한국농화학회지, Vol. 23, No.4 Dec. 1980
J. Korean Agricultural Chemical Society

Larqe guantity isolation of Ginsenoside -Rb_1, -Rb_2, -Rc, -Rd, -Re and -Rg_1 in *Panax ginseng* C.A. Meyer by High Performance Liquid Chromatography

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(Received Nov. 1, 1980)

高速液體 chromatography에 依한 Ginsenoside -Rb_1, -Rb_2, -Rc, -Rd, -Re 및 -Rg_1의 大量分離

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요 약

인삼의 유효약리성분으로 밝혀진 saponin중의 각 ginsenosides를 효과적이고 능률적으로 분리하기 위하여 대량분취전용 고속액체 chromatograph인 preparative HPLC의 응용을 검토하였다. 粗 saponin획분을 preparative HPLC인 Prep LC/system-500를 사용하여 부분분획을 하고 각 획분에 함유되어 있는 ginsenosides의 조성을 Analytical HPLC로 동정한 후 Semi-preparative HPLC를 사용하여 인삼주성분 saponin을 단리했다. 그 결과 인삼주성분 saponin인 ginsenoside-Rb_1, -Rb_2, -Rc, -Rd, -Re 및 -Rg_1은 약 20 mg / 2.0 ml / injection으로 chromatography를 행하여 300~400mg/day로 대량분취가 가능하였다. 따라서 ginsenosides의 약리 및 임상효능 연구에 크게 기여하게 될 것이다.

Introduction

Ginseng is one of the most important plant drugs which has been used as a medicine in Asia. Main effective components of ginseng have recently been believed to be saponins.[1~14] Biochemical and pharmacological effects of ginsenosides, saponins purified from *Panax ginseng* C.A. Meyer, were studied by a number of investigators.[15~19]

Twelve saponins has been separated and identified. The structures of ginsenosides, ginsenoside -Ro of its sapogenin is oleanolic acid, ginsenoside -Rb_1, -Rb_2, -Rb_3, -Rc and -Rd of whose sapogenin is 20(s) -protopanaxadiol and ginsenoside -Re, -Rf, -Rg_1, -Rg_2, -Rh_1 and 20-gluco-ginsenoside -Rf of whose sapogenin is 20(s)-protopanaxatriol, have been established.[1~11]

Studies on the isolation of ginsenosides have been made good progress with various chromatographic methods: thin-layer chormatogra-

phy(TLC),[20] gas liquid phase chromatography (GLC)[21,22] and high performance liquid chromatography (HPLC).[23,24] Recently, high performance liquid chromatography has been drawn a great attention to ginseng studies by natural-products chemist as well as biochemist and pharmacologist. Ginsenoside-Re, -Rf, -Rg_1, and -Rg_2 were separated by high perfo-rmance liquid chromatographic method.[23] However, the separation and determination of ginsenoside -Rb_1, -Rb_2, -Rc and -Rd have not been reported. Chen et al.[25] isolated ginsenosides by semi-preparative HPLC on silica gel column and reported that semi-preparative HPLC was applicable only to the isolation of ginsenoside-Rb_2, -Rd and-Re.

Notwithstanding the use of HPLC for the isolation of ginseng saponins, isolation procedures for pure ginsenosides are too time-consuming and laborious. Accordingly the result of this research was presented by Choi et al.[26]. The objective of this paper was to develope an highly efficient isolation technique of ginseng saponin in order to obtain large quantities of pure ginsenosides which can be used for biochemical and pharmacological studies.

Materials and Methods

Materials

Material used was four-year-old white ginseng (dried roots of *Panax ginseng* C.A. Meyer) which was cultivated at *Kum-San*, Korea.

Apparatus

Liquid chromatography was performed on a preparative HPLC(prep LC/System-500, Waters Associates, Inc., Milford, Mass., U.S.A.), analytical and semi-preparative HPLC(ALC-201, Waters Associates) equipped with a refractometer R-401 (RI detector). A prep PAK-500/silica cartridge (57mm ID×30cm), μ Bondapak C_{18} column(7.8mmID×30cm) and carbohydrate analysis column (3.9mm ID×30cm) prepacked (Waters Associates) were used for prep LC/System-500, semi-preparative and analytical identification, respectively.

