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Determination of ginsenosides in ginseng crude extracts by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method for the simultaneous determination of ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2, R_0 and malonylginsenosides Rb_1, Rb_2, and Rc was developed. Detection at 198 nm with linear gradient elution with dihydrogenphosphate buffer–far-UV-grade acetonitrile was found to be the most suitable and the contents of the ginsenosides in a non-pretreatment ginseng extract could easily be determined within 45 min. Validation of this method and differences in column selectivity are described.

1. Introduction

Ginseng, the roots of *Panax ginseng* C.A. Meyer, is one of the most commonly used Chinese herbal drugs, possessing CNS-stimulating, cardiotonic and hypotensive effects, etc. [1]. Active constituents of this plant are found to be a complex mixture of saponins often referred to as ginsenosides, and more than 30 ginsenosides are known. This study considered eight major neutral saponins, ginsenosides Rb_1 (**1**), Rb_2 (**2**), Rc (**3**), Rd (**4**), Re (**5**), Rf (**6**), Rg_1 (**7**) and Rg_2 (**8**), an acidic saponin, ginsenoside R_0 (**9**) [2,3], and three acidic malonates of the dammarane saponins, malonylginsenosides Rb_1 (**10**), Rb_2 (**11**) and Rc (**12**) [4], as shown in Fig. 1.

In the last 15 years, many attempts have been made to assay ginseng by high-performance liquid chromatography (HPLC) either in the normal-phase mode or more recently in the reversed-phase mode [5–13]. However, none of these methods is entirely adequate, as their resolution is limited to at the most six or even less than six of the ginsenosides. Yamaguchi et al. [14] have developed a method for the simultaneous determination of all twelve compounds on an amino column in 70 min, but failed to separate well **6** from **7**, **3** from **11** and **2** from **10**. Further, all of the previously reported methods require tedious pretreatment of ginseng extracts before analysis.

We describe here the development of a direct and rapid method for determining these twelve ginsenosides in ginseng crude extracts within 45 min. The column selectivity was also investigated by studying eleven kinds of commercial columns under the selected conditions.

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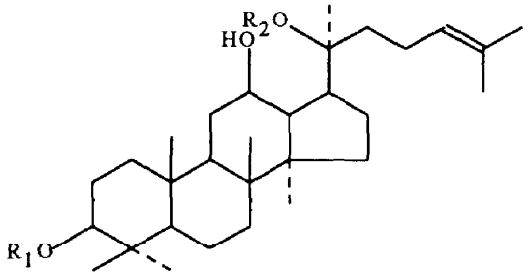
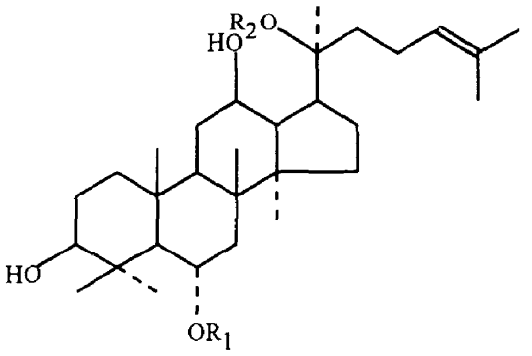


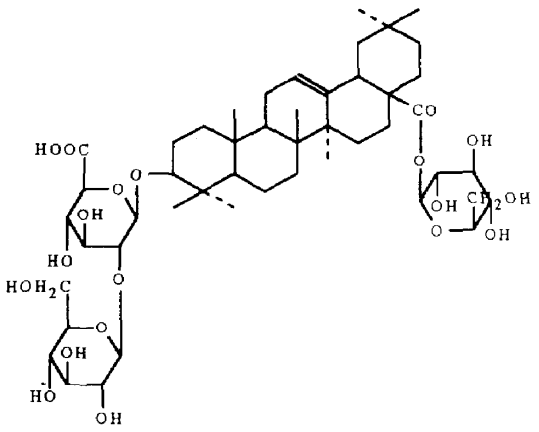
20-S-Protopanaxadiol(R1=R2=H)



