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Journal of Chromatography A, 898 (2000) 75–81

JOURNAL OF CHROMATOGRAPHY A

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Supercritical fluid extraction of catechins from *Cratoxylum prunifolium* Dyer and subsequent purification by high-speed counter-current chromatography

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Received 6 March 2000; received in revised form 18 July 2000; accepted 18 July 2000

Abstract

Supercritical fluid extraction of tea catechins including epigallocatechin-3-*O*-gallate (EGCG) and epicatechin-3-*O*-gallate (ECG) from *Cratoxylum prunifolium* Dyer was performed. The optimization of parameters was carried out using an analytical-scale supercritical fluid extraction (SFE) system designed in our laboratory. Then the extraction was scaled up by 100 times using a preparative SFE system under a set of optimized conditions of 40°C, 25 MPa and modified CO_2 with 80% ethanol aqueous solution. The combined yield of EGCG and ECG reached about 1 mg per 1 g of tea leaves where the solubility was near 1.4×10^{-4} mass fraction of CO_2 fluid. EGCG and ECG of high purity (>98%) were obtained from the crude preparative extract by high-speed counter-current chromatography. © 2000 Published by Elsevier Science B.V.

Keywords: Counter-current chromatography; *Cratoxylum prunifolium*; Tea; Food analysis; Catechins

1. Introduction

In view of increasing environmental and health concern about the use of organic solvents in the extraction of natural products, there has been growing interest in using supercritical fluids. Supercritical fluid extraction (SFE) has been demonstrated to be a valuable alternative for it requires less solvent, has a short extraction time and is capable of extracting thermally labile compounds under mild conditions. In addition, by selecting the fluid polarity and/or density, the solvating power of the fluid can be adjusted for selective extraction, and extraction fluids can be removed from the fractions by decompression into a suitable collection device. SFE has been applied to a wide range of non-polar biologically active constituents from natural products, including essential oils, other flavor and fragrance compounds, medicinal compounds, carotenes and alkaloids, but there is still a shortage of information on extraction of more polar compounds [1–3].

This paper reports our studies on SFE for epigallocatechin-3-*O*-gallate (EGCG) and epicatechin-3-*O*-gallate (ECG) from the leaves of *Cratoxylum prunifolium* Dyer, a green tea. These catechins have been known to possess anti-mutagenic [4] and anti-tumorigenic properties [5]. There are few publications describing the SFE for catechins

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0021-9673/00/$ – see front matter
PII: S0021-9673(00)00788-3

since a high polarity of these polyphenols makes the SFE inefficient. Only Li and Feng reported some studies on the solubility of a mixture of tea catechins in supercritical CO_2 fluid [6].

In our experiment, the extraction condition was optimized first by an analytical-scale SFE system made in our laboratory using an orthogonal test design. Then, the extraction was scaled up by 100 times by a preparative-scale SFE system. The obtained crude extract was purified by high-speed counter current chromatography (HSCCC).

2. Experimental

2.1. Reagents

Carbon dioxide (CO_2, with 99.95% purity) was obtained from Beijing Analytical Instrument Factory, Beijing, China. Organic solvents including ethanol, hexane, ethyl acetate, *N*,*N*-dimethylformamide (DMF), and methanol were all of analytical grade and purchased from Beijing Chemical Factory, Beijing, China.

EGCG and ECG (>98% purity by HPLC analysis) used for quantitation were prepared in our laboratory from a catechin mixture mainly containing EGCG, GCG and ECG (Chinese Green Tea Company) using HSCCC under a similar condition described in this article. Caffeine was purchased from the National Institute of the Control of Pharmaceutical and Biological products, Ministry of Health, Beijing, China. The leaves of *Cratoxylum prunifolium* Dyer used as the extraction sample were collected from Sanya, Hainan Province in China.

