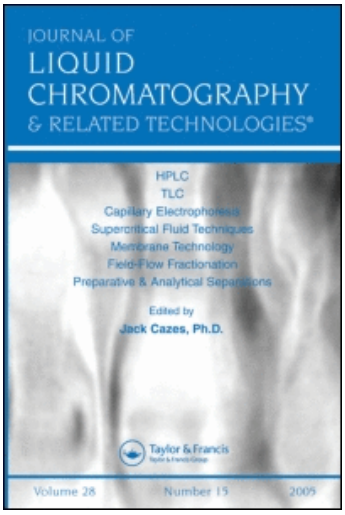


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation of Dammarane-Saponins from Notoginseng, Root of *Panax notoginseng* (Burk.) F. H. Chen, by HSCCC Coupled with Evaporative Light Scattering Detector

Xue-Li Cao[a]; Yu Tian[a]; Tian-You Zhang[a]; Qing-Hui Liu[a]; Li-Jun Jia[a]; Yoichiro Ito[b]
[a] Beijing Institute of New Technology Application, Beijing, P.R. China [b] Biophysical Chemical Laboratory, Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

Online publication date: 29 May 2003

To cite this Article Cao, Xue-Li , Tian, Yu , Zhang, Tian-You , Liu, Qing-Hui , Jia, Li-Jun and Ito, Yoichiro(2003) 'Separation of Dammarane-Saponins from Notoginseng, Root of *Panax notoginseng* (Burk.) F. H. Chen, by HSCCC Coupled with Evaporative Light Scattering Detector', Journal of Liquid Chromatography & Related Technologies, 26: 9, 1579 — 1591
To link to this Article: DOI: 10.1081/JLC-120021268
URL: http://dx.doi.org/10.1081/JLC-120021268

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, Nos. 9 & 10, pp. 1579–1591, 2003

Separation of Dammarane-Saponins from Notoginseng, Root of *Panax notoginseng* (Burk.) F. H. Chen, by HSCCC Coupled with Evaporative Light Scattering Detector

Xue-Li Cao,[1] Yu Tian,[1] Tian-You Zhang,[1,*] Qing-Hui Liu,[1] Li-Jun Jia,[1] and Yoichiro Ito[2]

[1]Beijing Institute of New Technology Application, Beijing, P.R. China
[2]Biophysical Chemical Laboratory, Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

ABSTRACT

High-speed countercurrent chromatography (HSCCC) coupled with evaporative light scattering detection, was applied to the separation of saponins from notoginseng, the main root of *Panax notoginseng* (Burk.) F. H. Chen. Five individual dammarane saponins have been isolated with the solvent systems composed of $CHCl_3$/MeOH/2-BuOH/H_2O (5 : 6 : 1 : 4, v/v/v/v) and EtOAc/1-BuOH/H_2O (1 : 1 : 2, v/v/v), successively. They were identified as ginsenoside-Rg_1, ginsenoside-Rd,

*Correspondence: Tian-You Zhang, Beijing Institute of New Technology Application, Xizhimen South Street, No. 16, Beijing 100035, P.R. China; E-mail: zhangty@public.east.cn.net.

DOI: 10.1081/JLC-120021268
1082-6076 (Print); 1520-572X (Online)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

notoginsenoside-R_1, ginsenoside-Re, and ginsenoside-Rb_1 by FAB-MS and ^{13}C NMR, together with HPLC and TLC analysis.

Key Words: CCC; Evaporative light scattering detector; Saponins; Ginsenosides.