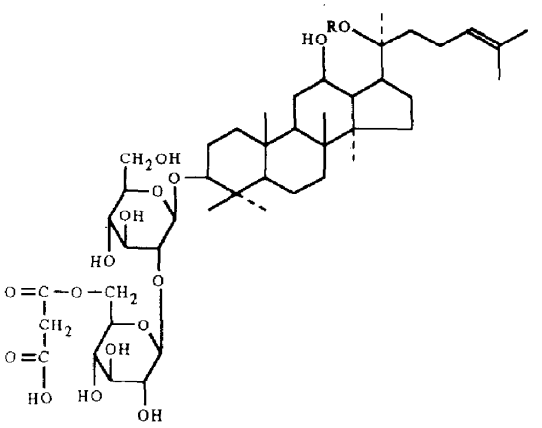
20-S-Protopanaxatriol(R1=R2=H)

1 Rb1: R1= D-Glc(β1-2)D-Glc-
R2= D-Glc(β1-6)D-Glc-
2 Rb2: R1= D-Glc(β1-2)D-Glc-
R2= L-Ara(pyr)(α1-6)D-Glc-
3 Rc: R1= D-Glc(β1-2)D-Glc-
R2= L-Ara(fur)(α1-6)D-Glc-
4 Rd: R1= D-Glc(β1-2)D-Glc-
R2= D-Glc-

5 Re: R1= L-Rha(α1-2)D-Glc-
R2= D-Glc-
6 Rf: R1= D-Glc(β1-2)D-Glc-
R2= H-
7 Rg1: R1= D-Glc-
R2= D-Glc-
8 Rg2: R1= L-Rha(α1-2)D-Glc-
R2= H-



Oleanolic acid



Malonyl-ginsenoside

9 R0

10 mRb1: R= D-Glc(β1-6)D-Glc-
11 mRb2: R= L-Ara(pyr)(α1-6)D-Glc-
12 mRc: R= L-Ara(fur)(α1-6)D-Glc-

Fig. 1. Structures of the twelve ginsenosides.

2. Experimental

2.1. Reagents and materials

Ginsenosides 1–7 were purchased from Extrasynthese (Genay, France) and potassium dihydrogenphosphate and acetophenone from Nacalai Tesque (Kyoto, Japan). Deionized water from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all buffers and sample solutions. Acetonitrile and methanol were of far-UV grade (Mallinckrodt, Paris, KY, USA). Ginseng was purchased from the Chinese herbal market in Taipei (Taiwan).

2.2. Preparation of ginseng extracts

A 2.0-g sample of pulverized ginseng was extracted by refluxing with 50% ethanol (7 ml) for 15 min, then centrifuged at 1500 *g* (Universal, Hettich Zentrifugen) for 5 min. Extraction was repeated three times. The extracts were combined and filtered through a No. 1 filter-paper. After adding 1 ml of internal standard solution (80 μl of acetophenone in 500 ml of 50% ethanol), the ginseng extract was diluted to 25 ml with 50% ethanol. A 20-μl volume of this solution was injected directly into the HPLC system.

2.3. Apparatus and conditions

The HPLC system consisted of two LC-6AD pumps, an SCL-6B system controller, a Rheodyne Model 7125 injector (20-μl loop) and an SPD-M6A photodiode-array detector, all purchased from Shimadzu (Kyoto, Japan).

The separations were obtained by linear gradient elution, using eluents A and B [A = 10 m*M* KH_2PO_4–CH_3CN (80:20); B = H_2O–CH_3CN (15:85)] according to the following profile: 0–15 min, 98–96% A, 2–4% B; 15–25 min, 96–85% A, 4–15% B; 25–40 min, 85–75% A, 15–25% B; 40–50 min, 75–0% A, 25–100% B; 50–62 min, 100% B. The flow-rate was kept constant at 1.0 ml/min.

The columns used are listed in Table 1. A precolumn of μBondapak C_{18} and Novapak silica (both from Millipore, Milford, MA, USA) was used to protect the column.