Extraction of crude saponin

Crude saponin was prepared by the modified method of Shibata et al.[1] and was further fractionated by the methods described in Figure 1. Ginseng powder was first extracted with methanol (at 80~90°C for 3hr), followed by evaporation in vacuum and dissolution in water. After extraction of aqueous solution with ethyl ether, the aqueous layer was extracted with n-butanol saturated with water. The n-butanol layer was washed with water, evaporated in vacuum and lyophilized.

Fractionation of crude saponin

Liquid chromatography was carried out with preparative HPLC using two prep PAK-500/Silica cartridges. A mixture of n-butanol-ethyl acetate-water(4 : 1 : 5, upper phase) was used as a mobile phase at a flow rate of 50m*l*/min.

The crude saponin obtained was redissolved in carrier solvent and filtered through a membrane filter TM-2P before injection. Thirty-two m*l* containing 4.5g of crude saponin was injected for each run. The crude saponin was devided into ten fractions (Figure 1 and 3) of which retent on time were compared with standard ginsenosides with using analytical HPLC. The ten fractions were combined into six group according to their similarity in saponin pattern as shown in Figure 1.

Isolation and identification

The isolation of ginsenosides was performed using semi-preparative HPLC. Prior to isolation of ginsenosides, each fraction was evaporated in vacuum, redissolved in carrier solvent containing small amount of methanol and then filtered through a membrane filter TM-2P. A reverse phase system with a μ Bondapak C_{18} column using various ratio of acetonitrile-water system as a mobile phase was employed for isolation of ginsenoside -Rb_1, -Rb_2, -Rc, -Rd, -Re and -Rg_1. The separated ginsenosides were determined by the RI detector at attenuation of 16x and the flow rate was 5 to 8 m*l*/min.

