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Separation and Purification of Sulforaphane from Broccoli Seeds by Solid Phase Extraction and Preparative High-Performance Liquid Chromatography

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A novel, rapid, and economical method to isolate and purify natural sulforaphane from broccoli seeds is described. The procedure involves solvent extraction of autolyzed seed meal, followed by separation by solid phase extraction (SPE) and purification by preparative high-performance liquid chromatography (HPLC). The SPE method provides higher yield of sulforaphane from crude extracts compared to conventional liquid–liquid extraction. High purity and recovery of sulforaphane product can be obtained by preparative HPLC with a C_{18} column and 30% methanol in water as the mobile phase. The purified compound was characterized by MS and ^{1}H and ^{13}C NMR. The techniques described here are useful tools in the preparative-scale isolation of sulforaphane in a fast, cost-effective, and waste-conscious manner.

KEYWORDS: Sulforaphane; broccoli seeds; separation; purification; solid phase extraction; preparative high-performance liquid chromatography

INTRODUCTION

Cruciferous vegetables contain compounds associated with protection against cancer. It has been shown that the cancer-preventive effects of cruciferous vegetables are related to their unique content in a large variety of glucosinolates (*1*). When vegetables are ground or chopped, myrosinase enzyme (thioglucoside glucohydrolase, EC3.2.3.1) 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 nonenzymetic, intramolecular rearrangement to yield isothiocyanates, thiocyanates, or nitriles.

Sulforaphane (4-methylsulfinybutyl isothiocyanate), an isothiocyanate derived from glucoraphanin (4-methylsulfinylbutyl glucosinolate), was initially identified as the principal inducer of phase II enzymes (*2*) and has subsequently been shown to possess anticarcinogenic activities (*3*, *4*). Recent data also suggest that sulforaphane may have a direct inhibitory action on cancer cells by inducing cell cycle arrest of prostate (*5*), leukemic (*6*), colon carcinoma (*7*), and medulloblastoma cells (*8*), leading to apoptotic cell death (*5*–*8*). These research results identify sulforaphane as a novel inducer of cancer cell apoptosis, supporting the potential clinical usefulness of diet-derived substances as chemopreventive agents.

Glucoraphanin has been successfully separated and purified by high-speed counter current chromatography (*9*) and preparative high-performance liquid chromatography (*10*, *11*). Although glucoraphanin can be hydrolyzed by gut microflora to sulforaphane, the rate of transformation in the human body has been shown to be very low in clinical trials (*12*). Therefore, it is very interesting and significant to separate and purify sulforaphane from plants directly.

Natural sulforaphane is mainly extracted from broccoli seeds. Due to the large amounts of oil contaminants in the seeds of broccoli, the traditional purification methods employ organic solvent liquid–liquid extraction (*13*–*15*). However, these processes are very time-consuming and require large amounts of solvents.

In order to perform studies evaluating the biological effects of sulforaphane in animal or clinical trials, it is necessary to obtain reasonably large quantities of highly purified compounds for experimental purposes. In studies to date, normal-phase liquid chromatography has been used for purifying sulforaphane (*13*, *15*), but the purity of the obtained sulforaphane has been inadequate for clinical trials. Moreover, the irreversible adsorption of impurities on normal-phase packing materials is a serious problem, which adversely influences the effectiveness of subsequent separations on the same column. Furthermore, the solvent consumption by normal-phase liquid chromatography is significantly high. Therefore, preparative reverse-phase high-

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performance liquid chromatography (RP-HPLC) is a valuable method for satisfying the demands of highly purified sulforaphane (*13, 14*). However, existing preparative RP-HPLC method for the purification of sulforaphane are costly and are not well suited for large-scale production. High purity and high recovery of sulforaphane cannot be simultaneously obtained by these methods because of the incomplete separation of sulforaphane and impurities when increasing the sample loading. Therefore, we have made an attempt to develop a suitable preparative RP-HPLC method for producing highly purified sulforaphane at a low cost and high efficiency.

