Simultaneous Analysis of Saponins in Ginseng Radix by High Performance Liquid Chromatography

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The presence of about 25 ginsenosides in Ginseng Radix and their structures have already been elucidated. Although some important neutral- and acidic-saponins have been detected by high performance liquid chromatography (HPLC), other saponins have not yet been examined by HPLC. Our present improved method for the detection of ginsenosides by HPLC with a C_{18} column and a CH_3CN–H_2O gradient system enabled the measurement of not only the representative ginsenosides in Red Ginseng [20(*R*)-ginsenoside-Rg_2 and -Rh_1, 20(*S*,*R*)-ginsenosides-Rg_3 and -Rs_1] but also the minor ginsenosides [ginsenosides-Rb_3, -Rg_2, -Rh_1 and quinquenoside-R1]. Furthermore, the application of this method after a little modification of the eluent in particular to an extract of Ginseng Radix achieved simultaneous separation of 22 neutral- and acidic-ginsenosides. The qualitative and quantitative analysis of ginsenosides using the present method offers an efficient means of evaluating Ginseng Radix and commercial products containing it.

Keywords ginseng saponin; simultaneous HPLC analysis; *Panax ginseng*; Ginseng Radix; White Ginseng; Red Ginseng

Ginseng Radix, which has been traditionally used as a tonic, sedative, tranquillizer, enhancer of gastrointestinal mobility and antihaemolytic activator is one of the most important Chinese herbal medicines, in particular, as an "elixir of life." In the Japanese Pharmacopoeia, the origin of Ginseng Radix is given as *Panax ginseng* C. A. MEYER, and two different types of ginseng, depending on their method of processing (White Ginseng and Red Ginseng) are listed there. White Ginseng is the dried root, whose peripheral skin is frequently peeled off, while Red Ginseng is the steamed and dried root, which has a caramel-like color, and both lateral roots have been also used. The bioactive principles in the radix have been regarded as ginseng saponins, some of which have been examined qualitatively by gas liquid,[1] thin-layer[2] and high performance liquid chromatography (HPLC).[3,4] Following studies on the chemical transformation caused by processing, Kitagawa *et al.* characterized four saponins [20(*R*)-ginsenosides-Rg_2 and -Rh_1; 20(*S*)-ginsenosides-Rg_3 and -Rh_2][5] as representatives of Red Ginseng, and four cognates (malonyl-ginsenosides-Rb_1, -Rb_2, -Rc and -Rd)[6] in White Ginseng. Although, so far, about twenty-five saponins have been identified in the radix, all of them have not been simultaneously determined qualitatively and quantitatively by HPLC. This is because multiple measurements on each sample under different conditions are required to avoid the effect of contaminants that spoil the separation of minor saponins in Red Ginseng, which is why no satisfactory comparison between White Ginseng and Red Ginseng has been made. Considering recent results describing the difference in bioactivities between the two Ginsengs such as their effects on blood flow, blood platelet aggregation and activation of fibrinolytic system,[7] there is a need for a detailed characterization of ginseng saponins in qualitative and quantitative terms.

Results and Discussion

Analysis of Characteristic and Minor Saponins in Red Ginseng Although the main saponins in Ginseng Radix, such as neutral-saponins (ginsenosides-Rb_1, -Rb_2, -Rc, -Rd, -Re and -Rg_1) and acidic-saponins (ginsenoside-Ro