INTRODUCTION

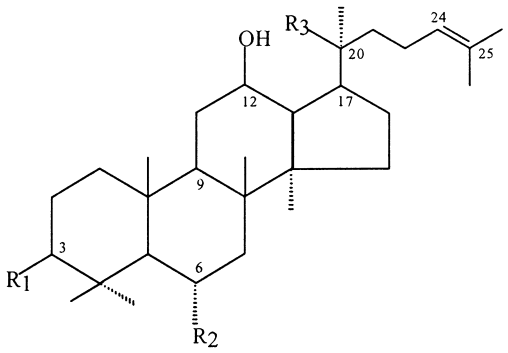
Notoginseng, which is also called "sanchi ginseng (or tienchi ginseng)," is a well-known Chinese traditional medicine used as a tonic and a haemostatic. Notoginseng is prepared from the main root of *Panax notoginseng* (Burk.) F. H. Chen and has been prescribed in several Chinese formulas including "Yunnan Bai Yao," which is used for treatment of trauma and bleeding due to internal and external injuries, and "Pien Tze Huang," which is known as a specific medicine against hepatitis. Saponins are the principal constituents of notoginseng. Extensive chemical studies have been made on saponins and their bioactivities.[1–8] As notoginseng is taxonomically related to *P. ginseng* C. A. Meyer, the root of which is a famous oriental medicine, "Ginseng," several of the ginseng dammarane saponins have been isolated from notoginseng along with its characteristic saponins, such as ginsenoside-Rb_1, ginsenoside-Rd, ginsenoside-Re, ginsenoside-Rg_1, and notoginsenoside-R_1 which are shown in Fig. 1. Later, many other minor dammarane saponins were also characterized from roots, leaves, and buds of *P. notoginseng*.

Traditionally, the isolation of ginsenosides and notoginsenosides uses silica-gel column chromatography and preparative HPLC.[1–3,5,6] The present paper describes the separation of these dammarane saponins by high-speed countercurrent chromatography (HSCCC), which is a unique liquid–liquid partition chromatography technique without a solid support matrix, thus eliminating irreversible adsorption of samples.[9] Since saponins lack chromophores, an evaporative light scattering detector (ELSD) was coupled with HSCCC for the detection of saponins. Evaporative light scattering detector has been seldom reported as a coupling detector of an HSCCC system, although it has been widely used as a part of HPLC system during recent years.[10]

EXPERIMENTAL

Materials

The dry crude powder of *P. notoginseng* was purchased from a local store of Chinese traditional medicines. The standard samples of ginsenoside-Rg_1,



	R_1	R_2	R_3
Ginsenoside-Rb_1	-O-Glc$^{2}_{1}$Glc	H	-O-Glc$^{6}_{1}$Glc
Ginsenoside-Rd	-O-Glc$^{2}_{1}$Glc	H	-O-Glc
Ginsenoside-Re	OH	-O-Glc$^{2}_{1}$Rha	-O-Glc
Ginsenoside-Rg_1	OH	-O-Glc	-O-Glc
Notoinsenoside-R_1	OH	-O-Glc$^{2}_{1}$Xyl	-O-Glc

Glc: β-D-glucopyranosyl, Xyl: β-D-xylopyranosyl, Rha: α-D-rhamnopyranosyl

Figure 1. The structures of the main dammarane saponins from *P. notoginseng*.

ginsenoside-Re, and notoginsenoside-R_1 were provided by the National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Health (Beijing, China).

Organic solvents, including petroleum ether (bp 60–90°C), chloroform, ethyl acetate, 1- and 2-butanol, 1- and 2-propanol, acetone, and methanol were purchased from Beijing Chemical Factory, Beijing, China.

Extraction of Crude Saponins from the Powder of *P. notoginseng*

The crude powder of *P. notoginseng* (100 g) was extracted with MeOH (750 mL) three times under ultrasonication. Evaporation of the solvent from the combined extract under reduced pressure gave the MeOH extract (25 g). The extract was partitioned with petroleum ether (bp 60–90°C)/H_2O mixture, and the H_2O-soluble portion was further extracted with 1-BuOH. Removal of the solvent from the 1-BuOH-soluble part yielded the 1-BuOH extract which was dissolved in a small amount of MeOH and precipitated with a large

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

amount of $(CH_3)_2CO$. The dried precipitate (5 g) containing a concentrated saponin mixture was subjected to HSCCC separation.

