JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Evaluation of the Efficiency of Three Different Solvent Systems to Extract Triterpene Saponins from Roots of *Panax quinquefolius* Using High-Performance Liquid Chromatography

STEFAN GAFNER,*,† CHANTAL BERGERON,† MEGAN M. MCCOLLOM,† LORENA M. COOPER,† KERRY L. MCPHAIL,‡ WILLIAM H. GERWICK,‡ AND CINDY K. ANGERHOFER†

Tom's of Maine, P.O. Box 710, Kennebunk, Maine 04043 and College of Pharmacy, Oregon State University, Corvallis, Oregon 97331

Despite the wide availability of liquid herbal extracts using mixtures of alcohol, glycerin, and water, or glycerin and water as solvents, no data on the chemical composition of such extracts is readily available. In this study, the amount and the stability of the major saponins in *Panax quinquefolius* root extracts, made either with 50% (v/v) aqueous ethanol, a mixture (v/v/v) of 20% ethanol, 40% glycerin, and 40% water, or with 65% (v/v) aqueous glycerin, were evaluated by HPLC-UV analysis. The amount of total saponins was highest in the 50% aqueous ethanol extract (61.7 ± 0.1 mg/g dry root), although similar to the ethanol−glycerin−water extract (59.4 ± 0.5 mg/g dry root). Saponins were significantly lower in the 65% aqueous glycerin extract (51.5 ± 0.2 mg/g dry root). Interestingly, the amounts of individual saponins were quite variable depending on the solvent. This is in part due to enzymatic cleavage of ginsenosides in the glycerin containing extracts during the maceration process. Storage of the extracts at 25 °C over the period of a year led to a 13−15% loss of saponins with all three types of extractions.

KEYWORDS: ***Panax quinquefolius*; saponins; extraction efficiency; stability; enzymatic degradation; HPLC-UV/MS/MS**

INTRODUCTION

Liquid herbal extracts are one of the most popular ways to deliver the benefits of an herb. For commercial products, extracts with mixtures of ethanol and water account for the majority of liquid products. In many countries, however, glycerin extracts, as well as mixtures of glycerin and ethanol in water at percentages commonly ranging from 25 to 75%, are becoming increasingly popular as pleasant-tasting alternatives. Because the solvent largely determines the phytochemical profile of a given plant extract, and because the chemistry influences the biological activity, we are interested in characterizing different extracts from identical plant materials.

Despite the wide use of glycerin extracts, data on the composition of glycerites is not available. The aim of this study was to fill this gap for North American ginseng, *Panax quinquefolius* L (Araliaceae). North American ginseng is generally used as a mild tonic; however, its mechanisms of action remain unclear. Recent publications indicated a weak CNS stimulant activity (*1*, *2*) and hypoglycemic activity (*3*) and showed immunomodulating properties (*4*). In general, saponins and polysaccharides are thought to be responsible for the biological activity. We investigated the extraction efficiency on the major ginsenosides and gypenoside XVII in extracts made with three different solvent systems. North American ginseng roots were extracted either with 65% (v/v) aqueous glycerin, an aqueous mixture (v/v/v) of 20% ethanol and 40% glycerin, or with 50% (v/v) aqueous ethanol, and the major saponins were quantified using HPLC-UV.

MATERIALS AND METHODS

Chemicals. HPLC grade acetonitrile was purchased from Fisher Scientific Co. (Pittsburgh, PA). Ginsenosides Rb_1, Rc, Rd, Re, and Rg_1 were obtained from Extrasynthèse, SA (Genay, France). Ginsenoside F_2 and gypenoside XVII were isolated from a *P. quinquefolius* glycerin extract as outlined below. The purity of the standards was evaluated by HPLC-UV analysis. Silica gel (170−400 mesh) and copper sulfate pentahydrate was from Fisher Scientific Co., Sephadex LH-20 was from Amersham Pharmacia Biotech (Uppsala, Sweden), and the high load C18 solid-phase extraction cartridges were from Alltech Corp. (Deerfield, IL).