3. Results and discussion

3.1. Analytical conditions

HPLC of the neutral saponins of ginseng has been studied extensively. Two methods for the separation of all the major acidic and neutral saponins have been developed by Yamaguchi et

Table 1
Columns used

No.	Column	Producer and location	Column I.D. (mm)
I	Cosmosil $5C_{18}$	Nacalai Tesque (Kyoto, Japan)	4.6
II	Cosmosil $5C_{18}$-MS	Nacalai Tesque	4.6
III	Cosmosil $5C_{18}$-AR	Nacalai Tesque	4.6
IV	Vercopak 5ODS	GL Science (Tokyo, Japan)	4.6
V	Vercopak ODS-2	GL Science	4.6
VI	Inertsil ODS-2	GL Science	4.6
VII	Inertsil ODS-80A	GL Science	4.6
VIII	Lichrosorb RP-18	Merck (Darmstadt, Germany)	4.0
IX	LiChrospher 60RP-SelectB	Merck	4.0
X	Purosphere RP-18	Merck	4.0
XI	Nucleosil 100 $5C_{18}$	Macherey–Nagel (Düren, Germany)	4.0

Length = 25 cm and particle size = 5 μm for all columns.

al. [14] and Kanazawa et al. [13]. The former used aqueous acetonitrile containing 1.0% H_3PO_4 as mobile phase whereas the latter used a mixture of acetonitrile and 50 m*M* KH_2PO_4 solution. In our preliminary study, we found that the addition of dihydrogenphosphate to the mobile phase and the selection of a suitable reversed-phase column are two main determining factors in achieving good resolution.

Buffer concentration

Among the five kinds of columns available in our laboratory (I, III, IV, VIII and X), we found that the Cosmosil $5C_{18}$ (Nacalai Tesque) gave the best separation in our preliminary study. This column was therefore chosen as the basis of our study in searching for the optimum eluent composition and flow-rate. First, we prepared eight KH_2PO_4 buffer solutions of different concentrations: 0 m*M* (pH 6.39), 2m*M* (pH 5.94), 5 m*M* (pH 5.92), 10 m*M* (pH 5.88), 20 m*M* (pH 5.76), 30 m*M* (pH 5.62), 40 m*M* (pH 5.55) and 50 m*M* (pH 5.55). Using these eluents, the capacity factors (k') of the column towards each ginsenoside were obtained. The results are given in Table 2.

From the results in Table 2, it can be seen that by using the mobile phase without KH_2PO_4, a good result was obtained only for neutral saponins and not for the acidic compounds. With the addition of a low concentration of KH_2PO_4 (2 m*M*) to the mobile phase, a much better separation of both kinds of saponins was obtained, which is in agreement with the result of Yamaguchi et al. [14]. Increasing the buffer concentration from 2 to 50 m*M* not only varies the retention time but also narrows the peak width of most of the components in ginseng. As shown in Table 2, satisfactory resolution was obtained with concentrations of 5, 10, 40 and 50 m*M*. However, when the individual chromatograms in Fig. 2 were closely examined, it was found that the separation of **5** from **7** and **6** from **11** using 5 m*M* buffer was incomplete, and the peak of **8** was partially overlapped with some minor impurities when using 40 and 50 m*M* buffers. Further, an appreciable amount of salt precipitated out in the course of analysis when a high concentration of buffer (e.g., 50 m*M* KH_2PO_4 or higher) was used, and thus contaminated the column. Hence a buffer solution containing 10 m*M* KH_2PO_4 was adopted in subsequent studies.

Column selectivity

The mobile phase and stationary phase, which are the factors governing HPLC separation, have mutual influences on each other. We therefore fixed the concentration of buffer at 10 m*M* KH_2PO_4 as discussed above and compared the

Table 2
Correlation of buffer concentration of KH_2PO_4 with capacity factor (k') of the saponins

Ginsenoside	KH_2PO_4 concentration (m*M*)							
	0	2	5	10	20	30	40	50
7	5.95	5.62	4.21	5.30	5.54	4.39	5.92	7.05
5	6.21	5.84	4.40	5.52	5.89	4.65	6.12	7.18
9	8.00	9.24	9.01	9.44	9.58	9.85	9.55	9.98
10	8.00	9.58	9.31	9.70	9.74	9.93	9.80	10.20
12	8.00	9.93	9.69	9.91	10.09	10.22	10.21	10.61
6	10.46	10.37	9.92	10.38	10.54	10.55	10.64	11.09
11	8.00	10.36	10.11	10.56	10.50	10.55	10.43	10.85
8	11.13	11.05	10.67	11.08	11.01	10.85	11.07	11.40
1	11.27	11.19	10.80	11.25	11.14	10.99	11.20	11.55
3	11.78	11.71	11.31	11.75	11.62	11.42	11.71	12.05
2	12.34	12.28	11.85	12.32	12.16	11.90	12.26	12.59
4	13.58	13.53	13.11	13.57	13.57	13.05	13.52	13.79