Preparation of Two-Phase Solvent Systems for High-Speed Countercurrent Chromatography

Several different two-phase solvent systems were tested, i.e., $CHCl_3$/MeOH/1-PrOH/H_2O (5 : 6 : 1 : 4, v/v/v/v), $CHCl_3$/MeOH/2-PrOH/H_2O (5 : 6 : 1 : 4, v/v/v/v), $CHCl_3$/MeOH/2-BuOH/H_2O (5 : 6 : 1 : 4, v/v/v/v), and EtOAc/1-BuOH/H_2O (1 : 1 : 2, v/v/v). Each solvent mixture was thoroughly equilibrated in a separatory funnel and separated shortly before use.

High-Speed Countercurrent Chromatography Separation and Evaporative Light Scattering Detector Detection of Dammarane Saponins

The present studies employed two different HSCCC units, i.e., a model GS20 analytical HSCCC and a model GS10A2 preparative HSCCC system, both manufactured by Beijing Institute of New Technology Application, Beijing, China. For the analytical model, the multilayer coil separation column was prepared by winding 0.8 mm I.D. tubing coaxially onto a spool. The β value ranges from 0.4 to 0.72, and the total capacity is 35 mL. For the preparative model, the multilayer coil was prepared by winding 1.6 mm I.D. tubing coaxially onto a spool-shaped column holder. The β value ranges from 0.5 to 0.75, and the total capacity is 230 mL. The detection of saponins was achieved by connecting the tail outlet of the coiled column with a model 75 ELSD, (SEDERE, France) through a split valve. An on-line filter was used before ELSD to eliminate particulates.

In each separation, the coiled column was first entirely filled with the upper stationary phase, then the lower mobile phase was pumped into the column at a flow-rate of 1 mL/min under 1800 rpm of column rotation for the analytical HSCCC and at 2 mL/min under 800 rpm for the preparative HSCCC. After the mobile phase front emerged and hydrodynamic equilibrium was established, the coil was connected with the ELSD. Then, the sample solution (sample dissolved in mobile phase) was injected through the sample loop. Peak fractions were collected according to the recorded elution profile.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

HPLC and TLC Analysis

The saponin mixture and peak fractions from the CCC were analyzed by HPLC and TLC. A Shimadzu LC-10AVP HPLC system, equipped with a model SPD-M10AVP photodiode array detector, was used for the analysis. The chromatographic separation was performed with a Phenomenex LUNA C_{18} column (150 × 4.6 mm I.D.). The mobile phase was a linear gradient of methanol and water, initial 50% (v/v) methanol in water, gradient to 75% in 25 min, isocratic at 75% for 13 min, then back to 50%. The flow-rate was 1 mL/min. Peak detection was carried out at 204 nm.

TLC was performed on silica-gel plate; the development solvent was the upper phase of the mixture of 1-butanol–ethyl acetate–water (4 : 1 : 5). After spraying 10% sulfuric acid in water, the saponin spots appeared by heating the plate at about 105°C for a few minutes.

MS and ^{13}C NMR Identification

All saponins obtained from the HSCCC were identified by FAB-MS and ^{13}C NMR. FAB-MS was performed on a Finnigan MAT711, Tabspec instrument in the Instrument Center, Academy of Military Medical Sciences, and ^{13}C NMR spectra were obtained with a Bruker AM-500 spectrometer (in C_5D_5N) in the Institute of Materia Medica, Chinese Academy of Medical Sciences, both in Beijing, China.

