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Short communication

Separation of catechin constituents from five tea cultivars using high-speed counter-current chromatography

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Abstract

Catechins were extracted from five different tea (*Camellia sinensis* L.) cultivars. High-speed counter-current chromatography was found to be an efficient method for the separation of seven catechins from the catechin extracts. High-performance liquid chromatography was used to assess the purity of the catechins isolated. Epigallocatechin gallate (EGCG), epicatechin gallate (ECG) and epigallocatechin (EGC) of high purity (91–99%) were isolated in high yield after a single high-speed counter-current chromatography run. The two-phase solvent mixtures used for the separation of the catechin extracts were hexane:ethyl acetate:methanol:water (1:6:1:6 for TRI 2023); (1:7:1:7 for TRI 2025 and TRI 2043); (1:5:1:5 for TRI 3079) and (1:6.5:1:6.5 for TRI 4006). Fresh tea shoots from the tea cultivar TRI 2023 (150 g) gave 440 mg of 96% pure EGCG while TRI 2025 (235 g) gave 347 mg of 99% pure EGCG and 40 mg of 97% ECG, and TRI 3079 (225 g) gave 432 mg of 97% pure EGCG and 32 mg of 96% pure ECG. Tea cultivar TRI 4006 (160 g) gave EGCG (272 mg, 96% pure) and EGC (104 mg, 90% pure). ^{1}H and ^{13}C NMR chemical shifts for catechin gallate (CG), EGC, ECG, EGCG and epigallocatechin 3,5-di-*O*-gallate (EGCDG) in CD_3OD were also recorded.

Keywords: Tea catechins; Flavan-3-ols; High-speed counter-current chromatography separation; Purity by high-performance liquid chromatography; ^{1}H and ^{13}C NMR

1. Introduction

Catechins (flavan-3-ol's) are the predominant components of young vegetative shoots of tea (*Camellia sinensis* L.). Tea catechins, which are responsible for most of the useful biological effects of tea [1–3] are powerful antioxidants that may help to offset chronic diseases such as cancer and cardiovascular diseases. Tea catechins have many other interesting effects including the ability to inhibit the biological activities of cariogenic streptococci such as *Streptococcus mutans* and *Streptococcus sobrinus* [4] and have been reported to be implicated in the reversing of methicillin resistance in the methicillin resistant 'super-bug' of *Staphylococccus aureus* [5]. Epicatechin gallate (ECG) and epicatechingallocatechin gallate (EGCG) from green tea leaves have been reported to exhibit 50% inhibition of the AIDS virus at 0.01–0.02 μg/ml [6]. Ready availability of pure samples of tea catechins would facilitate clinical and other studies that are required to exploit their biological properties. In this paper we report the results of a study carried out on the use of high-speed counter-current chromatography [7] to separate tea catechins. Catechin samples of high purity were isolated after a single high-speed counter-current chromatography run. Methods used previously for the separation of catechins include preparative Sephadex LH-20 column chromatography [8] and/or high-performance liquid chromatography [9]. These methods required large amounts of solvent, were time consuming and the catechin mixtures obtained required further chromatography to obtain catechin samples of high purity. Degenhardt et al. [10] separated green tea catechins using high-speed counter-current chromatography, but the purity of the catechins isolated was not reported. Three catechins of high purity were sepa-

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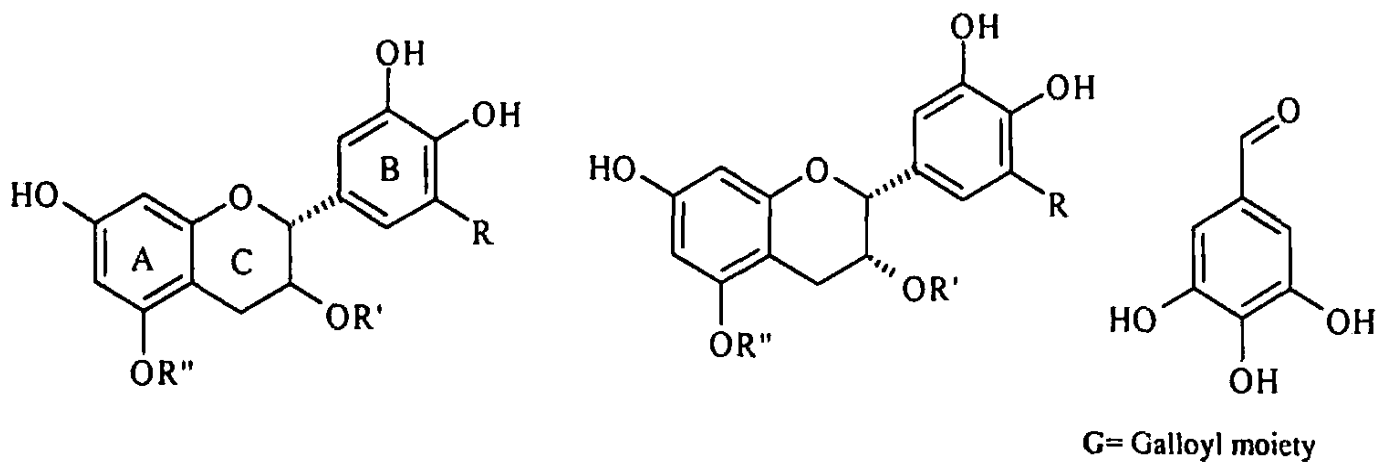

