

# A Comparative Study of High-Performance Liquid Chromatographic Method for Analysis of Ginseng Saponin

Jin Ho Choi, Kil Dong Park, Kang Wan Han\* and Sung Ki Oh\*\*

Korea Ginseng & Tobacco Reseach Institute, Seoul 110, \*Jeonbuk National

University, Jeongju, 580, \*\* College of Industry, Kyunghee

University, Seoul, 131

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## 人蔘사포닌分析을 위한 高速液體크로마토그래피法の 比較研究

崔鎮浩 · 朴吉童 · 韓康完\* · 吳成基\*\*

韓國人蔘煙草研究所, \*全北大學校 農科大學,

\*\*慶熙大學校 産業大學

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### Abstract

Ginseng saponins separated from *Panax ginseng* C.A. Meyer were analyzed by high-performance liquid chromatography using a carbohydrate analysis column. The effect on the resolution and retention time of each ginsenosides, as well as the addition effect of n-butanol on a acetonitrile/water system, was examined using various proportions of acetonitrile/water system (80/20-94/6) which have been used with typical solvent of carbohydrate analysis column.

The retention time of each ginsenosides was greatly affected by the compositions and mixture proportions of the mobile phase and also markedly increased as the proportion of acetonitrile in mobile phase increased. It was proved that acetonitrile/water system (80/20) and acetonitrile/water/n-butanol system (86/14/10) were very effective mobile phases for diol and triol saponin analyses, respectively.

According to the result obtained by this method, the PT/PD ratios of white and red ginseng saponins were 0.401% and 0.561%, respectively. Red ginseng increased PT/PD ratio, compared with white ginseng. This is attributed to the change of saponin pattern by processing method and the change of PT/PD ratio would greatly influence on the biochemical and pharmacological effects of ginseng and its products.

### Introduction

Korean ginseng (*Panax ginseng* C.A. Meyer) has been a mysterious cure-all medicine in Asia for several thousand years. The main effective components of ginseng have recently been believed to be

saponins. For the studies on biochemical and pharmacological effects of saponins, the isolation techniques of saponin components of ginseng and its products are indispensable and of great importance. A number of works have been reported in literature for the isolation and identification of ginsenosides

using chromatographic methods such as thin-layer chromatography (TLC)<sup>1-4)</sup>, droplet counter-current chromatography (DCC),<sup>5)</sup> and gas-liquid chromatography (GLC).<sup>6-7)</sup>

Recently, a new method using high-performance liquid chromatography (HPLC) has been accentuated as a useful tool for identification of natural products; HPLC is an instrumental technique which isolates complex mixtures into their components parts and also provides the quantitative and qualitative information of each component. High-performance liquid chromatographic techniques<sup>8-10)</sup> was used for identification of ginseng saponins. In previous papers, we found that the application of HPLC techniques to the rapid isolation of major and minor components of ginseng saponins<sup>11-16)</sup> sugars<sup>17)</sup> and water soluble vitamins<sup>18)</sup> was very effective.

The objective of this study was to develop a new separation method of crude saponin, selection of mobile phase system, and isolation techniques of diol and triol saponin using high-performance liquid chromatography equipped with carbohydrate analysis column. In this paper, we have further examined the relationship between the resolution and retention time of ginsenosides using various proportions of acetonitrile/water system as typical mobile phase, and the addition effect of n-butanol on the acetonitrile/water system.

## Materials and Methods

### Materials

Materials used were white and red ginseng manufactured with six years old fresh ginseng (*Panax ginseng* C.A. Meyer) which was cultivated at Pochun.

### Apparatus

The liquid chromatograph was a Waters Associates Model ALC/GPC-244 equipped with a R-401 refractive index (RI) detector. The column used for saponin analysis was a Waters Associates carbohydrate analysis column (3.9 mm i.d. × 30 cm) which has been used for the analysis of sugars and carbohydrates. The syringe filter was a 0.45 μm Fluoropore filter (Millipore, Inc., U.S.A.).