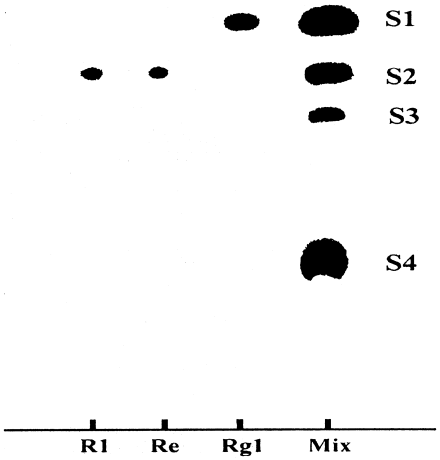


Figure 2. TLC chromatograms of standards and crude saponins.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

RESULTS AND DISCUSSION

Analysis of Saponin Extract

The crude saponins mixture, extracted from the *P. notoginseng* sample, was first analyzed by TLC. Four spots were found on silica-gel TLC (Fig. 2). Comparison of R_f values with those of standard samples revealed that one spot was ginsenoside-Rg_1 (S_1), and one was a mixture of ginsenoside-Re and notoginsenoside-R_1 (S_2). The other two spots were unknown. HPLC analysis also displayed four peaks, two of which were assigned as notoginsenoside-R_1 (P_1), mixture of ginsenoside-Rg_1 and ginsenoside-Re (P_2) by comparing with the retention times of standard samples (Fig. 3). The above results indicated that the sample contained at least five saponin components.

Solvent Effects

For HSCCC separation, several solvent systems, including $CHCl_3$/MeOH/1-PrOH/H_2O (5 : 6 : 1 : 4, v/v/v/v), $CHCl_3$/MeOH/2-PrOH/H_2O (5 : 6 : 1 : 4, v/v/v/v), $CHCl_3$/MeOH/2-BuOH/H_2O (5 : 6 : 1 : 4, v/v/v/v), and EtOAc/1-BuOH/H_2O (1 : 1 : 2, v/v/v) were tested by analytical HSCCC as shown in Fig. 4. Generally, these $CHCl_3$/MeOH/H_2O based solvent systems gave similar separation profiles of the crude saponin sample; five saponins could only be separated into three peak groups. However, distribution coefficients, K_D, of the compounds, seen as peaks presented in Table 1, indicated that

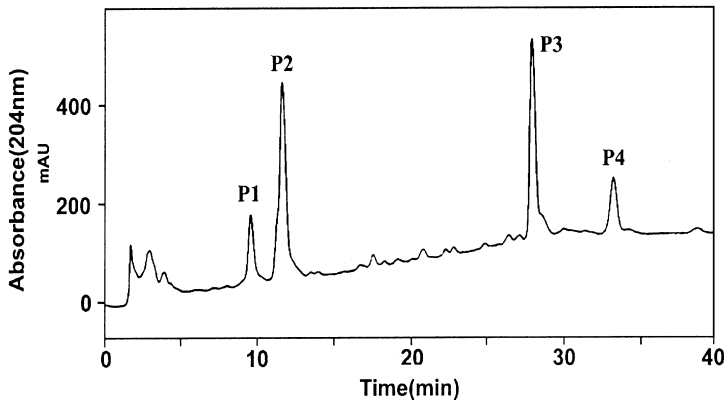


Figure 3. HPLC chromatograms of crude saponins. P1: notoginsenoside-R_1; P2: ginsenoside-Re; +ginsenoside-Rg_1.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