	R	R'	R''
Catechin(1)	H	H	H
CG (2)	H	G	H

	R	R'	R''
EC (3)	H	H	H
EGC (4)	OH	H	H
ECG (5)	H	G	H
EGCG (6)	OH	G	H
EGCDG (7)	OH	G	G

Fig. 1. Structure of tea catechins.

rated by Du et al. [11] using counter-current chromatography, while a low speed counter-current chromatographic system using a slowly rotating helical device, was used by Cao et al. [12] for the industrial separation of a crude tea extract.

In the present study catechins were extracted from five different tea cultivars and fractionated using high-speed counter-current chromatography [7]. Seven catechins (Fig. 1) including (−)-catechin (C, **1**), (+)-catechin gallate (CG, **2**), (−)-epicatechin (EC, **3**), (−)-epigallocatechin (EGC, **4**), (−)-epicatechin gallate (ECG, **5**), (−) epigallocatechin gallate (EGCG, **6**) and (−)-epigallocatechin-3,5-di-*O*-gallate (EGCDG,**7**) were isolated after high-speed counter-current chromatography. The identity of the catechins was established using ^{1}H and ^{13}C NMR. A significant result was the isolation of 440 mg of 96% pure epi-gallocatechin gallate (EGCG, **6**) from 800 mg of the ethyl acetate extract from the tea cultivar TRI 2023 after a single high-speed counter-current chromatography run. Similarly the tea cultivar TRI 2025 yielded 99% pure EGCG constituting 347 mg per 800 mg of ethyl acetate extract. The purity of the fractions isolated was estimated high-performance liquid chromatography. EGCG has been associated with many of the important biological effects of tea catechins.

2. Experimental

2.1. Collection of plant material

Fresh tea shoots (two leaves and a bud) of the tea cultivars TRI 2023, TRI 2025, TRI 2043, TRI 3079 and TRI 4006 were collected from the Tea Research Institute sub-station at Hantane, Kandy, Sri Lanka.

2.2. Preparation of catechin extracts

The crude catechin extracts from the shoots of cultivars TRI 2023 (150 g), TRI 2025 (235 g), TRI 2043 (85 g), TRI 3079 (225 g) and TRI 4006 (160 g) were extracted (5 min)with boiling aqueous 70% MeOH (500 ml × 2), concentrated on a rotavapor (40°) and partitioned with an equal volume of light petroleum (40–60). The aqueous layer was partitioned with EtOAc and the EtOAc layer was concentrated on a rotavapor (<40 °C). The sticky residue obtained was dissolved in water and freeze dried to obtain the catechin extracts as pale yellow solids from TRI 2023 (5.0 g), TRI 2025 (4.75 g), TRI 2043 (600 mg), TRI 3079 (6.0 g) and TRI 4006 (3.0 g).