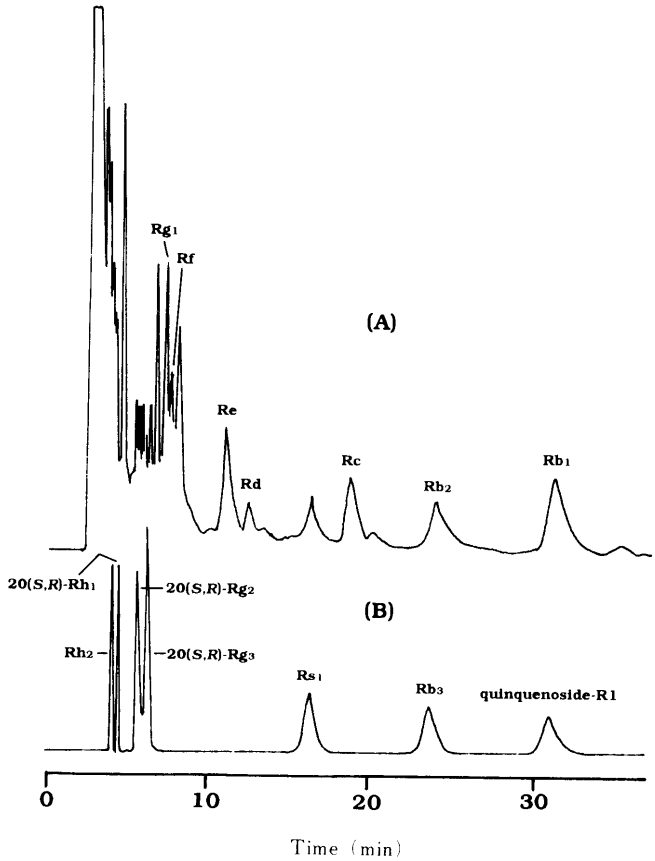

Fig. 1. HPLC Separation of Ginsenosides

(A) Red Ginseng, (B) standard ginsenosides. Column: Zorbax NH_2 (4.6 i.d. × 250 mm, Du Pont); eluent; CH_3CN–H_2O (84:16, v/v); flow rate: 0.8 ml/min; temperature: 40 °C; detection: UV 202 nm.