Instruments. The HPLC system consisted of an Agilent quaternary pump, UV−vis detector (DAD), and automatic sample injector, and LC-MS data were obtained with an Agilent 1100 series LC/MSD Trap

* To whom correspondence should be addressed. Tel.: ++1 207 985 2944. Fax ++1 207 985 2196. E-mail stefang@tomsofmaine.com.
† Tom's of Maine.
‡ Oregon State University.

10.1021/jf0307503 CCC: $27.50
Published on Web 02/20/2004

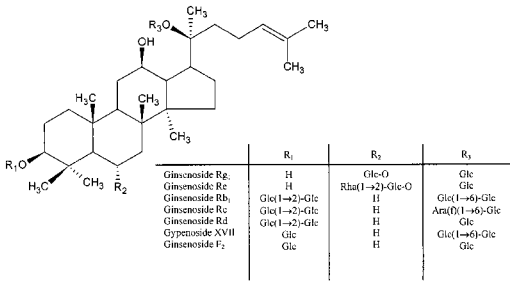

Figure 1. Structures of the saponins analyzed.

(Agilent Technologies, Burlington, MA) with an ESI interface. For the mass spectrometry, the following conditions were used: capillary voltage, 4.5 kV; skim 1, 67.9 V; capillary exit offset, 118.8 V; trap drive, 64.5. The drying temperature was set at 350 °C. The mass detector was used in positive ion mode (scan range *m*/*z* 400−1400) including an automated MS/MS operation. The HPLC separation was performed on a 150- × 2.0-mm i.d., 2-μm, Phenomenex Synergi Hydro-RP column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and water (solvent B), starting with 20% A, followed by a gradient to 22% A in 10 min., 28% A at 11 min., 42% A at 25 min., and 100% isocratic A from 26 to 30 min at a flow of 0.7 mL/min. The column temperature was set at 20 °C. The compounds were quantified with a UV detector at 203 nm.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX 600 instrument at 600 and 150 MHz, respectively. All spectra were recorded in pyridine-d_5 with the solvent used as an internal reference (δH 8.74, 7.58, 7.22; δC 150.35, 135.91, 123.87). For the statistical evaluation, StatView 5 software, version 5.0.1, was used.

Plant Material and Extraction. *Determination of Extraction Efficiency*. *P. quinquefolius* was purchased from Baumann Farms (Wausau, WI). The dried roots were cut and macerated for 6 weeks with either 50% (v/v) aqueous ethanol; a mixture (v/v/v) of 20% ethanol, 40% glycerin, and 40% water; or 65% (v/v) aqueous glycerin (ratio plant material/solvent 1:5 (w/v)).

Determination of Stability of Saponins in Glycerin Extracts During Maceration. Dried and cut *P. quinquefolius* roots were purchased from Blessed Herbs (Oakham, MA). The roots were either macerated with 65% glycerin or with 65% glycerin containing 100 mM of copper sulfate, as described above. After 6 weeks, solids were removed by filtration of the macerates through a screw press.

Isolation of Saponins. A 500-mL aliquot of a 65% aqueous glycerin extract of *P. quinquefolius* roots was partitioned three times with 500 mL of *n*-butanol. The *n*-butanol/glycerin partition fractions were combined, and the *n*-butanol was evaporated. The remaining glycerin was removed by solid phase extraction over an RP-18 cartridge using a gradient of water, water/methanol (4:1), and methanol. The saponins were found in the 100% methanol fractions. After evaporation of the solvent, the powder containing mainly saponins was fractionated by column chromatography on silica gel (280- × 41-mm i.d.) using mixtures of CH_2Cl_2/methanol/water (80:20:5 (lower layer) and 65:35:5) and methanol/water (8:2 and 6:4) to yield eight fractions. Fraction 2 was further separated by gel filtration on Sephadex LH-20 (260- × 19-mm i.d.) using methanol/water (1:1) to give ginsenoside F_2 (27.5 mg). Fraction 4 was separated by gel filtration on Sephadex LH-20 (methanol/water 1:1) to give four fractions (4a−4d). Gypenoside XVII (10.3 mg) was purified from fraction 4c using an RP-18 solid-phase extraction cartridge with a step gradient using mixtures of acetonitrile/water (1:4−1:1). The same compound (21.2 mg) was isolated from fraction 4b after gel filtration on Sephadex LH-20 (dichloromethane/methanol 1:1) and a final purification step using an RP-18 solid-phase extraction cartridge with a mixture of acetonitrile/water (3:7) as solvent.