This paper describes the separation and purification of sulforaphane by SPE and preparative RP-HPLC from crude extracts of broccoli seeds. The influence of the organic modifiers (acetonitrile or methanol) as a powerful selectivity tool for reversed-phase separations is also discussed. In addition, we describe the development and optimization of preparative RP-HPLC parameters for sulforaphane purification. A cost-effective strategy for maximizing the purification of sulforaphane is proposed.

MATERIALS AND METHODS

Materials. Broccoli seeds were kindly provided by Vegetables and Flowers Institute, China Academy of Agriculture Science. Sulforaphane standard was purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile and methanol were HPLC grade. Ethanol, ethyl acetate, hexane, and anhydrous sodium sulfate were of analytical grade. Silica gel (200–300 mesh, irregular) was obtained from Haiyang Chemical Group (Qingdao, China).

Preparation of Crude Extract from Broccoli Seeds. Fifty grams of seeds was homogenized in an analytical grinder. After ground seed was added to 300 mL of pure water, glucosinolates were hydrolyzed by myrosinase, and the mixture was allowed to spontaneously autolyze for 2 h at 25 °C. The resulting mixture was extracted 3 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 a crude extract. In order to remove impurities and enrich sulforaphane, the crude extract was treated by means of either liquid–liquid extraction or SPE.

Liquid–Liquid Extraction. The crude extract was dissolved in 300 mL of 10% ethanol (v/v) and washed 3 times with 300 mL of hexane to remove nonpolar contaminants. Following this, the aqueous phase was extracted 3 times with 300 mL of ethyl acetate. The ethyl acetate fractions were pooled, dried over 10 g of anhydrous sodium sulfate, and filtered through a 0.22 μm membrane. The filtrate was dried at 35 °C under vacuum in a rotary evaporator, to produce a sulforaphane-rich extract. The sulforaphane-rich extract was dissolved in 100 mL of methanol before quantitative analysis by HPLC.

Solid-Phase Extraction. As an alternative to the more traditional liquid–liquid extraction method, the crude extract was dissolved in 150 mL of hexane–ethyl acetate (8:2, v/v). Silica gel about 15 g (200–300 mesh) was activated for 60 min at 120 °C. Following this, silica gel was mixed with 50 mL of hexane, and the slurry was packed into an SPE column. The SPE column was conditioned with 50 mL of hexane prior to loading the sample. The solvent and sample flow rate through the column was controlled at 2–3 mL per minute under vacuum. The column was then washed with 30 mL of ethyl acetate. Sulforaphane was eluted with 50 mL of ethanol. The eluate was evaporated at 35 °C under vacuum in the rotary evaporator, to produce a sulforaphane-rich extract. The sulforaphane-rich extract was dissolved in 10% acetonitrile in water, and then filtered through the 0.22 μm membrane before subjection to preparative HPLC.

Table 1. Comparative Results and Solvent Consumption (Liquid–Liquid Extraction vs SPE)[a] for Extracting Sulforaphane

results	liquid–liquid extraction	SPE
content of sulforaphane (%)[b]	22.8 ± 0.5	49.9 ± 1.1
recovery of sulforaphane (%)[b]	83.8 ± 0.6	92.6 ± 0.8
	solvent consumption (mL)	
ethyl acetate	900	60
hexane	900	220
ethanol	30	50
total	1830	330

[a] 50 g broccoli seed meal used for each method. [b] Values represent mean ± S.D., $n = 3$.

Table 2. Effects of the Variations in the Mobile Phase on the Retention Time (t), the Retention Factor (K'), the Selectivity Factor (α), and the Resolution Factor (R_s) of Sulforaphane for HPLC Separation

	acetonitrile				methanol			
concentration	t	K'	α	R_s	t	K'	α	R_s
40%	4.724	0.845	1.26	1.59	9.739	2.80	1.15	1.65
35%	5.797	1.26	1.23	1.38	12.581	3.91	1.26	2.70
30%	7.181	1.80	1.20	1.97	16.841	5.57	1.42	4.13
25%	10.240	2.99	1.14	1.08	23.058	8.0	1.60	7.30

Analytical Chromatography. Analytical HPLC was performed with a reversed-phase C_{18} column (250 × 4.6 mm, 5 μm, Diamodsil) using Hitachi model L-7100 pumps. The analytical chromatography was carried out under isocratic conditions by varying the percentage of acetonitrile or methanol in water (from 25% to 40%) using a flow rate of 1.0 mL/min at room temperature. Chromatograms were recorded at 254 nm using a Hitachi L-7420 variable wavelength detector. The column hold-up time was determined by injection of an aqueous solution of sodium nitrate (26 mg mL^{-1}).