2.2. Optimization of SFE conditions

An analytical-scale SFE system is illustrated in Fig. 1. It was designed and fabricated in our laboratory for optimizing extraction conditions. The volume of extraction cell was 10 ml. In order to determine a suitable extraction condition in a wide range with a minimum number of trials, an orthogonal test design $L_9(3)^3$ was employed where temperature, pressure and modifier were considered to be three major factors for effective extraction. Supercritical CO_2 modified with an ethanol aqueous solution was used as the extraction fluid referring to report [6]. The combinations of three different levels of each factor were listed in Table 1. In each test, 2 ml of the modifier was directly added to 5 g of sample tea leaves which were placed into extraction cell. After l h of static extraction (no liquid flow), the sample was subjected to dynamic extraction for 1 h with flowing liquid CO_2 at a rate of 0.4 ml/min. The extract was trapped into a collection vessel containing about 20 ml of ethanol. The dissolved portion was concentrated and analyzed by HPLC.

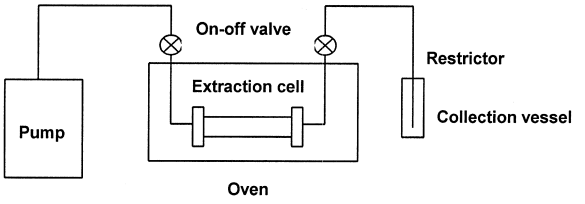

Fig. 1. Diagram of the analytical SFE system designed in our laboratory.

2.3. Scaling up SFE

Under the optimized SFE conditions determined above, the extraction was scaled up by about 100-fold using a preparative-scale SFE system (Fig. 2) manufactured by the Research Center for Eco-Environmental Science, Chinese Academy of Sciences, Beijing, China. A 485 g amount of sample leaves and 200 ml of 80% ethanol aqueous solution were placed into an extraction vessel with a 2-l capacity, and extracted statically for 1 h and dynamically for 1 h under the optimized condition at 40°C and 25 MPa

Table 1
L_9 $(3)^3$ orthogonal test design

Test no.	A: Temperature (°C)		B: Pressure (MPa)		C: Concentration of ethanol (aq) (%)	
Matrix 1	A_1	40	B_1	15	C_1	50
2	A_1	40	B_2	20	C_2	65
3	A_1	40	B_3	25	C_3	80
4	A_2	60	B_1	15	C_2	65
5	A_2	60	B_2	20	C_3	80
6	A_2	60	B_3	25	C_1	50
7	A_3	80	B_1	15	C_3	80
8	A_3	80	B_2	20	C_1	50
9	A_3	80	B_3	25	C_2	65

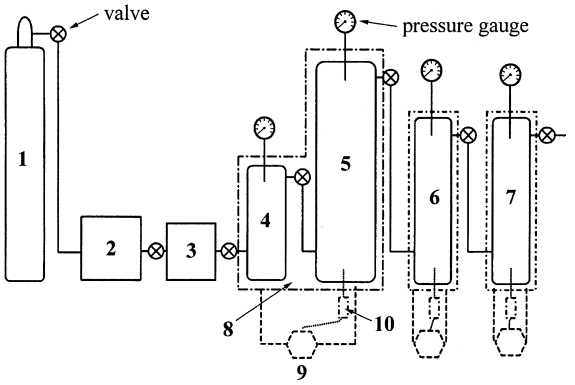

Fig. 2. Preparative-scale SFE system. 1: CO_2; 2: cryogenerator; 3: pump; 4: CO_2 preheater; 5: extraction vessel; 6: separator I; 7: separator II; 8: water circulator; 9: thermostatic control system; 10: temperature sensor.

with CO_2 containing 80% ethanol aqueous solution. The flow-rate of CO_2 supercritical fluid was set at 4 l/h and the extract in supercritical fluid was depressed directly into two separate vessels. The extract was collected and the ethanol soluble part was concentrated. Then it was cleaned by distributing between chloroform and water, and the water phase was extracted again with ethyl acetate to obtain a crude tea catechin extract. After concentrating and drying, it was subjected to HSCCC purification.