The identification of ginsenosides was perf-

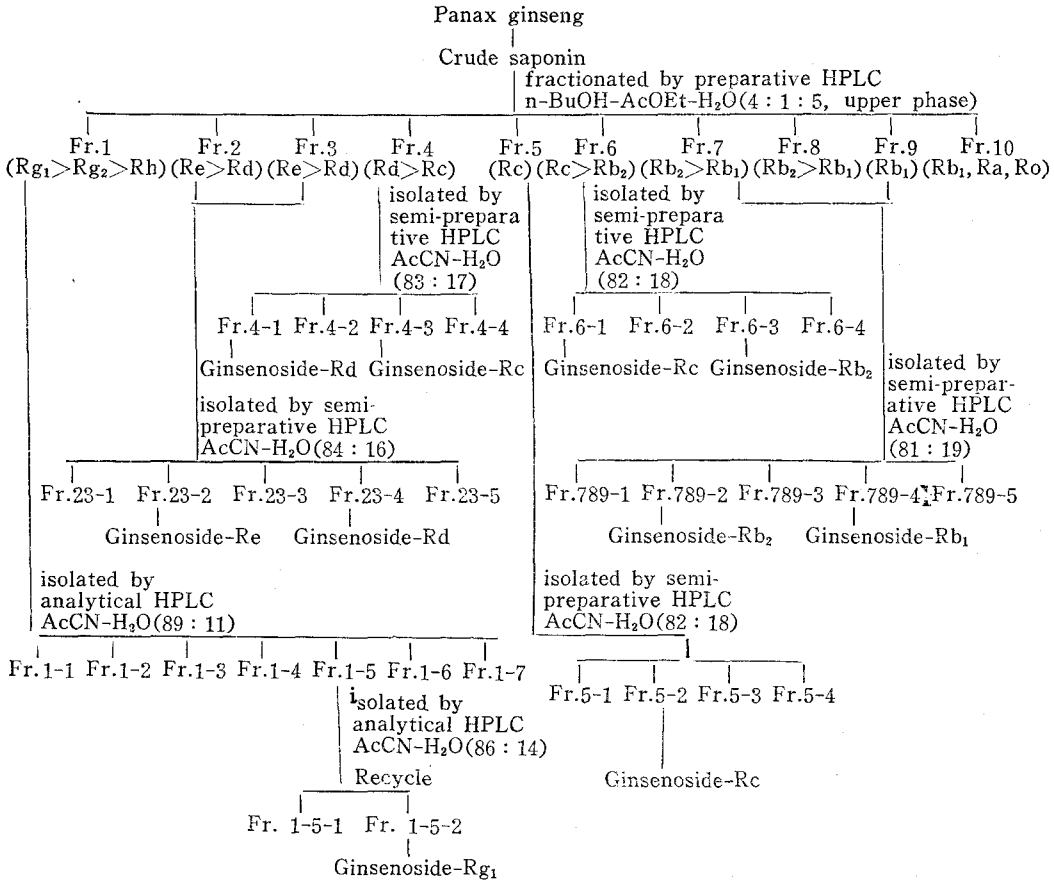

Fig. 1. Extraction and isolation of ginseng saponin

ormed using analytical HPLC. A special analysis system equipped with carbohydrate analysis column using various ratio of acetonitrile-water system as a mobile phase, was used for the identification of isolated ginsenosides. The retention times of isolated ginsenosides were compared to those of standard ginsenosides. The composition of mobile phase and other conditions used for each HPLC analysis are specified elsewhere in following chromatograms.

Results and Discussion

Analysis of crude saponin

A typical chromatogram of analytical HPLC for crude saponin prepared from white ginseng powder is shown in Figure 2. A mixture of acetonitrile-water(80 : 20) as a mobile phase, and a flow rate of 2 ml/min, were used for separation of saponin with using RI detector

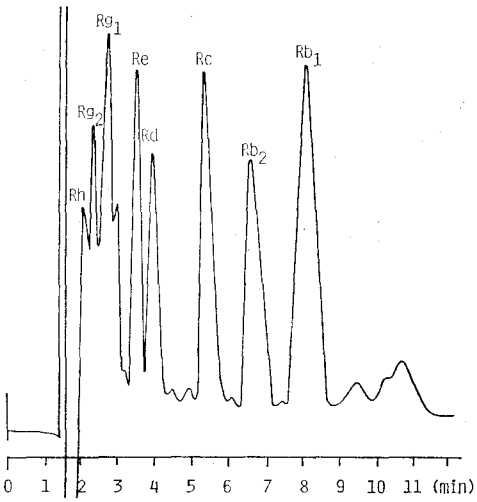

Fig. 2. Chromatogram of extracted crude saponin from *Panax ginseng* by Analytical HPLC
Condition; Packing : carbohydrate analysis
Column : 3.9 mm ID×30 cm
Mobile phase : AcCN : H_2O=80 : 20(v/v) Flow rate : 2 ml/min
RI detector: attenuation 8x

and carbohydrate analysis column.

The total separation time was shortened to approximately 10 min having satisfactory resolution and reproducibility. The carbohydrate analysis column also resulted in significant improvement for separation of triol group.

Application of HPLC for fractionation of crude saponin

Figure 3. shows a elution profiles of crude saponin when a large quantity of sample was injected into preparative column. A dual column packed with silica gel was found to be effective system for fractionation using a mobile phase of n-butanol-ethyl acetate-water(4 : 1 : 5, upper phase) at the flow rate of 50m*l*/min.

The total elution time was approximately 50 min and 10 fractions were obtained from 4.5g of crude saponin as shown in Figure 3.

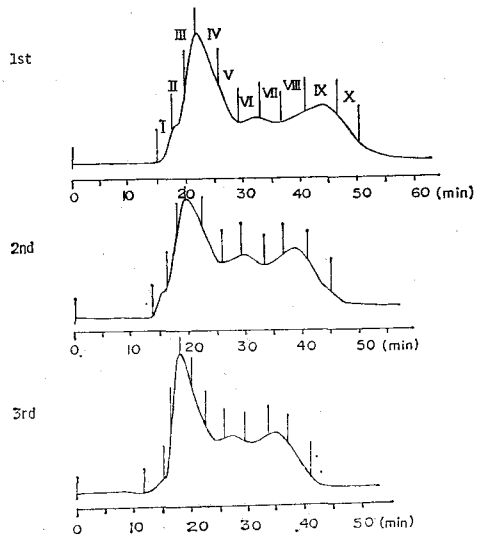

Fig. 3. Elution profiles of extracted crude saponin from *Panax ginseng* by preparative HPLC.
Conditions; Instrument : Prep LC/System-500
Packing/Column : Prep PAK-500/Silica Cartridge×2, 57 mm ID×30 cm
Mobile phase : n-BuOH : AcOEt : H_2O= 4 : 1 : 5 (upper phase)
Flow rate : 50 m*l*/min
Detection : RI detector
Sample load : 4.5 g/32 m*l*/injection

It was observed that the total elution time was reduced to about 5 min for each run. This fact signified, during the progress of fractionation, that deactivation of silica cartridges by water in carrier solvent affected the resolution as well as the retention time. Twenty gram of crude saponin dissolved in 120 *ml* was possible for fractionation at one time.

