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Reversed-phase high-performance liquid chromatographic determination of ginsenosides of *Panax quinquefolium*

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Abstract

A reversed-phase high-performance liquid chromatographic determination of ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rg_1, Ro, gypenoside XVII, pseudoginsenoside-F_{11}, and an indirect determination of the malonyl-ginsenosides Rb_1, Rb_2, Rc and Rd in *Panax quinquefolium* was developed. Ground ginseng samples were extracted with aqueous methanol in an ultrasonic bath. The neutral triterpene saponins were determined directly by liquid chromatography on the extract. The acidic ginsenosides were determined indirectly following the hydrolysis with aqueous potassium hydroxide. Separations were achieved with a phosphate buffer–acetonitrile gradient system using a C_{18} reversed-phase column.

Keywords: *Panax quinquefolium*; Ginsenosides; Terpenes; Saponins

1. Introduction

Panax quinquefolium L. (American ginseng) is cultivated extensively in North America. The roots of *Panax quinquefolium* and a related species, *Panax ginseng* (Asian ginseng), are widely used medicinal plants [1]. The most thoroughly examined active principles of ginseng are saponins usually referred to as ginsenosides (Fig. 1), and a large number of these triterpene saponins are known [1].

In recent years high-performance liquid chromatography (HPLC) has been used extensively for the analysis of the neutral saponins of ginseng. Although separations on silica gel [2], amino columns [3,4], and ion-exchange columns [5,6] have been reported, most procedures have employed reversed-phase columns [7–10]. The separation of the acidic saponins of ginseng has been studied much less frequently; however, Yamaguchi et al. [11] have analysed the neutral and acidic saponins in wild *Panax ginseng* with an amino column, and Kanazawa et al. [12,13] have determined these components on a reversed-phase column. More recently, Chuang and Sheu [14], and Chuang et al. [15] have also reported a reversed-phase separation of these constituents in *Panax ginseng*.

Kitagawa et al. [16] isolated four acidic saponins from *Panax ginseng*. These were the malonyl-ginsenosides Rb_1 (2), Rb_2 (4), Rc (6) and Rd (8). Malonyl-ginsenosides are unstable and are readily demalonylated on heating [11]. It is likely that this instability is, in part, responsible for the limited amount of information available on the concentrations of these constituents in ginseng; however, those studies which have examined the acidic saponins

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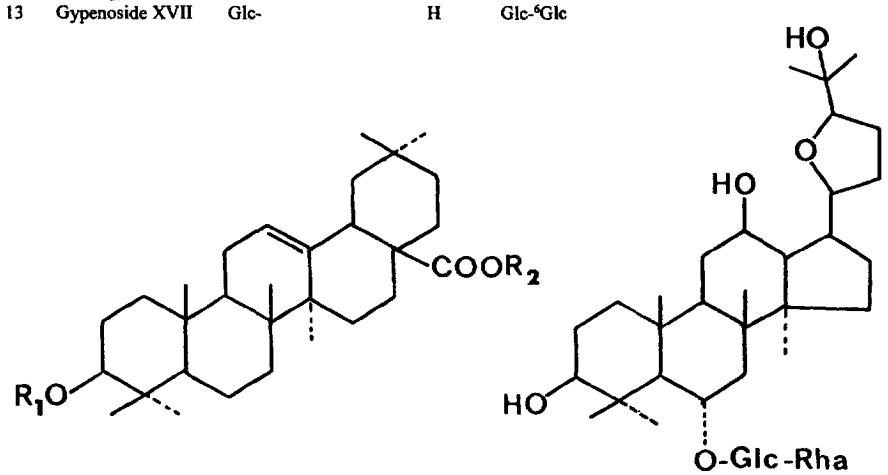
		R_1	R_2	R_3
1	Rb_1	Glc-^{2}Glc-	H	Glc-^{6}Glc-
2	m-Rb_1	Ma-^{6}Glc-^{2}Glc-	H	Glc-^{6}Glc-
3	Rb^2	Glc-^{2}Glc-	H	Ara(p)-^{6}Glc-
4	m-Rb_2	Ma-^{6}Glc-^{2}Glc	H	Ara(p)-^{6}Glc-
5	Rc	Glc-^{2}Glc-	H	Ara(f)-^{6}Glc-
6	m-Rc	Ma-Glc-^{2}Glc	H	Ara(f)-^{6}Glc-
7	Rd	Glc-^{2}Glc-	H	Glc-
8	m-Rd	Ma-^{6}Glc-^{2}Glc-	H	Glc-
9	Re	H	Rha-^{2}Glc-0	Glc-
10	Rf	H	Glc-^{2}Glc-0	H
11	Rg_1	H	Glc-0	Glc-
12	Rg_2	H	Rha-^{2}Glc-0	H
13	Gypenoside XVII	Glc-	H	Glc-^{6}Glc



