCTION

It is well known that green tea and its polyphenols (catechins) possess potent protective effects in cancer and cardiovascular disease development.[1,2] Recent studies suggest that black tea (a fermented tea), which accounts for almost 80% of the world tea production, also has some beneficial health properties, including antioxidative effects,[3–5] inhibition

Address correspondence to Youying Tu, Department of Tea Science, The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Zhejiang University, Hangzhou 310029, China. E-mail: youytu@zju.edu.cn

of carcinogenesis,[6,7] suppression of lipid peroxidation,[8] and protection against cardiovascular disease.[9] These beneficial effects are believed to be mainly due to antioxidant activities of theaflavins present in black tea, although theaflavins only account for 2–6% of the dry weight of solids in brewed black tea.[10]

Theaflavins contribute significantly to the color and "mouthfeel" characteristics of black tea infusions. The major theaflavins in black tea are TF_1, TF_2A, TF_2B, and TF_3 (Figure 1). All of the four individual theaflavins have one benzotropolone skeleton and two A-rings of flavonols linked by a fused seven-member ring.[11,12] These structural features may be responsible for strong antioxidant activities of theaflavins, for example, inhibition of LDL oxidation in mouse macrophage cells[13] and prevention for DNA oxidative damage in cell-free systems.[14] Theaflavins, especially TF_3, were more effective than a well-known antioxidant EGCG in suppression of intracellular reactive oxygen species in HL-60 cells[15] and protection against H_2O_2-mediated oxidative damage in HPF-1 cells.[4] The antioxidant mechanisms of theaflavins, like the main active sites of antioxidant action are still unclear, although theaflavins have shown strong antioxidant activities. This is because major individual theaflavins (TF_1, TF_2A, TF_2B, and TF_3) with high purities are not commercially available. Additionally, extraction of theaflavins from black tea requires a series of purification steps. As a result, a very limited yield of theaflavins can be obtained because of their low contents in black tea.

In our previous studies,[16,17] a model oxidation system using immobiled enzymes was developed to gain a higher yield of crude theaflavin mixture that was easier for further separation. In the present study, we

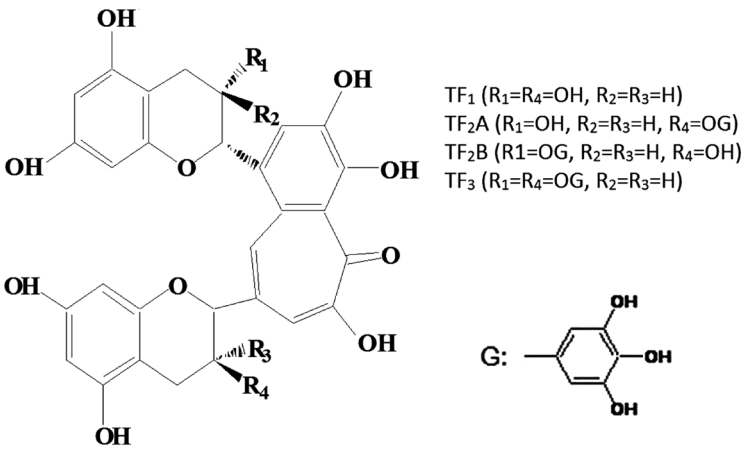

FIGURE 1 Chemical structures of the four main individual theaflavins.

developed a rapid approach to isolate and purify the four individual theaflavins (TF_1, TF_2A, TF_2B, and TF_3) from the crude theaflavins mixture by Mitsubishi SP-207 resin chromatography and semi-preparative high performance liquid chromatography. The purified TF_1, TF_2A, TF_2B, and TF_3 were identified by HPLC-UV and LC-MS.

EXPERIMENTAL

Reagents

Ethyl acetate, ethanol, acetic acid, citric acid, sodium alginate, glutaraldehyde, and $CaCl_2$ were of analytical grade and purchased from Zhejiang Medical and Chemical Company (Zhejiang, China). Acetonitrile and methanol for HPLC analysis or preparation were of HPLC purity and purchased from Tianjing Siyou Ltd. Company (Tianjing, China).