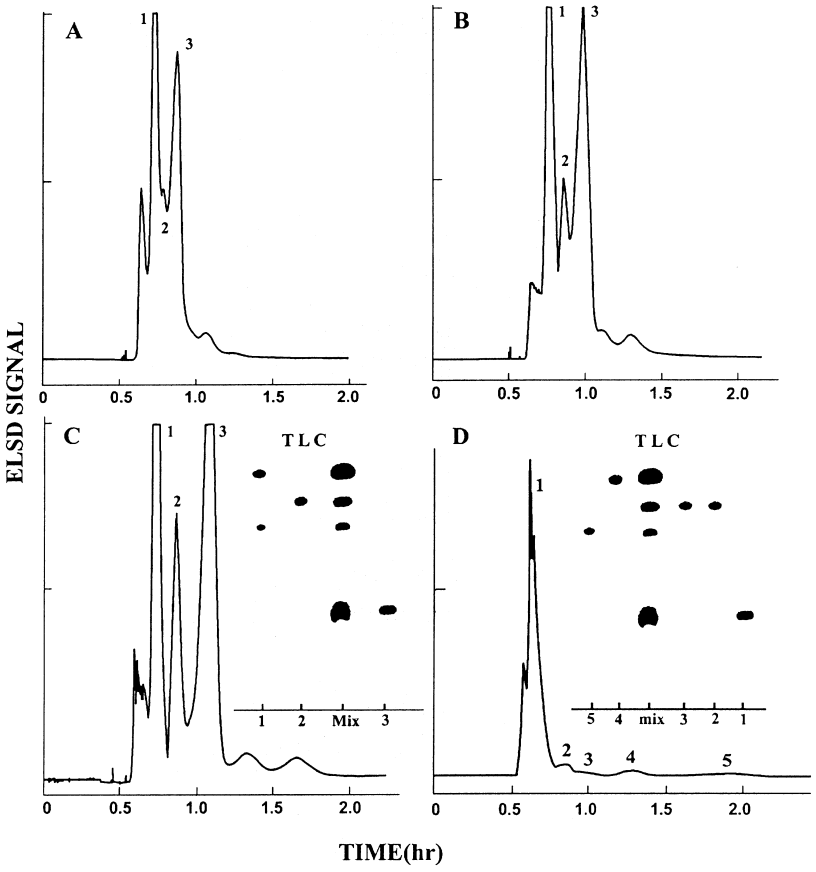


Figure 4. Test of different solvent systems for the separation of saponins by analytical HSCCC. A. $CHCl_3$–MeOH–1-PrOH–H_2O (5 : 6 : 1 : 4, v/v/v/v); B. $CHCl_3$–MeOH–2-PrOH–H_2O (5 : 6 : 1 : 4, v/v/v/v); C. $CHCl_3$–MeOH–2-BuOH–H_2O (5 : 6 : 1 : 4, v/v/v/v); D. EtOAc–1-BuOH–H_2O (1 : 1 : 2, v/v/v). Sample: 20 mg.

better resolutions were achieved as the change of modifier from 1-PrOH to 2-BuOH. Although the solvent system composed of EtOAc/1-BuOH/H_2O separated all five saponin components completely from each other, the same amount of sample loading gave very low chromatographic intensities compared with those from $CHCl_3$/MeOH/H_2O based solvent systems. This was probably due to the high boiling point of water and/or 1-BuOH in the mobile phase, which influences the sensitivity of the ELSD.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Table 1. The distribution coefficients of compounds under different solvent systems.

Solvent system/K_D	K_1[a]	K_2	K_3	K_4	K_5
A. $CHCl_3$–MeOH–1-PrOH–H_2O (5 : 6 : 1 : 4, v/v/v/v)	0.36	0.71	1		
B. $CHCl_3$–MeOH–2-PrOH–H_2O (5 : 6 : 1 : 4, v/v/v/v);	0.66	1.17	1.67		
C. $CHCl_3$–MeOH–2-BuOH–H_2O (5 : 6 : 1 : 4, v/v/v/v)	0.56	1.06	1.94		
D. EtOAc–1-BuOH–H_2O (1 : 1 : 2, v/v/v)	0.22	1.06	1.55	2.44	4.67

Note: $K_D = (V_R - V_{SF})/(V_T - V_{SF})$, in which V_R is the retention volume of the peak, V_{SF} is the solvent front volume, V_T is the machine volume and $V_T - V_{SF}$ is the stationary phase volume inside the machine.

[a]$K_1, \ldots, K_5$ corresponding to the peaks in Fig. 4.

Preparative Separation

Consequently, in the preparative separation, the solvent system composed of $CHCl_3$/MeOH/2-BuOH/H_2O (5 : 6 : 1 : 4, v/v/v/v) was first employed to separate the crude sample into three fractions, as illustrated in Fig. 5. HPLC analysis showed that the SI and SII fractions were mixtures of two saponins, and SIII mainly included one. Then the solvent system EtOAc/1-BuOH/H_2O (1 : 1 : 2, v/v/v) was used to separate each accumulated fraction into individual saponins, as illustrated in Fig. 6. HPLC analysis showed that five saponins have been separated completely from each other. This indicated that HSCCC was a powerful and effective tool for the separation of dammarane saponins, especially when coupled with the ELSD.

