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Application of High-Speed Countercurrent Chromatography for the Isolation of Sulforaphane from Broccoli Seed Meal

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In order to produce large amounts of pure sulforaphane for research purposes, a novel method using high-speed countercurrent chromatography (HSCCC) was developed. Without any initial cleanup steps, sulforaphane was successfully purified from the ethyl acetate extract of the broccoli seed meal by HSCCC. The separation was performed with two-phase solvent systems: *n*-hexane/ethyl acetate/methanol/water (1:5:1:5, v/v/v/v). From 850 mg of the ethyl acetate extract, 186 mg of sulforaphane was isolated with the solvent system. The purified compound was over 97% purity as determined by HPLC analysis, and the chemical structure was confirmed by MS and ^{1}H and ^{13}C NMR.

KEYWORDS: Sulforaphane; broccoli seeds; high-speed countercurrent chromatography; isolation; purification

INTRODUCTION

Epidemiological data show that a diet rich in *Brassica oleracea* vegetables, such as broccoli, cabbage, Brussels sprouts, cauliflower, and kale, can reduce the risk from a number of cancers. The possible reason for the reduction of cancer by *B. oleracea* vegetables is that these vegetables are rich in glucosinolates. When these vegetables are ground or chopped, myrosinase enzyme (thioglucoside glucohydrolase, EC 3.2.3.1) and glucosinolates come into contact. Myrosinase breaks the β-thioglucoside bond of glucosinolate molecules, producing glucose, sulfate, and a diverse group of aglycone products. The resultant aglycones then undergo nonenzymatic, intramolecular rearrangement to yield isothiocyanates, thiocyanates, or nitriles. Sulforaphane (4-methylsulfinybutyl isothiocyanate, SFN), derived from glucoraphanin (4-methylsulfinybutyl glucosinolate), is the most potent naturally occurring inducer of phase II enzymes, including quinone reductase and glutathione S-transferase (*1*) and has subsequently been shown to possess anticarcinogenic activities (*2, 3*). Recent data also suggest that SFN may have a direct inhibitory action on cancer cells by inducing cell cycle arrest of prostate (*4*), leukemic (*5*), colon carcinoma (*6*), and medulloblastoma cells (*7*), leading to apoptotic cell death (*4–7*). These research results identify SFN as a novel inducer of cancer cell apoptosis, supporting the potential clinical usefulness of diet-derived substances as chemopreventive agents.

With the increase in application of SFN in research, the demands for high purity sulforaphane are rapidly increasing. In studies to date, normal-phase liquid chromatography (*8, 9*) and preparative high-performance liquid chromatography (HPLC) (*8, 10, 11*) have been used for purifying SFN. In general, currently available methods are time-consuming and require multiple steps during the extraction, separation, and purification processes and often generate large amounts of organic solvent wastage.

High-speed countercurrent chromatography (HSCCC) is an advanced technique that is useful for scale-up separation with minimum sample preparation and cleanup procedures and permits both normal- as well as reversed-phase operation (*12, 13*). On the basis of the distribution of compounds between two immiscible liquids, this liquid−liquid chromatographic method offers the following advantages: (1) no adsorption and therefore complete recovery of the chromatographed sample, (2) simple technology (low-pressure method), and (3) low cost of operation (use of technical grade solvents) (*14*). The objective of this study was therefore to develop a novel method using HSCCC for more efficient purification and recovery of SFN from broccoli seed meal.

MATERIALS AND METHODS

Materials. Broccoli seeds of a high sulforaphane yielding variety (Zhongqing II) were kindly provided by Vegetables and Flowers Institute, China Academy of Agriculture Science. Sulforaphane standard at ≥98% purity was purchased from Sigma Chemical Co. (St. Louis, MO). All organic solvents used for sample preparation or HSCCC separation were of analytical grade and purchased from Beijing Reagent Co. (Beijing, China). Silica gel (200−300 mesh, irregular) was obtained from Haiyang Chemical Group (Qingdao, China). Solvents used for HPLC and MS analyses were of chromatographic grade purchased from Fisher Scientific. $CDCl_3$ used for NMR experiments was also from Fisher Scientific.