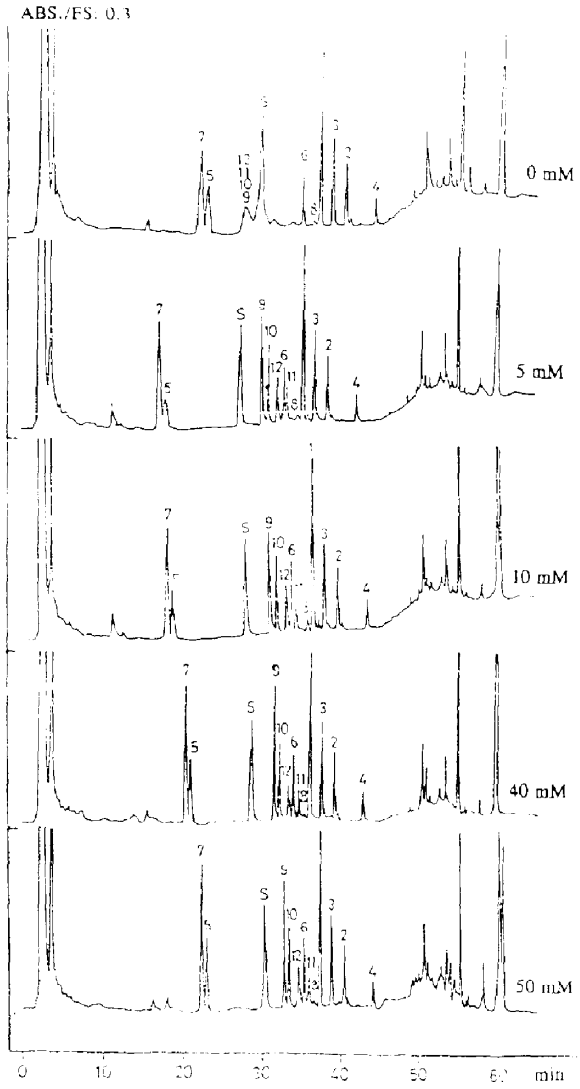


Fig. 2. HPLC of white-ginseng extract eluted with KH_2PO_4 buffers of different concentrations (column I).

selectivities of the eleven commercial C_{18} columns (all 25 cm in length). After a series of experiments, the capacity factors (k') of the ginsenosides were as shown in Table 3.

The data in Table 3 showed that **1–4** could be well separated no matter which column was used. However, great variations occurred in the separations of **5** from **7** and of **6** and **8–12**. Examining the individual chromatograms in detail, it was found that all the columns except I, III and XI failed to separate completely the compounds in the middle region (**8–12**). In addition, columns IV, V, VII and VIII did not give satisfactory separations of **5** and **7**.

Among columns I, III and XI, which are the columns that can give a fairly good separation for all the ginsenosides, column I was found to be the best one owing to a higher resolution in the separation of **8** from **11**. Therefore, column I was adopted (Fig. 3).

A precolumn is mainly used to protect the column, and the packing material used is normally in accordance with that in the analytical column. Our experiments revealed that precolumns of μBondapak C_{18} gave poorly reproducible retention times in the separation of **7** and **5** (19.20 ± 1.04 min, 20.18 ± 0.85 min, $n = 6$); an improvement was obtained using silica (19.37 ± 0.06 min, 20.26 ± 0.05 min, $n = 6$). Therefore, precolumns of the Novapak silica type were the best choice in this study. Moreover, the HPLC system should be completely washed for 17 min after each run to maintain the column reproducibility.

