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RESEARCH ARTICLE

The hexane-ethyl acetate-methanol-water system for the separation of theaflavins from black tea (*Camellia sinensis*) using high-speed counter-current chromatography

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Abstract: Theaflavins (TFs) from black tea leaves were separated using high-speed countercurrent chromatography (HSCCC). Isobutyl methyl ketone (4-methyl-2-pentanone) was used to separate the TFs from a hot water extract of black tea leaves. Three different compositions of the biphasic solvent system hexane–ethyl acetate-methanol-water (2:5:2:5 solvent system **A**) 1:4:1:4 (solvent system **B**) and 1:5:1:5 (solvent system **C**) were studied. The aqueous phase was used as the mobile phase in the head to tail elution mode. Flow rates of 2.0 – 2.8 mL min^{-1}, revolution speeds of 800-1000 rpm and a 200 mg sample of TF in 5 mL of the aqueous phase with settling times 17-18 secs (solvent systems **B** and **C**) gave the best separations. Three peaks were separated in the HSCCC traces obtained using solvent systems **B** and **C**, and each peak was monitored by high performance liquid chromatography (HPLC).Separation of theaflavin monogallates was not achieved, but solvent system Cresulted in a complete separation of theaflavin and theaflavin digallate.

Keywords: *Camellia sinensis*, Theaflavins, Theaflavin digallate, Counter current chromatography.

INTRODUCTION

Tea made from the tender leaves of *Camellia sinensis*, is one of the most widely consumed beverages in the world**.** Three principal types of tea **-** green tea, oolong tea and black tea are manufactured from the young green shoots of the tea plant *Camellia sinensis***.** Green tea contains high levels of catechins **(**flavanols and flavanol gallates**)** and proanthocyanidins **(**oligomers of flavanols**),** and has attracted considerable interest as a source of dietary antioxidants**.** A wide range of biological activities have been associated with both catechins and proanthocyanidins**.** Oxidative polymerisation of polyphenols takes place during oolong and black tea production, in the presence of the enzyme polyphenol oxidase**.**Oxidised or **'**fermented**'** teas contain the more complex flavanoids theaflavins **(**TFs**)** and thearubigins **(**TRs**)** formed by enzymic oxidation of the flavan-3-ols in the green tea shoots **(**Haslam, 2003**).**TFs are bright orange coloured pigments with acidic properties, and contribute to the colour and strength in the brew, andalso determine the quality of black tea**(**Smith and White, 1965**).**

We have used HSCCC, a support-free liquid-liquid partition system **(**Ito, 1996**)** to separate extracts of catechins **(**Kumar and Rajapaksha 2005**),** dimeric proanthocyanidin **(**Kumar *et al***.**, 2009**)** and oligomeric proanthocyanidins **(**Kumar *et al***.**, 2014 Kumar *et al***.**, 2015**)** from fresh tea leaves**.** Commercially available standard samples of TFs **[**Cao *et al***.**, 2004**),**TFs from commercial samples of fermented oolong and black tea**(**Yanagida *et al***.**, 2006**)** and TFs prepared by the enzymic oxidation of tea catechins using polyphenol oxidase isolated from pear fruit **(**Wang *et al***.**, 2008**)** havebeen separated using HSCCC**.** A combination of HSCCC and Sephadex LH-20 chromatography was used by Yang *et al* to separate TFs from a sample of black tea **(**Yang *et al***.**, 2008**).**

The use and versatility of the Hex-EtOAc-MeOH-water **(**HEMWat**)** system for the separation of different classes of natural products and a wide range of organic compounds of low and medium polarity has been reviewed by Friesen and Pauli**(**2005**).** In the HEMWat solvent system the organic phase is mainly composed of hexane and ethyl acetate **(**EtOAc**)** in the upper

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phase of the biphasic mixture, while the aqueous phase is mainly composed of methanol (MeOH) and water in the lower phase of the biphasic mixture.