Preparation of Crude Extract from Broccoli Seeds. Seeds (50 g) were homogenized in an analytical grinder. The ground seeds were added to 300 mL of pure water to allow the spontaneous hydrolysis of the glucosinolates by endogenous myrosinase for 2 h at 25 °C. The resulting

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Table 1. Partition Coefficient (*K*) Values of SFN in Different Biphasic Solvent Systems

solvent system	ratio (V/V)[a]	partition coefficient (*K*)
chloroform/methanol/water	2:1:1	0.11
ethyl acetate/*n*-butyl alcohol/water	4:1:5	4.02
	3:2:5	5.85
	2:3:5	6.11
	1:4:5	5.84
n-hexane/ethyl acetate/methanol/water	5:5:5:5	0.09
	4:5:4:5	0.18
	3:5:3:5	0.31
	2:5:2:5	0.61
	1:5:1:5	0.99
ethyl acetate/methanol/water	4:1:5	1.59
	3:2:5	1.34

[a] Solvent ratios were by volume.

mixture was extracted three times with 300 mL of ethyl acetate, following which all extracts were combined and dried at 35 °C under vacuum in a rotary evaporator, to produce the crude extract.

Selection of Two-Phase Solvent Systems. The two-phase solvent systems were selected according to the partition coefficient (*K*) of the target component of SFN. The solvent system was thoroughly mixed, vented, and allowed to separate into two distinct phases before use. The partition coefficient (*K*) was calculated as the ratio of the stationary and mobile phases of the target compound. The sample solutions were prepared by dissolving the crude extract in a mixture of upper and lower phases in a ratio 1:1 (v/v).

Preparation of Two-Phase Solvent System and Sample Solution. The two-phase solvent system used was composed of *n*-hexane/ethyl acetate/methanol/water (1:5:1:5, v/v/v/v). The solvent mixture was thoroughly equilibrated in a separation funnel at room temperature, and the two phases were separated shortly before use. The stationary phase was the upper organic phase and the mobile phase was the lower aqueous phase. The sample solution was prepared by dissolving 850 mg of crude extract in 20 mL of the upper phase.

HSCCC Separation Procedure. High-speed countercurrent chromatography (HSCCC) was performed on a model GS 10 J-type instrument (Beijing Institute of New Technology Application). This apparatus has three preparative coils connected in series (PTFE tube 110 m × 1.6 mm i.d., 250 mL of total volume). The average multilayer coil radius (*r*) was 6.0 cm, the rotation radius (*R*) was 8.0 cm, and the β value ($\beta = r/R$) was 0.75. The HSCCC system included an HPLC NS-1007 pump (Beijing Institute of New Technology Application), a model 450 UV detector (Alltech, Deerfield, IL), a model 3057 flat-bed recorder (Yokogawa, Sichuan Instrument Factory, Chongqing, China), and a sample injection valve with a sample loop of 20 mL. The HSCCC tubing was first filled with the (organic) stationary phase with no rotation. Then, the coils were rotated at 1000 rpm as the (aqueous) mobile phase was pumped at a flow rate of 1.5 mL/min from head-to-tail. In order to observe the stationary phase retention volume ratio in the column, the resulting effluent was collected in a graduated cylinder. When the volumes of the two phases of the eluant were approximately equal, the hydrodynamic equilibrium was considered to be established. After hydrodynamic equilibrium was reached (about 60 min later), the sample solution was injected into the separation column through the injection valve. The effluent from the tail end of the separation column was continuously monitored at 254 nm. After the sample injection, the data were collected immediately. The fractions were collected manually according to the obtained chromatogram and then evaporated under reduced pressure. The residuals were dissolved in methanol for subsequent purity analysis by HPLC.