2.4. HSCCC separation procedure

The HSCCC separation was performed with a Model GS10A2 multilayer coil planet centrifuge produced by Beijing Institute of New Technology Application, Beijing, China. The separation column was prepared by winding a 1.6-mm I.D. PTFE (polytetrafluoroethylene) tube coaxially onto the column holder. The total column capacity was 260 ml.

Two solvent systems were prepared for HSCCC: hexane–ethyl acetate–water (1:20:30 and 1:3:4, v/v/v). Each solvent system was thoroughly equilibrated in a separatory funnel and two phases separated shortly before use.

In each separation, the coiled column was first entirely filled with the upper organic stationary phase. Then the apparatus was rotated at 800 rpm, while the lower aqueous mobile phase was pumped into the column at a flow-rate of 2.0 ml/min. After the mobile phase front emerged and the two solvent phases established hydrodynamic equilibrium in the column, the sample solution was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were collected according to the chromatogram. During separation EGCG and ECG fractions were protected by constantly flushing with N_2, and after separation they were lyophilized in the dark.

2.5. HPLC analysis

HPLC analyses of EGCG, ECG and caffeine were performed using a Shimadzu LC-10A, Class-Vp

Table 2
L_9 $(3)^3$ test results

Test no.	A 1	B 2	C 3	Extraction yield (%)[a]	Concentration (%)			Extraction yield (mg/g)[b]		
					EGCG	ECG	Caffeine	EGCG	ECG	Caffeine
1	A_1	B_1	C_1	3.84	0.03	0.05	23.9	0.001	0.002	0.92
2	A_1	B_2	C_2	6.76	0.41	0.07	25.5	0.028	0.005	1.79
3	A_1	B_3	C_3	9.76	1.77	1.82	25.4	0.173	0.178	2.48
4	A_2	B_1	C_2	5.22	0.33	0.06	26.7	0.017	0.003	1.39
5	A_2	B_2	C_3	9.08	1.01	0.55	29.9	0.092	0.05	2.72
6	A_2	B_3	C_1	6.5	0.03	0.03	23.5	0.002	0.002	1.53
7	A_3	B_1	C_3	3.72	0.83	0.24	42.5	0.031	0.009	1.58
8	A_3	B_2	C_1	6.2	0.02	0.03	29.5	0.001	0.002	1.83
9	A_3	B_3	C_2	9.06	0.15	0.04	28.7	0.014	0.004	2.60

[a] Extraction yield (‰)=the amount of extract/sample mass.
[b] Extraction yield (mg/g)=the amount of EGCG, ECG or caffeine in extract/sample mass.

system with a Phenomenex LUNA C_{18} column (150×4.6 mm I.D.). The mobile phase composed of a 1:1 mixture of A (acetic acid–methanol–water, 1:1:98) and B (acetic acid–methanol–water–DMF, 1:2:48:50) was isocratically eluted at a flow-rate of 1 ml/min, and the effluent was monitored with a photodiode array detector.

3. Results and discussion

3.1. Optimization of temperature, pressure and modifier for maximizing SFE efficiency

The extract obtained from each test in analytical SFE was quantitatively analyzed by HPLC for the amount of EGCG, ECG and caffeine. Results of the $L_9(3)^3$ tests presented in Table 2 indicated that extraction yield was no more than 1% and the total concentration of EGCG and ECG in the extract was much lower than that of caffeine. This may be in part due to the difference in the contents in the original sample, but it may also be caused by low solubility of tea catechins in supercritical CO_2 fluid. In fact the concentration of tea catechins was at the level of 10^{-6} mass fraction while that of caffeine was at 4.4×10^{-4} mass fraction [6].

In our experiment, using different concentrations of ethanol aqueous solution as modifier, extraction efficiencies at different sets of temperature and pressure were examined under $L_9(3)^3$ test design. The results shown in Table 2 revealed great differences between each set of SFE conditions. If the extraction yields of EGCG, ECG and caffeine were expressed as control index, the results in Table 2 are transformed to Table 3.

