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Preparative Isolation and Purification of Glyceollins from Soy Bean Elicited with *Aspergillus sojae* by High-Speed Countercurrent Chromatography

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Abstract

High-speed countercurrent chromatography (HSCCC), a support free liquid-liquid partition chromatographic technique, has been used for the preparative isolation of isoflavonoid glyceollins from the ethanol extracts of elicited soy bean seeds. The preparative HSCCC instrument is a multilayer coil planet centrifuge equipped with three preparative coils, separation was performed with a two phase solvent n-hexane/EtOAc/MeOH/H_2O (3:4:2:1, v/v/v/v) at a flow rate of 1.0 mL/min in 'head-to-tail' mode. HSCCC separation yielded 6.0 mg of glyceollins and with purities > 86.90 %. Chemical structures of glyceollin compounds were elucidated by ESI– MS, UPLC—MS.

Keywords: High-speed countercurrent chromatography (HSCCC); Elicitor; Phytoalexins; Glyceollins; Soybean; Isoflavones

Introduction

The stress applied on plants is a well-known approach to induce changes in the chemical profile of secondary metabolites, including phytoalexins. This is a heterogeneous group of low molecular weight antimicrobial compounds that are de novo synthesized and accumulated in response to stress, biotic or abiotic elicitors [1]. Phytoalexins increasingly gain scientific interest due to their bioactivity and potential to enhance the nutraceutical value of crops [2]. Soy bean (Glycine max) is an important source of isoflavonoids.

Soy beans contain isoflavones, a subclass of isoflavonoids that are considered to be bioactive constituents responsible for some of the health-improving effects ascribed to soy bean [3]. The three main isoflavones in soy beans are daidzein, genistein and glycitein, and their respective glucosides, acetyl-glucosides and malonyl-glucosides [4]. Upon application of stress, such as fungal infection and other elicitors, soy bean seedlings produce coumestrol and glyceollin as the main phytoalexins [5]. Both coumestrol and glyceollin are derived from the precursor daidzein and belong to the isoflavonoid subclasses of the coumestans and pterocarpans, respectively. As glyceollin isomers (I, II, and III) have similar structure to other soybean isoflavonoids, there are good possibilities that they share common physiological properties with isoflavones including antioxidant, estrogen-like, or antiestrogenic activities. In fact, glyceollins have been reported to have inhibitory effects on growth of the human prostate cancer cells and proliferation of breast and ovarian carcinoma cells implanted in ovariectomized athymic mice [6,7]. In addition, previous studies have also confirmed the estrogenic and antidiabetic activities of the glyceollins [8,9].

High-speed countercurrent chromatography (HSCCC), a support free liquid-liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto solid support and has an excellent sample recovery [10]. It has been successfully applied to isolate and purify a number of natural products. This present paper describes a HSCCC method for separation of glyceollins from elicited soy bean seeds crude extract. The optimum conditions were obtained, which led to the successful separation of glyceollins HSCCC separation yielded 6.0 mg of glyceollins and with purities > 86.90 %. This method has been successfully employed in the analytical and semi preparative separation of glyceollins from other components found in extracted soy bean. However, Glyceollins have not been separated preparatively by means of high-speed counter-current chromatography (HSCCC) from other studies.

At present, Phytochemical Glyceollin is commercially gotten from Soy beans by several steps such as Thin Liquid chromatography (TLC) and other methods. Some of the methods are time consuming in terms of large-scale isolation and are not suitable for a large scale. The aim of this study was to develop an efficient preparative HSCCC method using a two phase solvent system with retention capabilities optimized for the first time to the preparative isolation and purification of Glyceollins from elicited Soy bean seeds. The identification of glyceollins were performed with the electro-spray ionization (ESI) mass spectrometry (MS) and the UPLC- MS.

Experimental

Apparatus

A preparative HSCCC instrument (Model CCC-1000, Pharma-Tech Research Corp., Baltimore, Maryland, USA) was used for the separation of glyceollins extract. It holds three multilayer coil separation columns connected in series (diameter of tube: 2.6 mm and total volume: 325 ml) and a 10 ml sample loop as shown in (Figure 1). The revolution radius or the distance between the holder axis and the central axis of the centrifuge (R) was 5 cm, and the β-value from 0.5 at the internal terminal to 0.8 at the external terminal (β=r/R, where r

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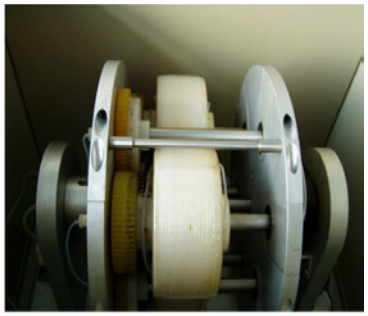

Figure 1: The main HSCCC multi-layer coil separation columns.

is the distance from the coil to the holder shaft). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1600 rpm. The system was also equipped with a constant flow pump (Pharma-Tech Research Corp.), a model SPD-10A UV monitor (SHIMADZU, Kyoto, Japan). The data were collected with a model EC-2000 chromatography work station (Dalian Yilite Apparatus CO. Ltd., Dalian, China).