2.3. Solvent system for high-speed counter-current chromatography

Two-phase solvent mixtures containing different ratios of hexane, EtOAc, MeOH and water were prepared (Table 1). Solvent mixtures which gave approximately equal volumes of each phase were used to determine the partition coefficient K for each catechin extract according to the procedure described by Ito and Conway [7]. A small amount of the cat-

Table 1
Two-phase solvent systems selected for HSCCC separation of catechin mixture isolated from each tea cultivar

Tea cultivar	Hex	EtOAC	MeOH	H_2O	K
TRI 2023	1	6	1	6	1.09
TRI 2025	1	7	1	7	1.00
TRI 2043	1	7	1	7	1.06
TRI 3079	1	5	1	5	1.06
TRI 4006	1	6.5	1	6.5	1.22

echin mixture was dissolved in a selected two-phase solvent system (1–2 ml of each phase) and equilibrated thoroughly in the test tube. After the two layers separated clearly, an aliquot of each phase was mixed with a solvent such as MeOH and the absorbance at 278 nm was determined using a Shimadzu UV-1601 UV–vis spectrophotometer. The partition coefficient was calculated from the equation

$$K = C_S/C_M$$

where C_S is the concentration of the solute in the stationary phase and C_M concentration of the solute in the mobile phase. K was then determined using the equation $K = A_S/A_M$ derived from Beer-Lambert's law, where concentration (C) $\propto$ absorbance (A). It was observed that the solvent system with a partition coefficient K close to 1, gave the most effective separation. The solvent system selected for TRI 2023 for example, was hexane (1): EtOAc (6): MeOH (1): H_2O (6) with $K = 1.09$ (Table 1).

2.4. High-speed counter-current chromatography

High-speed counter-current chromatograph Model CCC-1000-Pharma-Tech Research Corporation, USA was used for the separation. The preparative coil used in the separation had an i.d. of 2.6 mm, a β value of 0.8, volume capacity 300 ml, and a revolution radius of 3″. The solvent system selected was equilibrated at room temperature in a separatory funnel and the two phases separated shortly before use. In each separation, the multilayer coil was entirely filled with the upper organic phase (SP) at a flow rate of 9.5 ml/min. When the SP emerged from the tail end of the instrument, the flow was stopped and the coil planet centrifuge was allowed to rotate at 800 rpm, because a rotational speed of 800 rpm gave the best separation. When the speed stabilized, the reference cell of the UV detector was filled with the mobile phase (MP).

The lower aqueous phase was then pumped in to the inlet of the column at a flow rate of 1.5 ml/min. After the elution of 50–60 ml of SP, the sample (dissolved in 5 ml of MP) was injected in to the coil through the sample injection port. The effluent from the outlet of the column was continuously monitored with a UV detector and the fractions were collected manually, according to the absorbance plot obtained from the recorder (Fig. 2). A new fraction was collected when there was a change observed in the absorbance plot. After about 3–3.5 h separation in the head-tail mode, the elution mode was reversed to tail–head, by switching the solvent-changing valve from the head-tail elution mode to tail–head elution mode, and the SP was pumped through the inlet to obtain tail–head fractions. The tubes collected immediately after changing the elution mode to tail–head were green in colour (due to residual chlorophyll) and were discarded. The fractions collected were analysed by thin layer chromatography (TLC, 10% MeOH in EtOAc, Merck Silica Gel 60 F_{254}), combined accordingly and then concentrated on a rotavapor and freeze dried. Optical rotations were measured in methanol using a Bellingham and Stanley. ADP 220 polarimeter at 27 °C.

2.5. High-performance liquid chromatography

Analysis of fractions was carried out using high-performance liquid chromatography on a Waters Model 2690 analytical high-performance liquid chromatography system consisting of a photodiode array detector (Waters Model 996), a Millennium 2010 data processor, and a μ-Bond pack C_{18} column (30 cm). The mobile phase composed of *N*,*N*-dimethyl formamide:MeOH:AcOH:H_2O (20:1:0.5:78.5) was eluted in isocratic mode at a flow rate of 1.5 ml/min and the effluents were monitored at 278 nm. Standard reference samples of catechins for HPLC were obtained from the Tea Research Institute of Sri Lanka.