Preparative HPLC procedure. A preparative HPLC method preceded by solid-phase extraction (SPE) was facilitated for comparison purposes. The entire procedure was carried out according to Liang and coworkers (*11*) as follows. The crude extract was dissolved in hexane/ethyl acetate (8:2, v/v), while activated Silica gel was packed into a SPE column. The solvent and sample flow rate through the column was controlled at 2−3 mL/min under vacuum. The column was then washed with 30 mL of ethyl acetate, after which the sulforaphane was eluted with 50 mL of ethanol.

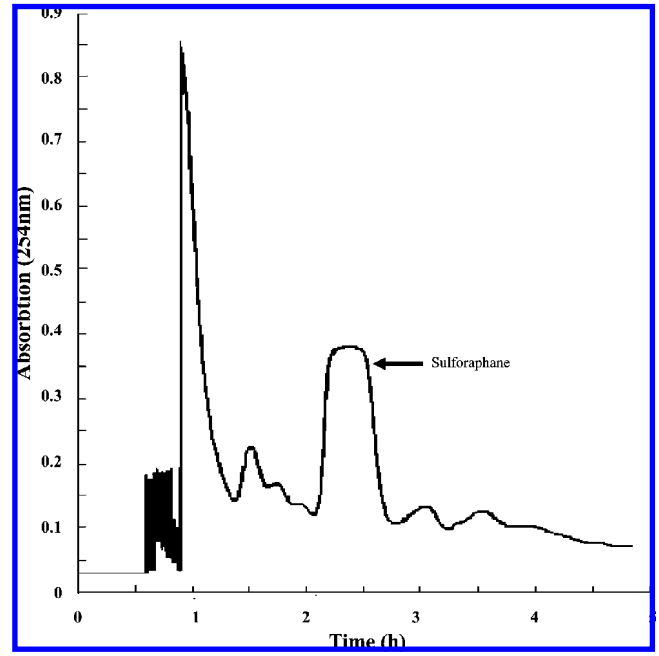

Figure 1. HSCCC chromatogram of the crude extract of broccoli seed meal in the *n*-hexane/ethyl acetate/methanol/water (1:5:1:5, v/v/v/v) solvent system.

Table 2. Comparison of HSCCC and HPLC in Separation and Isolation of SFN from Broccoli Seed Meal

item for comparison	HSCCC	HPLC
scale of apparatus	semipreparative	preparative
separation of column	250 mL	300 mm × 19 mm
flow rate	2 mL/min	12 mL/min
sample quantity	850 mg crude extract	300 mg crude extract
each separation time	240 min	60 min
total separation time	240 min	360 min
separation model	one-step separation	SPE + prep-HPLC
yield	186.0 mg	71.3 mg
recovery of the target	98.5%	87.4%
purity of the target	97%	95%
cost	lower	higher

The preparative HPLC separation was carried out on a Waters Prep 4000 liquid chromatography system fitted with a 2487 dual-wavelength absorbance detector (254 nm) with a preparative detection cell (Waters, Milford, MA). The chromatographic separation was performed on a Symmetry C_{18} column (300 mm × 19 mm, 7 μm).

Preparation of the Standard Curve. Quantification was based on the external standard method. A stock solution was prepared with 5.0 mg of SFN reference standard, which was dissolved and diluted to 10 mL with acetonitrile. Aliquots of the standard stock solution of SFN were pipetted into different 10 mL flasks and diluted to mark with acetonitrile. The final concentrations of SFN were in the range 2.5−17.5 μg/mL. Each solution was injected in duplicate. Peak areas were recorded for all the solutions.