The analytical HPLC chromatograms for 10 fractions are shown in Figure 4. It was observed that all fractions contained 2 or 3 ginsenosides. Ginsenoside -Rb_1 and -Rb_2 were found in the

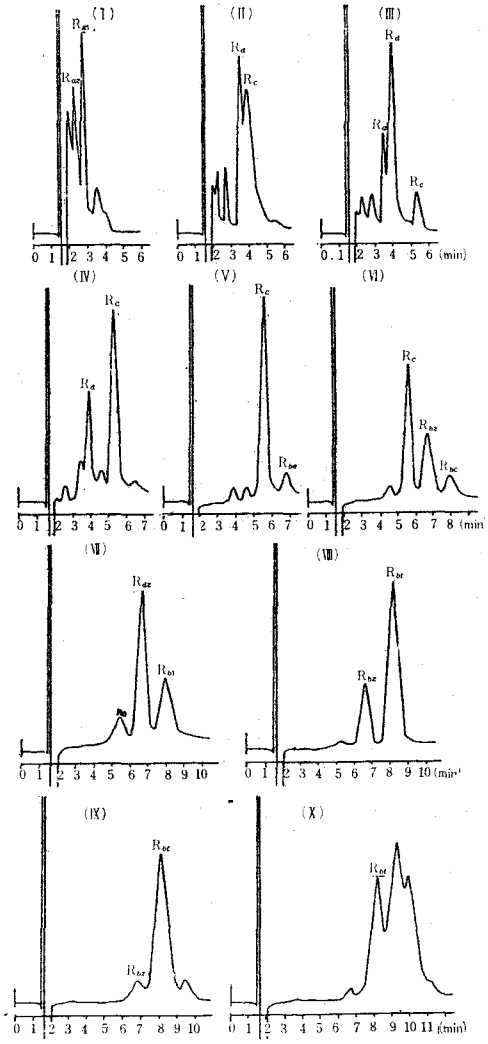

Fig. 4. Chromatograms of extracted each fraction from crude saponin by preparative HPLC
Conditions; Packing : carbohydrate analysis
Column : 3.9 mm ID×30 cm
Mobile phase : AcCN : H_2O=80 : 20(v/v)
Flow rate : 2 m*l*/min
RI detector : attenuation 8 x

fractions of VII, VIII and IX which were used for further separation of ginsenoside-Rb_1, and -Rb_2. Even though both fractions of V and VI contained ginsenoside-Rc, only the fraction V was used for separation of ginsenoside-Rc. Fraction II and III contained two major ginsenosides of Rd and Re.

Major ginsenosides isolated by large quantity treatment

Corresponding fractions which have the same ginsenosides were pooled and further fractionated by semi-preparative HPLC using acetonitrile -water system as a mobile phase (Figure 5, 6-A and 7). The combined fractions and the ratio of mobile phase are described in Figure 1.

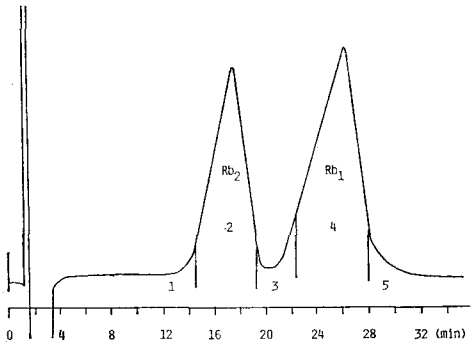

Fig. 5. Elution profile of fractionated saponin (Fr. Ⅶ+Ⅷ+Ⅸ) by semi-preparative HPLC: 1, Fr. 789-1; 2, Fr. 789-2; 3, Fr. 789-3; 4, Fr. 789-4; 5, Fr. 789-5,
Conditions; Packing : μ Bondapak C_{18}
Column : 7.8 mm ID×30 cm
Mobile phase : AcCN : H_2O=81 : 19(v/v)
Flow rate : 8 m*l*/min
RI detector : attenuation 16 x
Sample load : 17 mg/2.0 m*l*/injection