The analytical high performance liquid chromatography (HPLC) equipment used in the study was Waters Alliance series HPLC system including a Waters 2695 Separations Module with an automated injector, a Waters 2996 photodiode array detector, a Waters HPLC workstation, Agilent reverse C18 column , (ESI) mass spectrometry (MS) equipment, and the UPLC-MS equipment were used.

Reagents: Organic solvents, n-hexane, ethyl acetate, methanol and distilled water used for HSCCC separations were of analytical grade (Beijing Chemicals, China). For MS analysis, HPLC grade acetonitrile (Fair Lawn, New jersey, United States) were used.

Materials: The Soy bean (Glycine max) seeds were provided by Kefeng Group., Hei long jiang province, in China. *Aspergillus sojae* cultures were obtained from China General Microbiological Culture Collection Center (CGMCC) Beijing, China and cultured at the Feed Research Institute (CASS), Beijing, China.

Process for preparation of crude sample

Soybeans, which have an exceedingly high level of isoflavones were obtained, and subjected to elicitation by *Aspergillus sojae.* (*A. sojae*), an edible fungus, for de novo biosynthesis of glyceollins [6-8]. *A. sojae* cultures were grown at 25°C in the dark on potato dextrose agar media. After 5 days inoculum was prepared by harvesting conidia in 15 mL sterile distilled H_2O. Soybeans were surface-sterilized for 3 min in 70% ethanol, followed by a twice quick rinse with distilled water 2 min. Seeds were presoaked in sterile distilled water for 5hrs before placed in sterilized Petri dishes (diameter 150 mm), each compartment was lined with two autoclaved filter papers (Whatman, Clifton, NJ) moistened with 0.5 ml distilled H_2O, the soaked seeds were then cut using a sterilized knife and inoculated with *A. sojae* spore suspension (20 µL) was applied to the cut surface of each seed. Soybean seeds were stored in an incubator at 25°C in the dark for 72hrs. Soy bean seeds (50 g) exposed to *A. sojae* were grinded with mortar and pestle extracted with 80% (v/v) ethanol and heated at 50°C for 1 h with the magnetic apparatus, cooled, and then centrifuged at 12000 rpm for 10 min. After centrifuged, the extract were evaporated to paste by rotary vaporization at 45°C under reduced pressure and freeze dried with a digital freeze drying machine at 45°C for 4-5 days. About 5g of the crude extract was obtained and stored in a refrigerator at 4°C for the subsequent HSCCC separation.

Measurement of partition coefficient (K): The two-phase solvent system for HSCCC was selected according to the partition coefficient (K) of the target components. Different ratios of n-hexane-ethyl acetate-methanol-water (HEMW) were prepared and equilibrated in a separation funnel at room temperature. The K values were determined by HPLC analysis as follows: a small amount of the crude sample 1mg was added to 2.0 mL of the mixture containing equal volume of each phase of the two-phase solvent system in a test tube, and the contents were mixed thoroughly. After the equilibration was completed, the upper phase and the lower phase were each separately analyzed by HPLC. The peak area of the upper phase was recorded as A_U and that of the lower phase, as A_L. The partition coefficient (K) is expressed as the solute absorbance in the upper phase divided by that of the lower phase, or $K=A_{upper}/A_{lower}$.

Preparation of two-phase solvent system for HSCCC: The HSCCC experiments were performed with a two-phase solvent system composed of n-hexane–ethyl acetate–methanol–water (3:4:2:1 v/v/v/v). After thoroughly equilibrating the solvent mixtures in a separatory funnel at room temperature, two phases were separated shortly before use and degassed by ultrasonication for 3 min. The upper organic phase was used as stationary phase and the lower aqueous phase as mobile phase.

Isolation and purification of glyceollin with HSCCC: A two-phase solvent system composed of n-hexane-ethyl acetate-methanol-water (3:4:2:1 v/v/v/v) was applied for the HSCCC separation with the upper phase as the stationary phase and the lower phase as the mobile phase. In each separation, the separation coil tube was first filled with the stationary phase at the rate of 5 ml/min, and then the mobile phase was pumped into the head end of the column at the rate of 1 ml/min, while the HSCCC apparatus was run at a revolution speed of 800 rpm. After hydrodynamic equilibrium was established, as indicated by a clear mobile phase eluting at the tail outlet.