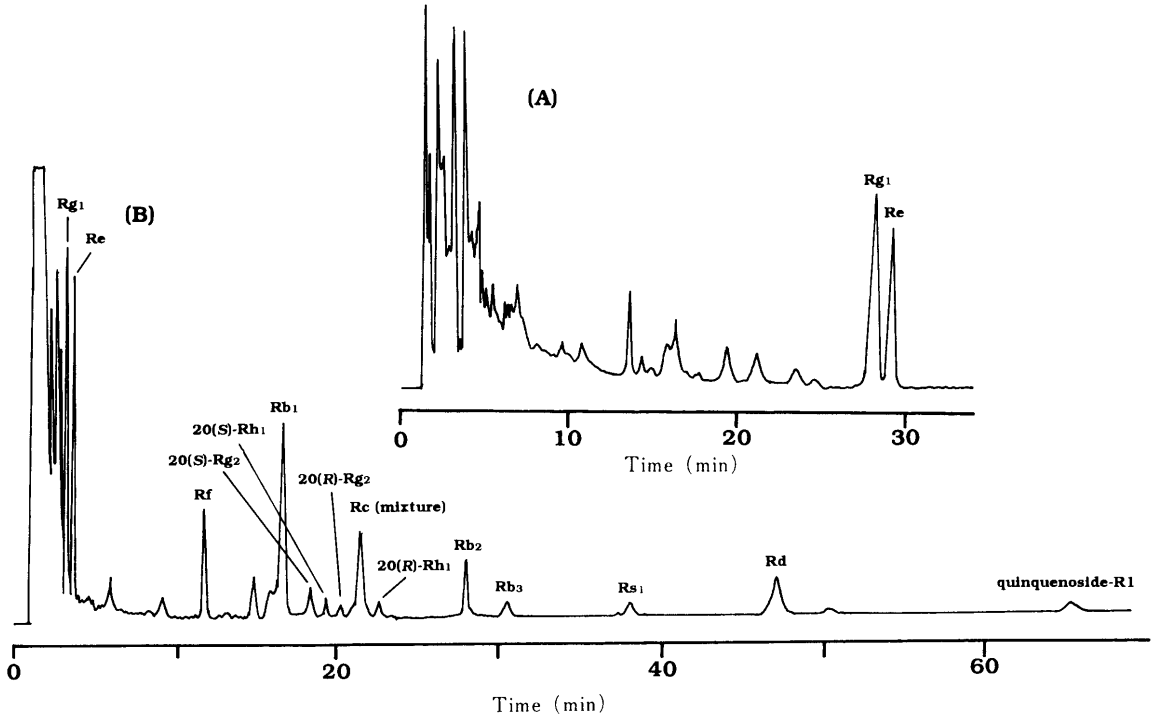

Fig. 2. HPLC Separation of Ginsenosides in Red Ginseng

Column: Superspher RP-18(e) (4.0 i.d. × 250 mm, Merck); eluent: (A) CH_3CN–H_2O (21 : 79, v/v), (B) CH_3CN–H_2O (32 : 68, v/v); flow rate: 0.8 ml/min; temperature: 40 °C; detection: UV 202 nm.

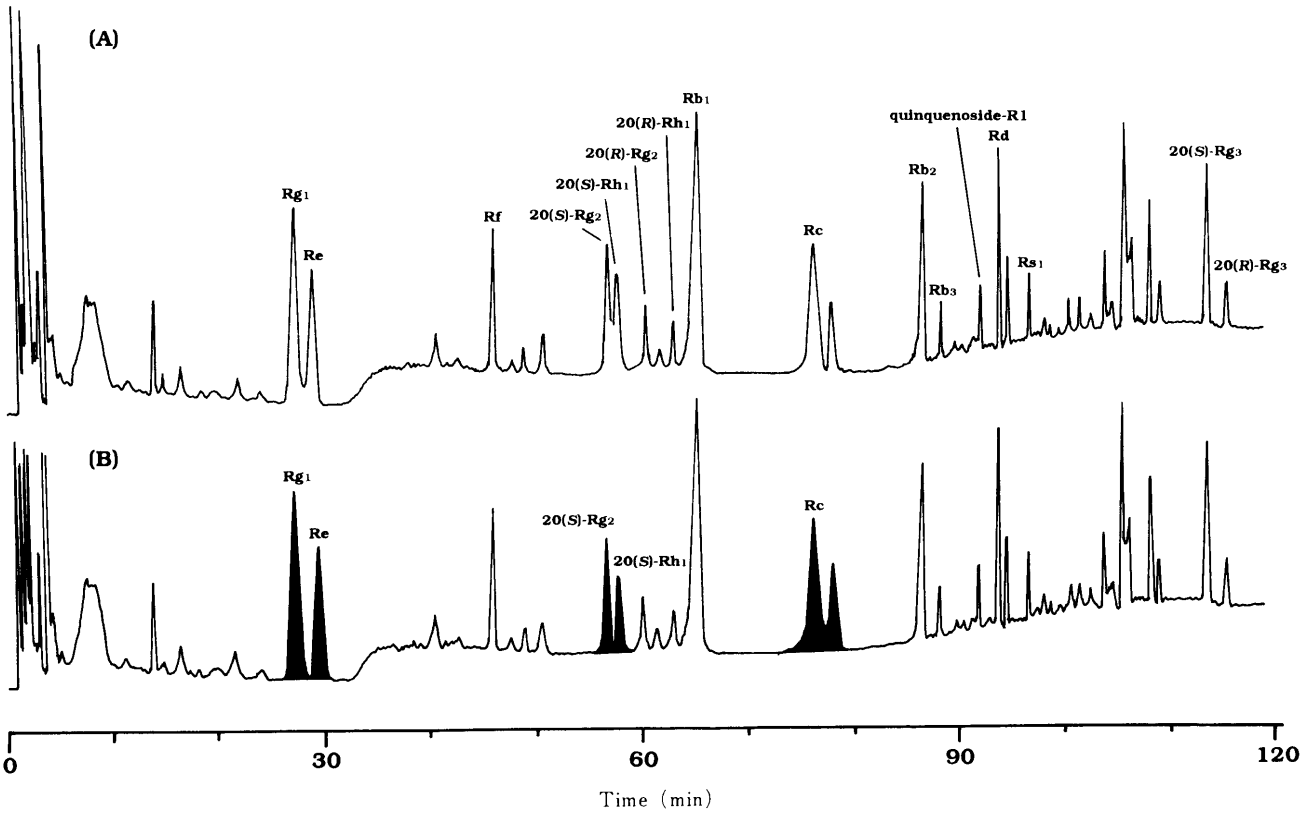

Fig. 3. Influence of Column Temperature on HPLC Separation of Ginsenosides in Red Ginseng