The structures of gypenoside XVII and ginsenoside F_2 (**Figure 1**) were elucidated by comparison of ^{1}H NMR and MS spectra with literature values (*5*, *6*) and were confirmed by interpretation of complete ^{13}C NMR and 2D datasets (HSQC, HMBC, and COSY90). The ^{13}C NMR data, which has not been published so far, is listed in **Table 1**.

Table 1. ^{13}C NMR Data of Gypenoside XVII and Ginsenoside F_2

aglycone			sugar moieties		
	gypenoside XVII	ginsenoside F_2		gypenoside XVII	ginsenoside F_2
			3-Glc		
C-1	39.6	39.6	C-1′	107.4	107.4
C-2	27.0	27.0	C-2′	76.2	76.2
C-3	89.2	89.2	C-3′	79.7	79.7
C-4	40.1	40.1	C-4′	72.3	72.3
C-5	56.8	56.8	C-5′	78.8	78.8
C-6	18.8	18.8	C-6′	63.5	63.5
C-7	35.5	35.5			
C-8	40.4	40.5	20-Glc		
C-9	50.6	50.6			
C-10	37.3	37.4	C-1″	98.5	98.7
C-11	31.2	31.3	C-2″	75.2	75.5
C-12	70.5	70.5	C-3″	80.2	79.2
C-13	49.9	49.9	C-4″	72.0	72.1
C-14	51.8	51.8	C-5″	77.5	78.7
C-15	31.1	31.2	C-6″	70.6	63.3
C-16	27.1	27.2	C-1‴	105.8	
C-17	52.0	52.0	C-2‴	75.7	
C-18	16.4	16.4	C-3‴	79.2	
C-19	16.7	16.7	C-4‴	72.1	
C-20	83.8	83.7	C-5‴	78.8	
C-21	22.8	22.8	C-6‴	63.2	
C-22	36.6	36.6			
C-23	23.6	23.6			
C-24	126.4	126.4			
C-25	131.4	131.3			
C-26	26.2	26.1			
C-27	18.1	18.1			
C-28	28.5	28.5			
C-29	17.2	17.2			
C-30	17.8	17.8			

RESULTS AND DISCUSSION

Several methods for the analysis of *Panax* spp., especially *P. ginseng*, have been published to date. Most of them use a gradient with acetonitrile and water, which gives an acceptable separation of the major saponins. To obtain a shorter run time, we used a modified version of the HPLC method published by Li et al. (*7*).

The method validation, which proved accuracy, repeatability, intermediate precision, and linearity, showed that this method is suitable for the analysis of the major saponins. Accuracy was determined by addition of 9 mL of standard solution to 1 mL of 65% glycerin extract; each analysis was performed in duplicate. The recovery rate was between 93.9 and 113.9% for all the saponins. Repeatability was evaluated on two samples, each sample injected six times in a row. The relative standard deviation of the results did not exceed 1.58% for any of the compounds. For intermediate precision (two workers, each preparing three samples), the relative standard deviation of the results was below 4.65% for the saponins analyzed. It is crucial, however, to have the specificity checked on a given sample, as the UV spectra of all the saponins are very similar, but the composition of the extract can be rather variable depending on the source of the roots. To verify that there had been no overlapping of peaks in the HPLC chromatogram, an LC-UV/MS analysis was carried out on every lot (**Figure 2**). In general, the main ions observed in the MS spectrum were the sodiated molecular ions $[M + Na]^+$, and to a lesser extent, the $[M + K]^+$ ions. The cleavage reaction in the MS/MS experiment occurs predominantly at the glycosidic linkage at C(20), as described by (*8*).

