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Comparative Study on Separation and Purification of Isoflavones from the Seeds and Sprouts of Chickpea by HSCCC

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Abstract

Chickpea is known as a plant that is rich in protein, carbohydrates, and nutrition, and its seeds and sprouts have been processed into various health foods. In the present study, four isoflavones were purified from the seeds and sprouts of chickpea by high speed countercurrent chromatography (HSCCC) using two biphasic solvent systems composed of n-hexane-ethyl acetate-methanol-water (5:5:5:5, v/v) and ethyl acetate-water (1:1 v/v). The results indicated that 14.2 mg of formononetin, 15.7 mg of biochanin A, 9.1 mg of ononin, 11.3 mg of biochanin A-7-O-β-D-glucoside were obtained from 150 mg of sprout extracts with the purity of 92.26%, 95.86%, 95.32%, and 96.56%, respectively. Compared with the sprouts, separation of seed extracts yielded less amounts of biochanin A-7-O-β-D-glucoside and biochanin A with lower purity. The results indicate that four main isoflavones in chickpea, i.e., isoflavones, formononetin, biochanin A, ononin, and biochanin A-7-O-β-D-glucoside, are substantially increased by biosynthesis during the seed germination.

Keywords

High speed countercurrent chromatography; Chickpea; Seed; Sprout; Biosynthesis; Isoflavone

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a traditional dish and a favorite food in Northwest China for centuries. It is known as a good source of protein, carbohydrates, and nutrition,[1] and nowadays seeds and sprouts of the chickpea are processed into all kinds of health foods. Furthermore, many pharmacological effects of the chickpea have been reported including reduction of the risk of diabetes and obesity,[2–4] and colonic cancer.[5] It is also used in the treatment of various diseases including bronchitis, leprosy, skin diseases, blood disorders, and biliousness,[6] diseases of the liver and spleen, and otitis.7]

Sprouting the seeds improves the nutritive value of seeds by increasing vitamin concentrations, bioavailability of trace elements, and minerals.[8] Isoflavonoids, having sterile and estrogenic

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activity[9] to protect against cancer, cardiovascular diseases, and osteoporosis,[10] were the main bioactivity compositions of chickpea. As is well known, the content and its bioactivity vary greatly between the seeds and sprouts of legumes,[11] and isolation of the isoflavone from them is essential for studying their chemical structure and potential activity.

Although preparative HPLC provides high partition efficiency, it often suffers from sample loss due to irreversible adsorption onto the solid support. High speed countercurrent chromatography (HSCCC) has an advantage over the conventional liquid solid chromatography, in that it yields higher sample recovery at near 100% by eliminating the use of solid support.[12]. Since the 1980s, the method has been widely used in the preparative separation of natural products.[13,14] The present study focuses on the separation of ononin, biochanin A-7-O-β-D-glucoside, formononetin, and biochanin A (structures see Figure 1) from the seeds and sprouts of chickpeas by HSCCC to compare their contents and compositions.

EXPERIMENTAL

Apparatus

The preparative HSCCC instrument employed in this study is a model TBE–300A high speed countercurrent chromatograph (Tauto Biotech, Shanghai, China) equipped with three polytetrafluoroethylene (PTFE) preparative separation coils connected in series (2.6 mm I.D. and total column volume, 290 mL). The revolution radius or the distance between the holder axis and the central axis of the centrifuge (*R*) was 5 cm, and the β-values vary from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$, where *r* is the distance from the coil to the holder shaft). An optimum speed of 850 rpm was used in this study.

The solvent was pumped into the column with a model TBP-50A constant flow pump (Shanghai, Tauto Biotech, China). Continuous monitoring of the effluent was achieved with a Model 8823A-UV monitor at 254 nm, and a manual sample injection valve with a 20 mL loop for the preparative HSCCC was used to introduce the sample into the column. Model N2000 workstation (Zhejiang University, Hangzhou, China) was used to draw the chromatogram.

The high performance liquid chromatograph (Dionex, USA) used was a Dionex system equipped with a P680 pump, an ASI-100 Automated sample injector, a TCC-100 Thermostatted column compartment, and UVD170U detector. The analysis was carried out with an inertsil ODS-SP column (5 μm, 4.6 × 250 mm GL Sciences Inc, Japan). Evaluation and quantification were made on a Chromeleon WorkStation.

Reagents

All organic solutions used for HSCCC were of analytical grade and purchased from Tianjin Chemical Factory (Tianjin, China). Methanol used for HPLC was of HPLC grade and purchased from Fisher Scientific Company (USA).

The *Cicer arietinum* sample was collected from Mulei County, Xinjiang, China and was identified by Shiming Duan, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences.