Evaporative Light Scattering Detector Detection

The above results indicated that ELSD is a very useful and sensitive detector for those non UV absorbing compounds and could be included as a part of the HSCCC successfully. Selection of the optimum temperature is very important when using ELSD. The temperature was set to 40°C when the solvent system EtOAc/1-BuOH/H_2O was used and 35°C when the solvent system $CHCl_3$/MeOH/2-BuOH/H_2O was used. For thermally labile compounds, a lower temperature could be used to improve the sensitivity to minimize thermal decomposition and loss. Lower temperature and particulate matter in the mobile phase will increase the background noise. So,

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

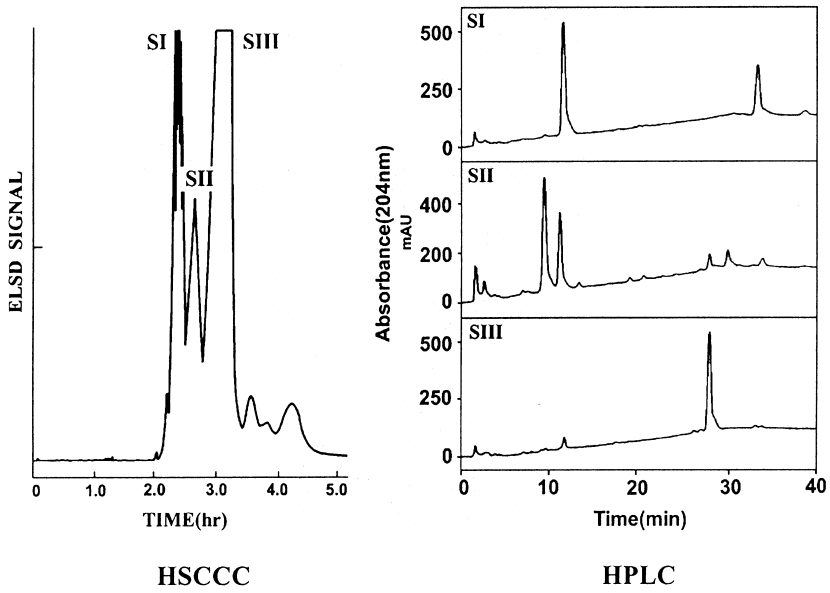


Figure 5. Preparative separation of crude saponin mixture by the solvent system $CHCl_3$–MeOH–2-BuOH–H_2O (5 : 6 : 1 : 4, v/v/v/v) and HPLC analysis of peak fractions. Sample: 200 mg.

the optimum ELSD temperature should always be found. In this HSCCC separation, filtered solvents were used and samples were also filtered before injection.

Compound Identification

The five individual saponins obtained from HSCCC were finally subjected to FAB-MS and ^{13}C NMR identification.

The positive-mode FAB-MS data of five saponins are summarized in Table 2. The detected ion peaks indicate that SI-1, SII-1, SII-2 were three (20S)-protopanaxatriol dammarane saponins. They were assigned as ginsenoside-Rg_1(SI-1), notoginsenoside-R_1 (SII-1) and ginsenoside-Re (SIII-2), respectively. This is consistent with the HPLC-based assignments. The other two were assigned as (20S)-protopanaxadiol dammarane saponis, which were ginsenoside-Rd (SII-2) and ginsenoside-Rb_1 (SIII).