Preparative Chromatography. Preparative HPLC separation was carried out on a Waters Prep 4000 liquid chromatography system equipped with a fluid handling unit (pump heads), controller (for solvent gradient, flow rate, external events, and sparging process) and a 2487 dual-wavelength absorbance detector with a preparative detection cell (Waters, Milford, MA). The chromatographic separation was performed on a Symmetry C_{18} column (300 × 19 mm, 7 μm). Detection was carried out at 254 nm.

Preparation of the Standard Curve. Quantification was based on the external standard method. A stock solution was prepared with 5.0 mg of sulforaphane reference standard, which was dissolved and diluted to 10 mL with acetonitrile. Aliquots of the standard stock solution of sulforaphane were pipetted into different 10 mL flasks and diluted to mark with acetonitrile. The final concentrations of sulforaphane 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. Sulforaphane was analyzed using an Hitachi HPLC apparatus equipped with Hitachi model L-7100 pumps, a L-7420 variable wavelength detector, and a reversed-phase C_{18} column (250 × 4.6 mm, 5 μm, Diamodsil). The solvent system consisted of 20% acetonitrile in water, then changing linearly over 10 min to 60% acetonitrile, and then raising to 100% immediately and running isocratically for 2 min to purge the column. The column oven temperature

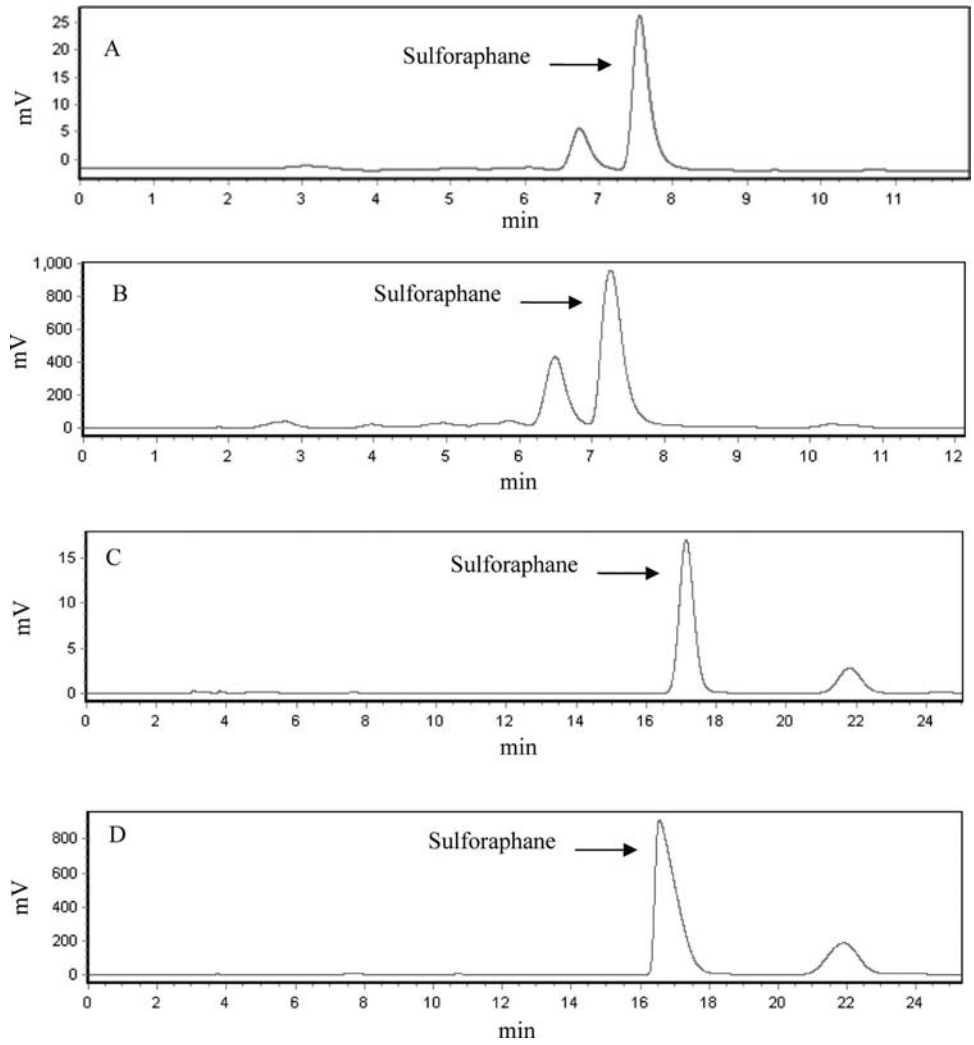

Figure 1. Comparison of the organic modifiers on separation of sulforaphane employing 30% acetonitrile (A,B) in water and 30% methanol in water (C,D). A and C are low mass loading (2.5 μg), and B and D are high mass loading (100 μg). The detected absorbance was set at 254 nm. The column was reversed-phase C_{18} (250 $\times$ 4.6 mm, 5 μm, Diamodsil). The column oven temperature was set at 30 °C. The flow rate was 1 mL/min.