100 mg of freeze dried soy bean crude extract in 4ml of upper phase and 4 ml of lower phase was injected into the separation column through the sample loop. Afterward the mobile phase was delivered into the column to elute the component. The effluent from the tail end of the column was continuously monitored with a UV detector at 285 nm, and collected by an ADVANTEC SF-3120 model fraction collector, collecting 3 ml of each fraction. Each peak fraction was collected according to the chromatogram. When the separation was completed, revolution was stopped and the column contents were collected into a graduated cylinder by connecting the column inlet to a nitrogen cylinder. The retention of stationary phase was computed by dividing the volume of the recovered stationary phase with the total volume collected. The fractions collected were sealed and stored in a refrigerator at 4°C for subsequent HPLC analysis and identification by ESI-MS, UPLC-MS.

Analysis of glyceollins by analytical HPLC: HPLC was performed with a Waters Alliance series HPLC system, including a Waters 2695 Separations Module with an automated injector, a Waters 2996 photodiode array detector, a Waters HPLC workstation and an Agilent

reverse C18 column (250 mm×4.6 mm id, 5 µm). A guard column containing the same packing was used to protect the analytical column. Elution was carried out at a flow rate of 1.0 ml/min with the following solvent system: A= acetic acid/water (pH 3.0); B = acetonitrile; 0% B to 45% B in 17 min, then 45% B to 90% B in 10 min followed by holding at 90% B for 6 min. The crude extract of Soy bean and each HSCCC peak fraction were performed at 40°C temperature. Glyceollins were monitored at 285 nm by a UV detector.

Mass spectrometry and ultra performance liquid chromatography (UPLC- MS) conditions: Mass spectral data were obtained for isolated isoflavonoids using a direct infusion technique at a flow rate of 3 mL/min using 50/50 acetonitirile/water (0.1% trifluoroacetic acid). The mass spectrometer used was a quadrupole ion trap (Finnigan LCQ instrument) equipped with a heated capillary electro spray interface. Positive ion mode was used with a sprayer needle voltage of 3.50 KV. Ion source temperature is 120°C and desolvation temperature is 250°C. Vacuum degree of Collision also was $3.0x10^{-3}$ mbar.

UPLC analyses were performed using a Waters Acquity Ultra Performance Liquid Chromatography system (Waters, Milford MA, US). UPLC separation was achieved using a Waters Acquity HSS T3 Column (100 × 2.1 mm i.d., 1.8 µm particle size) maintained at 40°C, Binary mobile phase:- 2% formic acid, 100% acetonitrile, flow rate:-0.3 ml/min.

Results and Discussion

This present study was to assess the applicability and possible use of high-speed countercurrent chromatography (HSCCC) for the isolation of phytochemical glyceollin from ethanol extract of elicited soy bean concentrate. In an initial step, the crude sample of the elicited soy bean was first analyzed by HPLC. The result indicated that it contained several isoflavonoids, including daidzein (retention time: 17.8 min), genistein (retention time: 20.1 min), coumestrol (retention time: 20.7 min), glyceollin III (retention time: 23.7 min), glyceollin II (retention time: 23.6 min), glyceollin I (23.7 min) and retention time for some other compounds as shown in (Figure 2a). An experiment to confirm the presence of glyceollin was carried out, where crude sample of elicited soy bean served as experiment A, and the non elicited crude sample of soy bean served as experiment B. HPLC chromatogram results for both experiments confirmed that glyceollins were found only in the *Aspergillus Sojae* elicited soy bean (Experiment A) at a retention time of (23.7 min), while for the non- elicited soy bean seed (Experiment B), glyceollins peak were not found to be visible as shown in (Figure 2b).

The HSCCC separated chromatogram and HSCCC separated peak fractions of the crude samples (*Aspergillus Sojae* elicited Soy bean seeds) is illustrated in (Figure 3). A two-phase solvent system composed of n-hexane-ethyl acetate-methanol-water (3:4:2:1 v/v) was applied for the HSCCC separation with the upper phase as the stationary phase