Preparation of TF50 and TF80

The TF50, containing catechins (mainly 7.03% EC, 8.80% EGCG, and 10.98% ECG), TF_1, TF_2A, TF_2B, and TF_3 (total theaflavins account for about 50%), was prepared according to our patent.[16] In brief, 75 mL PPO (polyphenol oxidase) solution of 1500 U mixed with 100 ml 2% sodium alginate solution was entrapped for 5 min, then the enzyme mixture was injected into 1000 mL 0.1 M $CaCl_2$ solution by injector. After shaping for 30 min, the particles were taken out and kept in 0.025% glutaraldehyde aqueous for 1 hr; an insoluble aggregate between PPO and cross-linking reagent was formed. These particles were conserved into the citrate buffer of pH 5.6 at 4°C. The operation of the model tea polyphenol oxidation system was the same as described by Tu et al.,[17] except that the reaction was carried out at at a defined pH 5.6 at 37°C and terminated after 30 min. The reaction solution was extracted by ethyl acetate and then put to vacuum dry to gain the crude theaflavins (TF50), which were analyzed by HPLC-UV.

The TF50 was then separated on a column (Φ5.5 cm × 100 cm) filled with Mitsubishi SP-207 resin by stepwise elution with ethanol and water to obtain a highly purified theaflavins mixture. An amount of 35 g TF50 was first loaded, and then stepwise elution was applied with 2 L of each at a flow rate of 33 ml/min: water – 20% aqueous ethanol – 30% aqueous ethanol – 40% aqueous ethanol – 50% aqueous ethanol – 70% aqueous ethanol. Four fractions were collected from the ethanol eluate. After removal of the solvent using a rotary evaporator at 40°C, all the fractions were extracted by ethyl acetate, dried with freeze-drying, and then analyzed by HPLC-UV. Finally, we gained 16 g theaflavins mixture with 80% purity (TF80).

Semi-Preparative HPLC System

Individual theaflavins were isolated from TF80 by a native preparative HPLC system (Dalian Elite Analysis Equipment Company, China) consisting of an Elite model P270 pump, an Elite Rheodyne 3725i-038 system controller, a manual injector fitted with 1 ml sample loop, and an Elite model UV230+ detector equipped with a preparative flow cell. A C18 preparative column (250 mm × 10 mm, i.d. 10 μm) (Elite, Dalian, China) was used for the separation and isolation of theaflavins. The mobile phase was distilled water/acetonitrile/glacial acetic acid (73.5:26:0.5, v/v/v), the isocratic flow rate was 5 mL/min, and the detector was set at 280 nm. Samples of 30 mg TF80 were injected and four fractions were collected separately and evaporated to solvent-free using a rotary evaporator at 40°C, then put to freeze-drying. Each of the fraction solids was dissolved with methanol for LC-MS analysis.

LC-MS Analysis

The LC-MS was measured with Agilent 1100 LC/MSD SL (Agilent Inc., USA) equipped with an atmospheric pressure chemical ionization (ApCI) interface. The LC/MS was performed on a Shimadzu VP-DOS C18 column (250 mm × 4.6 mm i.d. 5 μm) (Shimadzu, Kyoto, Japan), the flow rate was 1 ml/min, and the mobile phase was distilled water/acetonitrile/acetic acid (76:23.5:0.5, v/v/v). The effluent from the LC column was delivered to the ion source (150°C) through a heated nebulizer probe (400°C) using nitrogen as the drying gas (5 L/min, 350°C) and nebulizer pressure was set to 60 psi. The mass spectrometer was scanned from m/z 50 to 1000 in full scan mode.