Theaflavin (TF), theaflavin-3-gallate (TF3MG), theaflavin-3'-gallate (TF3'MG) and theaflavin-3,3'-digallate (TFDG), having a benzotropolone nucleus, are the four major TFs found in black tea formed at the fermentation stage of black tea manufacture by oxidative coupling of an appropriate pair of catechins. TFs constitute 3-5% (wt/wt) of extractable solids in brewed black tea. TRs are rusty brown pigments formed during black tea fermentation. It is believed that TRs constitute up to 20% of the black tea leaf and 60% of the solids in a black tea infusion (Haslam, 2003).

The four principal TFs can be separated from an extract of black tea using either ethyl acetate (EtOAc) orisobutyl methyl ketone (IBMK) (4-methyl-2-pentanone). EtOAc is the solvent that has been used commonly for this purpose(Yang *et al.*, Wang *et al.*, 2008, Degenhardt *et al.*, 2000).These two solvents extract TFs completely andalso extract part of the TR fraction present as free acids (Roberst and Smith 1963). The polarity of IBMK is similar to that of EtOAc, butIBMK has lower solubility (1.9% w/v at 25° C) than EtOAc (10% at 25°C) in water (USEPA 2003, USEPA 2006) and this makes IBMK a useful solvent in liquid-liquid extractions.The TRs extracted by IBMK are soluble in $NaHCO_3$ while TFs are almost insoluble. Separation of TFs byHigh-speed counter-current chromatography (HSCCC) of an extract of black tea was carried out by us in an effort to obtain pure reference samples of the four major TFs found in black tea.

MATERIAL AND METHODS

Reagents and Materials

Distilled solvents were used for HSCCC, while HPLC grade solvents (Merck, Mumbai, India) and water purified by a Milli-Q system (Millipore, Bedford, MA, USA) were used for HPLC. Freeze drying of extracts was performed with an Edwards Modulyo Freeze dryer. Authentic reference samples of TFs (Mitsui Norin Company Ltd, Japan) used for HPLC were provided by the Biochemistry Division of the Tea Research Institute, Talawakelle, Sri Lanka.

Extraction of TFs from black tea

Black tea dust No. 1 sample (60 g) from St. Coombs estate, Talawakelle, was extracted with boiling water (500 mL) for 4 min. The extract was filtered, partitioned with dichloromethane (400 mL x 3) and the aqueous phase was partitioned (500 mL x 3) with *iso*-butyl methyl ketone (IBMK)(Roberts and Smith, 1963). The organic phase was washed with 2.5% aq. $NaHCO_3$ (100 mL x 5), the IBMK layer was separated, concentrated on a rotavapor (< 40°C) and freeze dried to give the orange coloured TF extract (125 mg)

HSCCC; INSTRUMENTATION AND METHODS

The HSCCC set-up consists of the pump, multicoil CCC system, a UV detector (single path UV-1 detector 254)and a recorder.A Model CCC-1000HSCCC system (Pharma-Tech Research, Baltimore, MD, USA) was used for the separation. The preparative coil (volume capacity of 300 mL) had an I.D. of 2.6 mm, a β-value of 0.5-0.8 and a revolution radius of 7.5 cm. Spectrophotometric measurements were carried out with a Shimadzu UV-1601 UV-Vis spectrophotometer.

Three different two phase solvent systems containing different ratios of hexane, EtOAc, MeOH and water were prepared (Table 1).The partition coefficient (K_D) was determined by the simple test tube procedure described by Ito and Conway (Ito, 1986)(Table 1). A known quantity (~ 1 mg) of the TF extract was dissolved in a two phase solvent system (2 ml of each phase) in a test tube, shaken well, equilibrated, and separate the two phases. An aliquot of each phase was added to separate portions of MeOH (3 ml), and absorbance at 280 nm was determined using a UV-1601 UV-VIS spectrophotometer. K_D at 280 nm was determined using the equation (Ito 2005),

$$K_D = C_{\text{stationary phase}}/C_{\text{mobile phase}}$$

The settling times of the solvent systems were determined by placing each phase (2 mL) in a capped test tube, gently inverted a few times and immediately placed in an up- right position. The time taken to form clear layers was measured to give the settling time (Ito 1986) (Table 1).

Table 1: Two phase solvent systems, solvent composition, partition coefficient (K_D) and Settling time of TF extract.

