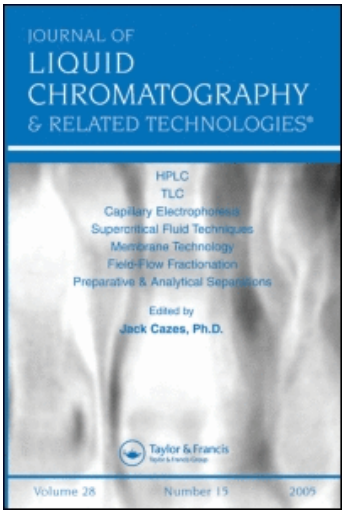



**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Application of High-Speed Countercurrent Chromatography to the Separation of Black Tea Theaflavins

Xueli Cao[ab]; John R. Lewis[b]; Yoichiro Ito[c]
[a] Beijing Key Lab of Plant Resource Research and Development, Beijing Technology and Business University, Beijing, P.R. China [b] Unilever Research, Sharnbrook, Bedford, UK [c] Laboratory of Biophysical Chemistry, NHLBI, National Institutes of Health, Bethesda, Maryland, USA

Online publication date: 27 May 2004

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 27, No. 12, pp. 1893–1902, 2004

# Application of High-Speed Countercurrent Chromatography to the Separation of Black Tea Theaflavins

**Xueli Cao,[1,2] John R. Lewis,[2] and Yoichiro Ito[3,*]**

[1]Beijing Key Lab of Plant Resource Research and Development, Beijing Technology and Business University, Beijing, P.R. China
[2]Unilever Research, Sharnbrook, Bedford, UK
[3]Laboratory of Biophysical Chemistry, NHLBI, National Institutes of Health, Bethesda, Maryland, USA



## ABSTRACT

High-speed countercurrent chromatography (HSCCC) has been applied for the separation of four theaflavins (TFs). The results indicated that pure TF and theaflavin-3,3′-*O*-digallate (TFDG) can be obtained by HSCCC using a solvent system composed of hexane–ethyl acetate–methanol–water (1.25 : 5 : 1.25 : 5, v/v/v/v). Although the separation of two TF monogallates is incomplete under all the solvent systems examined, peak cutting yielded a small amount of pure theaflavin-3-*O*-gallate (TF3MG) and theaflavin-3′-*O*-monogallate (TF3′MG). Thus, preparative

*Correspondence: Yoichiro Ito, Laboratory of Biophysical Chemistry, NHLBI, National Institutes of Health, Bldg. 50, Rm. 3334, Bethesda, MD 20892-8014, USA; E-mail: itoy@nhlbi.nih.gov.



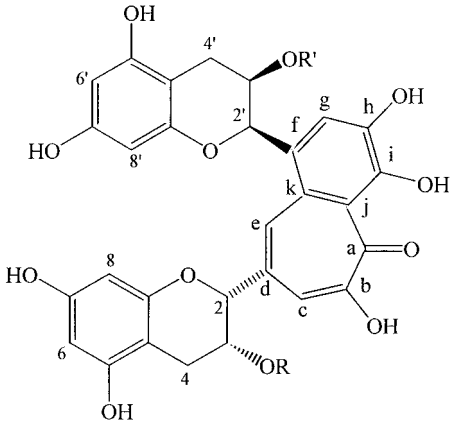
DOI: 10.1081/JLC-120038775 1082-6076 (Print); 1520-572X (Online)

HPLC has been employed as a complementary method for the separation of two TF monogallates. The HSCCC isolation of TFs from the solvent extract of black tea infusion has proved to be successful by gradient elution, based on the solvent system mentioned above.

*Key Words:* High-speed countercurrent chromatography; Theaflavins; Black tea; Theaflavin monogallates.