		R_1	R_2
14	Ro	Glc-^{2}GlcA-	Glc

15	F_{11}

Fig. 1. Structures of ginsenosides referred to in Section 1.

have found significant amounts of these constituents in the roots [3,11].

A number of approaches were considered for the analysis of the acidic ginsenosides of ginseng including the simultaneous determination of the acidic and neutral saponins; however, the aim of the present study was to evaluate indirect methods of determining the acidic saponins while determining the neutral saponins of *Panax quinquefolium*. In this paper, the results of this investigation are reported.

2. Experimental

2.1. Materials and reagents

Ginsenosides [Rb_1 (1), Rb_2 (3), Rc (5), Rd (7), Re (9), Rf (10) and Rg_1 (11)] were purchased from C. Roth (Atomergic Chemetals, Farmingdale, NY, USA), and Rb_1 (1), Rc (5) and Re (9) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (Acetonitrile-190, HPLC grade) and methanol (HPLC grade) were from Caledon (Georgetown, Canada). Water was from a Milli-Q water purification unit (Millipore, Mississauga, Canada). Potassium dihydrogenphosphate (KH_2PO_4) and disodium hydrogenphosphate (K_2HPO_4) were obtained from J.T. Baker (Phillipsburg, NJ, USA). The mobile phase (eluent A) was prepared by dissolving 2.80 g KH_2PO_4 in 2000 ml of water and adjusting the pH to 5.81 with a concentrated solution of K_2HPO_4 (35 g/100 ml). Potassium hydroxide (reagent grade) was obtained form Mallinckrodt (Canlab, Mississauga, Canada). Phenols (3,5-dimethoxyphenol, 3,4-dimethylphenol and *o*-, *m*- and *p*-cresol) were purchased from Aldrich (Milwaukee, WI, USA).

2.1.1. Sample preparation

Ginseng samples were ground in a Wiley Mill to pass a 40-mesh screen. Samples were freeze-dried before extraction.

2.1.2. Soxhlet extraction

Ground ginseng samples (1.500 g) were placed in cellulose extraction thimbles (10×50 mm, Whatman, Clifton, NJ, USA) and extracted in micro-Soxhlet extractors with 30 ml of methanol. After cooling the methanol was removed in vacuo. The residue was dissolved in acetonitrile–water (1:1) and filtered through a 0.45-μm filter prior to analysis.

2.1.3. Ultrasonic extraction

Ground ginseng samples (300 mg) were transferred to 15-ml centrifuge tubes followed by 10 ml of methanol–water (7:3). Sample tubes were placed in an ultrasonic bath (Labline No.9333, Melrose Park, IL, USA) at maximum ultrasonification for 15 min. Samples were stirred occasionally and rotated in the bath to ensure optimal extraction. Alternately, samples were mixed with a vortex mixer periodically during ultrasonification. Sample tubes were then centrifuged at 1500 rpm and the solvent was removed. The extraction was repeated two additional times and the combined extracts concentrated in vacuo at less than 35°C. The residue was dissolved in 2 ml water containing the internal standard (*m*-cresol). A portion of the sample was diluted 1:1 with acetonitrile, filtered through a 0.45-μm filter and analysed.