Column: Superspher RP-18(e) (4.0 i.d. × 250 mm, Merck); eluent: (a) CH_3CN–H_2O (21 : 79, v/v), (b) CH_3CN; flow rate: 0.8 ml/min (flow program: (a) 0→19 min: 100%, 19→20 min: 100→90%, 20→73 min: 90%, 73→103 min: 90→70%, 103→120 min: 70%); temperature: (A) 40 °C, (B) temp. program: 0→30 min: 35 °C, 30→60 min: 55 °C, 60→120 min: 35 °C; detection: UV 202 nm.

and malonyl-ginsenosides-Rb_1, -Rb_2, -Rc and -Rd), have been analyzed by HPLC using reversed phase NH_2 or C_{18} columns and CH_3CN–H_2O solvent systems,[3,4] characteristic and minor saponins in Red Ginseng have not yet been detected by HPLC. Our first trial used an NH_2 HPLC column for Red Ginseng analysis and resulted in an unsatisfactory separation between ginsenosides-Rg_1 and -Rf (Fig. 1A), which could not be improved by modifying the solvent system with changing pH, flow rate and so on. When authentic samples which are representative of Red Ginseng were chromatographed neither separation between isomers such as 20(*S*,*R*)-ginsenosides-Rg_2, -Rg_3 and -Rh_1, nor favorable retention times for ginsenosides-Rh_2, -Rs_1, -Rb_3 and quinquenoside-R1, which were overlapped by other cognates, was obtained (Fig. 1B). To improve the analysis, HPLC using a C_{18} column, which is selective for ginsenosides and sometimes needs two solvent systems, was carried out and the concentration ratio of CH_3CN and H_2O studied (Fig. 2). When an eluent composition of CH_3CN : H_2O = 32 : 68 (v/v) was used for HPLC, a good separation between the isomers mentioned above was achieved (Fig. 2B). One peak attributable to ginsenoside-Rc is a mixture because it was split under slightly different conditions. Although two stereoisomers of 20(*S*,*R*)-ginsenosides-Rg_3 and ginsenoside-Rh_2 were not separated under these conditions because of their high affinities for the column, increasing the concentration of CH_3CN allowed the three ginsenosides to be eluted. Following an investigation of the influence of column diameter and length, particle size, load of C_{18}, absence or presence of *endo*-capping and the particle size distribution on the separation, using five different columns [Superspher Rp-18(e) (4.6 i.d. × 250 mm, Merck), Superspher Rp-18 (4.0 i.d. × 250 mm, Merck), Puresil C_{18} (4.6 i.d. × 150 mm, Waters), Nova-Pak C_{18} (3.9 i.d. × 300 mm, Waters) and Cosmosil C_{18} (4.6 i.d. × 150 mm, Nacalai)], Superspher RP-18(e) was selected because of its excellent separation.

Simultaneous Analysis of Saponins in Ginseng Radix The above conditions were applied to the simultaneous analysis of ginsenosides in Ginseng Radix with only a little modification, in particular of the solvent gradient system. An excellent separation was obtained using the flow program of CH_3CN : H_2O = 21 : 79 (v/v) and CH_3CN in a linear gradient, and 16 ginsenosides were simultaneously analyzed (Fig. 3A).

Although ginsenoside-Rh_2, which is one of the characteristic components in Red Ginseng, could be separated by increasing the CH_3CN concentration, its characterization was outside our present scope because its content is below detectable concentrations.