## INTRODUCTION

Theaflavins (TFs) (Fig. 1) are a group of polyphenol pigments found in black tea formed by oxidative coupling of an appropriate pair of catechins at the fermentation stage of black tea manufacture. Although, TFs constitute only 2% of the dry weight (3–5% of soluble solids), they significantly contribute to the bright color and brisk taste of tea brews, and are generally considered to be most effective components for the inhibition of carcinogenesis.[1–3] Theaflavin-3,3′-gallate has been reported to be a better inhibitor of tyrosine receptor kinase than green tea polyphenol EGCG.[4,5] In addition to the four main TFs, which have been identified many years ago, several novel TF compounds have recently been isolated and characterized from black tea.[6]




Theaflavin R = R'= H
Theaflavin-3-O-gallate R= gallate, R'= H
Theaflavin-3'-O-gallate R'= gallate, R= H
Theaflavin-3,3'-di-O-gallate R= R'= gallate

***Figure 1.*** The structures of TFs.





MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

However, the preparative separation of TFs is of significant technical challenge because of their low-level amount in black tea. In the past, the isolation of TFs has been based on chromatography on Sephadex LH-20 and preparative HPLC. Although, NMR and MS data of these TFs have been published in detail,[7] their pure standards are still not commercially available. In order to promote studies on the effects of TF on human health, it is necessary to develop more efficient TF separation methods.

The separation of this group of compounds is ideal for the application of high-speed countercurrent chromatography (HSCCC), as this technique is a unique liquid–liquid partition chromatography using a liquid stationary phase without solid support and, therefore, offers many advantages over the currently used methodologies, such as no irreversible adsorption, low risk of sample denaturation, total sample recovery, large load capacity, and low cost. The high performance of HSCCC in the separation of TFs has been shown to be better than the conventional Sephadex LH-20 gel column chromatography.[8]

The present paper reports the method development for separation of four individual TFs, and the isolation of TFs (as a group) from other components in black tea infusion mainly by HSCCC.

## EXPERIMENTAL

### Reagents

All solvents used in this study were of analytical grade and purchased from Sigma Chemicals, Poole, Dorset, or Fisher Scientific, Loughborough, Leicestershire, UK.

### Materials

TF standard mixture solution, TF crude mixture sample, and soluble solid of black tea infusion were all prepared at Unilever Research, Colworth, Tea Sciences Unit.

### CCC Separation

A Quattro CPC manufactured by Brunel Institute for Bioengineering (Uxbridge, UK) was used for the separation. The CPC is equipped with two opposite bobbins containing two coils in each side. Several different ideal coil volumes can be achieved by joining the coils in series through the flying leads

externally. The total coil volume is 585.5 mL with 1.6 mm internal diameter (I.D.) of tubing. The minimum coil volume is 105.1 mL. The separations were run at a revolution speed of 800 rpm at 30°C. Lower phase was used as the mobile phase and eluted from the head to the tail of the multiplayer coil. Usually, in each separation, the coiled column was first filled with the stationary phase. Then, the mobile phase was pumped into the column at a flow-rate of 2.0–3.0 mL/min, while the CPC was rotated at 800 rpm. After the mobile phase front emerged and the system established a hydrodynamic equilibrium, the sample solution was loaded through an injection valve (with 2–6 mL loop). The samples were dissolved in the mobile phase, and in some cases a small amount of stationary phase was added to increase the solubility. The system is equipped with a Perkin Elmer Series 200 pump for gradient elution. The eluents were monitored by a Waters 486 tunable absorbance detector and collected using a Waters fraction collector. The data was processed by a chromatographic system.



### Preparative HPLC Separation

A Waters 486 preparative HPLC system equipped with a Hipersil 5 μ ODS 250 × 10 mm I.D. column has been used for the successive separation of two TF-monogallates from the HSCCC fraction corresponding to the second peak. The data was processed by a Shimadzu Class-*VP* system.