HPLC Analysis of Catechins and Theaflavins

The HPLC analysis of catechins and theaflavins was performed in a Shimadzu LC-20A high-pressure liquid chromatography (Shimadzu, Tokyo, Japan) equipped with a VP-DOS C18 reversed-phase column (250 mm × 4.6 mm i.d. 5 μm) (Shimadzu, Tokyo, Japan). A total of 10 μl of the sample solutions were analyzed using gradient elution with the solvent A [distilled water/acetonitrile/acetic acid (96.5:3:0.5, v/v/v)] and the solvent B [distilled water/acetonitrile/acetic acid (69.5:30:0.5, v/v/v)], at a flow rate of 1 ml/min at 28°C. The elution was performed using a linear gradient from 0% solvent B to77% solvent B in 35 min and sustaining 77% solvent B until 85 min, then back to initial conditions in 5 min. The UV spectra were recorded at 280 nm.

RESULTS AND DISCUSSION

Preparation of TF80

Our present experimental results indicated a simple and economic macroporous resin column chromatographic method to produce a highly purified theaflavins mixture (TF80). The total theaflavins content of TF80 reached 83.84%, including 10.24% TF_1, 15.08% TF_2A, 12.55% TF_2B, and 45.97% TF_3 according to the HPLC-UV analysis. There were less catechins in TF80 compared to TF50 which contained 29% catechins. The HPLC profiles of catechins standard (Figure 2a), TF50 (Figure 2b), and TF80 (Figure 2c) were illustrated. The other three fractions were identified as mainly caffeine, catechins without galloyl (EC, C, EGC, GC), and catechins with galloyl (ECG, CG, EGCG, GCG), respectively. Caffeine could be well removed after 20% aqueous ethanol elution. Our further study indicated 20% aqueous ethanol eluent with 5% acetic acid was much better for getting rid of caffeine. The decaffeinated and low-caffeine tea products are in an increased demand by consumers since caffeine is confirmed to exert adverse effects, including palpitations, gastrointestinal disturbances, anxiety, tremor, increased blood pressure, and insomnia.[18] Most catechins without galloyl (EC, C, EGC, GC) were collected after 30% aqueous ethanol elution, while most catechins with galloyl (ECG, CG, EGCG, GCG) came out after 50% aqueous ethanol elution. Finally, purified theaflavins were obtained during 70% ethanol eluting period. It was because the theaflavins were much more hydrophobic than catechins and caffeine. The yield of TF80 was 45.7%. Our method recovered almost the entire theaflavins from TF50.

Optimum Parameters of Semi-Preparative HPLC Separation System

To obtain optimum conditions for maximum amount of loaded sample (TF 80), we had to adjust the acetonitrile content in the mobile phase as well as adjust the flow rate. When isocratic elution with distilled water/acetonitrile/acetic acid [69.5:30:0.5 (v/v/v)] were applied, the peaks of TF_2B and TF_3 overlapped. However the two peaks were well separated with reducing acetonitrile concentration to 26% (Figure 3). We then studied the effect of flow rate ranging from 2.5 mL/min to 10 mL/min every 2.5 mL intervals with the mobile phase [distilled water/acetonitrile/acetic acid (73.5:26:0.5, v/v/v)]. Higher flow rate (7.5 mL/min, 10 mL/min) resulted in less retention time of theaflavins but reduced separation between the other two individual theaflavins. At the flow rate of 5 mL/min, four individual theaflavins were obtained in 45 min. Finally, 30 mg was determined