Sprouting Procedure

The fresh seeds were soaked by submerging in tap water in glass containers for 8 h at 30°C. Sprouting was carried out in the dark using 200 g quantities of seeds in dishes lined with filter paper. The temperature of the dish was maintained at 30°C. The distilled water was sprayed for 10 seconds, every 3 h, during germination. The sprouts were washed twice a day, for 3 days

to avoid microbial growth. The sprouts were pinched and dried. Finally, 50 g of the sprouts were obtained.

Preparation of Crude Sample

Preparation of Crude Sample I from the Seeds—The powdered seeds (5 kg) were refluxed with 70% ethanol (3 times). The extracts were concentrated in vacuum to a syrup yielding 230 g of extracts, which were dissolved with 1 L of distilled water. The solution was extracted three times with 2 L of light petroleum and then 2 L of ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness under reduced pressure. The above crude sample II (13 g) containing isoflavones as major components was directly subjected to HSCCC.

Preparation of Crude Sample II from the Sprouts—The dried sprouts (50 g) were refluxed with 70% ethanol (3 times). The extracts were combined and evaporated to dryness under reduced pressure, which yielded 25g extracts. The extracts were dissolved with 200 mL distilled water. The solution was extracted three times with 400 mL of light petroleum and then 400 mL of ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness under reduced pressure. The above crude sample II (4.5 g), also containing isoflavones as major components, was directly subjected to HSCCC.

Measurement of Partition Coefficient (K)

The two-phase solvent systems were selected according to the partition coefficient (K) of the target components. Various volume ratios of n-hexane, ethyl acetate, methanol, and water were equilibrated in a separation funnel at room temperature, each for determination of K values by HPLC analysis as follows: a suitable amount of sample (1 mg) was added to 4.0 mL consisting of equal volume of each phase of the two-phase solvent system in a test tube. The contents were then mixed thoroughly and separated into two layers. Then, equal volumes of the upper phase and the lower phase were analyzed by HPLC. The peak area of the upper phase was recorded as A_U and that of the lower phase was recorded as A_L. The *K* value was calculated according to the following equation: $K = A_U/A_L$, as listed in Table 1.

HSCCC Separation

The preparative HSCCC was performed with a model TBE-300A HSCCC instrument as follows: the multiplayer coiled column was first entirely filled with the upper phase as stationary phase. After rotation at 850 rpm, the sample solution (150 mg crude sample in 20 mL of a mixture of each phase) was injected through the sample port. The lower phase was pumped into the head end of the HSCCC coil column at a flow rate of 2 mL min^{-1}. The effluent from the outlet of the column was monitored with a UV detector at 254 nm. Peak fractions were manually collected according to the chromatogram.

HPLC Analysis and Identification of Crude Sample and Peak Fraction from HSCCC

The crude sample and the peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with a C_{18} column (4.6 mm I.D. × 250 mm, 5 μm) at the column temperature of 35°C. The mobile phase was eluted in a linear gradient of methanol (A) and 0.2% formic acid (B) with A–B (30:70, v/v) to A–B (70:30, v/v) in 60 min at a flow rate of 1.0 mL min^{-1}, while the effluent was monitored at 254 nm by a UV detector.

Identification of the HSCCC peak fraction was carried out by ^{1}H nuclear magnetic resonance (^{1}H NMR) and ^{13}C nuclear magnetic resonance (^{13}C NMR).

RESULTS AND DISCUSSION

Several elution modes were tested in the HPLC separation of crude samples, such as gradient elution of methanol-water, methanol-acid water, and acetonitrile-water and acetonitrile-acid water. When methanol-formic acid (0.2%) was used as the mobile phase in a gradient mode at 30% A: 70%B to 70%A: 30%B in 60 min, good resolutions of target isoflavones was obtained. The crude samples and peak fractions separated by HSCCC were analyzed by HPLC under the above optimum analytical condition (See Figure 2).

The crude samples I and II were first analyzed by HPLC. The chromatogram of crude sample I (extracts from the seeds) shows that biochanin A-7-O-β-D-glucoside (Retention Time: 30.367 min) and biochanin A (Retention Time: 49.065 min) are the main components in the seed, but formononetin and ononin are found in less amounts (Figure 2.A). The result of HPLC analysis of sample II (Figure 2.B) indicated that it contained several flavonoids including formononetin (Retention Time: 40.872 min), biochanin A (Retention Time: 49.065 min), ononin (Retention Time: 22.336 min), and biochanin A-7-O-β-D-glucoside (Retention Time: 30.367 min) with some unknown compounds.

