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Pengqun Kuang†[a]; Hao Liang†[a]; Qipeng Yuan[a]
[a] State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, Beijing, P.R. China

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Isolation and Purification of Glucoraphenin from Radish Seeds by Low-Pressure Column Chromatography and Nanofiltration

Pengqun Kuang†, Hao Liang†, and Qipeng Yuan
State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, Beijing, P.R. China

A new and low–cost method to isolate and purify glucoraphenin as a potassium salt from radish seeds was described in this article. Crude glucoraphenin was separated from radish seeds, and then used as raw material for further purification of glucoraphenin by low-pressure column chromatography. Compared with K_2SO_4 solution, KCl solution is a preferable eluent for increasing the purity and recovery of glucoraphenin, and reducing the usage of salt. In order to remove a large quantity of KCl, nanofiltration with NF270-400 membrane and precipitation with methanol were investigated. Consequently, compared with precipitation, the purity and recovery of glucoraphenin prepared by nanofiltration were both much higher. At the same time, nanofiltration also got better efficiency of desalination. The purity of glucoraphenin was 91.5%, the recovery of it was 89.0%, and the removal of KCl was 99.5%. The glucoraphenin product was identified by MS, ^1H, and ^{13}C NMR spectra.

Keywords desalination; glucoraphenin; low-pressure column chromatography; nanofiltration; purification

INTRODUCTION

Various investigations substantially illustrate that a diet rich in plant materials, such as fruits and vegetables, can reduce the risk of many cancers, because there are kinds of substances in fruits and vegetables that are important for maintaining health (1). Those secondary metabolites of plant materials have aroused scientists' great interest for their potential role in helping to maintain human health. And these beneficial substances thought to associate with cancer prevention are biologically active non-nutrients (2).

Glucosinolates (GSLs), which are commonly present in vacuoles of the Brassica genus and the family Cruciferae, have generated enormous interest among scientists in the field of cancer research (3). For example, glucoraphanin (4-methylsulfinyl glucosinolate) (Fig. 1) can be hydrolysed by myrosinase (thio-glucoside glucohydrolase, EC3.2.3.1) to sulforaphane after its release from vacuoles by stress (such as cutting or chewing). Sulforaphane can result in certain cancer prevention by inhibiting Phase I enzymes and inducing Phase II enzymes (4–6). Moreover, sulforaphane is able to reduce the incident of a number of forms of tumors, and induce cell cycle arrest and apoptosis in various experimental models (3, 7–10). Furthermore, as a structural analogue of sulforaphane (differs only at a carbon-carbon bond in the alkyl chain), sulforaphene which is hydrolyzed by myrosinase from glucoraphenin (Fig. 1) can also inhibit the growth of tumor cells and have strong antiproliferative activity (11–13). Glucoraphenin can be isolated and purified from radish seeds, which are particularly rich in glucoraphenin (90 μmol/g of dry weight) (14). Therefore, because of their great yield and high content of glucoraphenin, radish seeds can be the best feedstock for glucoraphenin.

A number of approaches to isolate and purify GSLs from plant materials have been reported. High-speed countercurrent chromatography (HSCCC) (15), preparative high performance liquid chromatography (preparative HPLC) (16,17), and strong ion-exchange centrifugal partition chromatography (SIXCPC) (18) have been successfully used to isolate large scale of GSLs. However, these techniques are not commonly available because of needing special apparatus. Although preparative HPLC is an attractive alternative to HSCCC and SIXCPC, it only can be used to prepare small quantities of glucoraphanin (16). Thus, these techniques cannot satisfy the increasing requirement considering great effects of GSLs in cancer prevention. Ion-exchange chromatography on DEAE-Sephadex A-25 (19,20) can isolate small quantities of glucoraphenin, but this method is not suitable for large-scale purification of glucoraphenin because of the high price of DEAE-Sephadex A-25. So far, there is no article that refers to the isolation and purification of large amount of glucoraphenin from plant materials which is suitable for industrial production.