### HPLC Analysis with Diode Array Detection (HPLC-DAD)

A Dionex summit system equipped with a model P580 pump, a GINA 50 autosampler, a UVD340S diode array detector, and a Chromeleon data system was used. Peak detection was carried out at 274 nm. The analysis of TFs was performed on Hypersil $C_{18}$ column (3 μm, 100 × 4.6 mm, Phenomenex, UK). The mobile phases were composed of solvent A, 2% acetic acid in acetonitrile and solvent B, 2% acetic acid in high pure water. The elution was programed as follows: initial, 85% B; gradient to 75% B in 20 min; at 20.01 min, back to initial condition 85% B and isocratic for 10 min; Flow-rate, 1.8 mL/min.

## RESULTS AND DISCUSSION

### Measurement of Partition Coefficient *K*

TF, theaflavin-3-*O*-gallate (TF3MG), theaflavin-3′-*O*-gallate (TF3′MG), and theaflavin-3,3′-*O*-digallate (TFDG) are the four main TFs in black tea.



MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

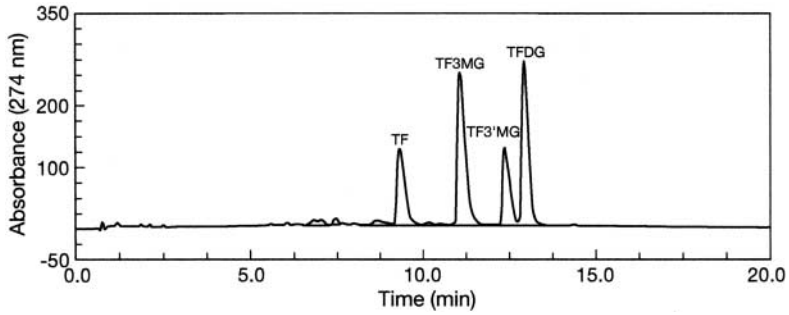



***Figure 2.*** HPLC chromatogram of the crude TF mixture.

As they are usually present together in low-level amounts in black tea samples, the separation of individual TFs has been of great difficulty. For the separation of TFs using HSCCC, several solvent systems have been examined to optimize the partition coefficients ($K$) of four main TFs by HPLC. The sample used for this purpose is a crude mixture of four TFs, as illustrated in Fig. 2. The results listed in Table 1 showed most of the TF $K$ values lied closely in the range of 0.5–2, and generally, the $K$ values of TF and TFDG are clearly different from that of TFMGs under most of solvent systems. This means that the separation between TF, TFMG and TFDG is relatively easier than that between TFMGs. Since the solvent systems with different compositions

***Table 1.*** Solvent selection for the separation of TFs by HSCCC.

| Solvent system | Volume ratios | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Hexane | 1 | 2 | 1 | 1 | 1 | 2 | | |
| Ethyl hexanoate | | | | | | | 5 | 10 |
| Ethyl propionate | | | 5 | 5 | | | | |
| Propyl acetate | | | | | 5 | 5 | | |
| Ethyl acetate | 5 | 5 | | | | | | |
| Methanol | 2 | 2 | 1 | 2 | 1 | 2 | 1 | 1 |
| Water | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 10 |
| Partition coefficient ($K$)[a] | | | | | | | | |
| TF | 3.57 | 0.88 | 1.12 | 1.39 | 3.23 | 0.71 | 0.42 | 1.09 |
| TFMG | 4.35 | 0.58 | 3.45 | 1.82 | 11.1 | 0.67 | 0.95 | 1.56 |
| TF3′MG | 4.17 | 0.83 | 3.45 | 2.56 | 11.1 | 0.87 | 0.86 | 1.92 |
| TFDG | 4.55 | 0.78 | 7.14 | 4.17 | 33.3 | 1.27 | 1.12 | 1.78 |

[a]$K$ is expressed as the solute concentration in the upper phase divided by that in the lower phase.





MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

did not make much difference, the common system composed of hexane–ethyl acetate–methanol–water was employed.