Using HSCCC, successful separation depends upon the selection of a suitable two-phase solvent system, which requires the following considerations: retention of the stationary phase should be satisfactory; the settling time of the solvent system should be short (i.e.,<30 s),[15] the partition coefficient of the target compound should be between 0.5 – 2.0.[16] Smaller K values would result in a loss of peak resolution, while large K values tend to produce excessive sample band broadening and long run times.[17] According to the properties of isoflavones, several two-phase solvent systems were tested and their K values are summarized in Table 1. Among them (Table 1), the two-phase solvent systems, including systems 7 and 8 that had K values suitable for separation of compound 1; systems 6, 7, and 8, were suitable for separation of compound 2; systems, 3 and 4, were suitable for separation of compound 3; and systems, 1, 2 and 3, were suitable for separation of compound 4. Therefore, system 3 was used for the simultaneous separation of compound 3 and 4, and system 7 and 8 for separation of compounds 1 and 2. Because of the small K value (0.51) of compound 1 in system 1, there is poor resolution between compound 1 and polar impurities. So, we decided to use system 8 for the separation of compound 1 and 2, although its separation factor ($\alpha = K_2/K_1 = 2.13$) is lower than system 7 ($\alpha = 3.86$).

Figure 3 shows HSCCC separation of 100 mg of the crude sample I (chickpea seeds) using a two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water (5:5:5:5, v/v and 0:1:0:1, v/v). The first solvent system was initially used for the separation of the crude samples to obtain fraction 1, and compound 4 with lower purity (4.2 mg, 81.12%) (figure 3A) in fraction 1 was further separated using the second solvent system to improve the purity of compound 2 (3.8 mg, 75.23%) (Figure 3B).

Figure 4 similarly shows HSCCC separation of the crude sample II (chickpea sprouts) using the same set of the two-phase solvent system. In this case, the first separation yielded fraction 2, compound 3 (14.2 mg, 92.26%) and compound 4 (15.7 mg, 95.86%) (Figure 4A and Figure 5), while the second separation of fraction 2 produced compound 1 (9.1 mg, 95.32%) and compound 2 (11.3 mg, 96.56%) (Figure 4B and Figure 5). Also, amorphous crystals were obtained from the fractions of the crude sample II due to their high purity of over 99%. But the products from the crude sample I can not be recrystallized because of its low purity. Table 2 indicates that contents of four target compounds were increased after germination. Biochanin A-7-O-β-D-glucoside and biochanin A were increased 22 and 25 times, respectively. But the increased amount of ononin and formononetin can not be compared since they are not separable from ethyl acetate extracts of seeds because of its lower contents in the seeds.

Identification of the compounds was carried out by ^{1}H NMR and ^{13}C NMR as follows:

Compound 1 (ononin):[18] 1H-NMR(DMSO-d6) δ:8.45(1H, s,H-2), 8.07(1H, d, J = 9.0Hz, H-5), 7.54(2H, d, J = 8.4 Hz, H-2', 6'), 7.25(1H, d, J = 2.4 Hz, H-8), 7.15(1H, dd, J = 9.0 Hz, J = 2.4 Hz, H-6), 7.01(2H, d, J = 9.0 Hz, H-3',5'), 3.79(3H, s, 4'-OCH3), 5.45 (1H, J = 7.5 Hz, H-1'').13C-NMR(DMSO-d6) δ: 174.7(C-4), 161.4 (C-7), 159.0(C-4), 157.1(C-9), 153.7(C-2), 130.1(C-2', 6'), 126.9(C-5), 124.0(C-1'), 123.4(C-3), 118.4 (C-10), 115.6(C-6), 113.6(C-3', 5'), 103.4(C-8), 100.0(C-1"), 77.2(C-5"), 76.5(C-3"), 73.1(C-2"), 69.6(C-4"), 60.6(C-6"), 55.2(4'-OCH_3).

Compound 2 (biochanin A-7-O-β-D-glucoside):[19] 1H-NMR(DMSO-d6) δ :12.91(1H, s, 5-OH), 8.48(1H, s, H-2), 7.54(2H, d, J=9.0Hz, H-2',6'), 7.02(2H, d, J=9.0 Hz, H-3', 5'), 6.73(1H, d, J = 2.4 Hz, H-8), 6.48(1H, d, J = 2.4 Hz, H-6), 3.80 (3H, s, 4'-OCH3), 5.43 (1H, = 5.4 Hz, H-1"). 13C-NMR(DMSO-d6) δ: 180.4(C-4), 163.1 (C-7), 161.6(C-5), 159.2(C-4'), 157.2(C-9), 154.9(C-2), 130.2(C-2', 6'), 122.7(C-3), 122.2(C-1'), 113.7 (C-3', 5'), 106.1(C-10), 99.8(C-6), 99.6(C-1"), 94.6(C-8), 77.2(C-5"), 76.4(C-3"), 73.1 (C-2"), 69.6(C-4"), 60.6(C-6"), 55.2(4'-OCH_3).