Solvent system	Hexane	EtOAc	MeOH	H_2O	K_D	Settling time (sec)
A	2	5	2	5	0.67	20
B	1	4	1	4	0.87	17
C	1	5	1	5	0.78	18

The coil was filled with the stationary phase and rotated at the required rotation speed (e.g. 800 rpm, 1000 rpm), while the mobile aqueous phase was pumped into the column at the required flow rate (e.g. 2.0 mL min^{-1} 2.5 mL min^{-1}, 2.8 mL min^{-1}. The effluent from the column was collected in a 500 mL measuring cylinder to measure the volume of stationary phase as well as the total volume of mobile phase eluted. The elution was continued until the total elution volume was more than 300 mL (capacity of the column) when the stationary phase was stabilized. The centrifuge was stopped and the column contents were emptied into a 500 mL graduated measuring cylinder to measure the retained volume of stationary phase.Stationary phase retention (SP_R %) after the HSCCC run was calculated using the equation:

$$SP_R\% = SP_R / C_v \times 100$$

Where SP_R = volume (mL) of stationary phase retained and C_v = total column volume.

Solvent systems **B** and **C** (Table 1) were selected for HSCCC, equilibrated at room temperature in a separatory funnel and the two phases were separated shortly before use. The multi layer coil was filled entirely with the upper organic stationary phase (SP) at a flow rate of 9.0 mL min^{1}. Flow was stopped when the SP emerged from the tail end of the instrument at a constant rate and the coil planet centrifuge was allowed to rotate at 1000 rpm for solvent **B** and at 800 rpm for solvent **C.** The crude TF mixture (150 mg) was dissolved in the aqueous mobile phase (5 mL) and injected into the column through the sample injection port. HSCCC carried out using the solvent systems **B** and **C** (Hex:EtOAc: MeOH:H_2O) at elution speeds of 2.8 mL/min and at 2.0 mL/min at rotation speeds of 1000 rpm and 800 rpm respectively. The effluent from the column outlet was monitored with a UV detector (280 nm) and fractions were collected manually according to the absorbance plot from the detector. Fractions collected were concentrated on a rotavapor (≤ 35°C), freeze dried and monitored by HPLC at the beginning and end of a peak in the HSCCC trace. If the HPLC was identical at the beginning and end of a peak, the fractions in between were combined and concentrated on a rotavapor (≤35°C).

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC instrumentation consisted of a Waters Alliance 2690XE separation module coupled to a Waters 996 photodiode array detector (PDA) and Waters Millennium 32 data system equipped with a built in column oven and a vaccum degasser. A Luna-5 µm Phenyl-Hexyl column 4.6 x 250 nm (Phenomenex Inc, USA) with a guard column made of the same material (Security-Guard, Phenomenex Inc, USA) was used for the analysis. A binary gradient with 9% (v/v) acetonitrile, 2% (v/v) acetic acid with 20 ug/mL EDTA (mobile phase A), 80% (v/v) acetonitrile2% (v/v) acetic acid with 20 ug/mL EDTA (mobile phase B), and the linear gradient programme described in ISO TC 34/SC 8 procedure (ISO, 1999) were used. The peaks eluted were monitored at 280 nm, and the conditions used were; Injection volume 10 uL, column temperature 35°C, flow rate 10 mL min^{-1}; the gradient elution profile started with 100% A for 10 min, and a linear gradient from 100% A to 68% A in 15 min, a linear gradient from 68% A to 100%A in 7 min. The column was re-equilibrated with 100% A for 10 min before the next injection. The total run time was 42 min.

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RESULTS AND DISCUSSION

Extraction and HSCCC parameters for separation of TFs

TFs are completely extracted from a tea extract by IBMK (or EtOAc) and part of the TR fraction which is acidic.However IBMK has a lower solubility in water and is therefore more effective than EtOAc, in liquid–liquid extraction. The higher boiling point of IBMK (116-117^{o}C at 760 mm Hg) than that of EtOAc (77^{o}C) was a disadvantage, but did not present problems when using a rotavapor. The IBMK extract from the black tea sample in the current study, was washed with aqueous 2**%** $NaHCO_3$ solution in order to remove the remaining TR fraction and obtain a TR-free TF extract (Roberts and Smith 1963). TFs are almost insoluble in the 2**%** $NaHCO_3$ solution.The orange coloured IBMK extract containing only TFs after concentration on a rotavapor, was subjected to HSCCC.Solvent systems **B** and **C** having K_D close to 1 and shorter settling times (Table 1) were selected for HSCCC of the TF extract(Ito, 1986).

