Purification of Secoisolariciresinol Diglucoside with Column Chromatography on a Sephadex LH-20

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Abstract

A simplified and efficient method is developed for the large-scale purification of the secoisolariciresinol diglucoside (SDG) from defatted flaxseed after aqueous ethanol extraction. Extractant from defatted flaxseed with aqueous ethanol is hydrolyzed with basic solution, concentrated under vacuum, subjected to Sephadex LH-20 column chromatography, and eluted with aqueous ethanol of different concentrations. Elution is monitored by a UV detector at 280 nm, and fractions containing SDG are pooled, concentrated, and applied to a second column chromatography under the same conditions. Elution with water results in a better resolution of SDG [94.5% by high-performance liquid chromatography (HPLC)] than that with pure ethanol or 50% (v/v) aqueous ethanol. HPLC–photodiode array detection-mass spectrometry and NMR are applied to identify SDG and to determine the purity of the eluted fraction. This simplified purification scheme avoids toxic organic solvent used in the common silica gel separation process and, thus, increases the safety of the process.

Introduction

Secoisolariciresinol diglycoside (SDG) (Figure 1) in flaxseed (*Linum usitatissimum* L.) was first described in 1956 (1). It is the main lignan in flaxseed with matairesinol, pinoresinol, and isolariciresinol as minor ones (2,3). Flaxseed contains the largest amount of SDG (11.7 to 24.1 g SDG/kg defatted flaxseed meal) among all the grains, legumes, fruits, and vegetables (2).

SDG has been of increasing interests for its potential anticarcinogenic, estrogenic, and antiestrogenic activities as well as for its antioxidant effect (4–10). It is digested in the colon by the action of bacterial flora to produce the mammalian lignans [enterolactone (EL) and enterodiol (ED) (11)] and protect against hormone-dependent cancers, especially breast and prostate cancers (5,6). Experimental and epidemiological studies suggested that high urinary excretion levels of mammalian lignans in humans and animals were associated with a decreased risk of developing hormone-dependent diseases (7,8). Exposure to purified SDG during early life showed a positive effect on developing bone due to the potential estrogenic effects of SDG (9). Both SDG and its metabolites, EL and ED, were demonstrated to possess antioxidant activity in lipid and aqueous systems at relatively low concentrations (10). Besides, SDG was also reported to represent a potential treatment for renal disorders (12) and inhibit the development of type-1 and type-2 diabetes (13,14).

Although whole flaxseed or its ground counterpart can be incorporated into foods, the rather high oil content and the mucilage component of the flax would contribute to excessive caloric intake and laxation, respectively (15). Isolation and purification of SDG from defatted flaxseed meal or hull provided more available products of SDG and added value to the byproduct of flaxseed meal or hull (15–17).

Silica gel chromatography, following preliminary separation on a C18 reversed-phase column, has been applied as the predominant method for isolation and purification of SDG (18,19). Although the purity and recovery of SDG is satisfactory, there exists a potential safety problem because of the volatile toxic solvents methanol and chloroform. High-performance liquid chromatography (HPLC) or preparative HPLC following solid-phase extraction has also been adopted for the isolation and purification of SDG (20,21). Although it is

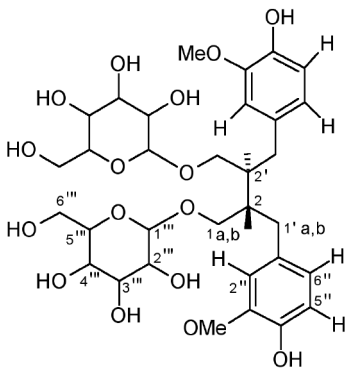

Figure 1. Chemical structure of SDG. Nomenclature was according to Chimini et al. (20).

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safe and offers satisfactory purity, it is time-consuming and expensive to recover pure SDG. High-speed counter current chromatography (HSCCC) was also introduced to purify SDG after enrichment with macroporous adsorption resin, and that method obtained a fraction with purity greater than 93% (22). Because HSCCC is based on the difference in partition behavior of solutes between the two immiscible liquid phases, the components that possess similar polarity with SDG have to be removed by other separation methods.

Hydroxypropylated dextran gel Sephadex LH-20 swells to different extents in different solvents and acts as a universal stationary phase for the separation of small molecules with a one-component eluent (23). To the best of our knowledge, there is no report on the use of Sephadex LH-20 for the isolation of SDG. Therefore, in this study, Sephadex LH-20 column chromatography was applied to the isolation and purification of SDG to improve purification efficiency, reduce or avoid safety problems, and prepare pure SDG in a large scale.