Compound 3 (formononetin):[20] 1H-NMR(DMSO-d6) δ :10.81(1H, s, 7-OH), 8.35(1H, s, H-2), 7.98 (1H, d, J = 9.0 Hz, H-5),7.50 (2H, d, J = 9.0Hz, H-2', 6'), 6.97 (2H, d, J = 9 Hz, H-3', 5'), 6.95(1H, dd, J = 9.0 Hz, J = 2.4 Hz, H-6), 6.87(1H, d, J = 2.4 Hz, H-8), 3.79 (3H, s, 4'-OCH3). 13C-NMR(DMSO-d6) δ: 174.6(C-4), 162.6 (C-7), 158.9(C-4'), 157.4(C-9), 153.2(C-2), 130.1(C-2', 6'), 127.3(C-5), 124.2(C-3), 123.1(C-1'), 116.6 (C-10), 115.2(C-6), 113.6(C-3', 5'), 102.1(C-8).

Compound 4 (biochanin A):[19,20] 1H-NMR(DMSO-d6) δ :12.93 (1H, s, 5-OH), 10.81 (1H, s, 7-OH), 8.38(1H, s, H-2), 7.51(2H, d, J = 9.0Hz, H-2',6'), 7.01 (2H, d, J = 9.0 Hz, H-3', 5'), 6.40(1H, d, J = 2.4 Hz, H-8), 6.24(1H, d, J = 2.4Hz, H-6), 3.79 (3H, s, 4'-OCH3). 13C-NMR(DMSO-d6) δ: 180.1(C-4), 164.3 (C-7), 162.0(C-5), 159.2(C-4'), 157.6(C-9), 154.3(C-2), 130.2(C-2', 6'), 122.9(C-3), 122.0(C-1'), 113.7(C-3', 5'), 104.5(C-10), 99.0 (C-6), 93.7(C-8) (see Figure 1.).

CONCLUSIONS

The seeds and sprouts of chickpeas are a very popular food in the world. As the main components, a variety of isoflavones, have many potential bioactivities. Our study showed that these isoflavones were biosynthesized during sprouting. The main ingredients of isoflavones, formononetin, biochanin A, ononin, and biochanin A-7-O-β-D-glucoside, in the sprouts were substantially increased compared to their counterparts present in the seeds

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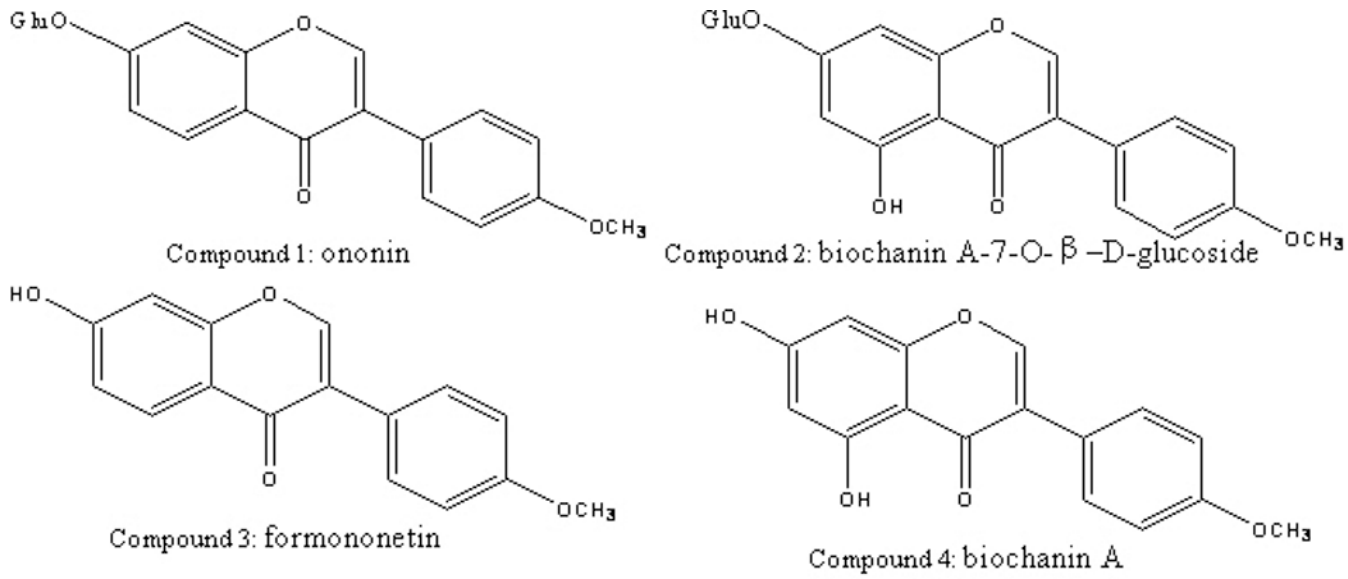

Figure 1.
Chemical structures of ononin, biochanin A-7-O-β-D-glucoside, formononetin and biochanin A.