Influence of Column Temperature on the Separation As far as the effect of column temperature on the

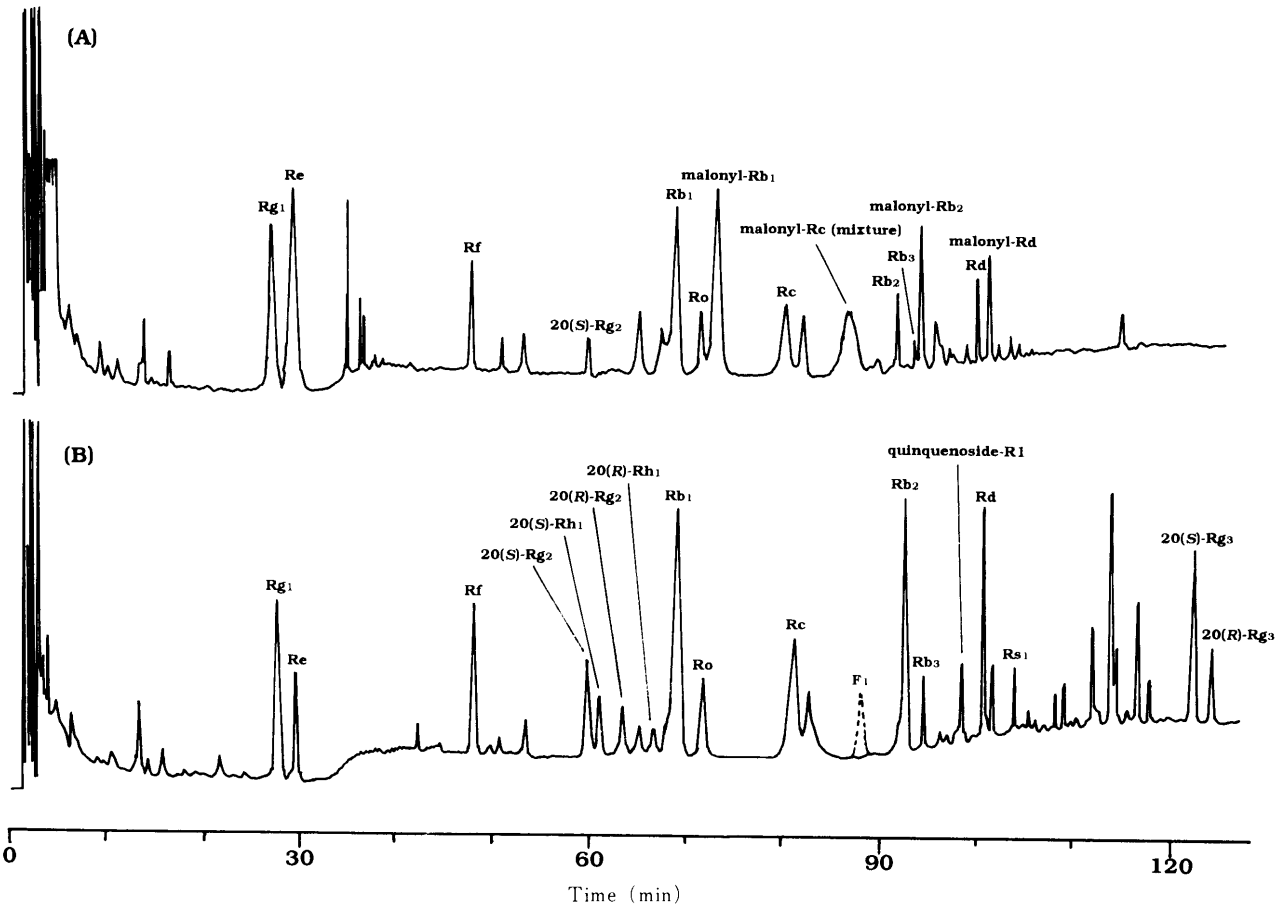

Fig. 4. Influence of pH on HPLC Separation of Ginsenosides in Ginseng Radix

(A) White Ginseng, (B) Red Ginseng. Column: Superspher RP-18(e) (4.0 i.d. × 250 mm, Merck); eluent: (a) CH_3CN–H_2O–0.1% H_3PO_4 (21 : 72 : 8, v/v), (b) CH_3CN; flow rate: 0.8 ml/min (flow program: (a) 0→19 min: 100%, 19→20 min: 100→90%, 20→73 min: 90%, 73→103 min: 90→70%, 103→120 min: 70%); temperature: temp. program (0→30 min: 35 °C, 30→60 min: 55 °C, 60→120 min: 35 °C); detection: UV 202 nm.

TABLE I. Quantitative Analysis Data

Ginsenosides	Recovery[a] (%)	Calibration curve	Limit of detection (μg)
Ginsenoside-Ro	99.67	$y=7.2\times10^{-6}x+0.1097818$ ($r=0.9998$)	0.20
Ginsenoside-Rb_1	99.58	$y=7.6\times10^{-6}x+0.0881716$ ($r=0.9999$)	0.10
Ginsenoside-Rb_2	99.45	$y=7.6\times10^{-6}x+0.0371787$ ($r=0.9999$)	0.09
Ginsenoside-Rb_3	100.62	$y=8.5\times10^{-6}x+0.1341795$ ($r=0.9966$)	0.09
Ginsenoside-Rc	98.76	$y=7.0\times10^{-6}x+0.1250410$ ($r=0.9999$)	0.20
Ginsenoside-Rd	99.79	$y=6.3\times10^{-6}x+0.0121816$ ($r=0.9999$)	0.09
Ginsenoside-Re	99.36	$y=6.4\times10^{-6}x+0.0733543$ ($r=0.9999$)	0.10
Ginsenoside-Rf	99.58	$y=7.2\times10^{-6}x+0.0164232$ ($r=0.9999$)	0.06