### Sample preparation

Ginsengs were ground in a Cut-Mill (Arthur H. Thomas Co., U.S.A.) to pass through a 80 mesh stainless steel sieve and extracted twice with 80% ethanol for 8 hours at 75~80°C. Ginseng extracts were evaporated in vacuum to 40° Bx at below 50°C, dissolved in distilled water and filtered through Whatman filter paper for ash-free. The filtrate defatted with n-hexane were separated with water-saturated n-butanol, followed by evaporation in vacuum at below 50°C to obtain crude saponin.

Crude saponin fraction was dissolved with distilled water containing a small amount of methanol, treated with active carbon, filtered on a glass filter for decoloration, evaporated in vacuum and lyophilized to give rise crude saponin powder. Crude saponin was dissolved in methanol filtered through a syringe filter. The solvents composed with various proportions of acetonitrile/water system and/or acetonitrile/water/n-butanol system as a mobile phase were filtered and degassed under vacuum just before the use. The standard solution of authentic ginsenosides was prepared with 5mg/ml methanol just prior to the use. A flow rate of mobile phase was maintained through the column at 1.2-2.0 ml/min as an occasion demands.

## Results and Discussions

### Extraction and separation

Saponin components and free sugars were extracted twice with 80% ethanol for 8 hours at 75~80°C. The ginseng components, which were resolved to some degree, were lipids and proteins. Ginseng was extracted with 80% ethanol to eliminate proteins, and then ginseng extracts were separated with water-saturated n-butanol.

Deproteinization with trichloroacetic acid (TCA) was not successful because of the overlap of TCA with ginsenosides peaks in high-performance liquid chromatography. But when use TCA for the deproteinization of ginseng extracts, the excess of TCA in solution should be removed first; the excess of TCA was removed with ethyl ether, and the ethyl ether-saturated solution was removed with n-hexane.

The lipid components of ginseng extracts were very effectively removed with n-hexane. Attempts

were made to eliminate the lipid components of ginseng extracts with benzene, ether, chloroform etc., but they were not successful because of emulsion between water and solvents, particularly, in the case of analysis of saponins and sugars.

The most of saponin components were almost separated by water-saturated n-butanol extraction. For the best saponin analysis, it is very important to remove non-saponin components such as sugars, colors and inorganic components, etc.. Sugars and inorganic components could be removed by gel filtration with sephadex, but should be washed several times with water.

In general, the exclusions of the non-saponin components of crude saponin, non-polar components, phenols and color components could be removed by precipitation with ethyl ether or acetone, and filtration through alumina and/or active carbon, respectively.

Particularly in saponin analysis by high-performance liquid chromatography, protein-free crude saponin improves the degree of resolution.

#### Selection of mobile phase

For analysis of natural products by high-performance liquid chromatography, it is very important to improve the degree of resolution for the determination of the selection and composition ratios (mixture proportions) of mobile phase. Acetonitrile/water system has been used as the mobile phase of carbohydrate analysis column was applied for saponin analysis.

For experiments, various proportions of acetonitrile/water system (80/20—94/6) prepared as a mobile phase were filtered and degassed under vacuum. And the standard solution (5mg/ml) was prepared with authentic ginsenosides. A flow rate of 1.2 ml/min was maintained through the column, and the attenuation of 8 x was set on RI detector. The aliquots (5—20  $\mu$ l) of standard solutions were injected onto the liquid chromatograph using the various proportions of acetonitrile/water system as a mobile phase.

Figure 1 shows relationship between the mobile phases and retention times of each ginsenosides at the flow rate of 1.2 ml/min to determine the opt-

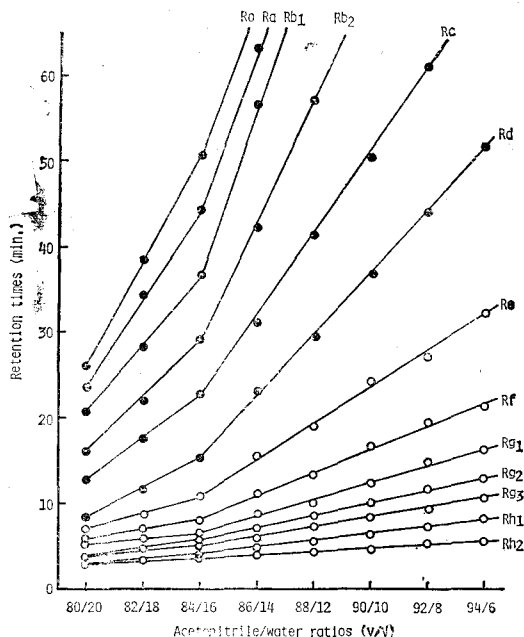


Fig. 1. Relationship between mobile phases and retention times to determine the resolution of each ginsenosides.