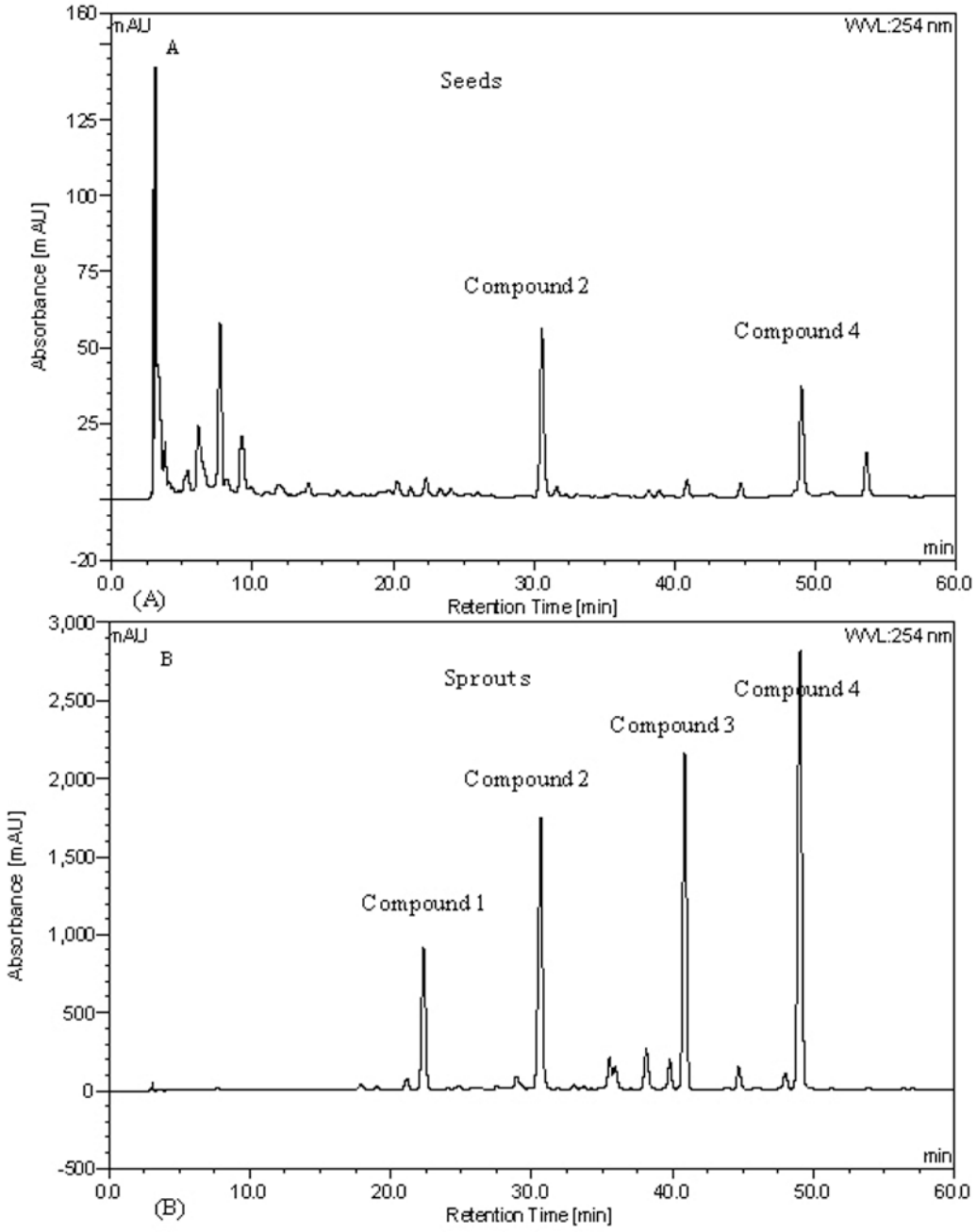

Figure 2.

HPLC analysis of the crude samples extracted from chickpea seeds and sprouts. Separation column: a C_{18} column (4.6 mm I.D. × 250 mm, 5 μm); column temperature: 35°C; detection wavelength: 254 nm; the mobile phase: a linear gradient of methanol (A) and 0.2% formic acid (B) that follows: A-B (30:70, v/v) to A–B (70:30, v/v) in 60 min; the flow rate: 1.0 mL min^{-1}. (A) crude sample I, (B) crude sample II.