Material and Methods

Preparation of SDG extract

Flaxseed was provided by Luqi Co. Ltd. (Cangji, Xinjiang Municipality, PR China). Mucilage was removed by soaking, leaching, and washing with water at 70°C. Flaxseed was dried, ground to flour (20–80 mesh), and defatted by two extractions with *n*-hexane under magnetic stirring for 1 h.

Portions of defatted flaxseed flour (50 g) were extracted with 50% (v/v) aqueous ethanol under 60°C with a solid–liquid ratio 1:20 (g/mL) by stirring for 3 h. The extract was obtained by centrifugation (3000 r/min for 20 min). The residue was re-extracted under the same conditions, but for 2 h. Both extracts were pooled, evaporated under vacuum at 45°C to approximately 15 mL, and subjected to alkaline hydrolysis at 25°C for 4 h under constant rotation using 0.25M aqueous sodium hydroxide. After hydrolysis, the samples were acidified to pH 4.0 using 6M hydrochloric acid and stored at 4°C before isolation. Microfiltration through 0.45-µm cellulose acetate membrane (Genosys Tech-Trading Co., Ltd, Beijing, China) or centrifugation (10,000 r/min and 15 min) was performed before sampling, and approximately 5 g solid content was kept in the filtrate or supernatant.

Sephadex LH-20 column chromatography

SDG extracts were separated using Sephadex LH-20 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) column chromatography. One hundred grams of Sephadex LH-20 was suspended in approximately 600 mL of aqueous ethanol (1:1, v/v) at 30°C for 24 h. Then the slurry was evaporated under reduced pressure in order to remove air from the slurry. The gel was allowed to equilibrate for 4–5 h at room temperature and then poured carefully into a glass column, and a continuous flow of solvent was maintained through the column. The column (95 × 1.6 cm) was conditioned with 50% (v/v) aqueous ethanol for 24 h.

The SDG extract was subjected to Sephadex LH-20 column chromatography after microfiltration or centrifugation and eluted with water, 50% (v/v) aqueous ethanol, or pure ethanol. The sample volume was 3 mL, and the flow rate was 0.8 mL/min. The UV detector was set at 280 nm. Fractions were collected every 7.5 min, and the wavelength scan was performed with a 1100 UV–vis spectrophotometer (Rayleigh Analytical Instrument Co., Beijing, China) for each fraction that possessed absorption at 280 nm. Fractions containing SDG were pooled, concentrated, and applied to Sephadex LH-20 column chromatography under the same conditions. Rinsing of the hydroxypropylated dextran gel was accomplished by elution with increasing concentrations of ethanol from 20% to 75% in water at an increment of 15–20%, and concentrations of ethanol were decreased to 20% for the a rinsing cycle.

Identification with HPLC–photodiode array detection-mass spectrometry

HPLC analysis was performed on a Waters 2690 HPLC system (Waters, Milford, MA) equipped with an in-line degasser, a 600E multisolvent delivery system, a 717 plus auto sampler, and a 996 photodiode array (PDA) detector. The PDA was set to a range between 200 and 400 nm, and chromatograms were recorded at 280 nm. The UV spectra of SDG peak was recorded by the PDA. A 150 × 2.1 mm i.d. Symmetry C8 reversed phase column (Waters) with 5-µm particle size was used for the analysis. Column temperature was set to 30°C, and the injection volume was set to 10 µL. The mobile phase consisted of two solvents: mobile phase A, 1% acetic acid solution (v/v); and mobile phase B, pure methanol. The separation was performed using the following linear gradient of A–B (v/v): 0 min, 5:95; 40 min, 55:45; and 60 min, 5:95. The flow rate was maintained at 0.5 mL/min. The mass spectrometry (MS) analysis was performed in both positive and negative electrospray ionization (ESI) mode on a Waters Platform ZMD 4000 (Waters). Typical running parameters were as follows: capillary voltage, +3.87 kV for positive mode and –3.88 kV for negative mode; cone voltage, 25 V for positive mode and 24 V for negative mode; and source temperature, 120°C. Spectra were scanned over a mass range of *m/z* 300–800.

^{1}H NMR and ^{13}C NMR

For the characterization of the SDG, an objective fraction eluted from Sephadex LH-20 column chromatography was dissolved in methanol-d_4 (99.8% D, Aldrich Chemical, Milwaukee, WI) and analyzed by ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectroscopy using a Bruker DRX400 instrument (Bruker, Coventry, UK), which was equipped with a 5-mm indirect detection probe with Z-gradient. NMR data were compared with literature data (19,20,24).