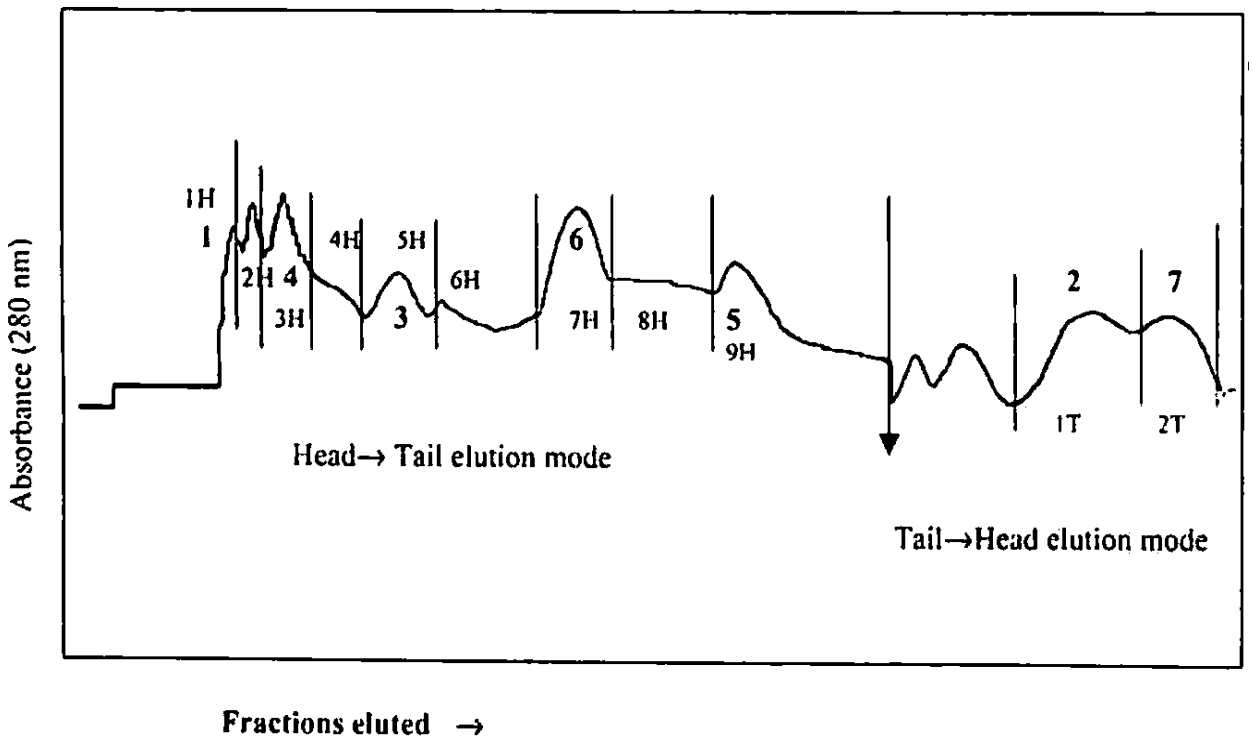

Fig. 2. HSCCC separation of the catechin crude extract from tea cultivar TRI 2025 Solvent system used Hex:EtOAc:MeOH:water 1:7:1:7. (+) Catechin **1**, (+) CG **2**, (−) EC **3**, (−) EGC **4**, (−) ECG **5**, (−) EGCG **6**, (−) EGCDG **7**, 1H–9H (head–tail fractions), 1T–2T (tail–head fractions).

2.6. ^{1}H and ^{13}C NMR spectroscopy

^{1}H and ^{13}C NMR spectroscopy were performed on a Varian Mercury 300 MHz instrument using CD_3OD as the solvent. All spectra were recorded at 30 °C. Chemical shifts are recorded in ppm.

3. Results and discussion

The aqueous MeOH extract of fresh moist tea leaves after concentration and partition with EtOAc afforded a pale yellow powder consisting mainly of catechins.

The crude catechin mixture (800 mg) from tea cultivar TRI 2025 on separation by high-speed counter-current chromatography gave six prominent peaks (1H, 2H, 3H, 5H, 7H and 9H) in the head-tail elution mode and two (without the cholorophyll containing peaks) in the tail–head elution mode (Fig. 2). The identities of the constituents of each fraction were established by comparison with a standard reference sample containing gallic acid, caffeine, theobromine, catechin (**1**), EC (**3**), EGC (**4**), ECG (**5**), EGCG (**6**). The identities of catechin gallate (CG, **2**, $[\alpha]_D + 30°$ MeOH c 0.44) and epi-gallocatechin 3,5-di-*O*-gallate (EGCDC, **7** $[\alpha]_D - 100°$ MeOHc 0.35), which were not in the standard reference sample, were established by 1D and 2D NMR experiments, and mass spectral data.