## Optimization of Solvent Systems and Preparative Separation of TFs

The above solvent series composed of hexane–ethyl acetate–methanol–water at various volume ratios, was first examined using a small volume coil. The starting sample is the TF mixture mentioned above. The results obtained with the above TF mixture, showed that slight differences between volume ratios of each composition can make a great difference in the separation (Fig. 3), and the complete separation of TF, TFMGs, and TFDG from each other was not difficult compared with the separation of two TFMGs. This was consistent with that predicted from the partition data in Table 1.

When one of the solvents was selected for the separation of TFs through a preparative coil on greater sample size (Fig. 4), better resolution was achieved under the same solvent condition, but unfortunately, two TFMGs were only partially resolved.

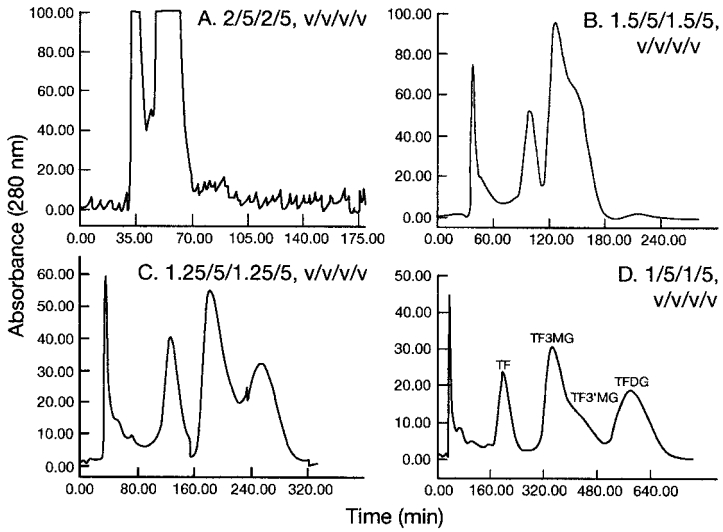



***Figure 3.*** HSCCC separation of four TFs through a small coil volume. Solvent system: hexane–ethyl acetate–methanol–water; flow-rate: 2 mL/min; coil volume: 208.9 mL; sample size: 10 mg.





MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

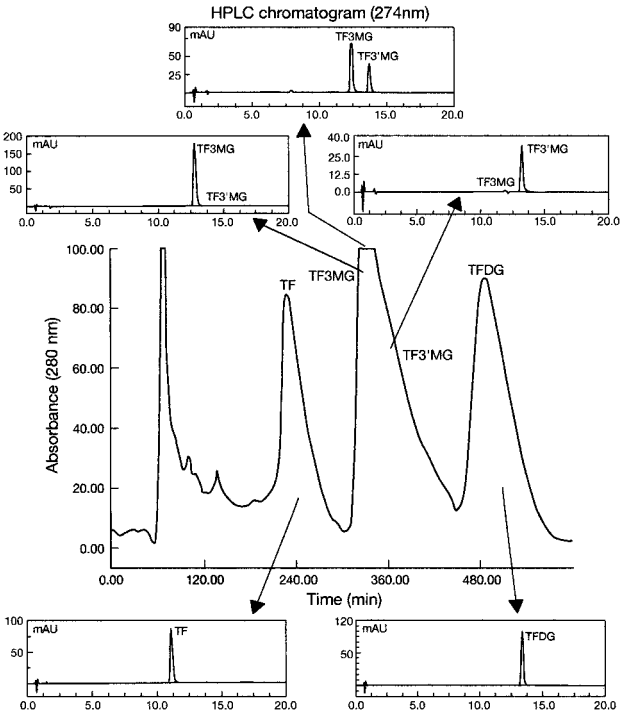



***Figure 4.*** Preparative separation of four TFs by HSCCC and HPLC analysis of each peak fraction. Solvent system: hexane–ethyl acetate–methanol–water (1.25/5/1.25/5, v/v/v/v); flow-rate: 3 mL/min; column volume: 585.8 mL; retention: 69%; sample size: 50 mg.