2.1.4. Base hydrolysis

Acidic ginsenosides were hydrolysed by adding 80 μl of 5% KOH to a portion (850 μl) of the ginseng extract. After 2 h at room temperature the base was neutralized by adding 80 μl of a KH_2PO_4 solution (14 g/100 ml). The mixture was diluted with 850 μl of acetonitrile, filtered through a 0.45-μm filter and analysed to determine total ginsenosides.

2.2. High-performance liquid chromatography

2.2.1. System 1

All analytical separations were performed on a Waters liquid chromatograph (Waters, Milford, MA, USA) consisting of a Waters Wisp 712A autosampler, a Waters 600E pumping system and Waters 490 programmable multi-wavelength detector at 203 nm. All computations were performed using a Waters Millennium data system. Injections were 5 μl. Separations were carried out using a 5 μm Waters Resolve C_{18} column (15 cm×3.9 mm) with a Symmetry C_{18} Sentry guard column. The ternary gradient employed the eluents (A) phosphate buffer solution, (B) acetonitrile and (C) water according to the following profile: 0–15 min, 81–79% A, 19–21% B, 0% C; 15–24.5 min, 79–73.7% A. 21–26.3% B, 0% C; 24.5–29 min, 73.7–73% A, 26.3–27% B, 0% C; 29–43 min, 73–66% A, 27–34% B, 0%C; 43–47 min, 66–64% A, 34–36% B, 0% C; 47–54 min, 64–57% A, 36–43% B, 0% C; 54–55 min, 57–0% A, 43–85% B, 0–15% C; 55–59 min, 0% A, 85% B, 15% C; 81% A, 19% B, 0% C. The flow-rate was 1.15 ml/min.

2.2.2. System 2

Separations were performed with a Hewlett-Packard 1090 liquid chromatography (Hewlett-Packard, Mississauga, Canada) equipped with an automatic injector and a 1040A diode array detector at 203 nm. Injections were 5 μl. Separations were carried out using a Waters cartridge system consisting of a carbohydrate cartridge (25 cm×4.6 mm) column and a carbohydrate Sentry guard column. The binary

Table 1
Percentage of selected ginsenosides extracted with ultrasonification[a]

Extraction	Rg_1	Re	Rb_1	Rc	Rb_2	Rd
Sample A						
1	90.20	90.18	89.57	89.99	91.40	90.42
2	8.12	8.62	8.89	9.11	8.58	8.61
3	1.49	0.78	0.91	0.84	-	0.89
4	0.15	0.26	0.33	0.07	-	0.08
5	-	0.15	0.29	-	-	-
Sample B						
1	91.40	87.66	85.72	88.09	94.20	88.49
2	8.60	8.81	8.74	8.27	5.80	8.23
3	-	1.80	3.77	3.64	-	3.28
4	-	0.83	1.18	-	-	-
5	-	0.91	0.59	-	-	-
Sample C						
1	91.80	90.63	91.04	91.36	92.34	91.41
2	8.20	8.35	7.95	8.10	7.66	8.01
3	-	0.53	0.67	0.54	-	0.58
4	-	0.21	0.17	-	-	-
5	-	0.27	0.17	-	-	-

[a] 10 ml CH_3OH–water (7:3) for 15 min.

gradient employed (A) water and (B) acetonitrile according to the following profile: 0–28 min, 9–19%A, 91–81%B; 28–30 min, 19–19%A, 81–81%B; 30–35 min, 19– 22%A, 81–78%B; 35–40 min, 22–25%A, 78–75%B; 40–41 min, 25–9%A, 75–91%B. The flow-rate was 1.5 ml/min.

3. Results and discussion

A wide variety of procedures have been utilized for the extraction of ginsenosides from root material; however, most methods employ multiple extractions by methanol or aqueous methanol [3,6,9,12,16]. Preliminary experiments comparing methanol versus aqueous methanol extractions at room temperature found that these triterpene saponins were more readily extracted with methanol containing water. Therefore, aqueous methanol was utilized for all extractions. A number of procedures have also employed some heat to facilitate the extraction process [9,14,17–19] even though malonyl-ginsenosides are thermally unstable and are demalonylated on heating [11]. Due to the thermal instability of the acidic ginsenosides, ultrasonification at room temperature was examined for extraction. Three extractions with 70% methanol in water were found to give satisfactory recovery of the ginsenosides (Table 1).