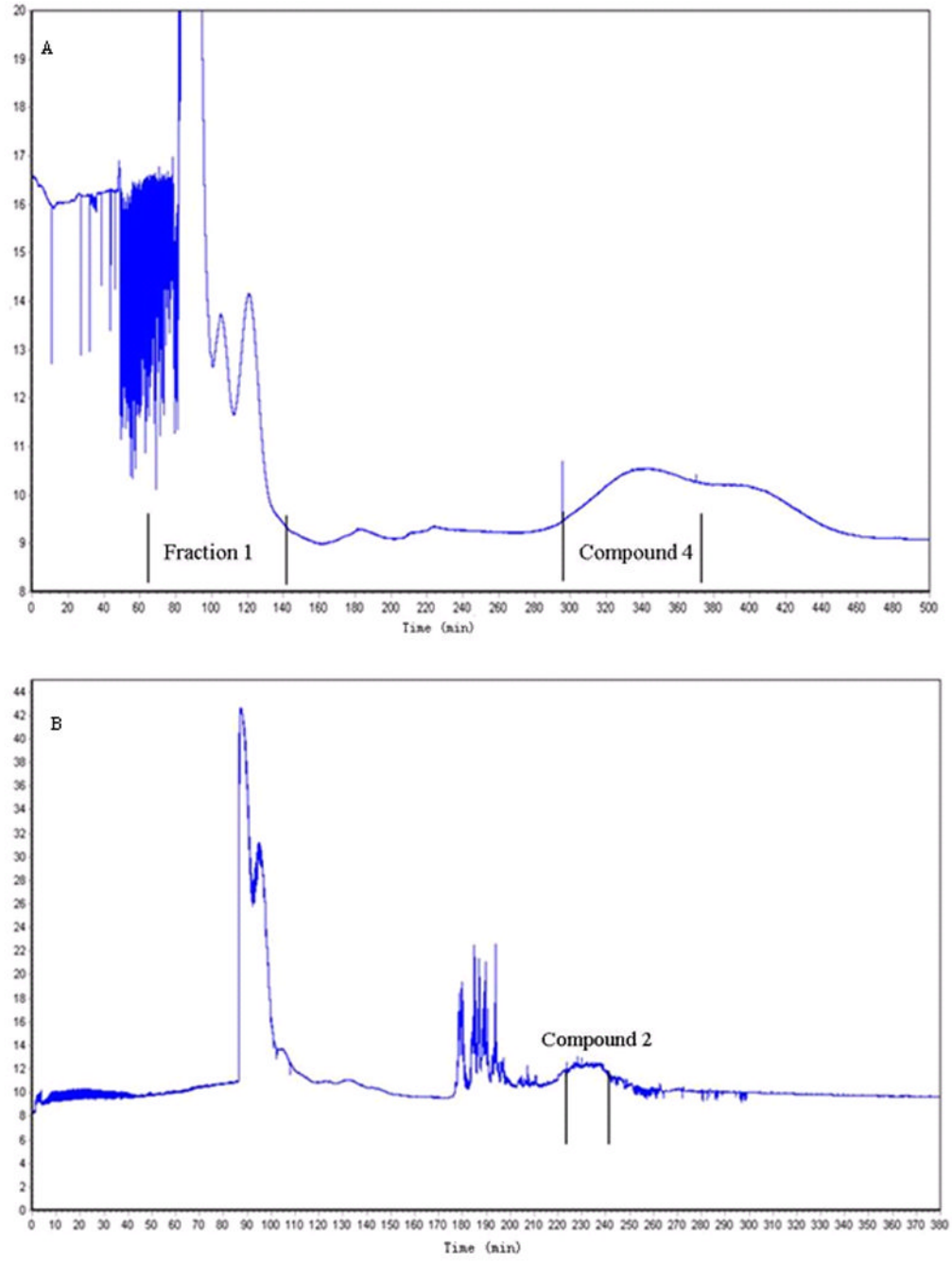

Figure 3.
HSCCC chromatograms of 100 mg crude sample I from the extracts of seeds. Solvent systems: (A) *n*-hexane-ethyl acetate-methanol-water (5:5:5:5, v/v), (B) *n*-hexane-ethyl acetate-methanol-water (1:1, v/v), flow rate: 2.0 mL min^{-1}; revolution speed: 850 rpm; detection wavelength: 254 nm; Samples: 100 mg crude sample I.