3.2. Method validation

Identification

Ginsenosides **1–7** in the samples were identified by comparing the retention times of authentic ginsenoside standards with those obtained from the sample chromatograms. As malonylginsenosides are known to be thermally unstable and will be decomposed on heating, the identification of **10–12** was carried out by comparing the chromatograms of heat-treated samples with those of the original samples, and also with those reported by Kanazawa et al. [13] and Yamaguchi et al. [14]. Ginsenoside R_0 (**9**), an acidic saponin of oleanolic acid, which has similar characteristics to malonyl ginsenosides except for its thermal stability, was identified by comparing the chromatograms with the published data [13,14]. On the other hand, on varying the detection wavelength from 195 to 220 nm, this acidic saponin was found to give the only large signal in the chromatogram at 220 nm (Fig. 4). Ginsenoside Rg_2 (**8**) was identified by repeating

Table 3
Correlation of column selectivity with capacity factor (k') of the saponins

Ginsenoside	Column										
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
7	5.30	3.94	3.54	7.08	4.29	4.41	3.32	3.78	3.58	6.09	5.47
5	5.52	4.20	3.82	7.23	4.50	4.71	3.34	4.07	3.86	6.38	5.77
9	9.44	8.98	8.70	10.41	9.44	9.49	8.43	8.97	8.67	0.98	9.43
10	9.70	8.98	8.90	10.41	9.56	9.61	8.17	9.33	8.67	0.98	9.68
12	9.91	9.11	9.23	10.41	9.96	10.02	8.76	9.67	8.90	0.98	10.04
6	10.56	9.58	9.38	10.92	10.10	10.07	9.07	9.39	9.45	10.25	9.86
11	10.38	9.98	9.59	11.23	10.40	10.43	9.07	10.00	9.38	0.98	10.36
8	11.08	9.98	9.79	11.67	10.40	10.43	9.33	10.14	9.80	12.08	10.85
1	11.25	10.29	10.00	11.87	10.70	10.78	9.47	10.84	9.93	12.25	11.18
3	11.75	10.76	10.44	12.40	11.19	11.28	9.88	11.29	10.37	12.84	11.68
2	12.32	11.63	10.93	12.89	11.73	11.81	10.28	11.77	10.77	13.41	12.09
4	13.57	12.45	12.03	14.10	12.97	13.01	11.34	12.78	11.73	14.23	13.18

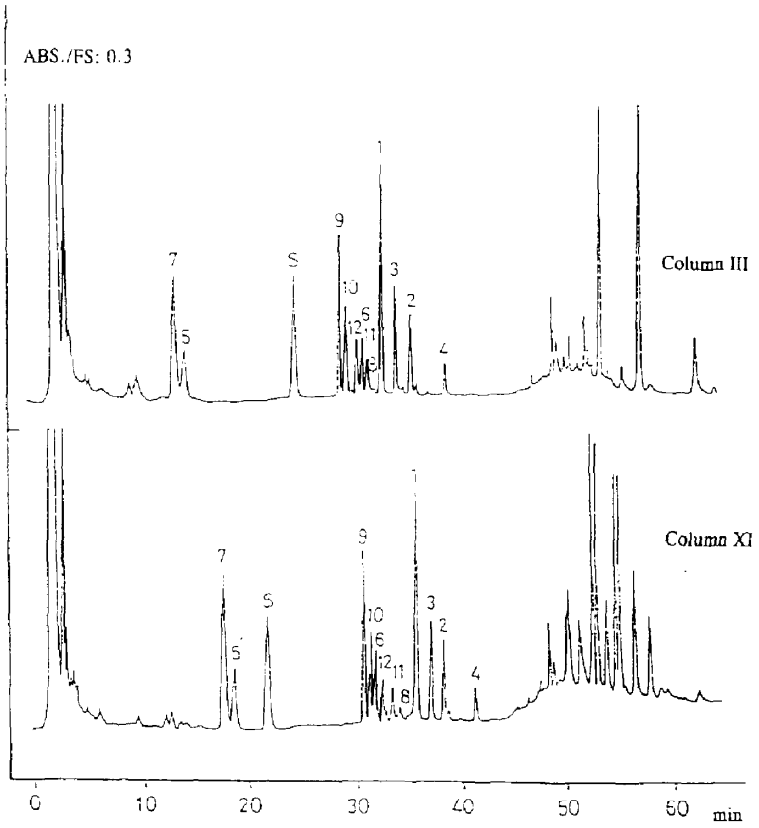


Fig. 3. HPLC of white-ginseng extract separated on different columns.