Quantitation of SDG

A linear HPLC calibration curve for SDG quantitation was obtained using the purified SDG (purity 94.54% by HPLC area) as external standard for the concentration range 0–150 µg/mL. The injection volume was 10 µL, and other conditions were the same with HPLC–PDA-MS analysis. Samples were micro-

fitered through a 0.45-µm cellulose acetate membrane before submitting to HPLC.

Results and Discussion

Identification of SDG

UV spectra of SDG recorded between 200 to 400 nm were obtained by a PDA detector with aqueous ethanol as the solvent (Figure 2). SDG presented maximum absorption at 228.60 and 280.60 nm by reason of its aromatic structure and electron-releasing substituents of –OH and $–OCH_3$. This result was close to those of Fritsche et al. (24), Coran et al. (25), and Madhusudhan et al. (17). They reported a maximum absorption at 280, 282, and 282 nm in order. This slight difference in maximum absorption could be attributed to differences in the UV detectors as well as the solvents used.

Mass spectra were recorded in both positive and negative ionization modes in order to obtain more information on the structural features of SDG (Figure 3). The mass spectra in negative mode showed a $[M–H]^-$ peak at *m/z* 686.3 corresponding to the molecular formula $C_{32}H_{46}O_{16}$. The ion at *m/z* 361.8 $[M–2Glu+2H_2O–H]^-$ was formed by loss of two glucose units while adding of two water units, representing the most prominent fragment ion in negative mode. Accordingly, the ions at *m/z* 343.8 $[M–2Glu+H_2O–H]^-$, *m/z* 524.1 $[M–Glu+H_2O–H]^-$, and *m/z* 722.3 $[M+2H_2O–H]^-$ were generated by loss of one or two sugar units and the addition of one or two water units. Besides, loss of a glucose unit and the addition of a water unit and Na^+ gave rise to a peak at *m/z* 546.2 $[M–Glu+H_2O+Na–2H]^-$ (Figure 3A).

In the case of positive mode, fragment ions at *m/z* 710.4 $[M+Na]^+$, *m/z* 726.3 $[M+K]^+$, *m/z* 548.1 $[M–Glu+H_2O+Na]^+$, and *m/z* 363.9 $[M–2Glu+2H_2O+H]^+$ were found in the mass spectra, among which ions at *m/z* 710.4 $[M+Na]^+$ represented the highest abundance (Figure 3B). These mass spectra were in accordance with literature data (24).

However, chances are that the occurrence of complex mixtures of glycosides and aglycones present in natural extracts represent similar or the same UV and mass spectra with SDG. Moreover, many natural products can undergo severe degradation when subjected to the elevated temperatures required for some MS interfaces. Therefore, it was difficult in many cases to sufficiently distinguish organic compounds by sole use of MS. NMR was adopted because it is a powerful tool for structural elucidation of complex compounds. Results of ^{1}H-NMR and ^{13}C-NMR spectra (Table I) were consistent with those data by Chimichi et al. (20) and Qiu et al. (19). Because only half of the expected number of proton and carbon signals were detected, there must be a symmetrical structure in the SDG molecule (19,20). Compared with the literature data (19), the definitive structure of purified SDG was recognized as 2,3-bis [(4-hydroxy-3-methoxyphenyl)methyl]-1,4-butanediyl bis-[R-(R*, R*)]-β-D-glucopyranoside.

Elution on Sephadex LH-20 column

Free SDG was obtained after alkaline hydrolysis of the extract because the existence of ester-bonds in the oligomer composed of SDG and 3-hydroxy-3-methyl glutaric acid (26). Centrifugation following acidification removed some undis-

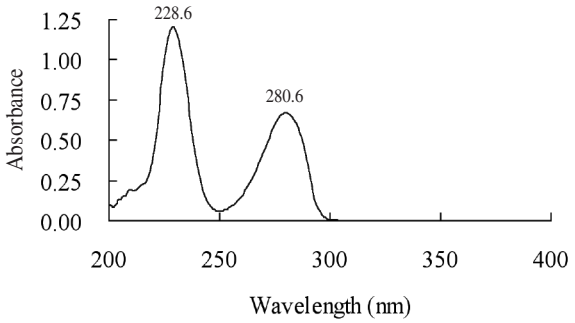

Figure 2. UV spectra of SDG. The solvent was the mobile phase for HPLC.