The composition and purity of seven catechin fractions separated from TRI 2025 are shown in Table 2. The levels of purity observed for EGCG (**6**, 99%), ECG (**5**, 97%), CG (**2**, 90%) and EGCDG (**7**, 93%) are noteworthy. Our results indicate that it is possible to separate a 99% pure fraction of EGCG from a single high-speed counter-current chromatography run. Tea cultivar TRI 2025 yielded 304 mg of 99% EGCG and 42.4 mg of 97% ECG from 800 mg of the EtOAc extract. The purine alkaloids theobromine and caffeine, were also separated during the same high-speed counter-current chromatography run.

Similarly the EtOAc fractions isolated from the tea cultivars TRI 2023, TRI 2043, TRI 3079 and TRI 4006 were separated by high-speed counter-current chromatography. The most abundant catechin isolated from all five tea cultivars was found to be EGCG (**6**). EGCG was isolated in high yield (440 and 432 mg respectively) from 800 mg of the EtOAc extract of TRI 2023 and TRI 3079. EGCG isolated from a single high-speed counter-current chromatography run of all five tea cultivars, was found to be 96–99% pure. Theobromine and EGC were found in all five tea cultivars. Catechin (**1**), CG (**2**) and EGCDG (**7**) were obtained in low yield from some of the tea cultivars.

Data on the ^{1}H and ^{13}CNMR of green tea catechins in DMSO-d_6 and acetone-d_6 have been reported previously by Davies et al. [13], Hashimoto et al. [8]. In this paper we report ^{1}H and ^{13}C NMR data for EGCG, ECG EGC, CG and EGCDG in CD_3OD (Tables 3 and 4, respectively). The assignments were achieved primarily by the means of proton-carbon correlation methods, specifically HMQC and HMBC experiments for long range correlations). NMR chemical shifts for EGCDG (**7**) given in Tables 3 and 4 have not been reported previously.

^{13}C NMR chemical shifts of the carbons in **7** were comparable with those of **6**. The two proton singlets at δ 6.97 and 7.21 ppm were assigned to the two galloyl moieties in **7**. A singlet at δ 6.54 ppm was due to the two protons of the flavan ring B and two ^{1}H doublets at δ 6.40 and 6.32 ppm to the two protons of the ring A. These observations suggest the presence of an epigallocatechin moiety with the substitution of another galloyl unit at either C-5 or C-7 position in ring A. The signal at δ 25.8 ppm in ^{13}C NMR was attributed to C-4 in ring C, while the signals at δ 77.2 and 68.6 ppm were assigned to C-2 and C-3, respectively. Carbons at C-6 and C-8 of ring A resonated at δ 103.1 and 101.3 ppm while C-5 and C-7 shifted further downfield (δ 150.6 and 156.7 ppm). The difference in the chemical shifts of C-5 in **6** and **7** are significant and may be attributed to the presence of a galloyl moiety at C-5 in **7**. Signals due to C-3′ and C-5′ atoms were shifted downfield (δ 145.2 ppm) as expected.

The CI mass spectrum of **7** showed a peak at *m/z* 628 ($M^+ + NH_3$) and peaks at *m/z* 611 (M^+), 476, 460 and 289. The peaks at *m/z* 476 and 289 were ascribed to fragment ions from the loss of one and two galloyl moieties respectively, with cleavage of the CO–O bond of a flavon-3-ol. Therefore, catechin **7** was identified as epigallocatechin-3-5-*di*-*O*-gallate.

The M^+ (*m/z* 442, $C_{22}H_{18}O_{10}$) and a major fragment peak at *m/z* 271 in the mass spectrum of **2** confirmed the presence of the flavon-3-ol moiety with a galloyl residue in the molecule. Although the ^{1}H NMR and ^{13}C NMR spectra of **2** and **5** were similar, compound **5** was eluted in the head-tail phase and compound **2** eluted in the tail–head phase.

Table 2
Weight (mg) of catechins from EtOAc extract of each tea cultivar and % purity of the catechins isolated from the EtOAc extracts from a single HSCCC run

Weight of catechins in mg and (% purity)									
Tea cultivar	Weight of extract (mg)	C (1)	CG (2)	EC (3)	EGC (4)	ECG (5)	EGCG (6)	EGCDG (7)	Theobromine
TRI 2023	800	–	–	–	24 (87)	–	440 (96)	12 (90)	7 (75)
TRI 2025	800	0.9 (40)	8 (90)	40 (76)	24 (52)	40 (97)	347 (99)	8 (93)	2 (87)
TRI 2043	200	–	–	18 (52)	Trace	8 (91)	48 (96)	–	2 (48)
TRI 3079	800	Trace	–	–	Trace	32 (96)	432 (97)	–	1 (98)
TRI 4006	800	Trace	16 (90)	–	104 (90)	–	272 (96)	–	6 (77)