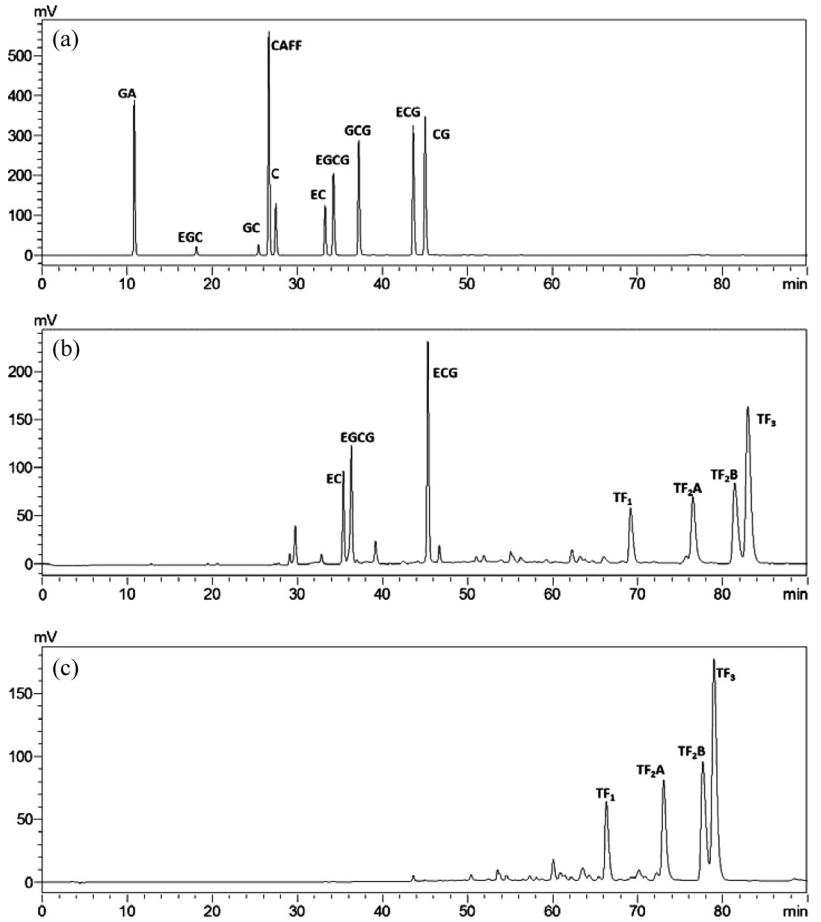

FIGURE 2 HPLC chromatogram of catechins, TF50 and TF80. a: catechins standard; b: TF50; c: TF80. Experimental conditions: Shimadzu VP-DOS C18 column (250 mm × 4.6 mm i.d. 5 μm); mobile phase: solvent A [distilled water/acetonitrile/acetic acid (96.5:3:0.5, v/v/v)] and solvent B [distilled water/acetonitrile/acetic acid (69.5:30:0.5, v/v/v)], a linear gradient elution from 0 to 77% solvent B in 35 min, keep 77% solvent B till 85 min, back to initial conditions in 5 min; The flow rate: 1 mL/min; detection wavelength: 280 nm; temperature: 28°C; injection volume: 10 μl.

to be the maximum loaded amount and TF80 was eluted with the mobile phase of distilled water/acetonitrile/acetic acid (73.5:26:0.5, v/v/v) at a flow rate of 5.0 mL/min.

Identification of Individual Theaflavins by LC-MS

The HPLC-UV and LC-MS chromatograms of four individual theaflavins are showed in Figure 4. The quasi-molecular ions were $[M+H]^+$ at m/z 565.1 for $TF_1(C_{29}H_{24}O_{12}=564)$, $[M+H]^+$ at m/z 717.1 for TF_2A

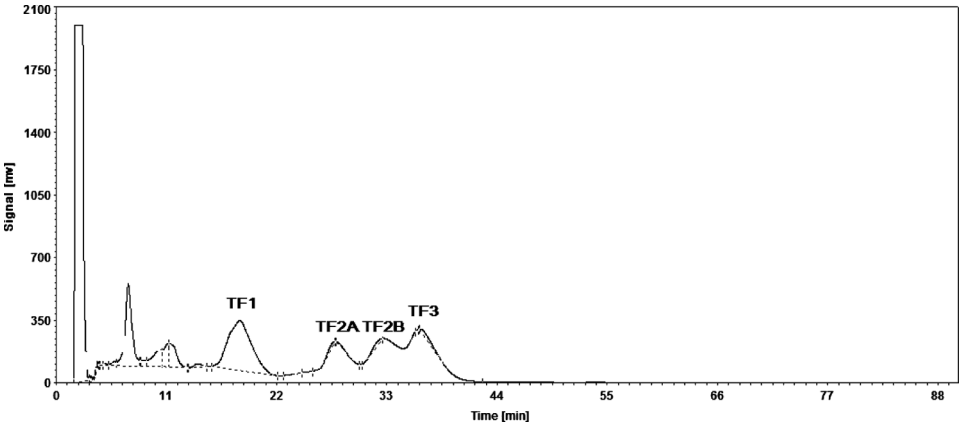

FIGURE 3 Preparative separation of the four individual theaflavins from TF80 by semi-preparative HPLC. Experimental conditions: C18 column (250 mm × 10 mm i.d., 10 μm); mobile phase: distilled water/acetonitrile/acetic acid (73.5:26:0.5, v/v/v) isocratic elution; flow rate: 5 mL/min; detection wavelength: 280 nm; loading sample amount: 30 mg TF80.