imum condition of isolation. The retention time of ginsenosides was greatly affected by the mixture proportion of mobile phase, and markedly increased with the increase of the proportion of acetonitrile. Triol saponins, ginsenoside-Re, -Rf, -Rg<sub>1</sub>-Rg<sub>2</sub>, -Rh<sub>1</sub>, etc., were not satisfactorily isolated with the various proportions of acetonitrile/water system (80/20—84/16) because of overlap, while diol saponins, ginsenoside-Rb<sub>1</sub>, Rb<sub>2</sub>, -Rc, Rd, etc., were well isolated with the acetonitrile/water system of 80/20 ratio.

Accordingly, in the analysis of diol saponins, it was inadequate to use above the acetonitrile/water system of 84/16 ratio because of poor resolution, time-consuming isolation and waste of mobile phase. By the same reason, the use of the acetonitrile/water system greater than 90/10 ratio was avoided for the analysis of triol saponin. However it was permitted to use acetonitrile/water system greater than 90/10 ratio for a special purpose such as isolation<sup>16)</sup> of ginsenoside -Rh<sub>1</sub> and -Rh<sub>2</sub>.

In fact, for the analysis of triol saponin, it was necessary to remove the diol saponins rapidly to keep from overlap with the next-run. Immediately after the triol saponins were identified by detector,

diol saponins were removed by changing the flow rate from 1.2ml/min to 4.0ml/min. For the removal of diol saponins, it was also very effective to inject a small quantity (200 $\mu$ l) of methanol through a U6K septumless injector.

#### Isolation of diol and triol saponin

Ginseng saponins are composed of neutral glycosides of bisdesmoside which has a basic structure of dammarane-type triterpene with the exception of

ginsenoside-Ro. Fourteen saponins, ginsenoside-Ro gave oleanolic acid, ginsenoside-Ra<sub>1</sub>, -Ra<sub>2</sub>, -Rb<sub>1</sub>, -Rb<sub>2</sub>, -Rb<sub>3</sub>, -Rc and -Rd gave 20(S)-protopanaxadiol, and ginsenoside-Re, -Rf, -Rg<sub>1</sub>, -Rg<sub>2</sub>, -Rh<sub>1</sub> and 20-gluco-ginsenoside-Rf give 20(S)-protopanaxatriol as the genuine aglycones have been established by the TLC and HPLC. Consequently, diol saponin and triol saponin should be analyzed differently, particularly in mobile phase and/or flow rate.