HPLC Analysis. SFN was analyzed using a Hitachi HPLC apparatus equipped with Hitachi model L-7100 pumps, a L-7420 variable wavelength detector, and a reversed-phase C_{18} column (250 mm × 4.6 mm, 5 μm, Diamonsil, Beijing, China). The solvent system consisted of 20% acetonitrile in water, then changing linearly over 10 min to 60% acetonitrile, rising to 100% immediately, and running isocratically for 2 min to purge the column. The column oven temperature was set at 30 °C. The flow rate was 1.0 mL/min, and 10 μL aliquots were injected into the column. SFN was detected at UV 254 nm.

MS and NMR. Electron impact mass spectra (MS) and nuclear magnetic resonance (NMR) spectra were obtained by analysts at the Center of Analysis, Beijing University of Chemical Technology. A Micromass 70-VSE mass spectrometer was used with an ion source

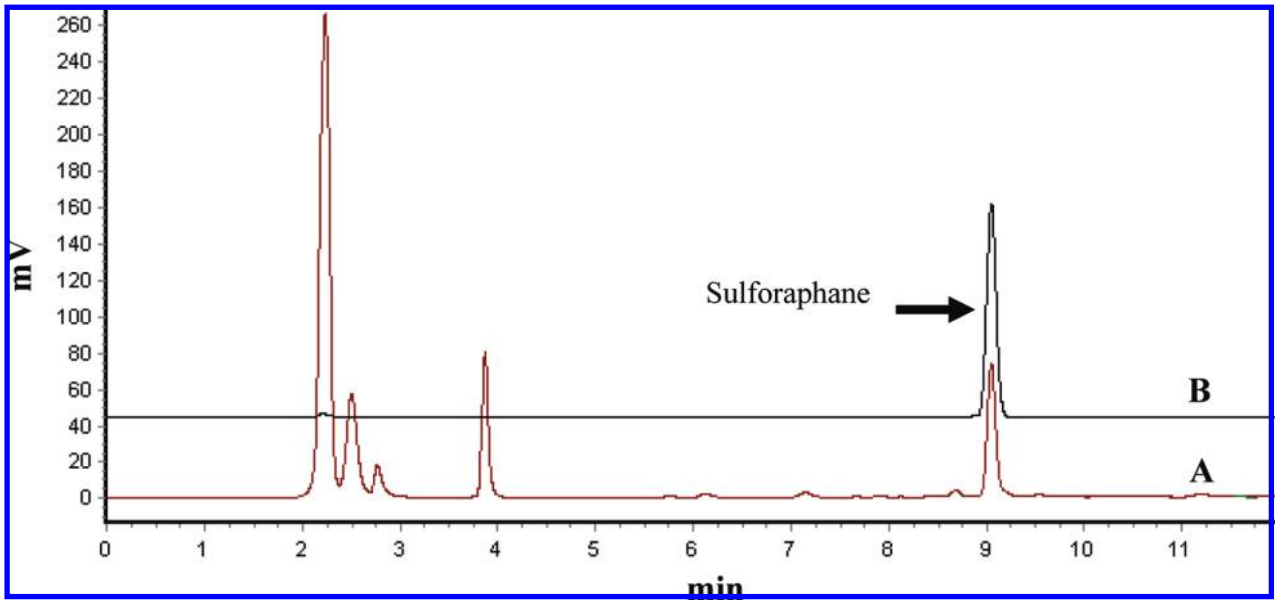

Figure 2. Analytical HPLC chromatograms of sulforaphane obtained by crude extraction (**A**) and HSCCC (**B**). The detected absorbance was set at 254 nm. The column was reversed-phase C_{18} (250 mm × 4.6 mm, 5 μm, Diamonsil). The solvent system consisted of 20% acetonitrile in water, then changed linearly over 10 min to 60% acetonitrile, and was subsequently maintained at 100% acetonitrile for 2 min to purge the column. The column oven temperature was set at 30 °C. The flow rate was 1 mL/min.