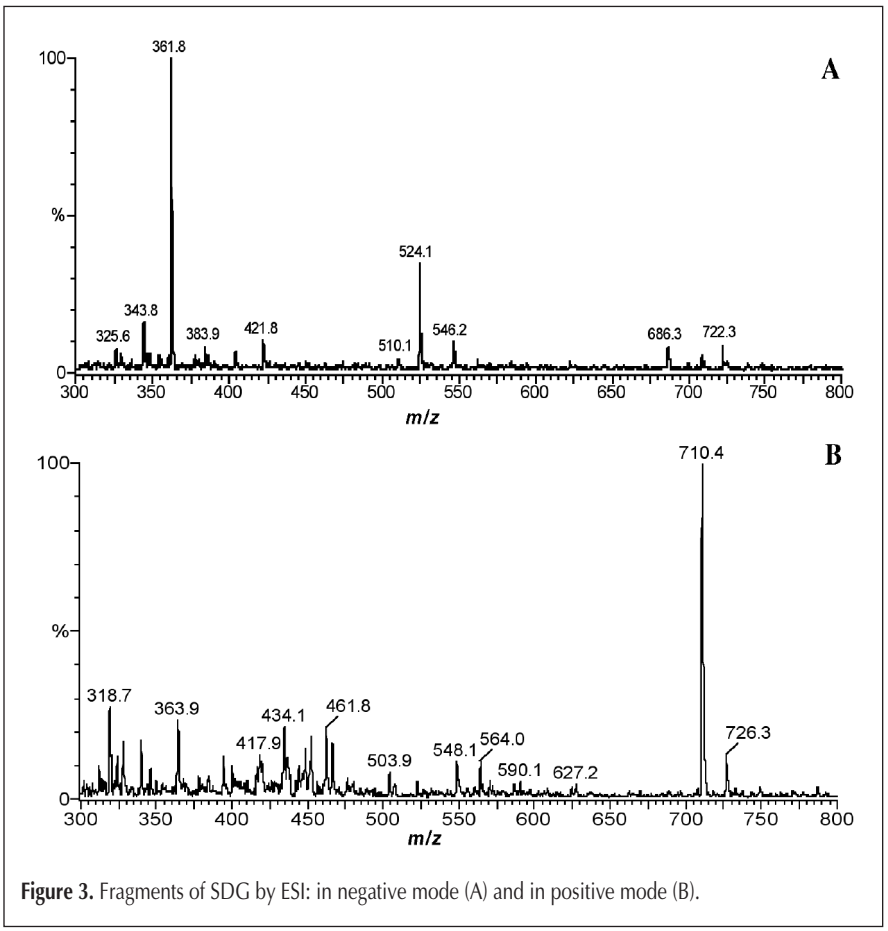

Figure 3. Fragments of SDG by ESI: in negative mode (A) and in positive mode (B).

Table I. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data for SDG in CD_3OD

	δ (ppm)				δ (ppm)		
		Literature data				Literature data	
Proton	Detected	Reported (19)	Reported (20)	Carbon	Detected	Reported (19)	Reported (20)
H1a	4.05	4.07	4.08	1	71.48	71.17	71.3
H1b	3.46	3.46	3.48	2	41.54	41.16	41.1
H-2	2.12		2.13	1'	35.92	35.59	35.6
H-1' a,b	2.59, 2.64	2.68, 2.60	2.69, 2.62	1"	134.24	133.90	134.2
H-2"	6.62	6.57	6.59	2"	113.93	113.49	113.7
H-5"	6.64	6.64	6.64	3"	149.01	148.68	148.9
H-6"	6.57	6.55	6.57	4"	145.67	145.32	145.8
3"-OMe	3.71	3.72	3.74	5"	115.99	115.63	115.8
H-1"'	4.22	4.23	4.24	6"	123.21	122.88	123.1
H-2"'	3.21		3.21	OMe	56.62	56.24	56.4
H-3"'	3.34	3.18–3.38	3.35	1"'	105.03	104.75	104.8
H-4"'	3.29	3.18–3.38	3.30	2"'	75.54	75.22	75.3
H-5"'	3.24		3.24	3"'	78.47	78.14	78.2
H-6"' a	3.84	3.85	3.86	4"'	72.00	71.64	71.7
H-6"' b	3.69	3.68	3.68	5"'	78.15	77.85	77.9
				6"'	63.13	62.77	62.8