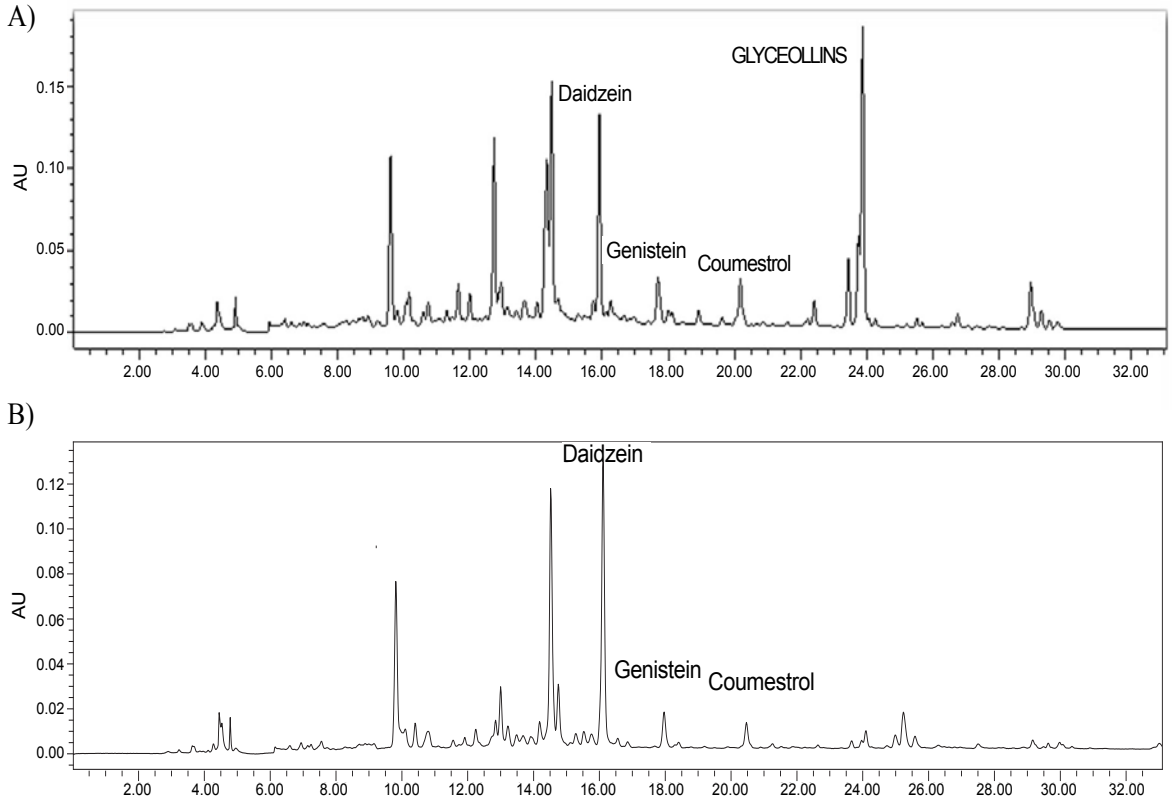

Figure 2: A) HPLC chromatogram of Aspergillus Sojaeelicited soybean extracted (Crude Sample). Column: reverse C18 column (4.6mm 250mm, 5µm); themobilephase A: acetic, B: acetonitrile Elution was carried out at a flow rate of 1.0mL/min with the following solvent system: A=acetic acid/water (pH3.0); B=acetonitrile; 0% B to 45% B in 17min, then 45% B to 90% B in 10min followed by holding at 90% B for 6min. Detection: 285nm; flow rate: 1.0mL/min. B) HPLC Chromatogram of crude ethanol extract of un-elicited soy bean seed which served as control.