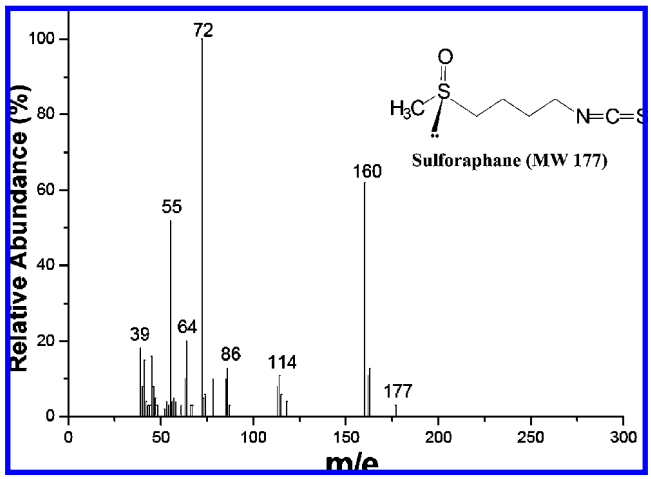

Figure 3. Mass spectrum of sulforaphane purified by HSCCC.

temperature of 200 °C and a probe temperature of 25 °C. The spectrum was scanned at 70 eV from *m/e* 30−300. NMR spectra were performed in $CDCl_3$ using a Bruker high-resolution AV600NMR spectrometer at 600 MHz (Bruker BioSpin Corporation, Billerica, MA).

RESULTS AND DISCUSSION

The solvent system is the most important factor that affects HSCCC separation. The compound of interest (SFN) must be stable and soluble in such a solvent system, and the solvent system must separate clearly and quickly into two phases. Moreover, the partition coefficient (*K*) of SFN should be in the range 0.5−2.0 (*15*). Various solvent systems with different ratios were tested. The measured *K* values are summarized in **Table 1**. Among them, four solvent systems, (a) *n*-hexane/ethyl acetate/methanol/water (2:5:2:5, v/v/v/v), (b) *n*-hexane/ethyl acetate/methanol/water (1:5:1:5, v/v/v/v), (c) ethyl acetate/methanol/water (3:2:5, v/v/v), and (d) ethyl acetate/methanol/water (4:1:5, v/v/v), provided acceptable *K* values at 0.61, 0.99, 1.34, and 1.59, respectively, in addition to meeting the other criteria (**Table 1**). Although all of these four systems maybe provide baseline separations for SFN, system (c) and (d) took much longer for SFN to be eluted due to their higher *K* values. With addition

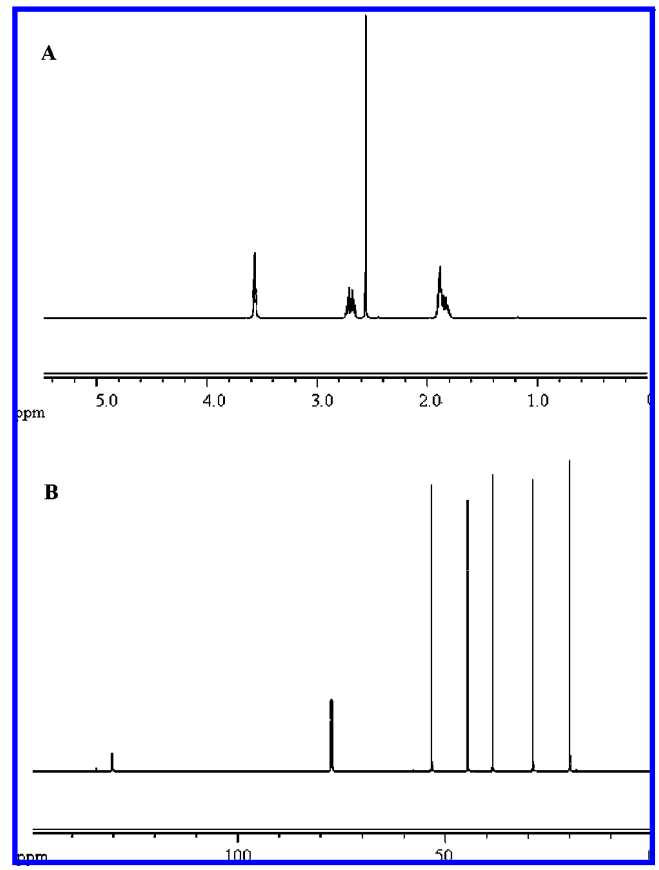

Figure 4. NMR spectra ($CDCl_3$, 600 MHz) of sulforaphane purified by HSCCC: (**A**) ^{1}H NMR spectrum; (**B**) ^{13}C NMR spectrum.