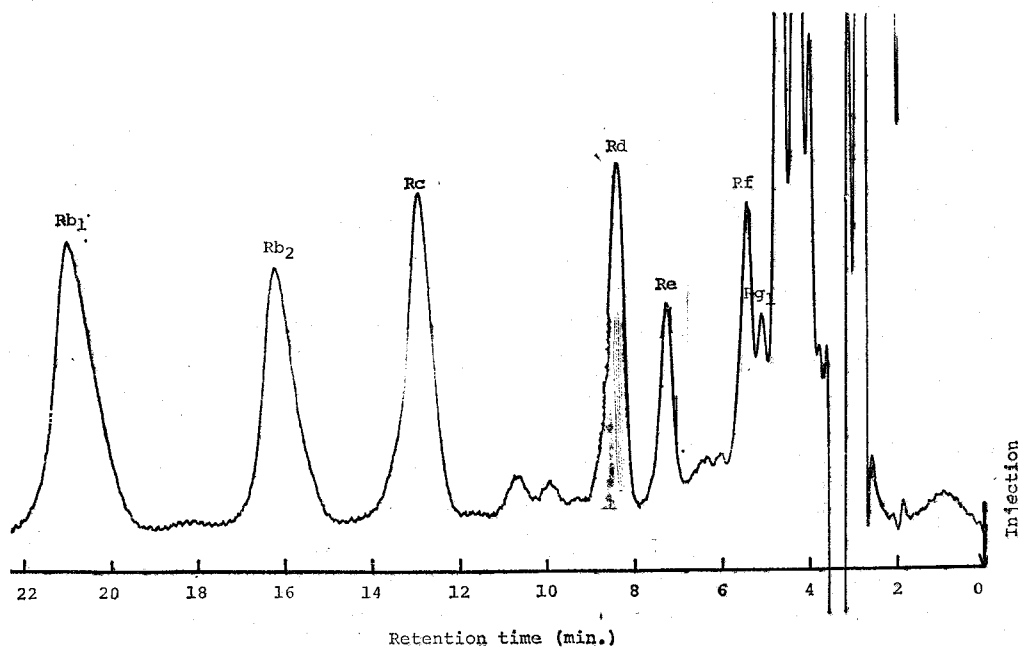


Fig. 2. HPLC chromatogram of crude saponin separated from red ginseng for diol saponin analysis.

As shown Figure 2, diol saponins could be isolated satisfactorily using the 80/20 ratio of acetonitrile/water, but triol saponins could not be isolated with this mobile phase because of overlap with ginsenosides. Therefore, triol saponins was examined on the kinds and mixture proportions of a mobile phase

at the flow rate of 2.0 ml/min (Table 1). Ginsenoside-Rg<sub>1</sub> and prosapogenin, ginsenoside-Rg<sub>2</sub> and ginsenoside-Rg<sub>3</sub>, could not be isolated satisfactorily with the acetonitrile/water of 84/16 ratio because of overlap, and ginsenoside-Rg<sub>1</sub> and prosapogenin could not be isolated with the acetonitrile/water of 86/14

Table 1. Relative retention times of triol ginsenosides to various mobile phases

Proportion of mobile phases (v/v and v/v/v)	Ginsenosides							
	Rh <sub>1</sub>	Rg <sub>3</sub>	Rg <sub>2</sub>	Rg <sub>1</sub>	Unknown	Prosapogenin	Rf	Re
Acetonitrile/water (84/16)	2.9	3.8	3.9	5.3	4.2	5.3	6.4	8.2
Acetonitrile/water (86/14)	3.0	3.9	4.2	5.9	4.7	6.0	6.7	10.2
Acetonitrile/water/n-butanol (86/14/10)	3.0	4.0	4.3	4.8	5.3	5.9	6.5	9.4

\* Flow rate: 2.0ml/min.

ratio because of same reason.

For the effective analysis, triol saponins were examined on acetonitrile/water/n-butanol system instead of acetonitrile/water system which has been used as a typical solvent for carbohydrate analysis column. The result obtained from the examination of the various mixture proportions of acetonitrile/water/n-butanol system was proved to be very effective for the resolution of triol saponin analysis using the 86/14/10 ratio of acetonitrile/water/n-butanol system (Table 1).

Especially, triol saponins were isolated with the sequence of unknown, ginsenoside-Rg<sub>1</sub>, prosapogenin using the 84/16 ratio and/or 86/14 ratio of acetonitrile/water system, and also isolated with the sequence of ginsenoside-Rg<sub>1</sub>, unknown, prosapogenin using the 86/14/10 ratio of acetonitrile/water/n-butanol system. Consequently, the isolation sequence of ginsenosides was found to be varied with the addition of n-butanol to acetonitrile/water system.

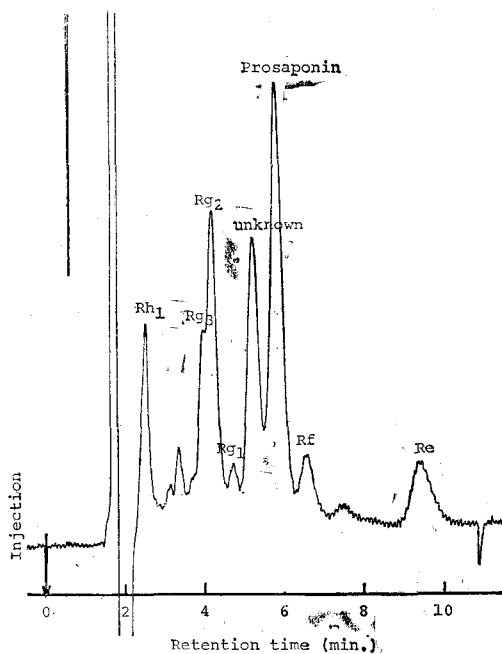


Fig. 3. HPLC chromatogram of crude saponin separated from red ginseng for triol saponin analysis.