was set at 30 °C. The flow rate was 1.0 mL/min, and 10 μL aliquots were injected into the column. Sulforaphane was detected at UV 254nm.

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 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, USA).

RESULTS AND DISCUSSION

Test Sulforaphane Content. Linear regression analysis of the peak area responses (y) versus the theoretical concentration (x) gave the following equation: $y = 149.36 + 160377.80x$, $r^2 = 0.9998$. The correlation coefficient demonstrated linearity of the method over the concentration range analyzed. The system precision was determined by chromatographing 6 injections of the standard solution and calculating the relative standard deviation (RSD) of the peak area responses. The method precision was established by assaying 6 different extracts of the same broccoli seed sample with the proposed chromatographic method. The RSD% for standard and samples were 0.96 and 0.98, respectively.

Table 3. Effects of Flow Rate and Loading Amount on the Purity of Sulforaphane Products[a]

	flow rate (ml/min)						
loading amount (20 mg)	8	10	12	14	16	18	20
purity (%)	92.3	95.8	99.1	95.6	92.8	91.0	90.2
	loading amount (mg)						
flow rate (12 mL/min)	20	40	60	80	100	150	200
purity (%)	99.1	98.5	97.9	97.2	96.9	95.3	93.3

[a] Different amounts of sulforaphane-rich extract obtained by SPE were dissolved in 10 mL of 10% acetonitrile in water, and then filtered through the 0.22 μm membrane before being injected into preparative HPLC.

Purification of Sulforaphane. The principal contaminants in broccoli seed meal extract are typically oil based, which should be removed in order to achieve high-purity sulforaphane. The traditional liquid–liquid extraction method is facilitated by resuspending the concentrated crude extract in 10% ethanol, which is then repeatedly washed with hexane to remove nonpolar impurities. The remaining aqueous phase