($C_{36}H_{28}O_{16} = 716$) and TF_2B ($C_{36}H_{28}O_{16} = 716$), and $[M+H]^+$ at m/z 869.1 for TF_3 ($C_{43}H_{32}O_{20} = 868$). The molecular weights were in agreement with those reported by Nonaka et al.[19] The purity was 92.48% for TF_2A, 90.05% for TF_2B, 92.40% for TF_3, and 73.02% for TF_1, respectively.

Comparison of Methods for Isolating Theaflavin Monomers

Chromatography is essential to the large-scale purification of natural products. Different types of chromatography such as Sephadex LH-20 column chromatography,[20–22] high-speed countercurrent chromatography (HSCCC),[21–26] polyamide column chromatography,[27–28] gel column chromatography,[29] and semi-preparative HPLC[30] were used for the separation of individual theaflavins (Table 1). Individual TF_1 and TF_3 could be obtained at one time by the methods illustrated. Only a mixture of TF_2A and TF_2B (TFG) was isolated using the conventional Sephadex LH-20 or silica gel column chromatography. Neither method could separate the chemical compounds with the same molecular weight. Their disadvantages also lay on repetitive tedious isolation processes as well as taking longer separation time because of a limited flow rate through the Sephadex column. For the method using the polyamide column, it also required too much time to gain four monomers though the loading sample size reached more than 1 g, yet the yields were unclear. Some papers[21–26] reported that HSCCC had a great potential for the preparative isolation of individual theaflavins from black tea extract. The HSCCC took a much shorter separation time and had a larger sample amount than the column chromatography; nevertheless,