Yamaguchi et al. [11] found that malonyl-ginsenosides are thermally unstable and are demalonylated on heating, and Kitagawa et al. [16] found that hydrolysis with base also readily removed the malonic acid. These observations offer opportunities for the conversion of the acidic ginsenosides to the corresponding neutral ginsenosides which are

Table 2
Influence of extraction procedures on selected ginsenosides

Method	Rg_1	Re	Rb_1	Rc	Rb_2	Rd
Sample A[a]						
Ultrasonification	1.00 (3.8)[b]	1.00 (1.9)	1.00 (1.5)	1.00 (2.5)	1.00 (4.9)	1.00 (1.4)
Ultrasonification + base	1.07 (4.2)	1.02 (0.8)	2.25 (0.8)	1.83 (2.0)	1.78 (3.9)	2.14 (1.1)
Soxhlet (h)						
5	0.99 (3.5)	0.96 (1.1)	1.65 (1.4)	1.43 (0.7)	1.35 (3.2)	1.57 (1.9)
10	0.99 (2.4)	0.98 (0.9)	1.96 (0.6)	1.65 (1.0)	1.57 (2.2)	1.92 (1.4)
20	1.00 (2.3)	0.98 (0.6)	2.10 (0.5)	1.73 (1.7)	1.56 (2.2)	2.04 (1.0)
30	1.01 (2.4)	0.98 (0.3)	2.11 (0.5)	1.74 (0.6)	1.59 (1.4)	2.02 (0.7)
40	1.00 (2.1)	1.00 (1.9)	2.15 (1.8)	1.80 (1.6)	1.62 (1.7)	2.08 (2.4)
Sample B						
Ultrasonification	1.00 (5.8)	1.00 (1.6)	1.00 (1.5)	1.00 (3.0)	1.00 (27)	1.00 (1.6)
Ultrasonification + base	1.07 (3.8)	1.02 (1.8)	2.06 (1.4)	1.72 (1.4)	1.66 (5.5)	1.76 (2.5)
Soxhlet (h)						
5	0.96 (1.5)	0.94 (0.6)	1.64 (0.6)	1.51 (1.4)	1.44 (3.2)	1.49 (0.6)
10	0.95 (2.2)	0.94 (0.7)	1.86 (0.8)	1.63 (1.9)	1.57 (2.2)	1.64 (0.9)
20	1.03 (4.4)	0.95 (0.4)	1.96 (0.4)	1.72 (0.4)	1.67 (1.8)	1.72 (0.4)
30	1.02 (3.3)	0.96 (1.0)	1.99 (0.5)	1.76 (1.9)	1.66 (1.7)	1.74 (1.4)
40	1.07 (3.7)	0.98 (1.5)	2.04 (1.2)	1.81 (1.9)	1.66 (3.2)	1.63 (1.2)
Sample C						
Ultrasonification	1.00 (4.2)	1.00 (1.5)	1.00 (1.4)	1.00 (1.7)	1.00 (4.2)	1.00 (1.2)
Ultrasonification + base	1.10 (3.2)	1.01 (2.5)	2.08 (1.9)	1.73 (3.3)	1.54 (6.2)	1.84 (1.1)
Soxhlet (h)						
5	0.95 (1.1)	0.94 (0.2)	1.64 (0.7)	1.44 (1.6)	1.33 (2.1)	1.57 (0.9)
10	1.08 (1.1)	1.03 (0.4)	1.99 (0.8)	1.74 (1.8)	1.58 (2.3)	1.88 (1.3)
20	1.08 (2.0)	1.02 (1.2)	1.08 (1.3)	1.79 (1.2)	1.60 (1.3)	1.96 (1.3)
30	1.12 (1.8)	1.03 (1.0)	2.10 (0.5)	1.85 (2.2)	1.72 (2.8)	1.99 (0.5)
40	1.12 (2.8)	1.04 (0.5)	2.15 (1.00)	1.83 (0.6)	1.68 (3.4)	2.03 (0.5)