The yields of EGCG and ECG are found to be increased by higher ethanol concentration in the modifier, higher pressure, and lower temperature. It is known that high temperature tends to polymerize tea catechins. Under the optimal conditions of 40°C, 25 MPa, and CO_2 fluid modified with 2 ml of 80% ethanol aqueous solution, the total yield of EGCG and ECG can reach up to 0.35 mg per 1 g of tea leaves, which is nearly equivalent to 8.6×10^{-5} mass fraction supercritical CO_2 fluid, since the density of CO_2 at 40°C and 25 MPa is calculated as 0.85 g/ml. Thus, in our procedure the solvability can be increased by ten times over the earlier report [6].

As to caffeine extraction, the pressure and modifier were two most important factors where higher pressure and increased ethanol concentration enhances its SFE efficiency. Although the temperature did not significantly influence its yield, higher temperature appears favorable for caffeine extraction, and this was quite opposite to EGCG and ECG. Therefore, under the optimal conditions described above, EGCG and ECG can be selectively extracted to some extent.

Effects of each key factor on the yield of EGCG, ECG and caffeine are shown in Fig. 3.

Table 3
Analysis of L_9 $(3)^3$ test results

	EGCG yield (mg/g)			EGG yield (mg/g)			Caffeine yield (mg/g)		
	A	B	C	A	B	C	A	B	C
K_1	0202[a]	0.049	0.004	0.185	0.014	0.014	5.189	3.893	4.275
K_2	0.111	0.121	0.059	0.055	0.057	0.012	5.637	6.338	5.784
K_3	0.046	0.189	0.296	0.015	0.185	0.237	6.009	6.604	6.776
k_1	0067[b]	0.016	0.001	0.061	0.005	0.005	1.729	1.298	1.425
k_2	0.037	0.040	0.020	0.018	0.019	0.004	1.879	2.113	1.928
k_3	0.015	0.063	0.099	0.005	0.061	0.079	2.003	2.201	2.259
R	0.052[c]	0.047	0.098	0.056	0.056	0.075	0.274	0.903	0.834
Optimal level	A_1	B_3	C_3	A_1	B_3	C_3	A_3	B_3	C_3

[a] $K_i^A=\Sigma$ extraction yield at A_i.
[b] $k_i^A=K_i^A/3$.
[c] $R_i^A=\max\{k_i^A\}-\min(k_i^A\}$.

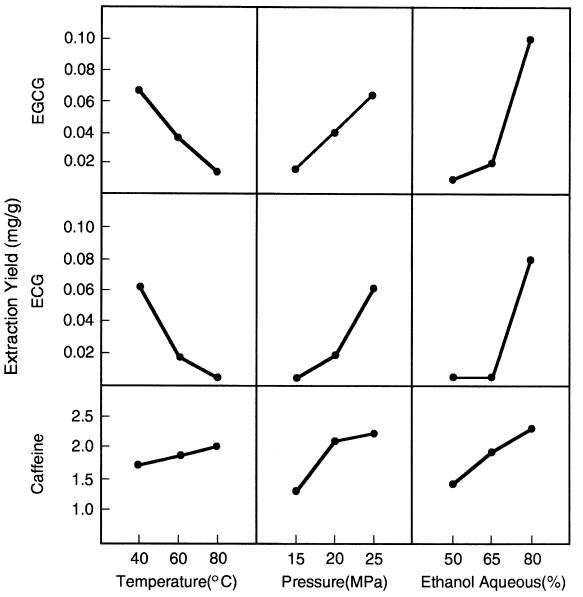

Fig. 3. Effects of temperature, pressure and modifier concentration on extraction yield.