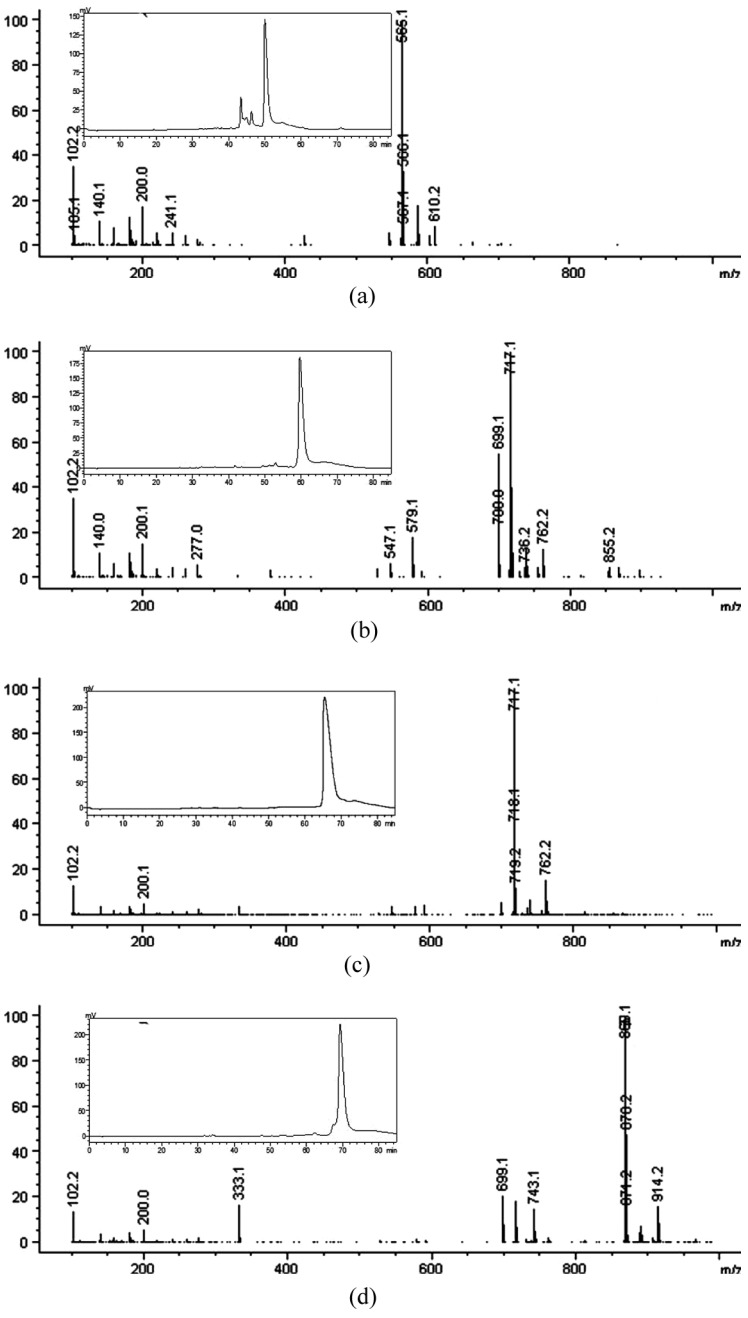

FIGURE 4 LC-MS and HPLC-UV profiles of the four individual theaflavins. a: TF_1; b: TF_2A; c: TF_2B; d: TF_3.

TABLE 1 Comparison of Methods for Isolating Individual Theaflavins Reported in Decade

Method	Cost Time	Max. Sample Loading	Individual Theaflavins Gained	Reference
Sephadex LH-20	21 h	100 mg	TF_1, TFG ($TF_2A + TF_2B$), TF_3	[21]
Sephadex LH-20	16 h	80 mg	TF_1 + ECG, TFG, TF_3	[22]
HSCCC	4 h	250 mg	TF_1, TFG, TF_3	[21]
HSCCC	3.75 h	400 mg	TF_1 + ECG, TFG, TF_3	[22]
HSCCC	6.7 h	54 mg	TF_1, TFG, TF_3	[24]
HSCCC	6.7 h	250 mg	TF_1, TF_2B, TFG, TF_3	[25]
HSCCC	8.3 h	30 mg	TF_1, TF_2A, TF_2B, TF_3	[26]
Polyamide column	36 h	250 mg	TF_1, TF_2B	[27]
Polyamide column	Several days	More than 1 g	TF_1, TF_2A, TF_2B, TF_3	[28]
Gel column and HSCCC	Several days	More than 1 g	TF_1, TF_2A, TF_2B, TF_3	[29]
Semi-preparative HPLC	0.75 h	30 mg	TF_1, TF_2A, TF_2B, TF_3	This work

only TF_1, TFG, and TF_3 could be well separated. Wang et al.[26] reported four theaflavin monomers were isolated by a HSCCC process applying the maximum loading amount of 30 mg, which was the same as we tested in this study. The semi-preparative HPLC isolation only spent about 10% time of HSCCC processing for the same sample loading amount and successfully gained four monomers. It proved to be a more rapid method. More importantly, TF_2A, TF_2B, and TF_3 were of good purity.

CONCLUSIONS

The present study optimized the parameters of the semi-preparative HPLC separation system, and the four purified individual theaflavins were efficiently obtained at one time. Finally, it took only 45 min for one separation process compared to other isolation methods which took over several hours. In addition, a column chromatography for preparing highly purified theaflavins mixtures based on a biosynthesis method by immobilized enzyme was developed in this study. In addition, further improvement on larger sample amounts needs to be investigated by future work. We studied applying molecular imprinting techniques to the isolation of individual theaflavins.

ACKNOWLEDGEMENTS

This study was partially supported by the Key program of Zhejiang Province of China (2007C12068) and the National Science Foundation for Distinguished Young Scholars of China (30901002). We thank Prof.