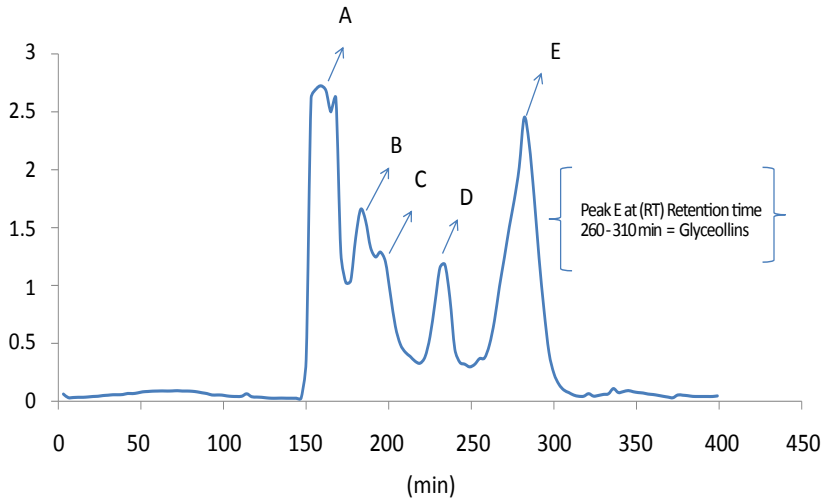

Figure 3: HSCCC chromatogram of the crude sample and HSCCC separated peak fractions from elicited Soybean seeds, Peak E at a retention time (RT) of 260–310 min from the HSCCC chromatogram gave a corresponding peak of glyceollins. A two phase solvent system composed of n-hexane ethyl acetate-methanol-water-(3:4:2:1v/v) was applied for the HSCCC separation with the upper phase as the stationary phase and the lower phase as the mobile phase. Detection by UV:285nm. Total volume: 325mL; sample loop: 25mL; revolution speed: 800rpm. Stationary phase: upper organic phase; elution mode in the coil system: 'headtotail'; flow rate: 1.0mL min).

and the lower phase as the mobile phase. Detection by UV: 285 nm. Total volume: 325 mL; sample loop: 25 mL; revolution speed: 800 rpm. Stationary phase: upper organic phase; elution mode in the coil system: ('head to tail'; flow rate: 1.0 mL/min). The HSCCC run yielded fractions of 5 separated peaks, Peak A, B, C, D and E were the identified peaks during the HSCCC separation. Peak A, B, C, D and E fractions collected in test tubes from the HSCCC chromatogram were analyzed by HPLC and Peak E at a retention time (RT) of 260–310 min from the HSCCC chromatogram gave a corresponding peak of glyceollins to that of the peak in the crude sample when analyzed by HPLC. The separated glyceollins were further confirmed by ESI– MS, UPLC––MS.

HPLC analysis and identification of HSCCC peak fractions

Following preparative isolation and purification of glyceollins from soy beans by high-speed counter-current chromatography (HSCCC), the collected fractions were generally analyzed by high-performance liquid chromatography (HPLC) to determine the relative purities of each fraction. HPLC chromatogram of HSCCC separated mixtures of phytochemical glyceollins that were not fully separated is illustrated in (Figure 4a), while HPLC chromatogram of HSCCC separated glyceollins, where the fraction was a mixture of phytochemical compounds is shown in (Figure 4b) and (Figure 4c) shows the HPLC chromatogram of HSCCC separated peak fraction of glyceollins. All HPLC analysis were performed with a Waters Alliance series HPLC system, including a Waters 2695 Separations Module with an automated injector. A Waters 2996 photodiode array detector was used for the detection at a UV wavelength of 285, and a Waters HPLC workstation and an Agilent reverse C18 column (250 mm × 4.6 mm id, 5 μm) were used. Repetition of the separation applying identical HSCCC conditions and injection of the same amount of sample confirmed the reproducibility of the preparative-scale chromatography.

Generally, in any high-speed counter-current chromatography condition, the two-phase solvent system used in HSCCC often contains large amount of low-polarity organic solvents. For example ethyl acetate and n-butanol, which if carried over from the column, tends to cause emulsification in the chromatographic column. In addition, the mobile phase of the HSCCC system is mainly composed of aqueous phase with such minor organic solvent. The results indicated that the tracing of the elution curve had not been disturbed when observed.

Selection of two -phase solvent system of HSCCC

In the HSCCC, a suitable two-phase solvent system is critical for a successful isolation and separation which requires the following considerations, retention of the stationary phase should be satisfactory, which is indicated by the short settling time of the solvent system in a test tube (<25 sec), and the partition coefficient of the target compound is between $0.5 \leq K \leq 1$ [11]. In this experiment, several kinds of solvent systems including n-hexane–ethyl acetate–methanol–water, n-hexane–ethyl acetate–ethanol–water, Chloroform- methanol- water and several other solvents at different volume ratios were tested. After trying the five kinds of solvent systems (Table 1), system composed of n-hexane-ethyl acetate-methanol-water (3:4:2:1 v/v/v/v) was applied for the HSCCC separation with the upper phase as the stationary phase and the lower phase as the mobile phase and was found to be satisfactory for the separation of phytochemical glyceollins. The separation was performed in the 'head-to-tail' mode using the lower aqueous solvent layer as mobile phase. The selected solvent system exhibited favorable retention capabilities of the organic stationary phase (90%) in the HSCCC coil-system during the complete separation even though large sample amounts were injected.