[a] Amount relative to ultrasonic extraction.
[b] Relative standard deviation ($n=6$).

more readily determined by HPLC. Conversion of the acidic ginsenosides to neutral ginsensosides can be readily achieved with heat (Table 2). Extraction of ginseng samples in a Soxhlet extractor demalonylated the acidic ginsenosides; however, a minimum 20 h were required for complete conversion which is not very efficient for handling a large number of samples. In addition, somewhat longer times were required when solutions of ginseng extracts were heated at 80°C in sealed vials. This contrasts with the complete conversion of the acidic ginsenosides to the neutral ginsenosides in less than 2 h with base (Table 2).

Several procedures for the determination of ginsenosides have found the the presence of KH_2PO_4 in the mobile phase enhanced resolution [11,12]. In addition, Chuang and Sheu [14] also found that the concentration of the KH_2PO_4 in the mobile phase was important for the resolution of the ginsenosides. In the present study, it was found that not only the presence of KH_2PO_4 was important but also the pH of the mobile phase. The best separation was obtained with a mobile phase containing KH_2PO_4 (1.4 g/1000 ml) adjusted to pH 5.81 with a concentrated solution of K_2HPO_4. In addition, Peterson and Palmqvist [7] examined a series of reversed-phase columns and found that column selectivity could be used in optimizing resolution of ginseng components. In order to examine this phenomenon a series of Waters Assoc. C_{18} reversed-phase columns (Resolve, Novapak, Symmetry and μBondapak) columns were examined with the present solvent system. Only the μBondapak column gave unacceptable results; however, the best separation was achieved with a Resolve column with a Symmetry Sentry cartridge guard column (Fig. 2). This system was used for all analyses.

In order to facilitate quantitation a variety of substances were evaluated as internal standards. A number of phenols including 3,5-dimethoxyphenol, 3,4-dimethylphenol and *o*-, *m*-, and *p*-cresol fitted into various open retention windows of the chromatogram. The preferred locations were 3,5-dimethoxyphenol (t_r=10.4 min) and *m*-cresol (t_r=13.6 min); however, 3,5-dimethoxyphenol was not stable in the basic sample mixture which left *m*-cresol as the preferred internal standard.

For those ginsenosides for which standards were available, ginsenosides in the sample were identified by comparing the retention times of the standards with those obtained in the samples on the reversed-phase column. Additional support for these assignments was obtained with the amino propyl column (System 2, see Section 2.2.2). The ginsenoside Ro (14) was identified by comparing the retention times with published data and by varying the detection wavelength [14]. Gypenoside XVII (13) and the pseudoginsenoside F_{11} (15) were isolated from *Panax quinquefolium* L. by thin-layer chromatography [20]. In addition, based on molecular mass and retention time the isolated gypenoside XVIII would