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

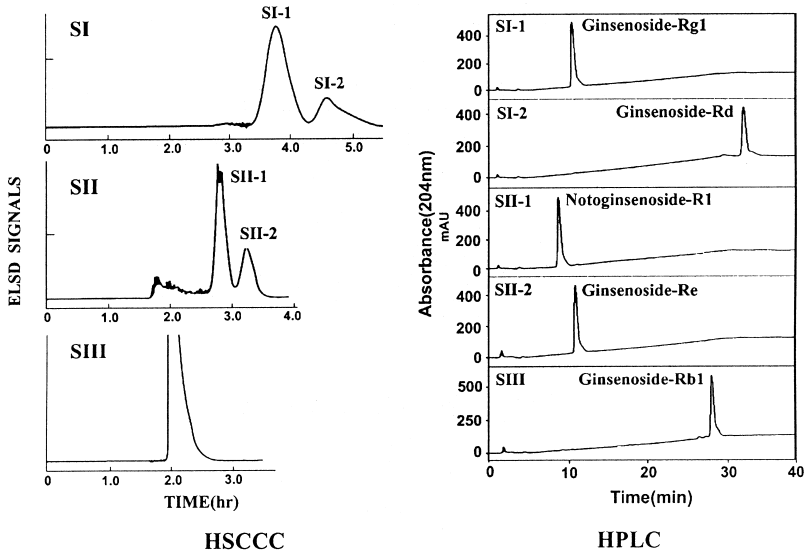


Figure 6. Further separation of each peak fractions accumulated from Figure 5 by the solvent system EtOAc–1-BuOH–H_2O (1 : 1 : 2, v/v/v) and HPLC analysis of each individual saponin fraction.

Table 2. Saponin ion peaks identified by positive-mode FAB-MS.

Saponins	m/z	Corresponding ions	m/z	Corresponding ions
Ginsenoside-Rg_1 (SI-1)	823	$[M+Na]^+$	423	$[M\text{-}2Glc]^+$
Ginsenoside-Rd (SI-2)	969	$[M+Na]^+$	407	$[M\text{-}(Glc^{2-1}Glc)\text{-}Glc]^+$
Notoginsenoside-R_1 (SII-1)	955	$[M+Na]^+$	423	$[M\text{-}(Glc^{2-1}Xyl)\text{-}Glc]^+$
Ginsenoside-Re (SII-2)	969	$[M+Na]^+$	423	$[M\text{-}(Glc^{2-1}Rha)\text{-}Glc]^+$
Ginsenoside-Rb_1 (SIII)	1,131	$[M+Na]^+$	407	$[M\text{-}(Glc^{2-1}Glc)\text{-}(Glc^{6-1}Glc)]^+$

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Table 3. ^{13}C NMR chemical shifts of the aglycone moities in C_5D_5N.

	Ginsenoside-Rg_1 (S-1)	Ginsenoside-Rd (S-2)	Notoginsenoside-R_1 (S-1)	Ginsenoside-Re (S-2)	Ginsenoside-Rb_1 (S)
C-1	40.4	39.2	39.7	40.0	39.2
C-2	27.9	26.8	27.8	27.8	26.8
C-3	78.7	89.0	78.3	79.3	89.0
C-4	39.7	39.7	40.2	39.4	39.7
C-5	61.4	56.4	61.3	60.9	56.4
C-6	78.2	18.5	79.5	74.6	18.4
C-7	45.2	35.2	45.0	45.9	35.2
C-8	41.2	40.1	41.1	41.2	40.0
C-9	50.2	50.2	49.9	49.6	50.2
C-10	39.7	36.9	36.1	39.7	36.9
C-11	31.0	30.8	30.7	30.8	30.7
C-12	70.2	70.2	70.1	70.2	70.3
C-13	49.2	49.5	49.2	49.1	49.5
C-14	51.4	51.6	51.5	51.7	51.6
C-15	30.7	31.0	31.0	31.0	30.8
C-16	26.7	26.7	26.6	26.7	26.6
C-17	52.0	51.4	51.4	51.4	51.4
C-18	17.8	16.3	17.8	17.8	16.3
C-19	17.6	16.0	17.5	17.5	16.1
C-20	83.3	83.6	83.3	83.3	83.5
C-21	22.3	22.4	22.3	22.3	22.4

(continued)

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Table 3. Continued.