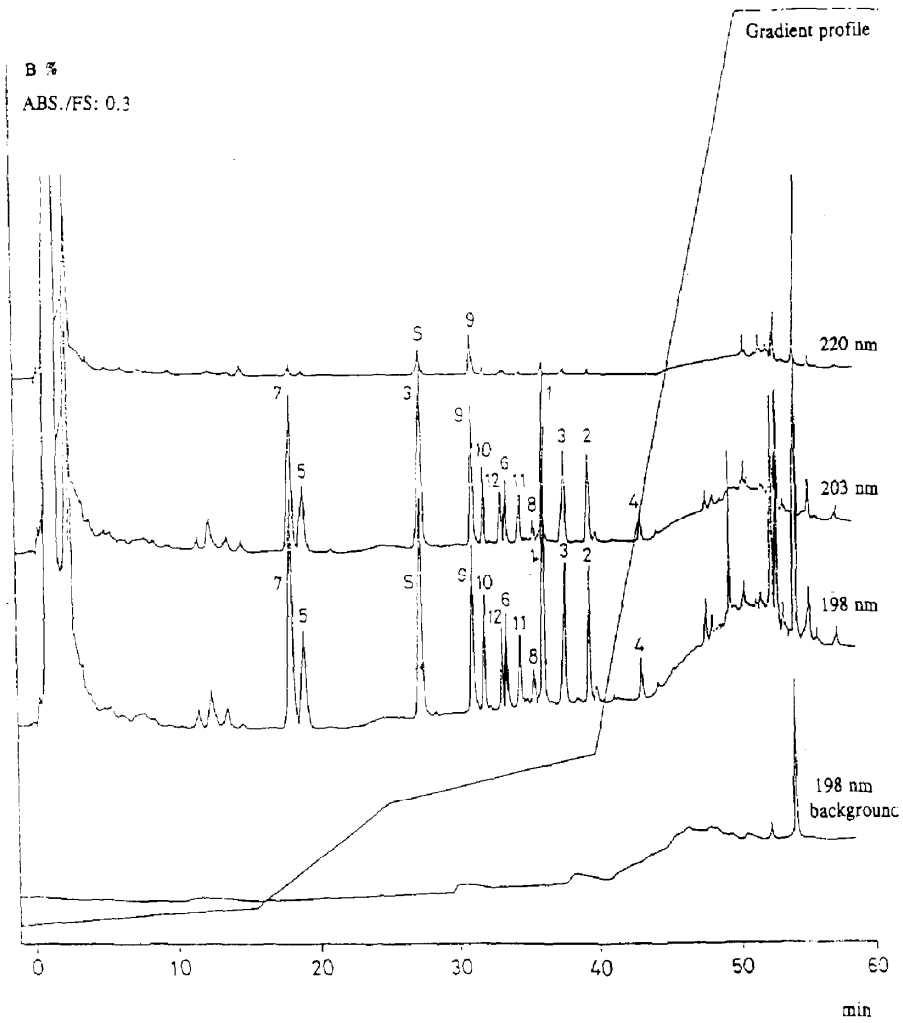


Fig. 4. HPLC of white-ginseng extract with detection at various wavelengths.

the methods reported by Pietta et al. [9] Kanazawa et al. [10].

Detection limit

For the HPLC detection of ginsenosides, the detection wavelength was 203 nm (the detection limit of each ginsenoside was about 5 μg [10,11]). With the use of far-UV-grade acetonitrile (Mallinckrodt), which has a weaker absorbance in the low wavelength region, detection at lower wavelengths was feasible in this study. After scanning with an SPD-M6A photodiode-array detector with wavelengths ranging from 195 to 380 nm, it was found that strong absorbance occurred only at wavelengths below 200 nm. In comparison, the peak sensitivity of ginsenosides monitored at 198 nm was about 1.5 times those at 203 nm, as shown in Fig. 4. Therefore, 198 nm was chosen as the detection wavelength. The detection limit of each ginsenoside at this wavelength was about 0.2 μg (0.01 mg/ml, signal-to-noise ratio = 2, sample size 8 μl). However, under such conditions, baseline drift occurred as shown in Fig. 4. Hence it was difficult to determine the peaks with retention times longer than 46 min.