The settling time of a two phase solvent system, the time required to separate two immiscible solvent phases, is related to the stationary phase retention. All three solvent systems studied had a settling time of 20 s or less. The settling times for the different ratios of Hex - EtOAc – MeOH – water (v/v) given in Table 1 were found to be 17-20 s and equal volumes of each phase was observed in the test tube. During the current study 55-65**%** stationary phase retention was obtained for the separation of TFs using solvent systems A, B and C (Table 1).Cao *et al.*,(2004) reported 69**%** retention of stationary phase (upper phase) in the separation of a standard mixture of TFs using the solvent system Hex: EtOAc: MeOH: Water (1:4:1:4).

HSCCC separation of TFs

Three fractions F-1, F-2 and F-3 were separated using solvent system **B** (Figure 1) when fractionation was carried out using a rotation speed of 1000 rpm and elution at 2.0 mL min^{1}, and 2.8mL min^{-1}. HSCCC using the solvent system **C** carried out using a rotation speed of 800 rpm and elution at 2.8 mL min^{-1}, and gave the three fractions F′-1, F′-2 and F′-3 (Fig 2) which were orange in colour. Elution speeds of 2.0 mL min^{-1}, at a rotation speed of 800 rpm gave the best separation.Fractionations were also carried out using a rotation speed of 1000 rpm and elution at 2.0 mL/min and 2.5 mL/min.

Use of a lower flow rate has been recommended when K_D value of the analyte is small and the settling time is around 20 s (Ito, 2005).The flow rates used in the present study were 2.8mL min^{-1}. and 2.0mL min^{-1}respectively for solvent systems **B** (revolution speed 1000 rpm) and **C** (revolution speeds 800 rpm), because separation of TFs was best at these flow rates. It was observed that flow rates greater than 2.9 mL min^{-1} led to loss of stationary phase while flow rates less than 2 mL min^{-1} were not studied since the time required for an HSCCC separation was too long.

Preparation of a sample solution for HSCCC depends on the solubility of the sample in the stationary phase, the mobile phase or in a mixture of two phases. In our study the sample (200 mg) was dissolved completely in 5 mL of the aqueous mobile phase, because it was readily soluble in this phase. After repeated HSCCC runs it was found that the maximum quantity of the TF sample that could be dissolved in 5 mL of the aqueous phase was 200 mg for effective separation. If the amount of sample added to 5 mL was too high (> 300 mg), the sample was only partially soluble and particles were observed in the sample solution. It was found that loss of stationary phase occurred when the concentration of solute was high. This was attributed to the particles formed which led to blocking of the column.In our study, the lower aqueous phase was used as the mobile phase since TFs are polar compounds and dissolved readily in the aqueous phase. It was observed that if the lower aqueous phase is used as the mobile phase, the system provided more stable retention of stationary phase and prevented trapping of air bubbles in the flow cell.Yang *et al.* (2008) have reported that 400 mg of the crude TF mixture was dissolved in 2 ml of the aqueous phase (used as the mobile phase), while using the HEMW at system 1:3:1:6 v/v. However in our hands, best HSCCC separations were obtained at much lower concentrations of the TF sample (150 -200 mg) dissolved in 5 ml of the aqueous mobile phase. Wang *et al.* (2008)used a 30 mg sample of crude TF in 5 ml of the mobile phase while the solvent system used for HSCCC was hexane-ethylacetate-methanol water-acetic acid (1:5:1:5:0.25 v/v). The weights of three fractions

obtained in TF separation 1 were F-1 **(**9 mg**)**, F-2 **(**11 mg**)** and F-3 **(**1.4 mg**).** Weights of three fractions obtained in TF separation 2 were F′-1 **(**8 mg**)**, F′-2 **(**2 mg**)** and F′-3 **(**9 mg**).** In both HSCCC separations TF3MG **(2)** and TF3'MG **(3)** were obtained as a mixed fraction and not separated from each other**.**