The amounts of total saponins in the 50% aqueous ethanol extract (61.7 ± 0.1 mg/g dry root) and the ethanol/glycerin/

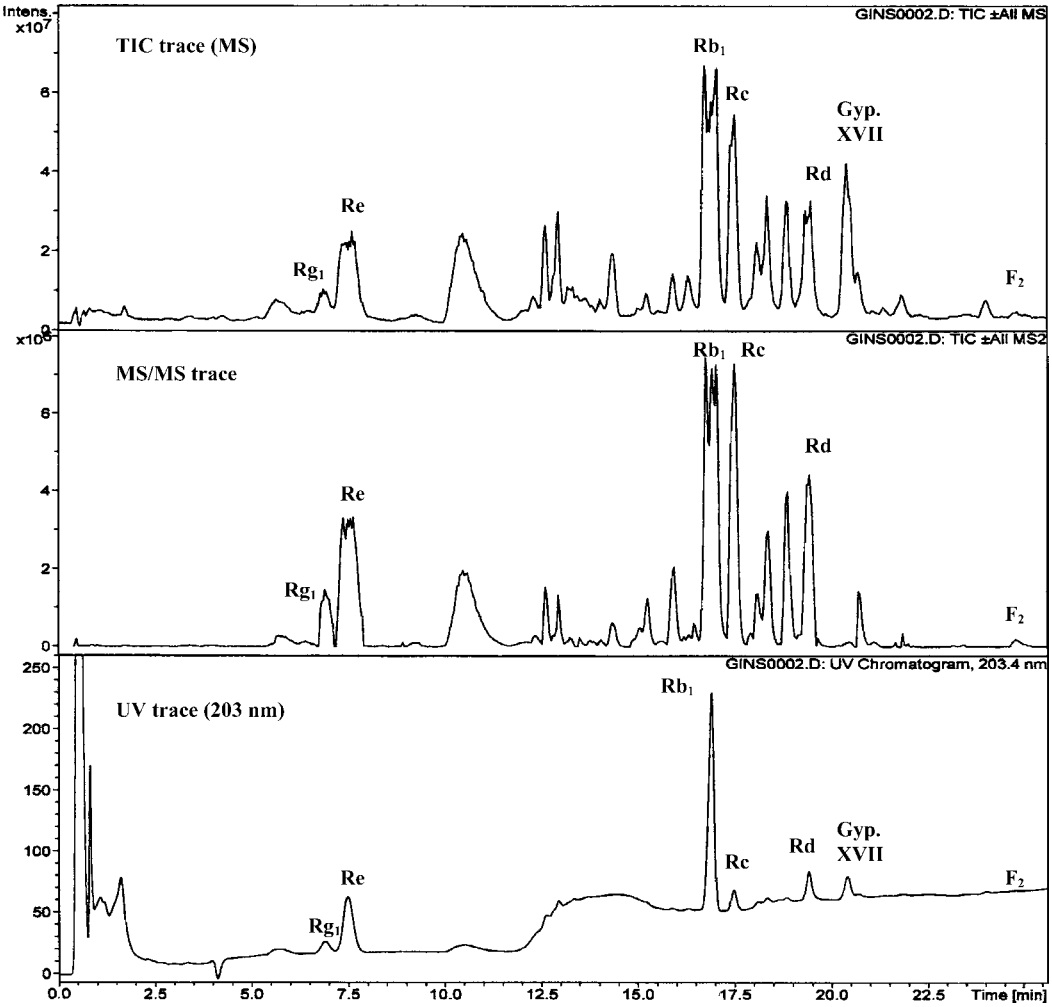

Figure 2. HPLC-UV/MS analysis of a 50% aqueous ethanol extract of *Panax quinquefolius* roots.

Table 2. Amount of Saponins (mg/g Dry Root) Extracted in Various Solvents after 6 Weeks of Maceration

	50% ethanol	ethanol/glycerin/water	65% glycerin
ginsenoside Rg_1	1.59 ± 0.03^a	1.63 ± 0.06^a	1.51 ± 0.03^b
ginsenoside Re	15.9 ± 0.1^a	15.7 ± 0.2^b	14.1 ± 0.0^c
ginsenoside Rb_1	28.7 ± 0.1^a	26.3 ± 0.1^b	15.3 ± 0.1^c
ginsenoside Rc	5.04 ± 0.13^a	5.01 ± 0.01^b	2.46 ± 0.09^c
ginsenoside Rd	8.40 ± 0.13^a	7.48 ± 0.05^b	4.41 ± 0.01^c
gypenoside XVII	1.63 ± 0.05^a	2.54 ± 0.01^b	10.9 ± 0.0^c
ginsenoside F_2	0.49 ± 0.01^a	0.77 ± 0.00^b	2.70 ± 0.01^c
total saponins	61.7 ± 0.1^a	59.4 ± 0.5^b	51.5 ± 0.2^c

[a–c] Values indicate mean ± SD ($n = 3$). Mean values of each row followed by a different superscript letter differ significantly at $P < 0.05$, according to Fisher's PLSD test.