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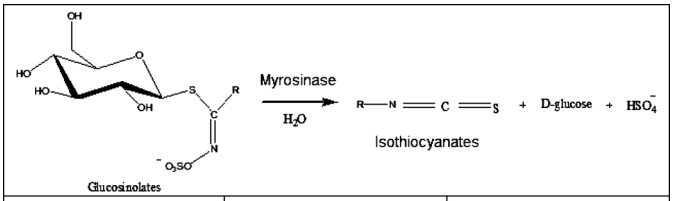
†These authors contributed equally to this work.
Address correspondence to Qipeng Yuan, State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, Beijing, P.R. China. Tel.: +86 10 6443 7610; Fax: +86 10 6443 7610. E-mail: yuanqp@mail.buct.edu.cn



Glucosinolates	Isothiocyantes	R side-chain
Glucoraphenin	Sulforaphene	$CH_3SOCH{=}CHCH_2CH_2$ -
Glucoraphanin	Sulforaphane	$CH_3SOCH_2CH_2CH_2CH_2$ -

FIG. 1. One pair of alkylthio glucosinolates and myrosinase-catalyzed reaction.

For those reasons mentioned above, an experiment using low-pressure column chromatography (LPCC) on acidic alumina was designed and investigated. What is more, nanofiltration technology and precipitation with methanol were used to remove salt from the product of glucoraphenin in our experiments. Our goal is to find a new and low-cost method for the isolation and purification of large amount of glucoraphenin from radish seeds by LPCC on acidic alumina and nanofiltration procedures.

EXPERIMENTAL

Materials

Radish seeds were purchased from Beijing Tong Ren Tang Co. Ltd (Beijing, China). Sinigrin standard was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol and Trifluoroacetic acid (TFA) were of HPLC grade. Ethanol, methanol, petroleum ether, potassium sulfate, and potassium chloride were of analytical grade. Acidic alumina (100–200 mesh) was obtained from Sinopharm Chemical Reagent Co. Ltd (Beijing, China). NF270-400 membranes were purchased from FilmTec Co, USA.

pH, KCl and HPLC Analysis

After being clarified by filtration with 0.22 μm microporous filters, pH values and KCl concentration of the samples were measured by an ion meter equipped with pH and Cl^{-1} electrodes (PXSJ-216, Precision & Scientific Instrument, China).

All the samples of glucoraphenin were measured using sinigrin as the internal standard with a Hitachi HPLC apparatus equipped with Hitachi model L-7100 pumps, L-7420 tunable absorbance detector and reversed-phase C-18 column (250×4.6 mm, 5 μm, Diamodsil^{TM}). Then the purity of the samples was calculated with the conversion factor between glucoraphenin and sinigrin (21). The solvent system consists of 1% methanol and 0.1% TFA in water. The column oven temperature was at 30°C. The flow rate was 1 ml/min, and 5 μl portions were injected into the column. Glucoraphenin and sinigrin was detected at 235 nm.

MS and NMR Analysis

To characterize the purified glucoraphenin, electrospray ionization (ESI) mass spectra (MS) and nuclear magnetic resonance (NMR) spectra were obtained from analysts at the Center of Analysis, Beijing University of Chemical Technology. A Micromass Quattro-Primier mass spectrometry was used with an ion source temperature of 100°C. The spectrum was scanned from m/e 150–550 in negative ion mode, and the sample was dissolved in pure water. The ^1H and ^{13}C NMR spectra were obtained by a Bruker high-resolution AV600NMR spectrometer at 600 MHZ (Bruker Biospin Corporation, USA). The samples were dissolved in D_2O, and the chemical shifts were determined with reference to a water signal at δ 4.70 in relation to that of TMS.

Preparation of Crude Extract from Radish Seeds

After being heated at 120°C for 2 hours to make myrosinase inactive, 200 g radish seeds were homogenized in an analytical grinder. The obtained seed meal was defatted with 3-fold excess (w/v) of hexane for 3 times. After being dried in a drying oven at 50°C, the seed meal was boiled and stirred for an half hour in a 10-fold excess (w/v) of boiling water. Then the clarified extract solution was obtained by filtration (with Whatman No.4 filter paper). The solid residue was extracted again and filtered. The combined boiling-water extracts were concentrated to about 50 mL by a rotary evaporator at 45°C under vacuum. In the first step precipitation, the concentrated extract was added 450 mL ethanol to remove proteins and polysaccharides. After the precipitate was removed by filtration, the clarified solution obtained was concentrated to about 10 mL by a rotary evaporator at 45°C under vacuum, then 190 mL ethanol was added. The ethanolic mixture was stored at 4°C overnight for the second precipitation. After centrifugation, about 8.5 g precipitate obtained by drying in an oven at 45°C under vacuum. The crude extract was dissolved in a measured volume of pure water and then quantitatively analyzed by HPLC.