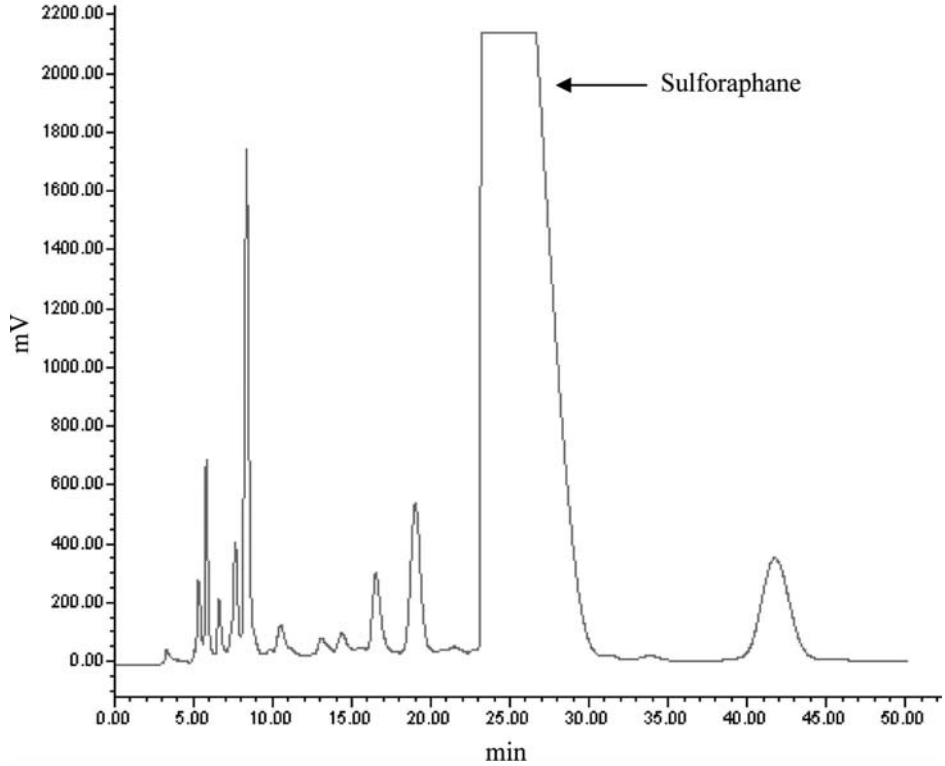

Figure 2. Preparative HPLC chromatogram of the sulforaphane-rich extract. The mobile phase was 30% methanol in water. 150 mg of sulforaphane-rich extract obtained by SPE was dissolved in 10 mL of 10% acetonitrile in water, and then filtered through the 0.22 μm membrane before being injected into preparative HPLC. The column was reversed-phase C_{18} (300 $\times$ 19 mm, 7 μm, Symmetry). The flow rate was 12 mL/min. The detected absorbance was set at 254 nm.

is extracted with ethyl acetate, after which a sulforaphane-rich extract is obtained by evaporating the ethyl acetate away.

Bertelli et al. (*16*) used SPE to enrich sulforaphane from the florets, stalks, and leaves of broccoli, but this method was only suitable for analyzing small quantities of samples. We describe a novel SPE procedure used to remove oil contaminants and other impurities from crude extracts of broccoli seeds.

The results for the comparison of liquid–liquid extraction and SPE are shown in **Table 1**. Despite using a triple solvent extraction, the liquid–liquid extraction did not achieve the same level of recovery as SPE. Moreover, the content of sulforaphane in the sulforaphane-rich extract by liquid–liquid extraction was less than that obtained by SPE, while the solvent consumption by liquid–liquid extraction was much greater than that by SPE (see **Table 1**). The SPE procedure described here allows for the removal of unwanted plant material that might potentially interfere with the separation or block the preparative HPLC column. Hence, this SPE procedure is a valuable alternative to liquid–liquid extraction for sulforaphane prepurification, resulting in significant savings with regard to solvent use, time, and labor.

Effects of the Organic Modifiers on the Separation of Sulforaphane. Organic solvents such as acetonitrile and methanol are powerful tools for enhancing selectivity during separations with an aqueous mobile phase in RP-HPLC. The effects of the variations in the mobile phase on the retention factor (k'), the selectivity factor (α), and the resolution factor (R_s) of sulforaphane are shown in **Table 2**. When developing an analytical method for scaling up to the preparative mode, it is desirable to have the resolution factor (R_s) greater than 2, and the selectivity factor (k') less than 10 (*17*, *18*). Considering the better separation, the shorter run time, and the consequent increase in the throughput, the use of 30% acetonitrile and 30% methanol, respectively, resulted in the most effective mobile phases. However, when increasing the sample loading at 30% acetonitrile as the mobile phase, there was a deterioration in the baseline resolution between sulforaphane and the impurity; in addition, the resolution decreased sharply because of excessive peak broadening (see **Figure 1A,B**). In contrast, much better retention and chromatographic resolution were obtained when the analytical chromatography was performed using 30% methanol in water while increasing loading (see **Figure 1C,D**). Thus, 30% methanol in water was chosen as the mobile phase when considering the preparation of large amounts of samples.