In order to obtain good peak resolution within acceptable separation time, the flow rates were evaluated. The flow rate was tested from 1 mL/min to 3 mL/min with 100 mg sample size. It was observed that the high flow rate decreased resolution (Rs) between fractions, apparently due to lower retention of stationary phase, though the retention time was reduced. With an increased flow rate from 1.0 to 3.0 mL/min, the stationary phase retention decreased from 89.9% to 45.6%. At the flow rate of 1.0 mL/min, peaks A - E were eluted within

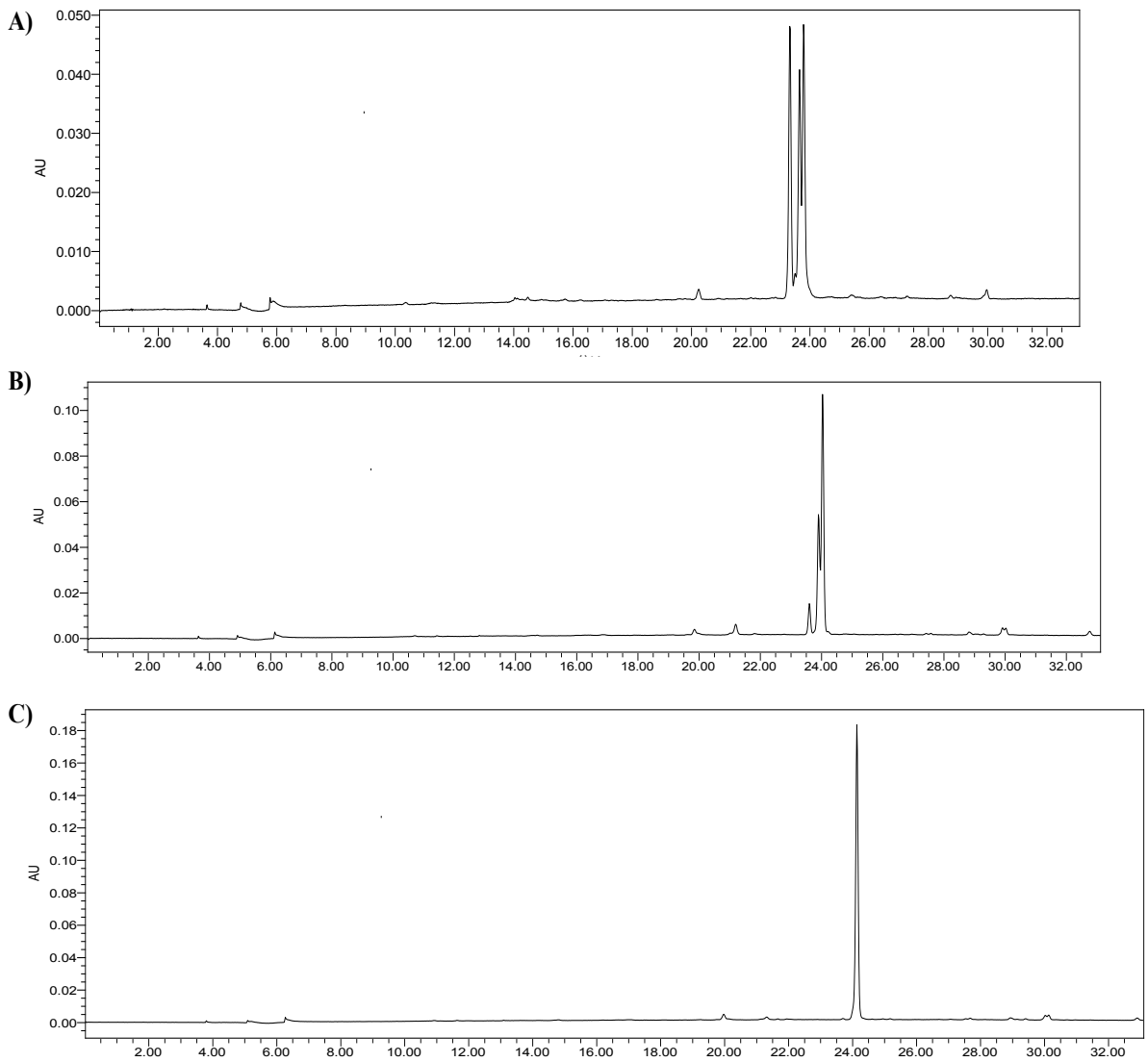

Figure 4: A) HPLC chromatogram of HSCCC separated mixtures of phytochemical glyceollins not fully separated. B) HPLC chromatogram of HSCCC separated glyceollin mixtures. C) HPLC chromatogram of separated HSCCC peak fraction of glyceollins.

450 min with the retention of stationary phase at 90%. When the flow rate was increased to 3.0 mL/min, the whole retention time was shortened, while the retention of stationary phase was reduced to 45%, resulting in loss of peak resolution between peaks (figure not shown). These observations indicated that retention of the stationary phase was affected or decreased by increased flow rate in this HSCCC separation. Therefore, the flow rate of 1.0 mL/min was used for the separation because of the acceptable resolution of target fractions.