Preparative Low-Pressure Column Chromatography

The crude extracts obtained by the two-step precipitation were purified by preparative low-pressure column chromatography. A 500×18 mm glass tube was used as the preparative chromatography column. Acidic alumina (100–200 mesh) about 120 g was completely washed with deionized water and then slurry packed into the column. Elution was performed using a pump to deliver a constant flow rate. The separation procedure was monitored with a UV absorbance detector at 220 nm.

Crude extracts (1.5 g) were dissolved with 10 mL deionized water, and injected into the acidic alumina column. The acidic alumina column was washed exhaustively

with deionized water, then eluted with 100 mM KCl at a flow rate of 5 ml/min and monitored at 220 nm in the whole procedure. The fractions containing glucoraphenin were combined together. The volume of the eluate was determined with a measuring cylinder, and the recovery of glucoraphenin was determined by HPLC. The recovery of a given product was evaluated by dividing the quantity of glucoraphenin in the eluate obtained by that in the loading sample.

Separation of KCl by Precipitation with Methanol

300 mL eluate obtained by elution with 100 mM KCl was dried under vacuum at 45°C by the rotary evaporator. The obtained white powder containing glucoraphenin was dissolved with the initial volume of boiling methanol. Then the mixture solution was stored at 4°C to allow KCl to settle. After filtration to remove the remaining particles of KCl, the methanol phase was dried under vacuum at 45°C by the rotary evaporator. The purity and recovery of the glucoraphenin product were determined by HPLC.

Separation of KCl by Nanofiltration

Dead-end filtration experiments were conducted with a magnetic stirred cell which was purchased from Nitto Denko Corporation (Japan). The maximal volume of the magnetic stirred cell was 380 mL, which could be fitted with a membrane disc having an effective diameter of 75 mm within the module, and the effective membrane surface area was 0.0032 m^2. The operation temperature was controlled by the water bath, and the operation pressure was adjusted by a valve and showed with a pressure gauge. All the experiments were performed at constant pressure (about 10 bar) of nitrogen so as to exclude pressure variation throughout those procedures. The commercial NF270-400 membrane was used in the present work, based on the manufacturer's data and literatures (22–24). The properties of this nanofiltration membrane are shown in Table 1.

Fresh membranes used for desalination were soaked in pure water for at least 48 h prior to use, and all the feed and pure water were filtered through 0.22 μm microporous filters before use. In order to diminish the effect of pressure on membrane performance in the subsequent tests, the membranes were pre-pressured for at least 30 min at 25°C and 10 bar with pure water until a constant flux (about 135.0 $L \cdot m^{-2} \cdot h^{-1}$) was obtained. After that, 300 mL eluate obtained by LPCC was added into the magnetic stirred cell. When the volume of the permeate was 250 mL, 250 mL pure water was added into the magnetic stirred cell. The above procedures were repeated until the third nanofiltration procedure was finished. All permeates and corresponding retentate were collected for subsequent analysis. The corresponding retentate containing glucoraphenin obtained by nanofiltration procedures was dried under vacuum at 45°C by the rotary evaporator. The purity and recovery of the glucoraphenin product were determined by HPLC.

TABLE 1
The properties of NF270-400 membrane

Membrane	NF270-400
Manufacturer	Filmtec
Surface material	Polyamide
Molecular weight cut-off	150–200 (22)
L_p ($Lm^{-2}h^{-1}bar^{-1}$) 25°C	13–14 (23)
Max. temperature (°C)	45
Max. pressure (bar)	41
PH range	3.0–10.0
Isoelectric piont (PH)	about 5.3 (24)

RESULTS AND DISCUSSION

Preparation of Crude Extract

Heating the fresh radish seeds at 120°C for 2 h to make all the myrosinase inactive before smashing is very important, if not, nearly 30% of glucoraphenin would be lost during smashing and extraction due to the hydrolysis action. The primary contaminants of radish seeds meal were fatty acids, so the procedure of defatting by hexane is really important to improve the purity of extracts. After the hexane was removed exhaustively, the recovery of extraction with boiling water for two times was more than 95%. By the first step precipitation with ethanol, proteins and water-soluble polysaccharides were isolated from the extracts. By the second step precipitation with ethanol, the ethanol-soluble impurities were removed by filtration and the precipitate was the crude extract mainly containing glucoraphenin. After the two-step precipitation procedure, the purity of glucoraphenin in the crude extracts was up to 45%.