Table 3. Amount of Saponins (mg/g dry root) Extracted in Various Solvents after Storage of Extracts for One Year at 25 °C

	50% ethanol	ethanol/glycerin/water	65% glycerin
ginsenoside Rg_1	1.26 ± 0.10^a	1.37 ± 0.06^b	1.28 ± 0.05^a
ginsenoside Re	12.6 ± 0.1^a	12.7 ± 0.1^a	11.4 ± 0.1^b
ginsenoside Rb_1	24.7 ± 0.4^a	22.7 ± 0.3^b	13.6 ± 0.2^c
ginsenoside Rc	4.56 ± 0.11^a	4.57 ± 0.05^a	2.40 ± 0.05^b
ginsenoside Rd	7.61 ± 0.14^a	6.94 ± 0.09^b	4.15 ± 0.02^c
gypenoside XVII	1.44 ± 0.03^a	2.26 ± 0.06^b	9.64 ± 0.09^c
ginsenoside F_2	0.44 ± 0.03^a	0.76 ± 0.01^b	2.44 ± 0.03^c
total saponins	52.6 ± 0.8^a	51.2 ± 0.5^b	44.9 ± 0.5^c

[a–c] Values indicate mean ± SD ($n = 6$). Mean values of each row followed by a different superscript letter differ significantly at $P < 0.05$, according to Fisher's PLSD test.

water extract (59.4 ± 0.5 mg/g dry root) were similar, but saponins were significantly lower in the 65% aqueous glycerin extract (51.5 ± 0.2 mg/g dry root). Interestingly, the amount of individual saponins varied depending on the solvent (**Table 2**). Ginsenosides Rb_1 and Rd were best extracted in 50% aqueous ethanol, while the highest levels of gypenoside XVII and ginsenoside F_2 were found in the glycerin extract. The ethanol/glycerin/water extract contained amounts of saponins similar to the 50% aqueous ethanol extract with the exceptions of ginsenosides Rb_1, Rd, and gypenoside XVII. The amount of total saponins decreased over the period of one year in all three extracts, with a loss in the range of 13−15% (**Table 3**).

The fact that the amounts of gypenoside XVII and ginsenoside F_2 were high while the amounts of ginsenosides Rb_1, Rc, and Rd were low in the glycerin extract compared to the ethanol-containing products suggested that a possible cleavage of the terminal sugars in ginsenoside Rb_1, and to a lesser extent in Rc and Rd, might occur. This could be due to enzymatic activity. It has been reported that plant enzymes can be active even after harvesting and drying of the plant material (*9*). Two enzymes that can catalyze a cleavage of terminal sugars have been isolated recently (*10, 11*) from roots of Asian ginseng (*P. ginseng* C. A. Meyer). A hydrolytic cleavage, which has been described by Karikura et al. (*12*), seems unlikely. The hydrolysis