To assess the applicability of the new technique, comparison was made between the results obtained using the conventional Thin liquid chromatography, HPLC and HSCCC method. Principal advantages of the HSCCC methodology resulted in a relatively separation time of 450 min and the immediate isolation of pure compounds. The HSCCC technique shows superior chromatographic properties compared to conventional Thin Liquid chromatography (TLC). It can be learnt that the purity results obtained from this study by HSCCC and HPLC were compactable but the HSCCC technique shows superior

No	SOLVENTSYSTEM	VOL/RATIO	(K) VALUE
1.	Chloroform(CHCl3)–methanol–water	(4:3:2)	0.071
2.	Ethyl acetate–ethanol–water	(10:2:10)	1.233
3.	n-Hexane–ethyl acetate–ethanol–water	(4:4:3:2)	1.579
4.	n-Hexane–ethyl acetate–methanol–water	(3:4:2:1)	0.821
5.	n-Hexane–ethyl acetate–methanol–water	(6:8:4:2)	0.862

Experimental protocol: A small amount of the crude sample 1mg was added to 2.0mL of the mixture containing equal volume of each phase of the two-phase solvent system in a test tube, and the contents were mixed thoroughly. The test tube was caped and shaken vigorously for 2min to equilibrate the sample thoroughly. An equal volume of each phase was then analyzed by HPLC to obtain the partition coefficient (*K*). The *K*-value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.

Table 1: The K (partition coefficient) values of solvent systems tested.

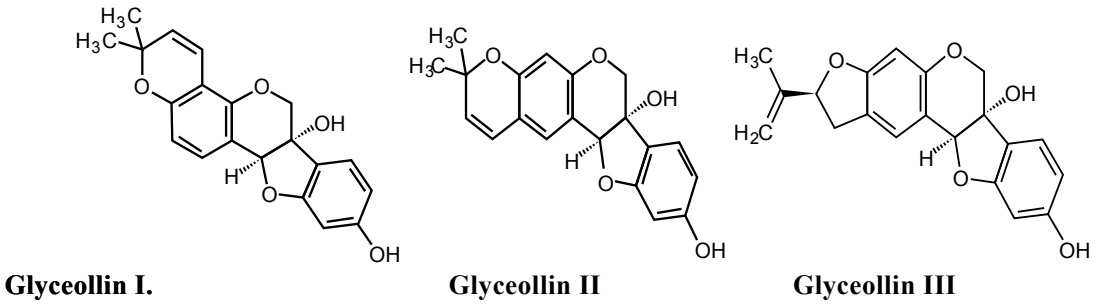

Figure 5: Chemical Structures of glyceollin I, II, and III in challenged soybeans seeds.