HPLC of fractions and identification of TFs

The HPLC traces of the three fractions are shown in Figure 3 and Figure 4**.** F-1 in Figure 3 was identified to be TF **(**1**)**, F-2 and F-3 were a mixture of TF3G**(**2**)** and TFDG **(**4**).** F-3 was found to be a mixture of TF3′G **(**3**)** and TFDG **(**4**).**The TF fractions of HSCCC separation-2 were identified by HPLC analysis and combined to give F′-1, F′-2 and F′-3**.** The HPLC trace shown in Figure 4 shows that the purity of the F′-1and F′-3 were approximately 90**%-**95 **%** while F'-2 was a mixed fraction**.**F'-1was identified as TF**(**1**).** F'-2 was a mixture of TF3G **(**2**)** and TF3′G **(**3**)**, and F'-3 was TFDG**(**4**).** Each HPLC fraction was identified by comparison with authentic HPLC standards available at the TRI, Talawakelle**.** Structures of the major TFs in black tea TFs **(**1**) – (**4**)** are given in Figure**.** 5**.**

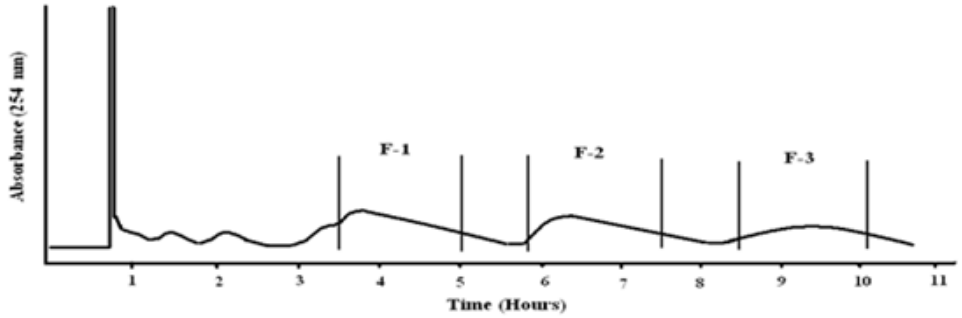

Figure 1: HSCCC separation 1 of theaflavin crude extract from black tea (254 nm), Solvent system Hex:EtOAC:MeOH:water (1:4:1:4), elution 2.8 ml/min, 1000rpm, sample size 200mg

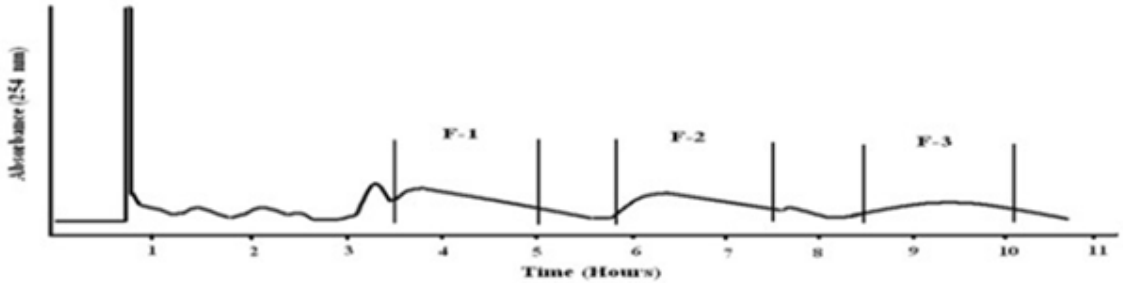

Figure 2: HSCCC separation 2 of theaflavin curde extract from black tea (254 nm), Solvent system Hex:EtOAc:MeOH:water (1:5:1:5) elution 2.0 mL/min, 800 rpm, sample size 200mg