Ginsenoside-Rg_1	99.42	$y=5.7\times10^{-6}x+0.0778712$ ($r=0.9999$)	0.09
20(S)-Ginsenoside-Rg_2	102.16	$y=4.9\times10^{-6}x+0.0361426$ ($r=0.9999$)	0.07
20(R)-Ginsenoside-Rg_2	102.68	$y=4.5\times10^{-6}x+0.0359725$ ($r=0.9999$)	0.06
20(S)-Ginsenoside-Rg_3	101.63	$y=4.7\times10^{-6}x+0.0250572$ ($r=0.9999$)	0.07
20(R)-Ginsenoside-Rg_3	99.79	$y=5.2\times10^{-6}x+0.0114887$ ($r=0.9999$)	0.03
20(S)-Ginsenoside-Rh_1	99.96	$y=4.0\times10^{-6}x+0.0206970$ ($r=0.9999$)	0.07
20(S)-Ginsenoside-Rh_1	102.87	$y=3.9\times10^{-6}x+0.0365324$ ($r=0.9999$)	0.06
Ginsenoside-Rs_1	100.54	$y=8.7\times10^{-6}x+0.0746179$ ($r=0.9984$)	0.09
Quinquenoside-R1	101.67	$y=8.5\times10^{-6}x+0.1172577$ ($r=0.9971$)	0.09

a) Average of five measurements.

separation of ginsenosides was concerned, the lower the temperature, the better the separation for ginsenosides-Rg_1 and -Re and -Rc, conversely, the higher the temperature, the better the separation between 20(S)-ginsenoside-Rg_2 and -Rh_1, and between ginsenoside-Rc and an unidentified substance (Fig. 3B) (the corresponding peaks are shown by shadow) without affecting other separations. In the present system, the column oven did not need to be cooled to 35 °C quickly after 60 min, and this did not spoil the quantitative evaluation.