	Ginsenoside-Rg_1 (S-1)	Ginsenoside-Rd (S-2)	Notoginsenoside-R_1 (S-1)	Ginsenoside-Re (S-2)	Ginsenoside-Rb_1 (S)
C-22	36.2	36.2	36.1	36.1	36.2
C-23	23.2	23.2	23.2	23.2	23.2
C-24	126.0	126.0	126.0	126.0	126.0
C-25	130.9	130.9	130.9	130.9	131.0
C-26	25.8	25.8	25.8	25.8	26.6
C-27	18.0	17.8	17.5	18.8	18.0
C-28	31.8	28.1	31.7	32.2	28.1
C-29	16.5	16.6	17.5	17.5	16.6
C-30	17.2	17.4	16.7	17.3	17.4

Note: Chemical shift values are given in (ppm).

Comparison of the ^{13}C NMR data of the SI-1, SII-1, SII-2, and SIII, illustrated in Table 3, with that reported for Rg_1, R_1, Re, and Rb_1 in the literature with regard to the chemical shifts of the aglycone moieties[3] confirmed the assignments from FAB-MS. The ^{13}C NMR data of the SII-2 showed similar signals to that of SIII. Both of them displayed two attributable signals at δ88.9 (C-3) and δ18.4 (C-6), which were different from that of SI-1, SII-1, and SII-2. SII-2 was finally assigned as ginsenoside-Rd. This also supports the assignment of FAB-MS.

Thus, five saponins obtained from HSCCC were proved to be ginsenoside-Rg_1 (SI-1), ginsenoside-Rd (SI-2), notoginsenoside-R_1 (SII-1), ginsenoside-Re (SII-2), and ginsenoside-Rb_1 (SIII), which correspond to P_2, P_4, P_1, P_2, and P_3 in the HPLC chromatogram (Fig. 3), and S_1, S_3, S_2, S_2, and S_4 in TLC chromatogram (Fig. 2), respectively.

REFERENCES

1. Zhou, J.; Wu, M.Z.; Taniyasu, S.; Besso, H.; Tanaka, O.; Saruwatari, Y.; Fuwa, T. Chem. Pharm. Bull. **1981**, *29*, 2844–2850.
2. Yang, T.; Kasai, R.; Zhou, J.; Tanaka, O. Phytochemistry **1983**, *22*, 1473–1478.
3. Zhou, L.G.; Zheng, G.Z.; Gan, F.Y.; Wang, S.L.; Yang, C.R.; Xu, C. Acta Pharm. Sinica **1991**, *26*, 876–880.
4. Gan, F.Y.; Zheng, G.Z. Chinese Pharm. J. **1992**, *27*, 138–143.
5. Zhao, P.; Liu, Y.Q.; Yang, C.R. Phytochemistry **1996**, *41*, 1419–1422.
6. Yoshikawa, M.; Murakami, T.; Ueno, T.; Yashiro, K.; Hirokawa, N.; Murakami, N.; Yamahara, J.; Matsuda, H.; Saijoh, R.; Tanaka, O. Chem. Pharm. Bull. **1997**, *45*, 1039–1045.
7. Yoshikawa, M.; Murakami, T.; Ueno, T.; Hirokawa, N.; Yashiro, K.; Murakami, N.; Yamahara, J.; Matsuda, H.; Saijoh, R.; Tanaka, O. Chem. Pharm. Bull. **1997**, *45*, 1056–1062.
8. Tanaka, O.; Han, E.C.; Yamaguchi, H.; Matsuura, H.; Murakami, T.; Taniyama, T.; Yoshikawa, M. Chem. Pharm. Bull. **2000**, *48*, 889–892.
9. Ito, Y.; Conway, W.D. Eds. *High-Speed Countercurrent Chromatography, Chemical Analysis*; Wiley Interscience: New York, 1996.
10. Elfakir, C.; Lafosse, M.; Dreux, M. J. Chromatogr. **1990**, *513*, 354–359.

Received April 21, 2002
Accepted October 7, 2002
Manuscript 6044P

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016