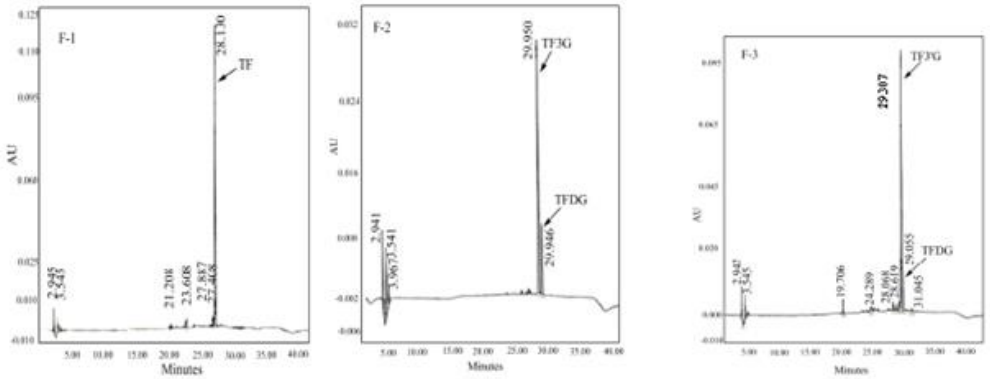

Figure 3: HPLC traces of fractions F-1, F-2, F-3 after HSCCC separation-1 using solvent system B Hex:EtOAc:MeOH:water (1:4:1:4)

Conditions: Luna-5 µm Phenyl-Hexyl colum 4.6 ×250 mm with guard column of the same material: binary gradient with 9% (v/v) acetonitrile, 2% (v/v) acetic acid with 20 µg/ml EDTA (mobile phase A) 80% (v/v) acetonitrile, 2% (v/v) acetic acid with 20 µg/ml EDTA (mobile phase B).

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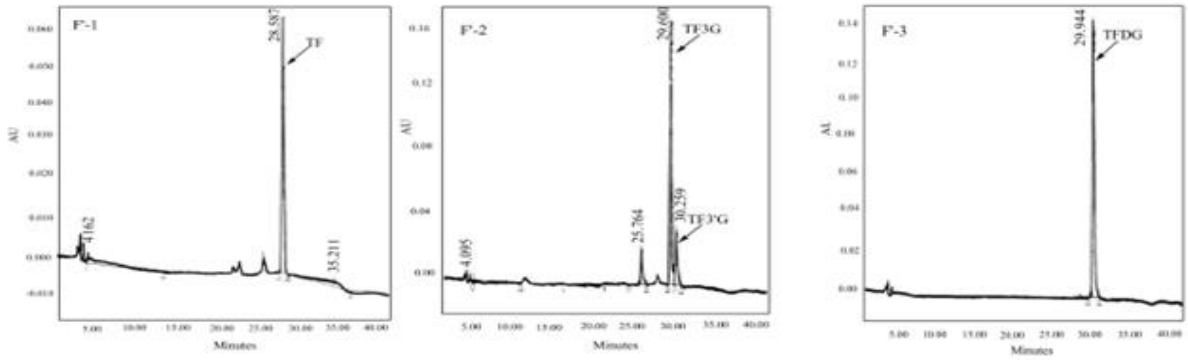

Figure 4. HPLC traces of fractions F'- 1, F'-2, F'- 3 after HSCCC separation- using the solvent system C Hex:EtOAc:MeOH:water (1:5:1:5)

Conditions: Luna - 5µm Phenyl-Hexyl column 4.6×250nm with a guard column of the same material: binary gradient with 9% (v/v) acetonitrile, 2% (v/v) acetic acid with 20 µg/ml EDTA (mobile phase A) 80% v/v) acetonitrile, 2% (v/v) acetic acid with 20 µg/ml EDTA (mobile phase B).