3.2. Preparative-scale SFE

Under the optimal SFE conditions at 40°C, 25 MPa, and 80% ethanol concentration in the modifier, 485 g of sample tea leaves was extracted with 4 l of supercritical CO_2 containing 200 ml of modifier (corresponding to 4.7%, w/w modifier) yielding 3.7 g of ethanol soluble extract. HPLC analysis in Fig. 4 shows that the extract contained 22% caffeine, 6.8% EGCG and 6.5% ECG. The combined yield of

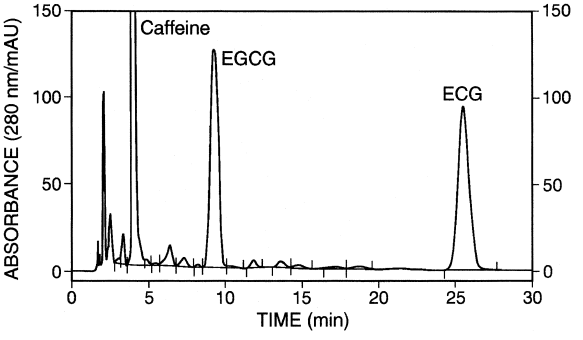

Fig. 4. HPLC analysis of the extract from preparative SFE. Sample: ethanol soluble part of the extract from preparative SFE extraction without any further treatment. Concentration was about 1 mg/ml.

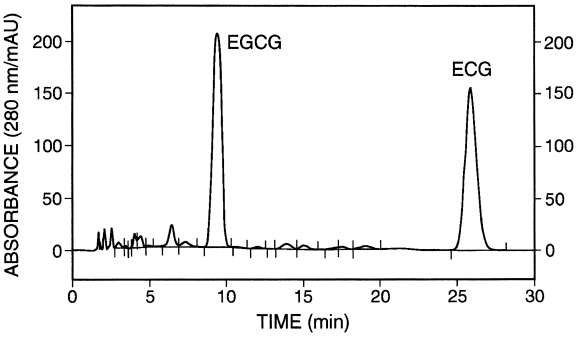

Fig. 5. HPLC analysis of the crude catechin mixture derived from SFE extract after cleaning-up.

EGCG and ECG can reach ca. 1 mg per 1 g of tea leaves, which is about equal to 1.4×10^{-4} mass fraction of supercritical CO_2 fluid. Relative yields of EGCG and ECG were found to be greater than that obtained from analytical SFE. After cleaning up the extract by distributing it between water and organic solvent, 1.4 g of crude tea catechin was produced, in which EGCG and ECG were present at 35% according to the HPLC analysis (Fig. 5). The amount of CO_2 fluid (4 l) used here was only twice the volume of the extraction vessel, so that if the extraction time is increased at the same flow-rate, the extraction yield may be further increased.

3.3. HSCCC separation of EGCG and ECG

HSCCC is a unique liquid–liquid partition chro-

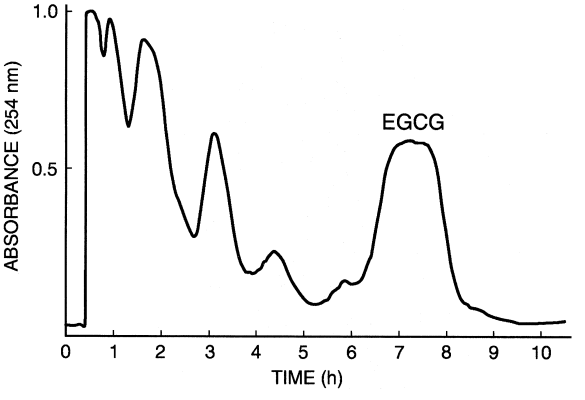

Fig. 6. HSCCC separation profile of EGCG from cleaned SFE extract of *Cratoxylum prunifolium* Dyer. Solvent system: hexane–ethyl acetate–water (1:20:30, v/v/v); sample size: 1.4 g.