Preparative Low-Pressure Column Chromatography

Many suitable separation methods reported in some literatures could be used for the isolation of glucoraphenin, but those methods, such as preparative HPLC, HSCCC, SIXCPC, and ion-exchange chromatography, are really expensive for industrial production of glucoraphenin. Therefore, preparative LPCC on acidic alumina was chosen to isolate and purify glucoraphenin. Although preparative LPCC procedure on acidic alumina column which was used for isolating glucoraphenin was essentially based on the method described by Fahey (15) for the initial purification of glucoraphanin, glucoerucin, and 4-(Rhamnopyranosyloxy)-benzyl glucosinolate, our slight modifications were of crucial importance to obtain the pure product of glucoraphenin. Briefly, the two-step precipitation procedure was used before the preparative LPCC procedure to increase the initial purity of glucoraphenin.

TABLE 2
The result of separation with two different eluents for purifying glucoraphenin

	Glucoraphenin			
Eluent	Collection time (min)	Purity (%)[a]	Recovery (%)	Usage of salt (g)
57 mM K_2SO_4	238–286	30.4	89.2	4.7
100 mM KCl	238–296	36.0	91.9	3.7

[a]Mention: the purity of the product is really low because of containing much salt.

More importantly, to improve the salt removal of nanofiltration, KCl solution was used as the eluate to take the place of K_2SO_4 solution which was hard to remove by nanofiltration. Because of these modifications, all the properties of the eluate obtained by preparative LPCC were perfectly acceptable for the separation of KCl. Using KCl solution as eluent to take the place of K_2SO_4, the purity of the product which contained glucoraphenin and much salt was improved by 16%, the recovery of glucoraphenin was increased by 3%, and the usage of salt was decreased by 20%. Table 2 shows the comparisons of elution effects between KCl solution and K_2SO_4 solution. Figure 2 shows the diagram of the low-pressure chromatography on acidic alumina eluted with KCl solution.

Separation of KCl

The structure of GSLs (Fig. 1) shows that GSLs are ionic compounds and have a β-thioglucoside bond which will be easily broken when they meet with myrosinase, but they are more stable as a salt. Therefore, some salt has been successfully used in all kinds of methods for the isolation and purification of GSLs. As preparative HPLC, HSCCC, SIXPCC, and ion-exchange chromatography, salt has been used in preparative LPCC on acidic alumina to purify the product of glucoraphenin. Because of containing much salt, the purity of glucoraphenin obtained by preparative LPCC on acidic alumina was very low. Precipitation with methanol is a traditional method to remove salt from GSLs, so at the beginning of the experiment, the procedure which was described in 2.5.1 was used. However, the purity and recovery of the product of glucoraphenin were both lower than 83.2%, because the high temperature of distillation makes glucoraphenin much prone to degradation, and KCl cannot be exhaustively removed by precipitation with methanol. What is worse, this method would result in environmental pollution, high production cost, and residue of methanol.

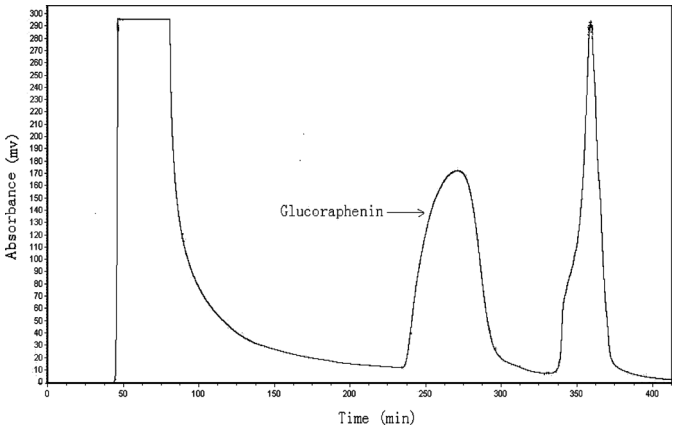


FIG. 2. LPCC chromatogram for preparing glucoraphenin by isocratic elution. The isocratic elution was performed by A and B solvent system. From 0 min to 200 min, using 100% B to wash the column exhaustively, and then using 100% A to obtain the product of glucoraphenin. The eluent A was 100 mM KCl, and the eluent B was water. The flow rate was 5 ml/min. The detected absorbance was at 220 nm.