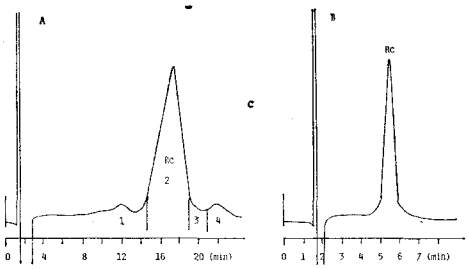

Fig. 6. Elution profile(6-A) of fractionated saponin(Fr. V) by semi-preparative HPLC and chromatogram(6-B) of isolated ginsenoside-Rc(Fr. 5-2) by analytical HPLC: 1, Fr. 5-1; 2, Fr. 5-2; 3, Fr. 5-3; 4, Fr. 5-4,
Conditions; (6-A) Packing : μ Bondapak C_{18}
Column : 7.8 mm ID×30 cm
Mobile phase : AcCN : H_2O=82 : 18(v/v)
Flow rate : 8 m*l*/min
RI detector : attenuation 16 x
Sample load : 16 mg/1.0 m*l*/injection
(6-B) Packing : carbohydrate analysis
Column : 3.9 mm ID×30 cm
Mobile phase : AcCN : H_2O=80 : 20(v/v)
Flow rate : 2 m*l*/min
RI detector : attenuation 8 x

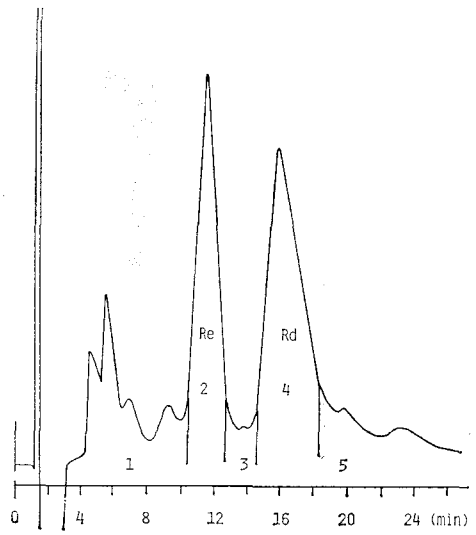

Fig. 7. Elution profile of fractionated saponin (Fr. Ⅱ+Fr. Ⅲ) by semi-preparative HPLC: 1, Fr. 23-1; 2, Fr. 23-2; 3, Fr. 23-3; 4, Fr. 23-4; 5, Fr. 23-5,
Conditions; Packing : μ Bondapak C_{18}
Column : 7.8 mm ID×30 cm
Mobile phase : AcCN : H_2O=84 : 16(v/v)
Flow rate : 8 m*l*/min
RI detector : attenuation 16 x
Sample load : 15 mg/1.5 m*l*/injection

The sub-fractionated sample was identified again for their homogeneity as shown in Figure 6-B. It was found all ginsenosides were obtained in pure from by sub-fractionation except ginsenosides -Rg_1. In order to obtain pure ginsenoside -Rg_1, the fraction I which contained ginsenoside-Rg_1 and -Rg_2, was further fractionated into 7 subfractions using semi-preparative HPLC(Figure 8). Because of overlapping difficulty, the subfraction 1-5 was further separated by recycle chromatographic method using semi-preparative HPLC(Figure 9) and divided into two compon-

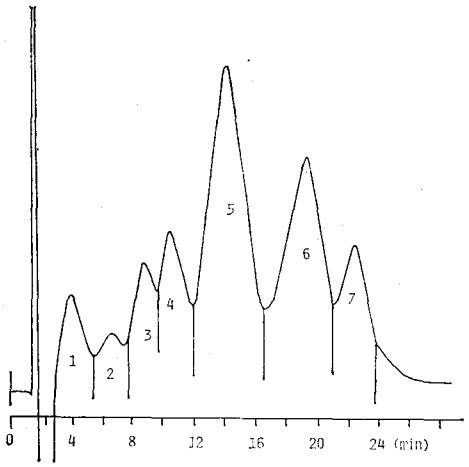

Fig. 8. Elution profiles of fractionated saponin (Fr. 1) by semi-preparative HPLC: 1, Fr. 1-1; 2, Fr. 1-2; 3, Fr. 1-3; 4, Fr. 1-4; 5, Fr. 1-5; 6, Fr. 1-6; 7, Fr. 1-7.
Conditions; Packing : μ Bondapak C_{18}
Column : 7.8 mm ID×30 cm
Mobile phase : AcCN : H_2O=89 : 11(v/v)
Flow rate : 8 m*l*/min
RI detector : attenuation 16 x
Sample load : 30 mg/0.75 m*l*/injection