Effects of Flow Rate and Sample Loading on Separation of Sulforaphane. When performing separation on preparative HPLC, the flow rate and the sample loading may have very important roles. The effects of the flow rate and sample loading on the separation of sulforaphane are shown in **Table 3**. Twenty milligrams of sulforaphane-rich extract obtained by SPE was separated by using 30% methanol as the mobile phase at different flow rates (from 8 to 20 mL/min). Furthermore, varying quantities of sulforaphane-rich extract (from 20 to 200 mg obtained by SPE) were separated by 30% methanol at a constant flow rate of 12 mL/min. **Figure 2** shows a preparative HPLC chromatogram of sulforaphane extract using 30% methanol in water as the mobile phase at a flow rate of 12 mL/min. The fractions containing sulforaphane should be combined to guarantee the recoveries of sulforaphane to be more than 95%. The purity of a given product was defined for this study as the sulforaphane percentage of the product. The most efficient separation (high apparent purity) can be obtained at a sample loading of 20 mg at a flow rate of 12 mL/min. Once the sample

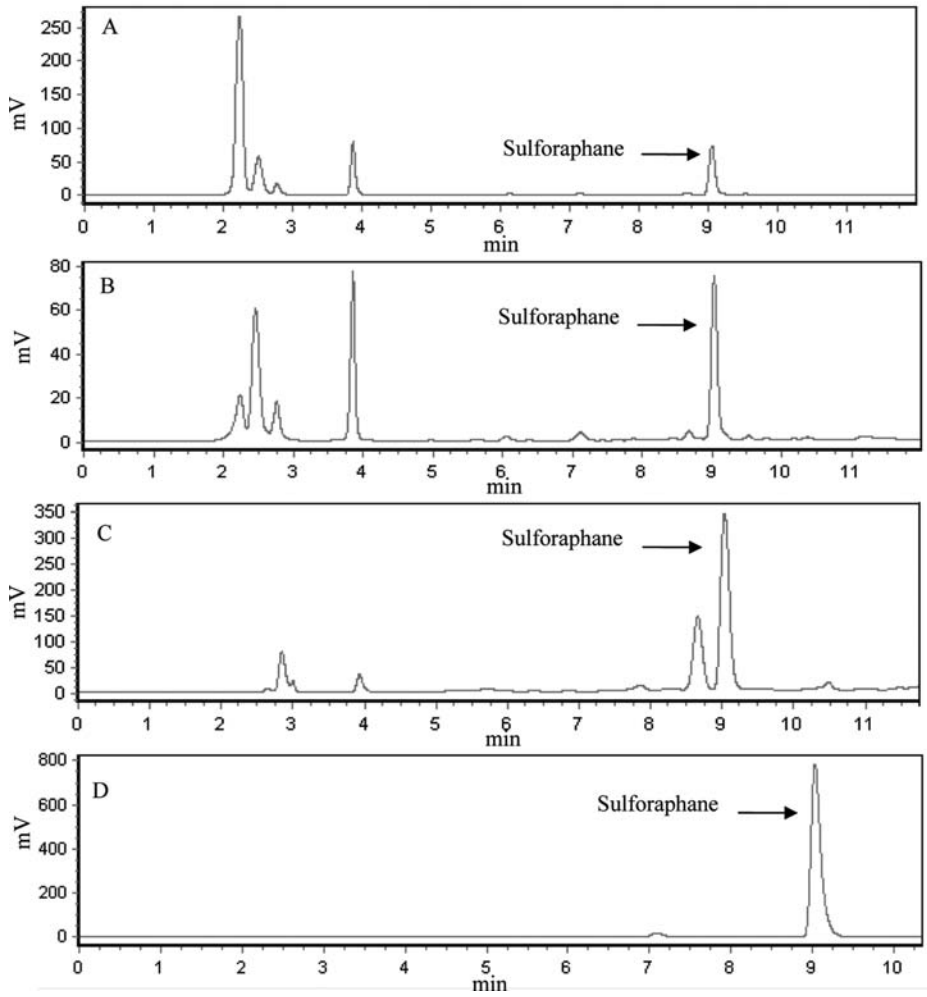

Figure 3. Analytical HPLC chromatograms of sulforaphane obtained by crude extraction (A), liquid–liquid extraction (B), SPE (C), and preparative HPLC (D). The detected absorbance was set at 254 nm. The column was reversed-phase C18 (250 × 4.6 mm, 5 μm, Diamodsil). The solvent system consisted of 20% 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.

loading surpasses 150 mg or the flow rate exceeds 14 mL/min, the separation efficiency of the sulforaphane decreased sharply (**Table 3**). Consequently, for preparation of the purified sulforaphane product from the sulforaphane-rich extracts, the maximal loading amount should be limited to less than 150 mg, and the appropriate flow rate should be controlled at 12 mL/min.

Chromatograms of sulforaphane obtained by crude extraction, liquid–liquid extraction, SPE, and preparative HPLC are shown in **Figure 3**. On the basis of the above procedures, the crude extract of 50 g broccoli seeds can produce 342 ± 5.6 mg of sulforaphane-rich extract of about 50% sulforaphane after separation by SPE, and then 162 ± 2.8 mg of 95% sulforphane can be obtained after purified by preparative HPLC. Therefore, each kilogram of broccoli seeds can be expected to yield 3.2 g of highly purified sulforaphane.