Influence of pH on the Separation To improve further the separation of ginsenosides, in particular, the acidic ones, the influence of pH was studied. To separate the acidic saponins in White Ginseng, the eluent was usually adjusted to an acid pH by a buffer solution. A gradient system of $CH_3CN:H_2O:0.1\%$ $H_3PO_4=21:72:8$ (v/v) and CH_3CN produced a good separation of an acidic-saponin, ginsenoside-Ro, without affecting the separation of other neutral saponins. These conditions also were applied to the separation of the malonyl-ginsenosides-Rb_1, -Rb_2, -Rc and -Rd that are characteristic of White Ginseng with good results (Fig. 4A). A broad peak at about 87 min was a mixture of malonyl-ginsenoside-Rc and an unidentified component. Ginsenoside-F_1, which is regarded as a decomposed glycoside from ginsenoside-Rg_1 and is not contained in the radix of the intact plant, was detected as a good-shaped peak in an extract of leaves under the present HPLC conditions (Fig. 4B).

Our present improved methods for the qualitative and quantitative analysis of ginseng saponins using HPLC allowed separation of 22 saponins when authentic samples were subjected to analysis, and 21 saponins, except malonyl-ginsenoside-Rc, were also detected in the raw material of Ginseng Radix. The HPLC data allows almost all the ginsenosides in Ginseng Radix to be measured, which rationalizes the processing the preparation of Red Ginseng that is not regulated at the moment. It also allows monitoring of *Panax ginseng* cultivated in different locations and Ginseng Radices processed by different methods. Furthermore, this method will allow the proper evaluation of White Ginseng and Red Ginseng as a medicinal agents, because the genuine bioactive components have been suggested to be decomposition products of the intact main ginsenosides.

The calibration curves for seventeen ginsenosides prepared by the present method are shown in Table I. The recovery ratios are 99.42—102.87%, relative coefficients are 0.9971—0.9999, the limits of detection are less than 0.1 μg, except for ginsenosides-Ro and -Rc (0.2 μg). Because of the instability of malonyl-ginsenosides, they are only shown by their retention times and not listed in Table I. As the content of ginsenoside-Rh_2 below the limit of detection and ginsenoside-F_1 is absent in the roots, these two saponins are not shown in Table I.

Experimental

Material Authentic samples of ginsenosides-Ro,[5] -Rb_1,[5] -Rb_2,[5] -Rb_3,[5] -Rc,[5] -Rd,[5] -Re,[5] -Rf,[5] and -Rg_1,[5] 20(S,R)-ginsenosides-Rg_2,[5] -Rg_3[5] and -Rh_1,[5] ginsenosides-Rh_2[5] and -Rs_1[8] and quinquenoside-R1[8] were obtained from 6-year old Red Ginseng in Korea and ginsenoside-F_1 was obtained by enzymatic hydrolysis of ginsenoside-Rg_1.[9] Malonyl-ginsenosides-Rb_1, -Rb_2, -Rc and -Rd were provided by Prof. Kitagawa. The respective structures had been confirmed. Ginseng Radices used analysis were a 5-year old White Ginseng and a 6-year old Red Ginseng both from Korea.

Apparatus The apparatus used in the present study was as follows: Shimadzu liquid chromatograph LC-6A, Shimadzu UV spectrophotometric detector SPD-6A, Shimadzu system controller SCL-6A, Shimadzu column oven CTO-6A and Shimadzu chromatopac C-R3A. The detailed conditions are described in each Fig. CH_3CN (HPLC grade), distilled water (HPLC grade) and H_3PO_4 (guaranteed reagent grade) were used.

Preparation of Samples Crushed Ginseng Radix about 3 g was accurately weighed, and extracted with 70% MeOH (20 ml × 5) for 20 min at room temperature. The combined extracts were concentrated at room temperature and an aliquot was dissolved in 30 ml CH_3CN–H_2O (21 : 80) and filtered through Hlcdisk 25 (i.d. 0.45 mm, Kanto Chemical Co., Inc.), and the filtrate (20 μl) subjected to HPLC separation.

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