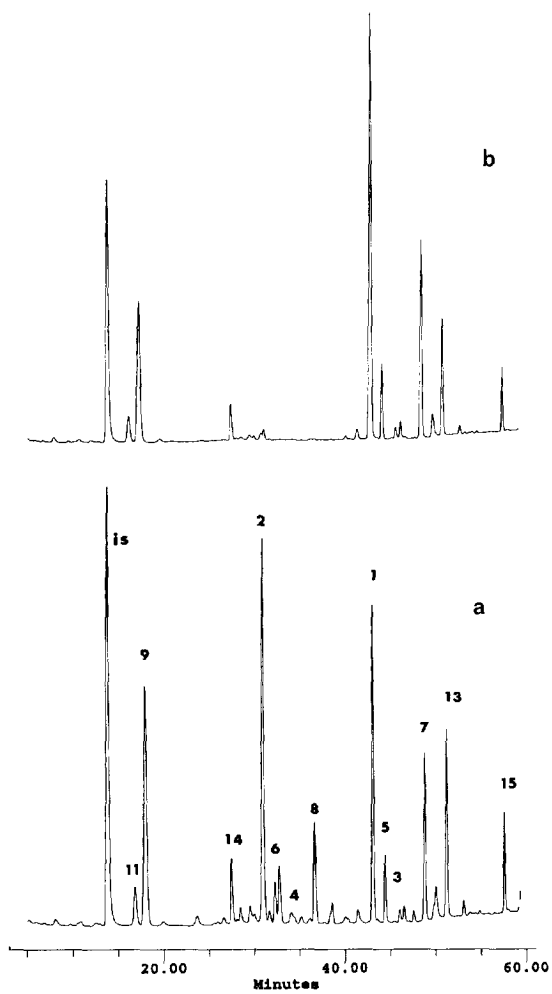


Fig. 2. HPLC of ginsenosides of *Panax quinquefolium*: (a) aqueous methanol extract; (b) base hydrolysed extract.

Table 3
Reproducibility data for replicate analyses of ginsenoside

Ginsenoside	Sample A		Sample B	
	mg/g[a]	R.S.D. (%)[b]	mg/g	R.S.D. (%)
Rb_1	16.75	1.2	15.17	3.3
m-Rb_1[c]	23.15	1.8	16.92	3.2
Rb_2	0.49	4.6	0.44	2.8
m-Rb_2[c]	0.35	8.3	0.27	1.2
Rc	2.54	3.3	2.13	2.1
m-Rc[c]	2.09	3.7	1.56	3.3
Rd	4.53	6.1	4.11	1.9
m-Rd[c]	4.65	5.5	4.08	3.4
Re	14.81	1.1	12.34	1.3
Rf	-	-	-	-
Rg_1	1.96	3.8	1.39	3.1
Ro[d]	3.65	8.7	3.16	5.0
F_{11}[d]	0.65	3.7	0.51	6.3
Gypenoside XVII[d]	3.16	1.3	1.21	1.3

[a] Mean value of six determinations.
[b] Relative standard deviation.
[c] Represents difference between ginsenoside content of extract and base hydrolysed extract.
[d] Determined as Rb_1.

appear to be the unknown constituent (t_r=16.0 min) reported by Van Breeman et al. [21]. The malonyl ginsenosides were tentatively identified by comparing the chromatograms of the extracts with those of the heat treated and base hydrolysed samples, and by the retention times reported earlier ([11–14]). Since the locations of these constituents in the chromatograph were not critical to this method these tentative assignments were not confirmed.

The major ginsenosides found in *Panax quinquefolium* grown in Ontario were mRb_1 (2) and Rb_1 (1) with the total of the neutral and acidic forms of this saponin often exceeding one half of the total ginsenosides present in the root (Table 3). Significant amounts of Re (9) were also present in most samples. Intermediate concentrations of Rg_1 (11), Ro (14), *m*-Rc (6), Rc (5), *m*-Rd (8) and Rd (7) were found in root samples. Ginsenosides *m*-Rb_2 (4) and Rb_2 (3) were present in all samples as minor constituents. The concentrations of gypsenoside XVII (13) and the pseudoginsenoside-F_{11} (15) were quite variable and concentrations were dependent upon the drying process of the root. The smallest amounts were found in freeze-dried root samples. Differences in drying procedures probably explains some of the variations in the concentrations of these two saponins that have been reported in *Panax quinquefolium* [20,22].

In summary, this method affords the opportunity for determining neutral saponins directly on a ginseng extract. Except for several of the minor constituents acceptable reproducibility (relative standard deviation) was obtained (Table 3). It also provides the means to determine total ginsenosides in the extract after hydrolysis with base. In addition, it enables the indirect estimate of the acidic ginsenosides in the extract. This is an estimate since derivatives other than malonic acid have been reported. For example, a small amount of an acetate of Rb_1 has been reported in *Panax quinquefolium* [22] although at very small concentrations compared to *m*-Rb_1 (2). In addition, it is apparent that additional small unidentified peaks are present in the chromatogram of the extract which are hydrolysed with base; however, these observations do not detract from advantages of the method for estimating the malonyl ginsenosides, which are present in significant amounts in *Panax quinquefolium* using standards that are readily available.

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