Optimizing the Preparative Purification Strategy. In order to raise the production rate and decrease solvent consumption, additional modification and refinements were carried out. Once the target compound was eluted and collected (about 30 min), the concentration of methanol was increased to 100%, achieving a complete elution of undesired compounds in only 2 min. Thus, cross-contamination between sequential injections was avoided. To minimize equilibration time, the system is held under the initial conditions (30% methanol in water) for 8 min before the next injection. The cycle time for this approach, including column equilibration time, is 40 min. Consequently, the purification strategy has been found to be efficient and robust and is well-suited for the separation and purification of sulforaphane when evaluating the biological effects of sulforaphane in our research laboratories.

MS and NMR. Purified samples were analyzed by MS and NMR. The mass spectrum (EI) of sulforaphane, in *m*/*z* is 39, 55, 64, 72, 86, 114, 160, and 177. Electron impact mass spectrometry (see **Figure 4**) gave a small molecular ion (M^+) signal at 177, and revealed prominent fragment ions with masses of 160 and 72. Precise masses of molecular and fragment ions obtained by electron impact mass spectrometry were 177.0286 (calculated for $C_6H_{11}NOS_2$, 177.0283), 160.0257 (calculated for $C_6H_{10}NS_2$, 160.0255), and 71.9909 (calculated for C_2H_2NS, 71.9908). MS (EI) analysis of the purified sulforaphane sample was consistent with those previously reported (*13*, *14*, *16*). ^{1}H NMR parameters are (see **Figure 5A**) δ 3.60 (t, 2H, CH_2NCS), 2.80–2.66 (m, 2H,CH_2SO), 2.60 (s, 3H,CH_3SO), and 1.99–1.86 ppm (m, 4H, CH_2CH_2). ^{13}C NMR parameters are (see **Figure 5B**) δ 53.5, 44.6, 38.7, 29.0, and 20.1 ppm. NMR analysis of the purified sulforaphane sample produced results that also were consistent with those previously reported (*2*, *13*) and did not show the presence of compounds other than sulforaphane.