H. L. Xia and Dr. Z. Y. Yang for reviewing the manuscript and for helpful discussions.

REFERENCES

1. Zhu, Y. X.; Huang, H.; Tu, Y. Y. A Review of Recent Studies in China on the Possible Beneficial Health Effects of Tea. *Int. J. Food Sci. Technol.* **2006**, *41* (4), 333–340.
2. Yang, C. S.; Ju, J.; Lu, G.; Xiao, H.; Hao, X.; Sang, S.; Lambert, J. D. Cancer Prevention by Tea and Tea Polyphenols. *Asia Pac. J. Clin. Nutr.* **2008**, *245* (Suppl 1), 245–248.
3. Luczaj, W.; Skrzydlewska, E. Antioxidative Properties of Black Tea. *Prev. Med.* **2005**, *40* (6), 910–918.
4. Yang, Z. Y.; Jie, G. L.; Dong, F.; Xu, Y.; Watanabe, N.; Tu, Y. Y. Radical-Scavenging Abilities and Antioxidant Properties of Theaflavins and Their Gallate Esters in H_2O_2-Mediated Oxidative Damage System in the HPF-1 Cells. *Toxicol. In Vitro.* **2008**, *22* (5), 1250–1256.
5. Yang, Z. Y.; Tu, Y. Y.; Xia, H. L.; Jie, G. L.; Chen, X. M.; He, P. M. Suppression of Free-Radicals and Protection Against H_2O_2-Induced Oxidative Damage in HPF-1 Cell by Oxidized Phenolic Compounds Present in Black Tea. *Food Chem.* **2007**, *105* (4), 1349–1356.
6. Tu, Y. Y.; Tang, A. B.; Watanabe, N. The Theaflavin Monomers Inhibit the Cancer Cells Growth in vitro. *ABBS* **2004**, *36* (7), 508–512.
7. Pan, M. H.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. H.; Lin, J. K. Suppression of Lipopolysaccharide-Induced Nuclear Factor-Kappa B Activity by Theaflavin-3,3′-digallate from Black Tea and Other Polyphenols Through Down-Regulation of I_kB Kinase Activity in Macrophages. *Biochem. Pharmacol.* **2000**, *59* (4), 357–367.
8. Leung, L. K.; Su, Y. L.; Chen, R. Y.; Zhang, Z. H.; Huang, Y.; Chen, Z. Y. Theaflavins in Black Tea and Catechins in Green Tea are Equally Effective Antioxidants. *J. Nutr.* **2001**, *131* (9), 2248–2251.
9. Gardner, E. J.; Ruxton, C. H. S.; Leeds, A. R. Black Tea – Helpful or Harmful? A Review of the Evidence. *Eur. J. Clin. Nutr.* **2007**, *61* (1), 3–18.
10. Balentine, D. A. *Manufacturing and Chemistry of Tea*, American Chemical Society: Washington, D.C. 1992; 102.
11. Geissman, T. A. *Chemistry of Flavonoid Compounds*, Pergamon: Oxford, UK, 1962; 468.
12. Runeckles, V. C.; Tso, T. C. *Recent Advances in Phytochemistry*, Academic Press: New York, 1972; 247.
13. Yoshida, H.; Ishikawa, T.; Hosoai, H., et al. Inhibitory Effect of Tea Flavonoids on the Ability of Cells to Oxidize Low Density Lipoprotein. *Biochem. Pharmacol.* **1999**, *58* (11), 1695–1703.
14. Shiraki, M.; Hara, Y.; Osawa, T.; Kumon, H.; Nakayama, T.; Kawakishi, S. Antioxidative and Antimutagenic Effects of Theaflavins from Black Tea. *Mutat. Res.* **1994**, *323* (1–2), 29–34.
15. Lin, J. K.; Chen, P. C.; Ho, C. T.; Lin-Shiau, S. Y. Inhibition of Xanthine Oxidase and Suppression of Intracellular Reactive Oxygen Species in HL-60 Cells by Theaflavin-3,3′-digallate, (-)-epigallocatechin-3-gallate, and Propyl Gallate. *J. Agric. Food Chem.* **2000**, *48* (7), 2736–2743.
16. Tu, Y. Y.; Xu, X. Q.; Xia, H. L.; Watanabe, N. Optimization of Theaflavin Biosynthesis from Tea Polyphenols Using an Immobilized Enzyme System and Response Surface Methodology. *Biotechnol. Lett.* **2005**, *27* (4), 269–274.
17. Tu, Y. Y.; Xia, H. L. Method for Producing High-Purity Theaflavin from Tea Polyphenol by Using Immobilized Polyphenol Oxidase as Catalyst. China Patent, 2004, CN 1403580 A.
18. Eskenazi, B. Caffeine – Filtering the Facts. *New Engl. J. Med.* **1999**, *341* (22), 1688–1689.
19. Nonaka, G. I.; Hashimoto, F.; Nishioka, I. Tannins and Related Compounds. *Chem. Pharm. Bull.* **1986**, *34*, 61–65.
20. Su, Y. L.; Xu, J. Z.; Ng, C. H.; Leung, L. K.; Huang, Y.; Chen, Z. Y. Antioxidant Activity of Tea Theaflavins and Methylated Catechins in Canola Oil. *J. Am. Oil Chem. Soc.* **2004**, *81* (3), 269–274.
21. Du, Q. Z.; Jiang, H.; Ito, Y. Separation of Theaflavins of Black Tea. High-Speed Countercurrent Chromatography vs. Sephadex LH-20 Gel Column Chromatography. *J. Liq. Chrom. Rel. Technol.* **2001**, *24* (15), 2363–2369.