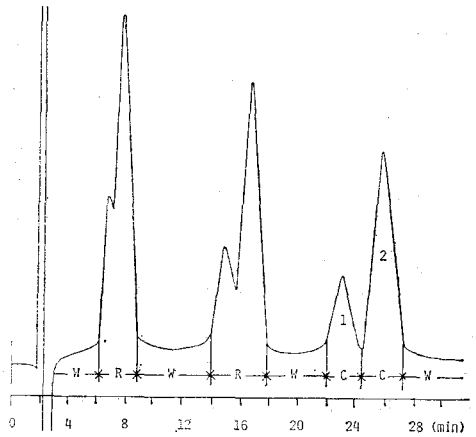

Fig. 9. Elution profile of fraction 1-5 with recycle chromatography by semi-preparative HPLC: 1, Fr. 1-5-1; 2, Fr. 1-5-2.
Conditions: Packing : μ Bondapak C_{18}
Column : 7.8 mm ID×30 cm
Mobile phase : AcCN : H_2O=86 : 14(v/v)
Flow rate : 5 m*l*/min
RI detector : attenuation 16 x
Sample load : 20 mg/0.5 m*l*/injection
*R; recycle C; collect W; waste

ents (Fraction 1-5-1 and 1-5-2). It was found that recycling method was satisfactory for obtaining purified ginsenoside-Rg_1 which was confirmed by co-chromatographic technique.

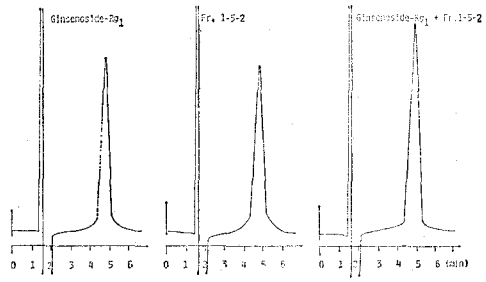

Fig. 10. Chromatograms of standard ginsenoside -Rg_1 and fration 1-5-2 by analytical HPLC
Conditions; Packing : carbohydrate analysis
Column : 3.9 mm ID×30 cm
Mobile phase : AcCN : H_2O=85 : 15(v/v)
Flow rate : 2 m*l*/min
RI detector : attenuation 8 x

The maximum amount of loading sample which can be separated effectively by semi-preparative HPLC system was approximately 20 mg/2.0 m*l*. Those isolated ginsenosides were recrystalized using the mixtures of ethanol-butanol for ginsenoside-Rb_1, -Rb_2 and -Rc, and ethanol-ethyl acetate for ginsenoside-Rd, and 50% ethanol for ginsenoside-Re in order to obtain pure gisenosides.

The capacity of isolating pure ginsenosides were 30~50 mg/hr(300~400 mg/day) idicating that the application of high performance liquip chromatography for isolation of the major comporents of ginseng saponins, ginsenoside-Rb_1, -Rb_2, -Rc, -Rd, -Re and -Rg_1, was very efficient and useful method for biological and pharmacological studies at ginsenoside level.

Acknowledgement

The author express my gratitude to Mr. Jae-Koo Ha, Director General, Office of Monopoly, Seoul, Korea, who approved of this research work in the laboratory of Professor Oura, Department of Biochemistry, Research Institute for WAKAN-YAKU, Toyama Medical and Pharmaceutical University, Toyama, Japan.

Abstract

Relatively large quantity of the major components of saponin, ginsenoside-Rb_1, -Rb_2, -Rc, -Rd, -Re and -Rg_1 from *Panax ginseng* C.A. Meyer were isolated using preparative and semi-preparative high performance liquid chromatography, and analyzed by analytical HPLC. The application of HPLC for isolation of ginsenosides was not only very effective for rapid analysis but also reduced the isolation time. The isolation capacity of pure ginsenosides was 30~50 mg/hr.

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