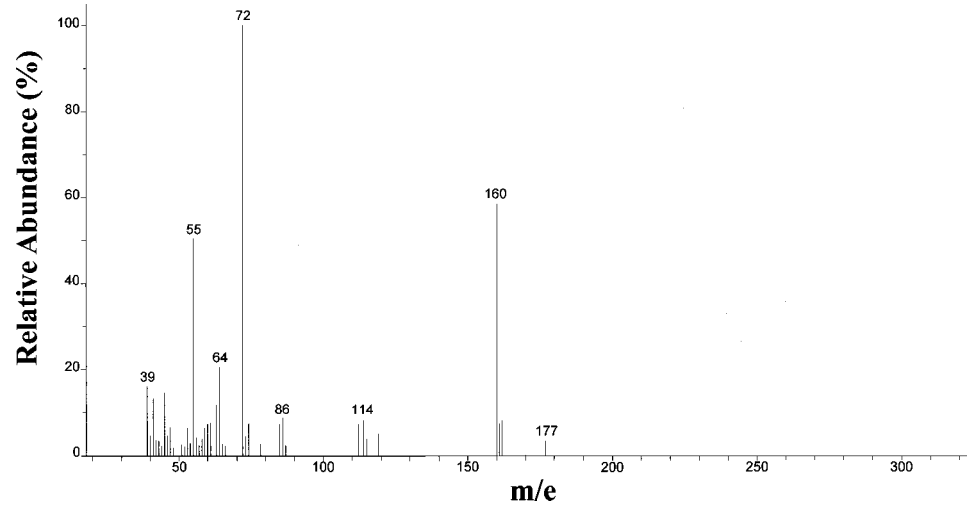

Figure 4. Mass spectra of sulforaphane purified by preparative HPLC.

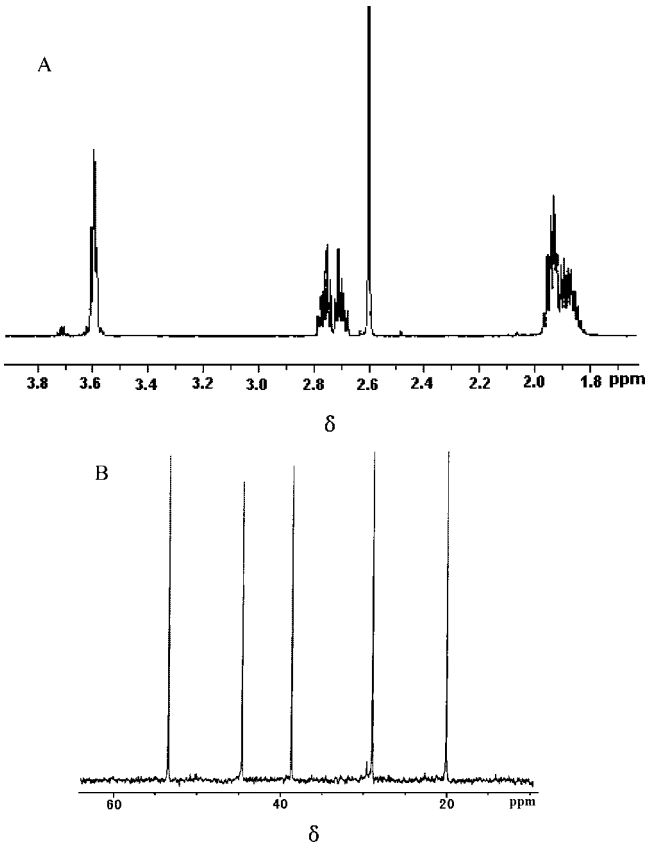

Figure 5. NMR spectra ($CDCl_3$, 600 MHz) of sulforaphane purified by preparative HPLC: (A) ^1H NMR spectrum (B) ^{13}C NMR spectrum.

A novel method for separation and purification of sulforaphane from the seeds of broccoli has been described in this research. In order to remove large quantities of oil contaminants and other impurities, a solid-phase extraction (SPE) method was applied. The SPE method provides the advantage of yielding a higher content of sulforaphane from the crude extract and will therefore be a valuable alternative to the more conventional liquid–liquid extraction. Pure sulforaphane was obtained by preparative C_{18} HPLC using a mobile phase consisting of 30% methanol in water. The preparative purification strategy was optimized by significant improvement in the loading amount, flow rate, and cycle time. The purity was assessed by HPLC, MS, and NMR. The results reported here indicate that the proposed process could provide effective purification, high recovery, and sustained usability of materials in a fast, cost-effective, and waste-conscious manner.

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