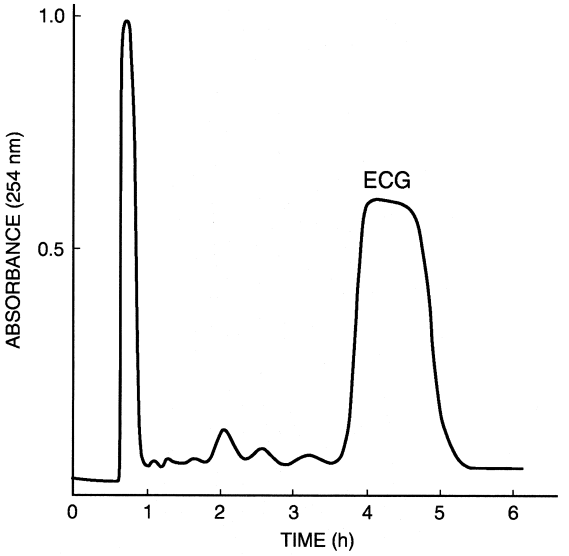

Fig. 7. HSCCC separation profile of ECG from the residues of SFE extract after the separation of EGCG in Fig. 6. Solvent system: hexane–ethyl acetate–water (1:3:4, v/v/v).

matographic method without a solid matrix so that it eliminates various complications arising from interaction between solutes and solid surface. The method has been increasingly used for purification of natural products in recent years [7,8]. In our previous studies, two solvent systems of ethyl acetate–ethanol–water and hexane–ethyl acetate–water were examined in detail for the separation and purification of EGCG, GCG and ECG from a crude tea polyphenol fraction [9]. Both of these solvent systems can be used for preparative separation of tea catechins by selecting suitable volume ratios. In the present study, the hexane–ethyl acetate–water system at two different volume ratios of 1:20:30 and 1:3:4 was successively used for purification of EGCG and ECG, the results of which are given in Figs. 6 and 7, respectively. The peak fractions of EGCG and ECG were freeze-dried to yield white amorphous powder. HPLC analysis shown in Fig. 8 indicates that EGCG and ECG were both over 98% pure.

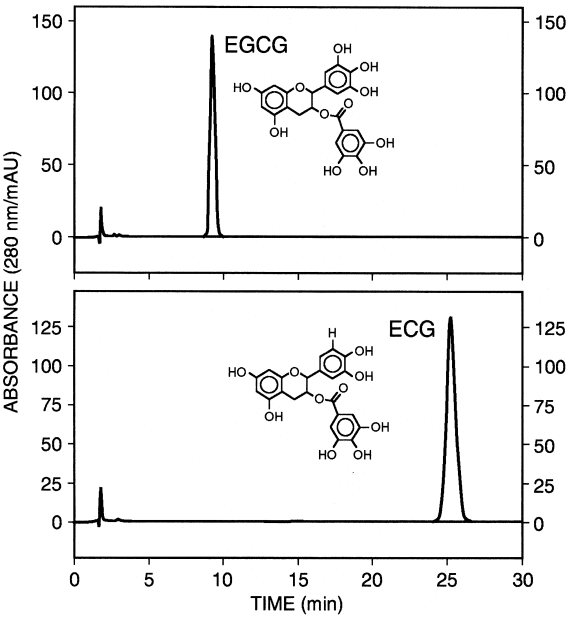

Fig. 8. HPLC analysis of EGCG and ECG obtained from HSCCC fractions.

4. Conclusion

The extraction of tea catechins by SFE is difficult because its solubility is only 10^{-6} in pure supercritical CO_2 fluid by weight fraction. However, our studies indicate that the extraction of these compounds can be improved by optimizing extraction conditions by applying low temperature and high pressure and adding a small amount of ethanol as a modifier. It was demonstrated that under a set of optimized SFE conditions of 40°C, 25 MPa and modifying supercritical CO_2 fluid with 80% ethanol aqueous solution the combined yield of EGCG and ECG can reach about 1 mg per 1 g of tea leaves where solvability of these compounds is as high as about 1.4×10^{-4} mass fraction of CO_2 fluid. The overall results of our studies suggest that the yield of these catechins may be further increased by increasing extraction time and/or using absolute ethanol as modifier. From a crude SFE extract over 98% pure EGCG and ECG were obtained by HSCCC.

Acknowledgements

The authors thank Beijing Municipal Committee of Science and Technology for financial support of New Star Candidate. They are also indebted to Professor. W.-X. Yao of Research Center for Eco-Environmental Science, CAS for his kind coordination.

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