## HPLC Separation of TFMGs

Preparative HPLC was employed as a supplementary technique for the separation of TFMGs (Fig. 5). It was proven that preparative HPLC could separate individual TFMGs successfully. However, the resolution goes down quickly as the sample size is increased, and this will limit its application on a large preparative scale.

## Separation of TFs as a Group from Black Tea Extract

In tea and health studies, sometimes a mixture of TFs is required. So the possibility of separating TFs as a group from catechins has also been explored.





Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

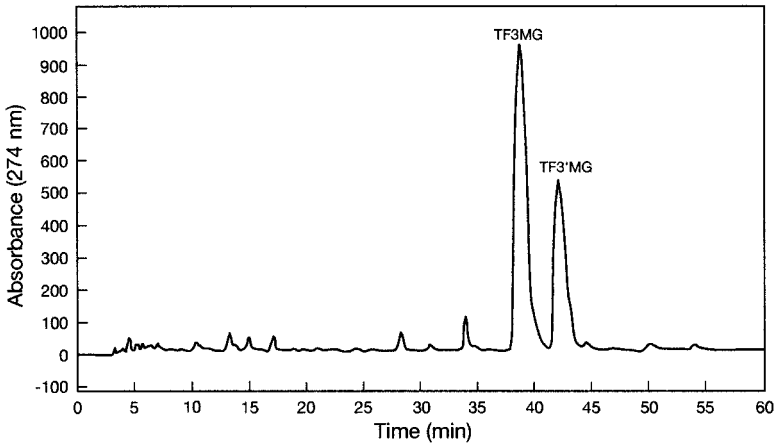



***Figure 5.*** Preparative HPLC separation of TF-monogallates. Sample: TFMG mixture derived from HSCCC fractions corresponding to the second peak (Fig. 4), 1 mg/0.1 mL; Column Hypersil 5 mm, ODS 250 × 10 mm I.D.; solvent system: C—5% CAN., in 0.1% aqueous TFA, D—50% ACN in water; flow rate: 4.8 mL/min; gradient program: starting with 70% C and changed gradually to 50% C in 50 min, then kept eluting for 10 min, finally back to 70% C at 61 min and get ready for the next run. ACN—acetonitrile; TFA—trifluoroacetic acid.



Figure 6 illustrated a separation profile of TFs from an ethyl acetate extract of black tea infusion by HSCCC. HPLC analysis of peak fractions revealed that the shadow area only contained TFs. This indicates that TFs can be completely separated from catechins. Our studies also suggested that if TF can be separated from ECG under certain separation conditions, others may be also completely resolved from catechins.

## CONCLUSION

The results indicated that pure TF and TFDG can be obtained from one HSCCC run by the method developed. However, the separation of TFMGs has proven to be incomplete under all the solvent systems examined and a number of gradient elution profiles evaluated within the hexane : ethyl acetate : methanol : water system. However, peak cutting allows the collection of a small amount of pure TF3MG and TF3′MG. Thus, preparative HPLC