22. Yang, C. J.; Li, D. X.; Wan, X. C. Combination of HSCCC and Sephadex LH-20 Methods – An Approach to Isolation and Purification of the Main Individual Theaflavins from Black Tea. *J. Chromatogr. B* **2008**, *861* (1), 140–144.
23. Degenhardt, A.; Engelhardt, U. H.; Wendt, A. S.; Winterhalter, P. Isolation of Black Tea Pigments Using High-Speed Countercurrent Chromatography and Studies on Properties of Black Tea Polymers. *J. Agric. Food Chem.* **2000**, *48* (11), 5200–5205.
24. Cao, X. L.; Lewis, J. R.; Ito, Y. Application of High-Speed Countercurrent Chromatography to the Separation of Black Tea Theaflavins. *J. Liq. Chromatogr. Relat. Technol.* **2004**, *27* (12), 1893–1902.
25. Yang, Z. Y.; T. Y. Y.; Zhao, Q.; He, P. M. Study on Separation of Theaflavins Monomer by High-Speed Countercurrent Chromatography. *Food Sci.* **2005**, *26*, 87–90 (in Chinese).
26. Wang, K. B.; Liu, Z. H.; Huang, J. A.; Dong, X. R.; Song, L. B.; Pan, Y.; Liu, F. Preparative Isolation and Purification of Theaflavins and Catechins by High-Speed Countercurrent Chromatography. *J. Chromatogr. B* **2008**, *867* (2), 282–286.
27. Ding, Y. P.; Liu, Z. H.; Huang, J. N. Study on Separation and Purification of Theaflavins by Polyamide. *Food Sci.* **2007**, *28*, 55–57 (in Chinese).
28. Jiang, H. Y.; Wang, C. P.; Gao, Q. Q. Method for Preparing Four Theaflavins. China Patent. 2008, CN 101190909 A.
29. Cao, X. L.; Dong, Y.; Huang, D.; Li, P.; Li, T. Separation and Preparation of High Purity Theaflavin Monomer Includes Dissolving Black Tea Crude Extract by Eluent and Performing Gel Column Chromatography and High-Speed Countercurrent Chromatography. China Patent. 2007, CN 101081844 A.
30. Davies, A. L.; Cai, Y.; Davies, A. P. ^{1}H and ^{13}C NMR Assignment of Theaflavin, Heaflavin Monogallate and Theaflavin Digallate. *Magn. Reson. Chem.* **1995**, *33* (7), 549–552.