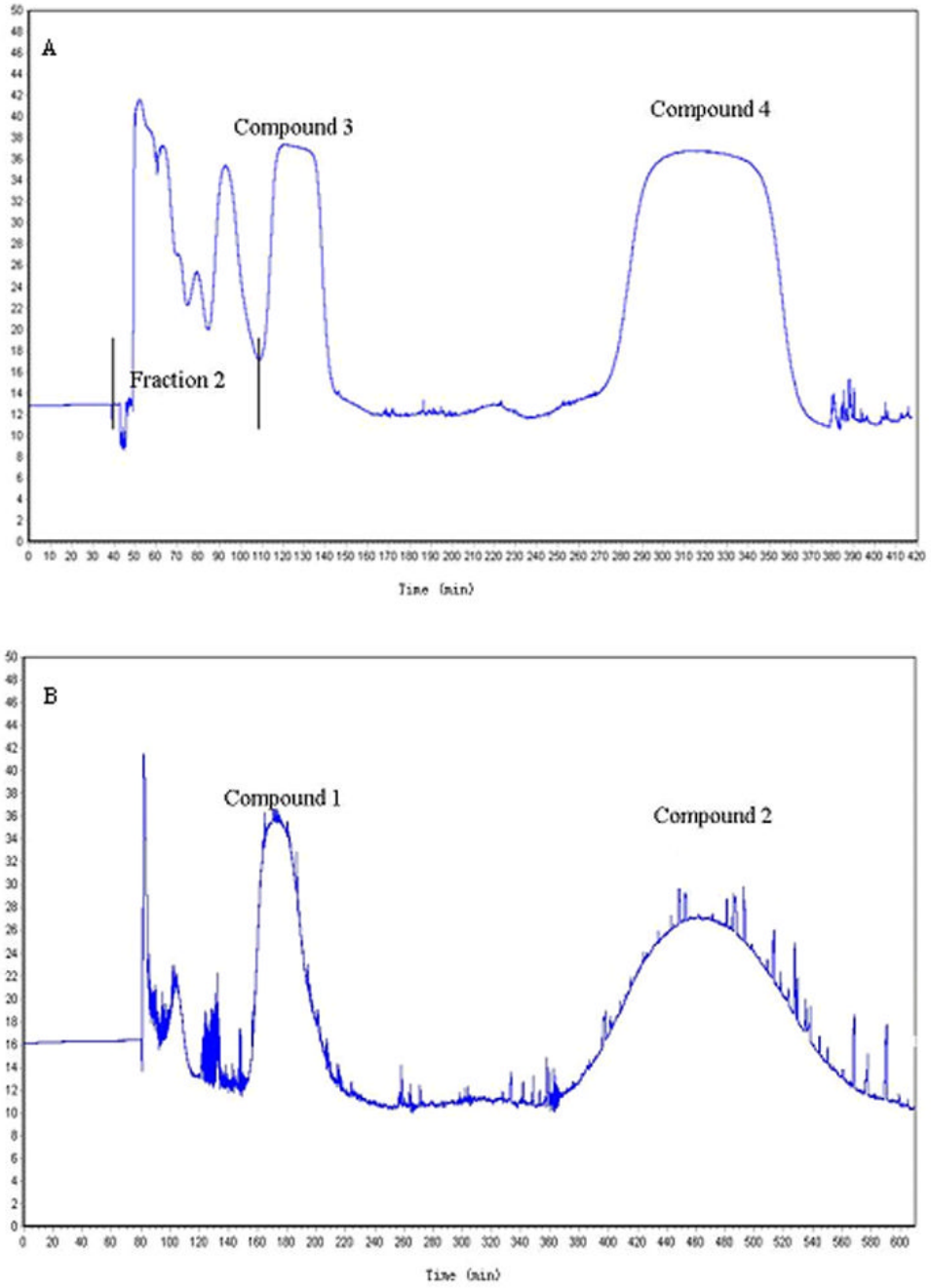

Figure 4.
HSCCC chromatograms of 150 mg crude sample II from the extracts of sprouts. Solvent systems: (A) *n*-hexane-ethyl acetate-methanol-water (5:5:5:5, v/v), (B) *n*-hexane-ethyl acetate-methanol-water (1:1, v/v), flow rate: 2.0 mL min^{-1}; revolution speed: 850 rpm; detection wavelength: 254 nm.