Nanofiltration is a membrane separation technology based on the Donnan effect and sieving effect. It is well-known that nanofiltration technology can separate low molecular weight solutes from inorganic salt solution, showing great potential in desalination. In the field of desalination, compared with other methods, nanofiltration technology gets higher salt removal, especially for univalent salt. To our knowledge, nanofiltration technology has not been used to remove salt in the case of isolation of GSLs. Therefore, nanofiltration procedures were used for removing salt and avoiding product degradation by heating. In the nanofiltation procedures, NF270-400 membrane was chosen because of its suitable properties shown in Table 1. Consequently, compared with precipitation with methanol, using nanofiltration with NF270-400 got a better effect on desalination. The purity of glucoraphenin was improved from 80.3% to 91.5%, the recovery of the product of glucoraphenin was increased from 83.2% to 89.0%, and the KCl removal was increased from 82.3% to 99.5%. Moreover, the cost of the pure glucoraphenin production was decreased, as the consumption of electronic power used in dry procedures was reduced largely and a large quantity of methanol used in precipitation was saved. Table 3 shows the comparisons of desalination result between these different methods. Figure 3 shows the HPLC chromatogram of purified glucoraphenin obtained by nanofiltration with NF 270-400, in which, one main peak with a retention time greater than sinigrin (the internal standard compound) was shown. The purity of the purified glucoraphenin determined by HPLC was 91.5%.

MS and NMR

A purified sample was analyzed by MS (Fig. 4), and it is in agreement with the structure of the compound. ESI mass spectrometry gave a large molecular ion ($[M\text{-}H]^-$) in negative ion mode, and precise mass of molecular

TABLE 3
The result of salt separation with two different methods

Method	Glucoraphenin				
	Usage of energy	Consumption of methanol	Purity (%)	Recovery (%)	Removal of KCl (%)
Precipitation	high	great	80.3	83.2	82.3
Nanofiltration	low	no	91.5	89.0	99.5

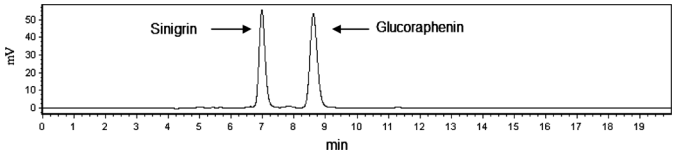


FIG. 3. HPLC chromatogram of purified glucoraphenin prepared by nanoflitration. The detected absorbance was at 235 nm. The column was reversed-phase C_{18}(250 × 4.6 mm, 5 μm, Diamodsil™). The solvent system consists of 1% methanol and 0.1% TFA in water. Column oven temperature was at 30°C. The flow rate was 1 ml/min.

obtained by ESI mass spectrometry was 434 (calculated for $[glu\text{-}S\text{-}C_6H_9NS_2O_5]^-$). In the ^1H NMR spectrum (Fig. 5), the signal at δ 2.69 (3 H, s) is attributable to a methyl group attached to a sulfur atom, the signal at δ 2.69 (2 H, m) to the methylene protons attached to a double bond, and the signal at δ 2.90 (2 H, t, J = 7.2 Hz) to the methylene protons next to the quaternary carbon. Only one signal was present in the vinylic proton region, and the signal at δ 6.54 (2H, s) can be assigned to vinyl proton 10 and vinyl proton 11 attached to the Methylthio-bearing carbon atom. The complex group of signal between δ 3.41 and δ 3.85 and the signal at δ 5.01 is characteristic of the glucose moiety. The multiplet at δ 3.41–3.53 (4 H) can be attributed to protons 2, 3, 4, and 5 of the glucose. The signal at δ 3.66 (1 H, dd, J = 12.5, 5.5 Hz) and δ 3.85 (1 H, dd, J = 12.5, 1.6 Hz) can be attributed to the methylene group of glucose. Then, the

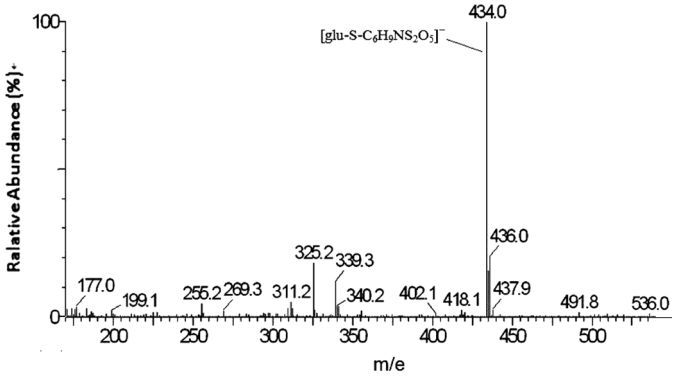


FIG. 4. Mass spectra of purified glucoraphenin.