Precision

The reproducibility (relative standard deviation) of the proposed method, on the basis of peak-area ratios for six replicate injections, was 0.04–3.09%, as shown in Table 4. The variation of the retention time of each peak was less than 0.5% for six replicate injections.

Linearity

The linearity of the peak-area ratio (y) vs. concentration (x, mg/ml) curve for each of the seven ginsenosides was investigated in the range 0.01–1.00 mg/ml. Results of the regression analysis and the correlation coefficients (r) were as follows: **1**, $y = 2.34x + 0.03$ ($r = 0.9988$); **2**, $y = 2.09x + 0.04$ ($r = 0.9990$); **3**, $y = 3.03x + 0.05$ ($r = 0.9992$); **4**, $y = 2.13x + 0.04$ ($r = 0.9987$); **5**, $y = 2.60x + 0.01$ ($r = 0.9998$); **6**, $y = 2.31x + 0.03$ ($r = 0.9992$); **7**, $y = 3.45x + 0.02$ ($r = 0.9988$).

Accuracy

The results of a standard addition recovery study for the seven ginsenosides from ginseng are summarized in Table 5. The recoveries were 98.5–103.2%, with the exception of **6** (95.5% and 96.8%). All the tailing factors of the peaks are very close to unity.

Table 5
Data for recoveries of ginsenosides ($n = 3$)

Ginsenoside	Added (mg)	Recovery (%)
7	0.1000	100.3
	0.2000	99.5
5	0.1000	101.2
	0.2000	102.3
6	0.1000	95.5
	0.2000	96.8
1	0.1000	98.5
	0.2000	100.5
3	0.1000	101.5
	0.2000	100.9
2	0.1000	101.2
	0.2000	103.2
4	0.1000	99.4
	0.2000	98.8

3.3. Determination of ginsenosides in various ginseng samples

When the test solutions of various ginsengs were analysed by HPLC under the selected conditions, the calculated contents of ginsenosides given in Table 6 were obtained. These

Table 4
Reproducibility of separation of ginsenosides

Ginsenoside	Intra-day		Inter-day	
	Ratio[a]	R.S.D. (%)[b]	Ratio[a]	R.S.D. (%)[b]
7	1.14	2.53	1.13	1.18
5	0.50	3.09	0.51	2.43
9	0.67	0.96	0.67	0.95
10	0.38	0.41	0.37	0.40
12	0.30	0.73	0.31	1.01
6	0.30	2.18	0.29	2.81
11	0.19	1.54	0.15	0.78
8	0.02	2.08	0.02	1.98
1	1.01	1.90	1.03	1.50
3	0.49	0.56	0.49	0.82
2	0.33	0.70	0.32	0.33
4	0.15	0.33	0.14	0.45

[a] Ratio = peak area of ginsenoside/peak area of internal standard (acetophenone).
[b] $n = 6$.

Table 6
Contents (mg/g) of ginsenosides in ginseng samples (±S.D., $n = 3$)

Ginsenoside	Sample[a]		
	1	2	3
7	3.96 ± 0.05	3.56 ± 0.08	4.23 ± 0.04
5	2.22 ± 0.02	2.91 ± 0.03	6.77 ± 0.01
6	0.84 ± 0.04	1.45 ± 0.04	2.51 ± 0.03
1	3.31 ± 0.06	5.67 ± 0.01	11.34 ± 0.06
3	1.71 ± 0.03	3.86 ± 0.04	9.47 ± 0.08
2	1.93 ± 0.02	3.51 ± 0.01	8.13 ± 0.02
4	0.43 ± 0.01	1.77 ± 0.04	7.53 ± 0.02
Total	14.40 ± 0.17	22.71 ± 0.10	49.96 ± 0.13

[a] 1 = White-ginseng; 2 = red-ginseng; 3 = Ginseng hair-root.

results indicate that the proposed HPLC method is suitable for the determination of ginsenosides in various ginseng samples. Moreover, no pre-treatment is needed in this method.

Acknowledgement

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