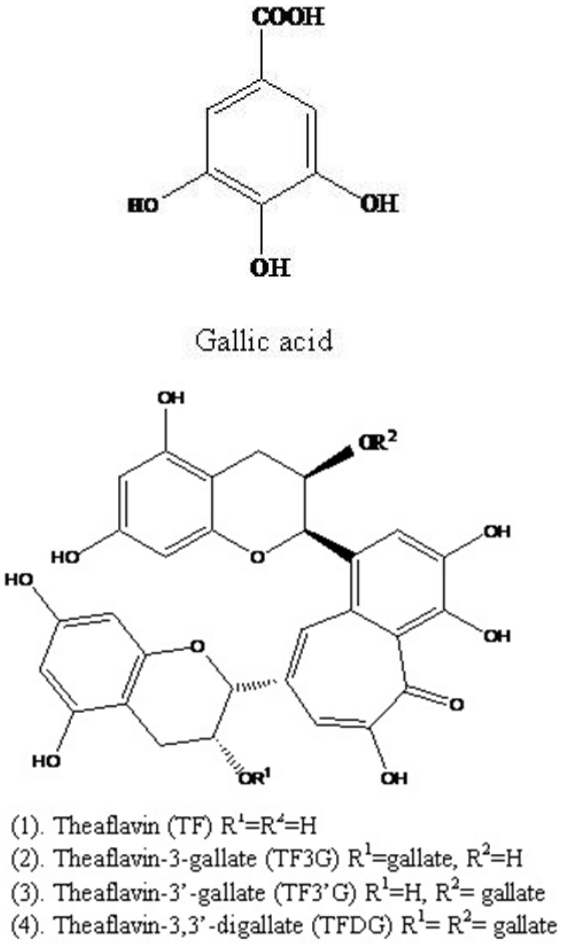

Figure 5: Four major theaflavins found in black tea.

A black tea sample boiled with water, then partitioned with EtOAc was used by **(**Degenhardt *et al.*, 2000**)** to separate TFS using the solvent system, Hex**:** EtOAc**:** MeOH**:** Water **(**1**.**5**:**5**:**1**.**5**:**5**).** In this study a clean-up procedure of the EtOAc extract on Sephadex LH-20 was carried out prior to HSCCC**.** TF **(**1**)** was isolated in high purity whereas TF3G **(**2**)** and TF3′G **(**3**)** were isolated as a mixture and isolation of TFDG was not reported**.**Cao *et al***. (**2005**)** determined $K_D$of monomeric TFs and investigated several different compositions of solvent systems for the separation of a crude mixture of TFs from black tea, optimized conditions for the HEMW at system **(**1**.**25**:** 5**:** 1**.**25**:** 5**)** and obtained similar results**.** In this study too, TF**(1)** and TFDG**(**4**)** were isolated in high purity whereas TF3G **(**2**)** and TF3′G **(**3**)** were isolated as a mixture**.**A similar result was obtained in our study where HSCCC was carried out without a preliminary step involving Sephadex LH-20 chromatography, using the HEMWat solvent systems C**.** The work-up procedure was therefore minimized by leaving out the clean-up procedure using Sephadex LH-20**.** Results of these separations indicated that two monogallates, TF3G**(**2**)** and TF3′G **(**3**)** are not separated in high purity by a single HSCCC run using the HEMW at solvent systems used in these studies**.**It may be possible to separate TF and TFDG from the two TFMGs by HSCCC, separate the fractions, reduce the sample size and then carry out a second HSCCC of the mixed TFMG fractions, using a different biphasic solvent system**.** Cao *et al***.**, **(**2004**)**suggested that HPLC can be used for the separation of the two TF monogallates but resolution was decreased with increase in sample size and therefore application for the separation of TFs on a large scale was limited**.**

CONCLUSIONS

Our study indicated thatisobutylmethyl ketone is a useful solvent for the separation of TF extracts from a water infusion of black tea leaves**.** A smaller sample size **(**150-200 mg**)** dissolved in 5 ml was found to give the best HSCCC separation**.** Butit is not possible to separate all four TFs by a single HSCCC run using the HEMW at solvent systems 1**:**5**:**1**:**5 and 1**:**4**:**1**:**4 **(**v/v/v/v**).** It may be possible to separate TF and TFDG from the two TFMGs by HSCCC, separate the fractions, and then carry out a second HSCCC of the TFMGs, using a different biphasic solvent system**.**

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Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper**.**

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