Table 3
^{1}H NMR data of tea catechins in deuterated methanol

Chemical shift (δ_H, ppm) in CD_3OD					
Chemical unit	CG (2)	EGC (4)	ECG (5)	EGCG (6)	EGC3, 5G (7)
Flavon					
H-2	5.03, brs (1H)	4.65, brs (1H)	5.10, brs (1H)	4.86, brs (1H)	5.02, brs (1H)
H-3	5.53, m (1H)	4.06, m (1H)	5.42, m (1H)	5.42, m (1H)	5.41, m (1H)
H-4	2.67–2.92, m (2H)	2.59–2.78, m (2H)	2.67–2.92, m (2H)	2.71–2.92, m (2H)	2.80–3.02, m (2H)
H-6	5.98, s (2H)	5.81, d (1H) $J=2$ Hz	5.91, s (2H)	5.87, s (2H)	6.32 d (1H), $J=2.4$ Hz
H-8	5.98, s (2H)	5.83, d (1H) $J=3$ Hz	5.91, s (2H)	5.87, s (2H)	6.41 d (1H), $J=2.4$ Hz
H-2′	6.94, d 1H $J=2$ Hz	6.41, s (2H)	6.90, d (1H) $J=2$ Hz	6.42, s (2H)	6.54, s (2H)
H-6′	6.83, dd 1H $J=2$ Hz	6.41, s (2H)	6.80, dd (1H) $J=2$ Hz	6.42, s (2H)	6.54, s (2H)
H-5′	6.72, d 1H $J=8$ Hz	–	6.69, d (1H) $J=8$ Hz	–	–
Galloyl					
H-2, H-6(1)	6.96, s (2H)		7.00, s (2H)	6.86, s (2H)	6.97, s (2H)
H-2, H-6(2)					7.21, s (2H)

brs—broad signal, m—multiplet, d—doublet, dd—double doublet, s—singlet.

Table 4
^{13}C NMR data for tea catechins in CD_3OD

Chemical shift/δ_C (ppm)					
Chemical unit	CG (2)	EGC (4)	ECG (5)	EGCG (6)	EGC-3, 5G (7)
Flavon					
C-2	77.5	78.7	77.6	77.5	77.5
C-3	68.9	67.4	68.4	68.9	68.6
C-4	25.7	29.2	25.7	25.9	25.8
C-4a′	98.3	99.9	98.3	98.3	104.0
C-6	95.5	96.2	95.6	95.4	103.1
C-8	94.8	95.7	95.9	94.7	101.3
C-5, C-7, C-8a	156.7, 156.7, 156	157.4, 157.1, 156	156.2, 156.2, 156	156.4, 156.4, 155.8	150.6, 156.7, 155.9
C-1′	130.3	131.2	129.9	129.5	129.3
C-2′, C-6′	114.9, 113.9	106.8	114.6, 113.8	105.7	106.3
C-3′, C-5′	145.1, 118.3	146.3	145.0, 118.2	145.3	145.2
C-4′	138.6	133.2	131.2	132.5	132.6
Galloyl					
C-1	120.4	–	120.4	120.3	120.5, 119.5
C-2, C-6	109.1	–	109.1	109.1	109.6, 110.1
C-3, C-5	14408	–	144.8	144.9	138.4, 139.1
C-4	144.7	–	138.6	138.4	144.7, 145.2
C=0	166.5	–	166.5	166.4	166.5, 165.6

Comparison of the specific rotation of **2** ($[\alpha]_D$ +30 c 0.5) and **5** ($[\alpha]_D$ − 114 c 0.8), and by high-performance liquid chromatography indicated that the compounds were different. Since catechin **5** was identified as epicatechin gallate by comparison with the high-performance liquid chromatography reference sample, catechin **2** was identified as its 3-epimer, catechin 3-*O*-gallate.

Our results indicate that high-speed counter-current chromatography provides a convenient and efficient method for the separation of tea catechins. It is possible to obtain a high yield of highly pure EGC (**4**), ECG (**5**), and EGCG (**6**), from a single high-speed counter-current chromatography run. The method could be extended, through repeated high-speed counter-current chromatography runs, to obtain pure samples of the less common catechins present in fresh tea leaves.

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