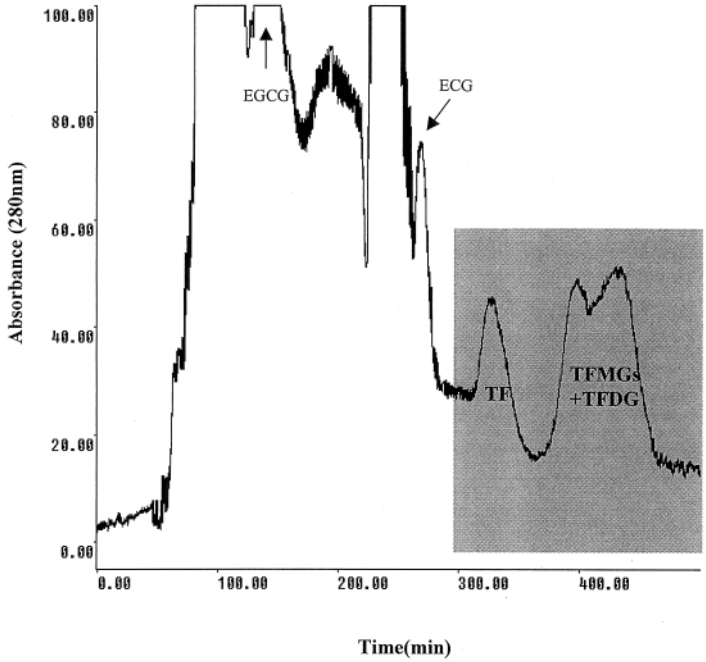



***Figure 6.*** Isolation of TF mixture from ethyl acetate extract of black tea infusion. Solvent system: hexane–ethyl acetate–methanol–water, A—1/5/1/5, v/v/v/v; B—2/5/2/5, v/v/v/v. Started with 100% A and eluted with the lower phase of A for 100 min, and then change the mobile phase in a gradient manner from 100% A to 100% B (lower phase) in 50 min, finally keep eluting with 100% B for another 350 min; flow rate, 3 mL/min; column volume, 585.9 mL; sample size, 54 mg.

has been employed as a supplementary method for the separation of two TFMGs. The HSCCC isolation of TFs from the extract of black tea infusion has proven to be successful by gradient elution based on the solvent system mentioned above.

## REFERENCES

1. Lea, M.A.; Xiao, Q.; Sadhukhan, A.K.; Cottle, S.; Wang, Z.Y.; Yang, C.S. Inhibitory effect of tea extracts and (-)-epigallocatechin gallate on DNA-synthesis and proliferation of hepatoma and erythroleukemia-cells. Cancer Lett. **1993**, *68*, 231–236.





MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

2. Lee, B.L.; Ong, C.N. Comparative analysis of tea catechins and theaflavins by high-performance liquid chromatography and capillary electrophoresis. J. Chromatogr. A **2000**, *881*, 439–447.
3. Lu, Y.P.; Lou, Y.R.; Xie, J.G.; Yen, P.; Huang, M.T.; Conney, A.H. Inhibitory effect of black tea on the growth of established skin tumors in mice: effects on tumor size, apoptosis, mitosis and bromodeoxyuridine incorporation into DNA. Carcinogenesis **1997**, *18*, 2163–2169.
4. Chen, Y.C.; Liang, Y.C.; Lin-Shiau, S.Y.; Ho, C.T.; Lin, J.K. Inhibition of TPA-induced protein kinase C and transcription activator protein-1 binding activities by thearflavin-3,3′-digallate from black tea in NIH3T3 Cells. J. Agric. Food Chem. **1999**, *47*, 1416–1421.
5. Liang, Y.C.; Chen, Y.C.; Lin, Y.L.; Lin-Shiau, S.Y.; Ho, C.T.; Lin, J.K. Suppression of extracellular signals and cell proliferation by the black tea polyphenol, theaflavin-3,3′-digallate. Carcinogenesis **1999**, *2*, 733–736.
6. Lewis, J.R.; Davis, A.L.; Cai, Y.; Davies, A.P.; Wilkins, J.P.G.; Pennington, M. Theaflavate B, isotheaflavin-3′-O-gallate and neotheaflavin-3-O-gallate: three polyphenolic pigments from black tea. Photochemistry **1998**, *49*, 2511–2519.
7. Davies, A.L.; Cai, Y.; Davies, A.P. 1H and 13C NMR assignment of theaflavin, heaflavin monogallate and theaflavin digallate. Magn. Reson. Chem. **1995**, *33*, 549–552.
8. 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. and Rel. Technol. **2001**, *24*, 2363–2369.

Received February 3, 2004
Accepted February 28, 2004
Manuscript 6313

# Request Permission or Order Reprints Instantly!