solvable impurities. The sample submitted to identify SDG by HPLC–PDA-MS and NMR was twice purified by the column chromatography on a Sephadex LH-20. In consideration of the dissolving capacity of SDG and toxicity, as well as safety of a mobile phase, three different concentrations of ethanol were used: pure ethanol, 50% (v/v) aqueous ethanol, and water. Elution curves were plotted according to the absorbance of each eluted fraction at 280 nm (Figure 4). Under the following conditions: column temperature, 25°C; and flow rate, 0.8 mL/min, better resolution of SDG was obtained when water was used as the mobile phase. The elution volume of the SDG peak with water as the mobile phase (270 mL) was significantly bigger than that when 50% (v/v) aqueous ethanol (222 mL) or pure ethanol (204 mL) were used as the mobile phase. Also, when ethanol was replaced with water as the mobile phase, nearly baseline separation was achieved. The elution curves showed that with the increasing ratio of water–ethanol in mobile phase, fractions containing the SDG component were separated better from fractions containing other components. This indicated that the retention behavior of SDG was greatly affected by the mobile phase. The reason may be that hydroxypropylated dextran gel swells to a different degree with a different concentration of aqueous ethanol, and a lower swelling degree would cause smaller pore volume (23). Therefore, with water as the mobile phase, the separation efficiency was greater due to the bigger swelling ability of Sephadex LH-20 gel.

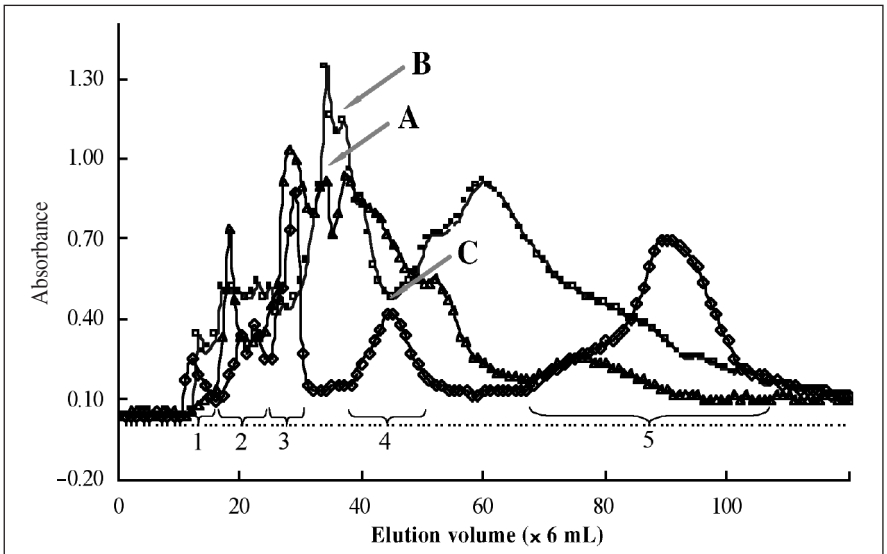

Figure 4. Separation of SDG extract on Sephadex LH-20 column chromatography with different mobile phase: pure ethanol (A); 50% aqueous ethanol (B); and water (C). Arrows point to where the SDG component eluted. Numbers 1–5 mean different fractions pooled according to UV absorption. Separation was carried out under the conditions that flow rate 0.8 mL/min and UV detection at 280 nm. Fractions were collected every 7.5 min. Absorbance was at 280 nm.

Wavelength scan between 240–400 nm for each fraction was carried out to judge whether SDG was eluted in the fraction. Similar results were obtained for fractions in the same peak (Figure 5). Therefore, fractions that possess similar absorption with SDG (peak 4 in Figure 4) were pooled, evaporated under vacuum to a concentration of approximately 0.1 g/mL, and re-sampled. According to the UV absorption characteristics, the fractions containing SDG were collected, evaporated under