The HPLC chromatogram of crude saponin separated from red ginseng for the analysis of triol saponins was shown in Figure 3. All the triol sap-

onins, with the exception of ginsenoside-Rg<sub>3</sub>, were isolated satisfactorily using acetonitrile/water/n-butanol system (86/14/10). By this method, triol saponins could be satisfactorily isolated within about ten minutes.

**Saponin pattern of white and red ginseng**

For quantitative analysis, ginseng saponins were isolated using two mobile phase systems; namely, the 80/20 ratio of acetonitrile/water system and 86/14/10 ratio of acetonitrile/water/n-butanol system were used for diol saponin and triol saponin, respectively. Chromatographic peak areas obtained were quantitatively calculated by comparing with the standard calibration curve of each ginsenosides detected.

Table 2. Compositions of white and red ginseng saponin

Aglycone	Ginsenosides	White ginseng (%)	Red ginseng (%)
Oleanolic acid	Ro	0.18	0.17
20(S)-proto panaxadiol	Ra <sub>1</sub> +Ra <sub>2</sub>	0.12	0.11
	Rb <sub>1</sub>	1.48	1.35
	Rb <sub>2</sub>	0.72	0.65
	Rb <sub>3</sub>	—	—
	Rc	0.65	0.60
	Rd	0.42	0.36
	Total	3.39	3.08
20(S)-proto panaxatriol	20-gluco-Rf	—	—
	Re	0.52	0.37
	Rf	0.18	0.16
	Rg <sub>1</sub>	0.37	0.24
	Rg <sub>2</sub>	0.18	0.32
	Rh <sub>1</sub> +Rh <sub>2</sub>	0.05	0.09
	Prosapogenin*	0.02	0.18
	Unknown*	0.04	0.37
	Total	1.36	1.73
PT/PD ratio		0.401	0.561

\* Hydrolyzates obtained during extraction

The composition of white and red ginseng saponin was shown in Table 2. The diol saponin contents of white and red ginseng were 3.39% and 3.08%, respectively. In contrast, the triol saponin contents of white and red ginseng were 1.36% and 1.73%, respectively. The diol saponin contents of white ginseng was very close to that of red ginseng. However red ginseng showed greater triol saponin content than white ginseng. The total saponin con-

tents of white and red ginsengs were 4.75%, and 4.81%, respectively, and thus white and red ginseng were no difference in the total saponin content.

The PT/PD ratios of white and red ginseng were 0.401% and 0.561%, respectively. Red ginseng increased the PT/PD ratio compared with white ginseng. The great ratio difference between white and red ginseng is due to the change of saponin pattern by the method of ginseng processing, and thus the change of PT/PD ratio is believed to be related to the biochemical and pharmaceutical effects of red ginseng.

### 要 約

人蔘사포닌종의 각 ginsenosides를 効果의으로 分離定量하기 위하여 carbohydrate analysis column을 사용한 HPLC로 典型的인 溶媒 system인 acetonitrile/water의 混合比率를 80/20에서 94/6까지 調整하여 retention time과 分離能과의 關係를 比較試驗하였고 또 n-butanol의 添加效果도 調査하였다. 基本構造가 다른 diol saponin과 triol saponin을 같은 mobile phase로 만족하게 分離定量하기는 어렵다.

따라서 diol saponin은 acetonitrile/water system (80/20), triol saponin은 acetonitrile/water/n-butanol system(86/14/10)을 mobile phase로 하여 分析함이 効果의이었다. 이 方法에 따라 白蔘과 紅蔘을 定量한 結果, diol saponin 含量은 큰 差異가 없으나 triol saponin 含量은 紅蔘이 白蔘보다 增加하였다. 特히 PT/PD ratio가 白蔘은 0.401인데 비해 紅蔘은 0.561로서 紅蔘이 白蔘보다 약 1.4 배나 높았다. 이것이 紅蔘의 生化學的藥理效能과 깊은 關係가 있다고 判斷되며 熱處理에 의한 製造工程과 關係가 있다.

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