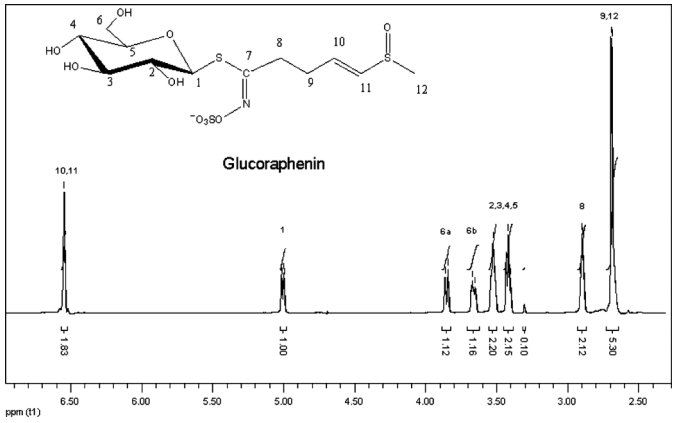


FIG. 5. ^1H NMR spectrum of purified glucoraphenin.

signal at δ 5.01 (1H, d, J = 9.5 Hz) is that of the anomeric proton. All these data about ^1H NMR analysis of the pure glucoraphenin sample was consistent with the previous report (20,25). The signals of the ^{13}C NMR spectrum (Fig. 6) were consistent with the presence of one methyl, three methylenes, two vinyls, five CH carbons, and one quaternary carbon group, and all these data about ^{13}C NMR analysis of the pure glucoraphenin sample was consistent with the previous report (20,25).

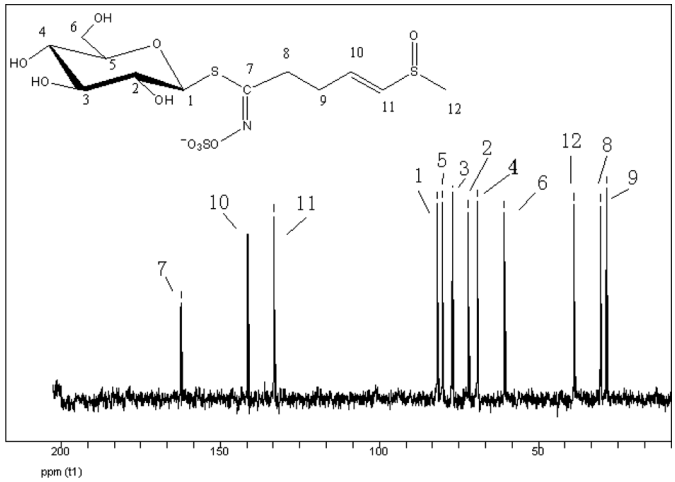


FIG. 6. ^{13}C NMR spectrum of purified glucoraphenin.

CONCLUSIONS

A new and low–cost method for the separation and purification of glucoraphenin from the radish seeds was described in our work. In order to remove a large quantity of the contaminants, the defatting procedure and the two-step precipitation were applied. At the preparative LPCC on acidic alumina, KCl solution was a good choice as the eluent to take the place of K_2SO_4 solution for higher recovery, higher purity of glucoraphenin, and lower salt consumption. Nanofiltration procedures with NF 270-400 membrane and precipitation with methanol were both used to remove KCl. Consequently, compared with precipitation with methanol, the purity and recovery of glucoraphenin obtained by nanofiltration were both much higher, with a higher removal of KCl. Meanwhile, the consumption of electronic power and organic solvent was much less in the nanofiltration procedure. These results indicate that this method for separation and purification of glucoraphenin from radish seeds could provide effective purification, high purity, high recovery, sustained usability, and economy.

ACKNOWLEDGEMENTS

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