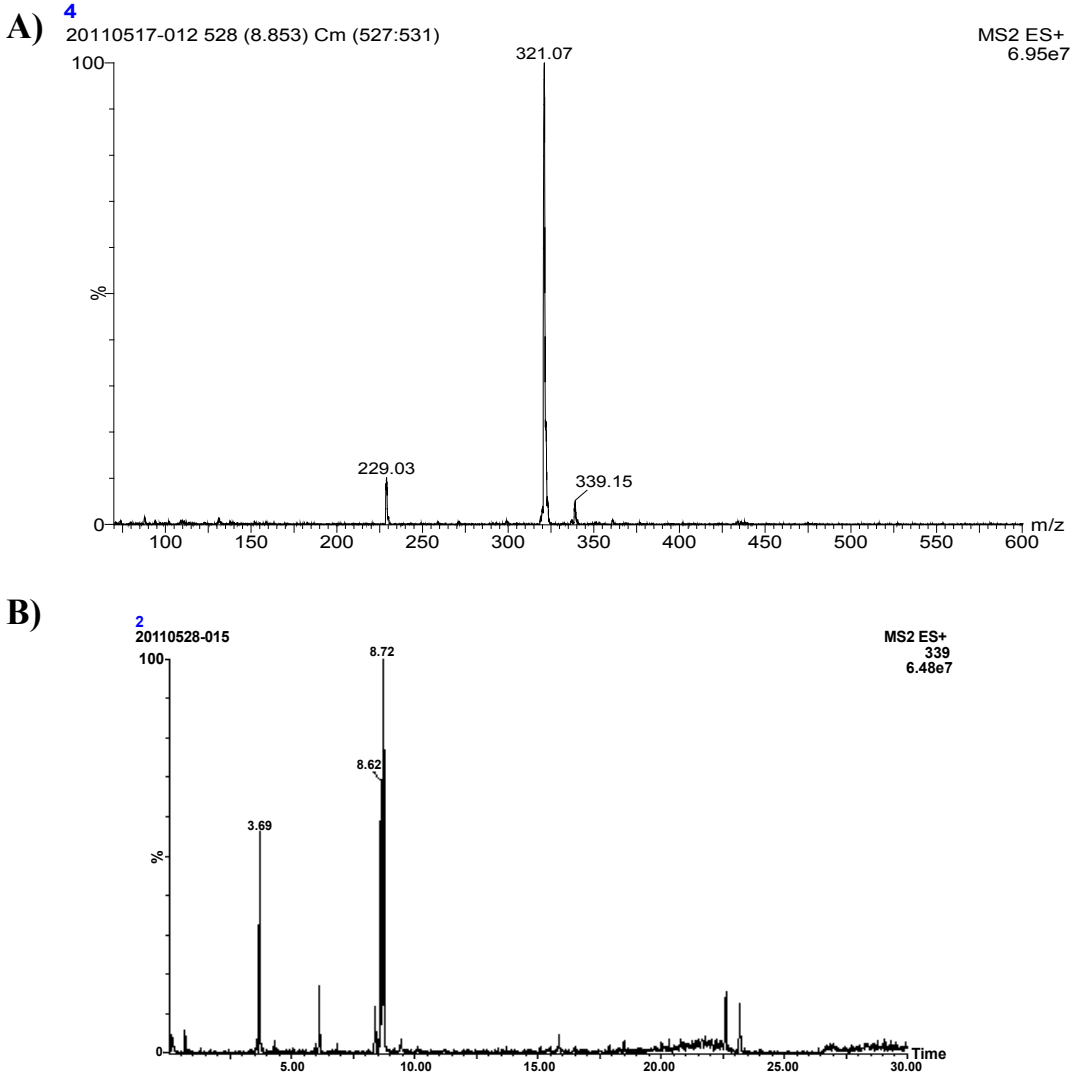

Figure 6: A) Positive ion electro spray ionization-MS analysis of glyceollins isolated from soybean cotyledons inoculated with *A. sojae*. Separated by HSCCC. (ESI) Voltage of 3.50 KV, Ion source temperature is 120˚ C and desolvation temperature is 250˚ C. **B)** UPLC–MS analysis of glyceollins isolated from soybean cotyledons inoculated with *A. sojae* , Separated by HSCCC and analyzed by HPLC. UPLC separation was achieved using a Waters Acquity HSS T3 Column (100 × 2.1 mm i.d., 1.8 µm particle size).

chromatographic properties compared to conventional analytical HPLC for separating and resolving components with differing polarities in large preparative scale procedures and isolation. Though the 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 close to 100% by eliminating the use of solid support [10]. Since the 1980s, the High-speed counter-current chromatography (HSCCC) has been a powerful tool used worldwide for the preparative separation of natural products. [10,12-14].

Confirmation of structure

In order to confirm the glyceollin isomers (I, II, and III) having similar structure (Figure 5), several analytical techniques were carried out which includes the UV-vis absorption and electro spray ionization (ESI) mass spectrometry. The positive ion ESI mass spectrum of glyceollins is shown in (Figure 6a). The base peak in the spectrum is the ion at m/z 321 detailing the loss of one water molecule, and the molecular ion at m/z 339 represents protonated glyceollin (M + H)+. Similar mass spectra were obtained for glyceollin I, II, III isomer which is in agreement with the molecular formula C20H18O5. Despite the great analytical potential of HPLC, some compounds have such similar chromatographic properties that they co-elute from the columns, affecting the ability to identify them using (MS) mass spectrometry. The use of UPLC-MS has enabled the resolution of several positional isomers of isoflavonoid and this was achieved in much shorter chromatographic run times than with standard HPLC. Using UPLC-MS and drawing single ion chromatograms, it was possible to resolve three (isomers I, II, III of glyceollin) as shown in (Figure 6b).

Conclusion

The HSCCC study on the isolation of glyceollins with purity from *Aspergillus sojae* elicited soy beans seeds demonstrates the high effectiveness of the methodology for selective preparations, and indicates its advantage for providing significant large amounts of natural products that are then available for further biological and scientific studies. In the HSCCC separation, the effect of flow rate on the stationary phase retention and peak resolution was studied. The results indicated that flow rate is a factor to determine the stationary phase retention and the peak resolution. A lower flow rate provides a higher retention level of the stationary phase, hence improving the peak resolution, although it requires a longer separation time.

Overall results of our studies demonstrated that the use of HSCCC and the HPLC could be adopted as a technical platform for a wide range or large scale isolation and purification of natural products.

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