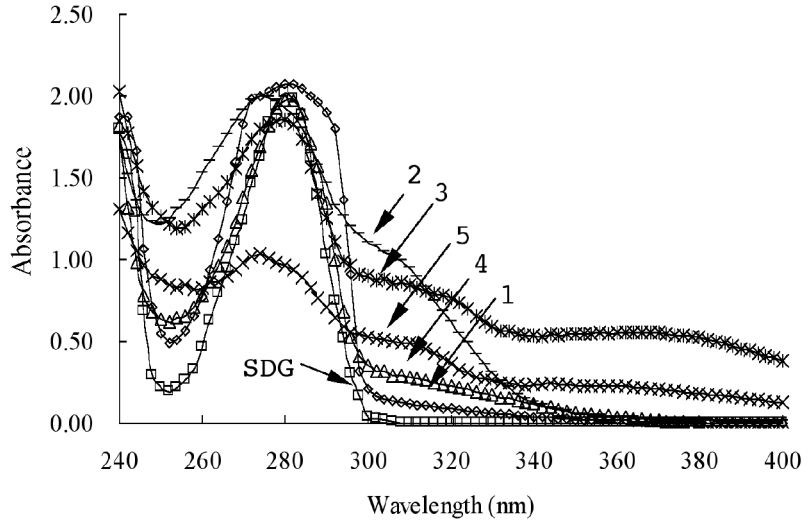

Figure 5. UV absorption of main fractions after pooling according to UV absorption of each fraction. These fractions were collected when water was used as elution solvent.

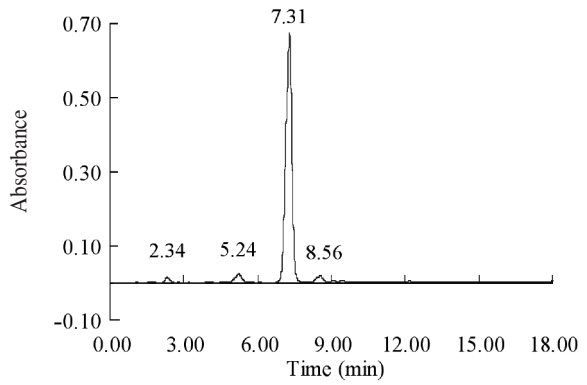

Figure 6. HPLC chromatogram of purified SDG. Retention time was marked on the figure. A Symmetry C8 column was used as the stationary phase. Separation was performed using a linear gradient mobile phase consisting of two solvents: 1% acetic acid solution (v/v) and pure methanol. The flow rate was 0.5 mL/min. Absorbance was at 280 nm.

vacuum, and freeze-dried before submitting to HPLC–PDA-MS analysis. Although the energy costs for evaporation may be high for use of inexpensive distilled water as the mobile phase, the substance-free fractions can be discharged into the waste water system. In addition, water is safer than methanol and chloroform.

One of the disadvantages of column chromatography is a time-consuming process in a rinsing cycle. In our experiments, rinsing of Sephadex LH-20 gel with aqueous ethanol immediately following the elution of SDG fraction was found to save time to a great extent. Rinsing with increasing and decreasing concentrations of aqueous ethanol takes approximately 8 h for a rinsing cycle and that with water, approximately 16 h. However, the concentration of aqueous ethanol should be increased by a gradient method way and all the solvent must be degassed under vacuum before use. Aqueous ethanol for rinsing was collected in a container and recovered by evaporation for reuse. Also, our study revealed that rinsing of Sephadex LH-20 with gradient aqueous ethanol to a final concentration of 75% (v/v) avoided significant change of bed volume (less than 1%).

Purity of SDG by HPLC–PDA-MS

Purity of SDG fraction was determined with HPLC. According to UV absorption by PDA and MS spectra in both ESI^+ and ESI^-, the peak eluted at 7.31 min was identified as SDG. Content of SDG in a purified sample was 94.54% (Figure 6). With water as the mobile phase, SDG with satisfactory purity was obtained after isolation by two Sephadex LH-20 column chromatography runs.

Recovery of SDG by Sephadex LH-20 column separation

A calibration curve was obtained by plotting peak area versus SDG content as A = 40.601C – 12.002, where A refers to response peak area and C refers to content of SDG (ng). The repeatability of the quantitation method was tested by simultaneous analysis of five replicates, and the coefficient of variation was 3.42%. The regression coefficient of the calibration plot (R^2) was 0.9997.

To determine recovery of SDG in the isolation and purification process on Sephadex LH-20 column chromatography, SDG content of 5 repeated samples was determined before and after the run. An average SDG recovery of 97.2 ± 1.6% was obtained. Therefore, the loss of SDG in the purification step was insignificant and acceptable.

Conclusion

This study demonstrated that the separation of fractions containing the SDG component with water as the mobile phase was better than that with aqueous ethanol or pure ethanol. This is probably attributed to the bigger swelling capacity of Sephadex LH-20 in water. This study also revealed that increasing and decreasing concentrations of ethanol in water saved time greatly in rinsing Sephadex LH-20 gel. Because column chromatography on Sephadex LH-20 is easy to scale-up, a large amount of purified SDG can be obtained to evaluate its bioactivity or act as a standard for HPLC quantitation.

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