of *n*-hexane to ethyl acetate/methanol/water, the system could improve the phase separation and *K* values of the target compound, but the system is so sensitive that the large ratio of *n*-hexane dramatically changes the distribution of SFN in the lower phase. Since SFN is hardly soluble in *n*-hexane, adding large amounts of *n*-hexane would prevent SFN from dissolving in the upper phase.

Therefore, system (b) was chosen for the final preparative HSCCC separation of SFN.

One of the most important parameters in determining the peak resolution is the retention of the stationary phase. Effects of flow rates and revolution speeds on the retention volume of the stationary phase were studied. A preliminary test was conducted at two flow rates, 1.5 and 2.0 mL/min, and it was found that the former (1.5 mL/min) gave higher retention of the stationary phase and better separation. When the flow rate of 2.0 mL/min was employed in HSCCC separation, it caused a considerable loss of the stationary phase. Two different revolution speeds (800 and 1000 rpm) were also used to separate the crude extracts at either flow rate. Higher retention of the stationary phase and better separation of SFN were obtained at 1000 rpm. A flow rate of 1.5 mL/min and a revolution speed of 1000 rpm were therefore used in the present study, and the retention of the stationary phase was 52% (**Figure 1**). A low flow rate and a high revolution speed help increase the retention volume of the stationary phase (V_s) and result in an improved peak resolution (R_s) (*15, 16*). Chromatograms of sulforaphane obtained by crude extraction and HSCCC are shown in **Figure 2**. On the basis of the above procedures, the crude extract of 50 g of broccoli seeds can produce 850 mg of crude extract by ethyl acetate extraction, and then, 186 mg of 97% sulforphane can be obtained after being purified by HSCCC. The purity of a given product was defined for this study as the sulforaphane percentage of the product. Purified samples were analyzed by MS and NMR (see **Figures 3** and **4**). MS and NMR analysis of the purified sulforaphane sample produced results that were consistent with those previously reported (*1, 8–11*) and did not show the presence of compounds other than sulforaphane.

The yields, purities, and recoveries of SFN by HSCCC and preparative HPLC (*11*) methods and their differences are summarized in **Table 2**. Through the data, we can find that the purity (97%) of the SFN obtained via HSCCC was slightly higher than that from preparative HPLC (95%), but there was no difference between them. In consideration, the yields and recoveries of the product (186 mg of sulforaphane with 98.5% recovery from HSCCC was obtained compared to 71.3 mg with 87.4% recovery from the combined use of solid-phase extraction and preparative HPLC) confirmed that HSCCC, a free liquid−liquid partition chromatographic technique, could eliminate irreversible adsorption of the sample onto solid support. Furthermore, the separation mechanism of HSCCC is different from HPLC, and it can provide an alternative method when a satisfactory separation cannot be obtained by methanol, acetonitrile, or other solvents on C_{18} or C_8 columns. Compared with HPLC, HSCCC requires no sample treatment before separation. Moreover, only 240 min of the total separation time and 360 mL of solvent were required compared to 360 min and 2000 mL in preparative HPLC. By taking into account the time required for the purification steps before preparative HPLC, the productivity of HSCCC is higher than that of HPLC. In view of the yield, purity, and recovery of the obtained compound, separation time, and solvent consumption, it can be seen that HSCCC is a compelling technique for purifying SFN from broccoli seed meal.

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