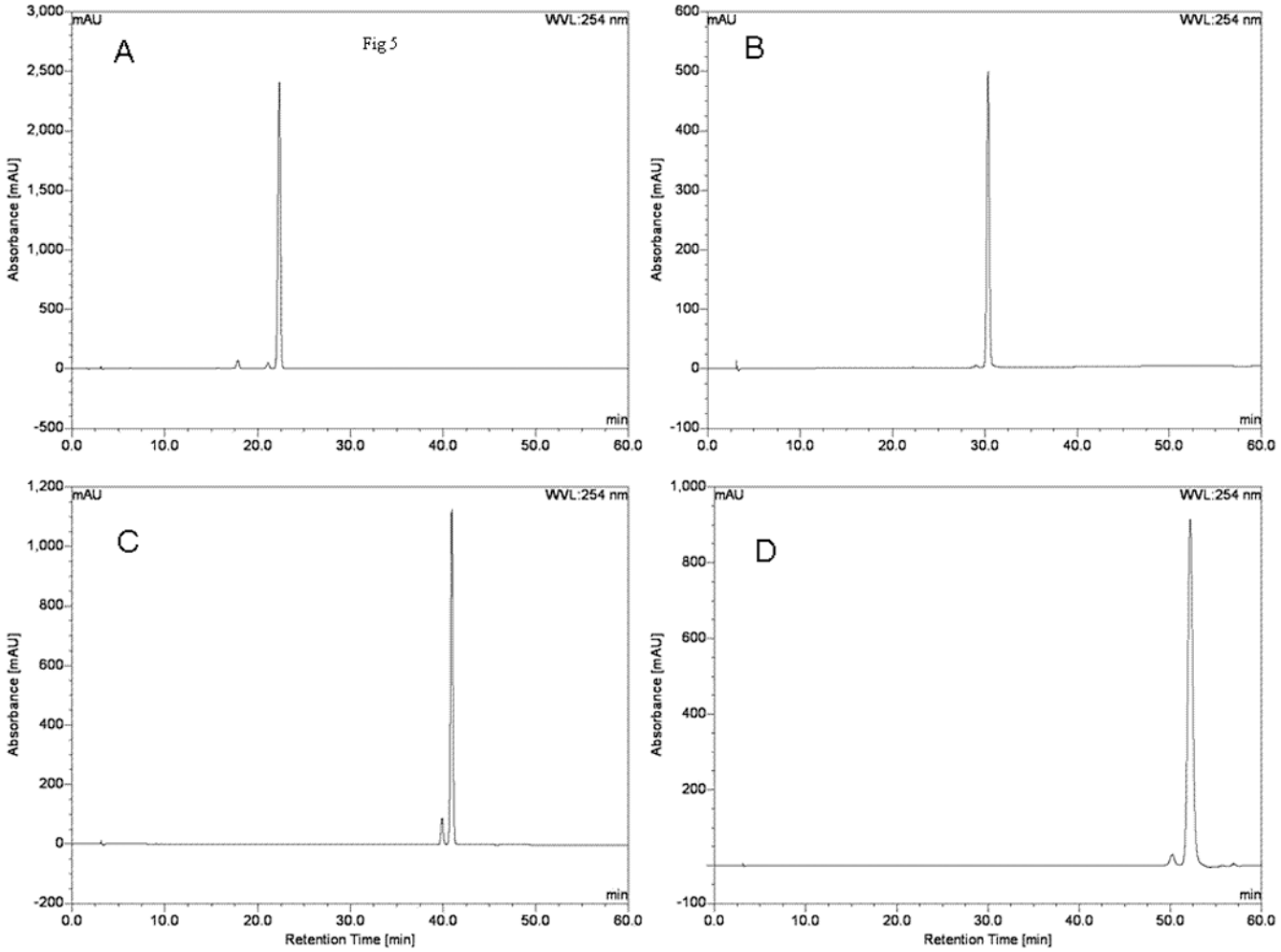

Figure 5.

HPLC analysis of four compounds separated from the crude sample II. Separation column: a C_{18} column (4.6 mm I.D. × 250 mm, 5 μm); column temperature: 35°C; detection wavelength: 254 nm; the mobile phase: a linear gradient of methanol (A) and 0.2% formic acid (B) that follows: A–B (30:70, v/v) to A–B (70:30, v/v) in 60 min; the flow rate: 1.0 mL min^{-1}. (A) compound 1 in Figure 4B, (B) compound 2 in Figure 4B, (C) compound 3 in Figure 4A, (D) compound 4 in Figure 4A.

Table 1

Partition coefficient (K) of four isoflavones in different solvent systems of *n*-hexane–ethyl acetate–methanol–water

NO.	Solvent system (v/v)	K_{ononin}	$K_{biochanin\ A\text{-}7\text{-}glucoside}$	$K_{formononetin}$	$K_{biochanin\ A}$
1	7:5:7:5	--	--	0.31	1.09
2	6:5:6:5	--	--	0.36	1.62
3	5:5:5:5	0.003	0.02	0.90	3.02
4	4:5:4:5	0.01	0.04	1.81	4.71
5	3:5:3:5	0.16	0.30	14.13	41.72
6	2:5:2:5	0.15	0.64	29.13	108.09
7	1:5:1:5	0.51	1.97	70.66	--
8	0:1:0:1	1.35	2.88	79.56	--

Table 2

Comparison of yields and purities between the seeds and sprouts by HSCCC

Compound	seeds Source (g)	Crude sample I (mg)	Yield (mg)	Purity (%)	sprouts source (g)	Crude sample II (mg)	Yield (mg)	Purity (%)
1	38.46 g seeds	100	0	--	6.67 g seeds or 1.67 g sprouts	150	9.1	95.32
2			3.8	75.23			11.3	96.56
3			0	--			14.2	92.26
4			4.2	81.12			15.7	95.86