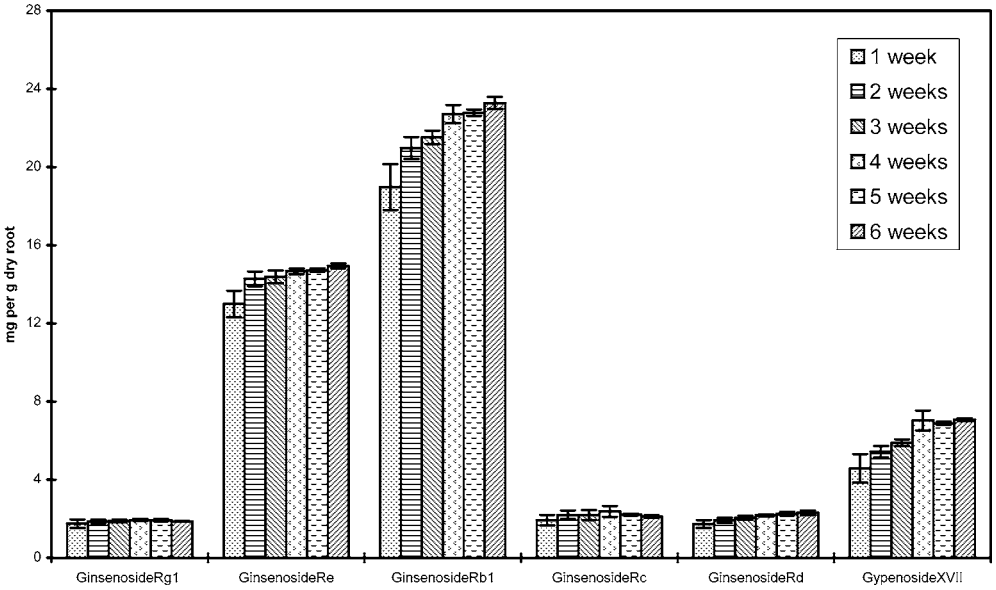

Figure 3. Changes in the amounts of saponins in a 65% aqueous glycerin extract of *Panax quinquefolius* roots over a maceration period of 6 weeks.

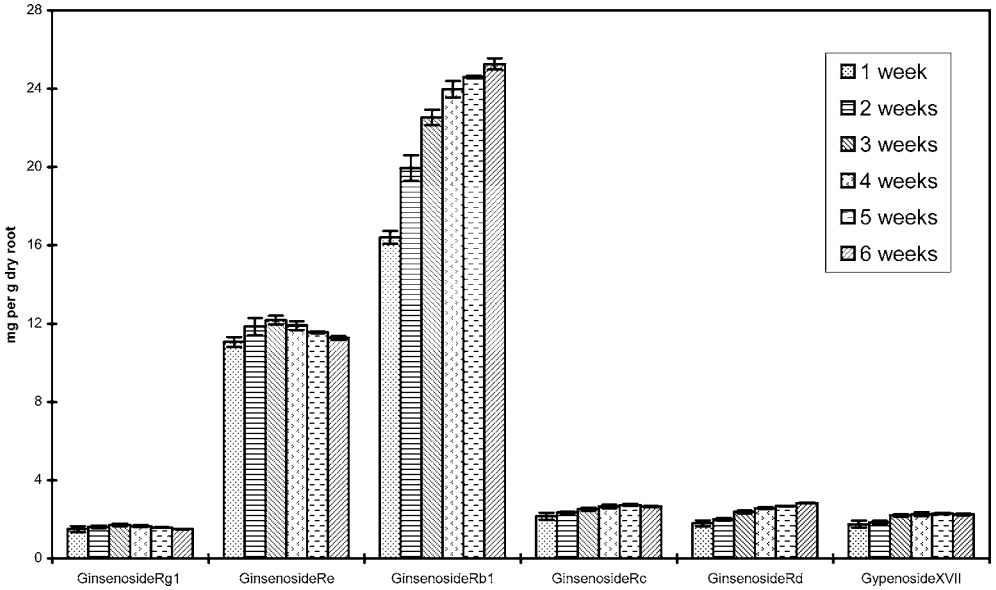

Figure 4. Changes in the amounts of saponins in an extract of *Panax quinquefolius* roots over a maceration period of 6 weeks using 65% aqueous glycerin containing 100 mM $CuSO_4$ as solvent.

of ginsenoside Rb_1 with 0.1 N HCl favors cleavage between the C(20)−OH and the sugar moieties, leading to ginsenoside 20(*S*)-Rg_3. Furthermore, the evaluation of the extracts after 1 year of storage at 25 °C shows that there is only a slight decrease in ginsenoside Rb_1 and no increase in gypenoside XVII after the end of the maceration period (**Table 3**).

To determine if degradation was occurring during the maceration process, American ginseng roots were macerated with 65% glycerin extract or with 65% glycerin containing 100 mM copper sulfate. It has been shown that enzymes from Asian ginseng, which hydrolyze ginsenoside Rc or ginsenoside Rg_3, were effectively inhibitied by Cu^{2+} at levels of 50 and 100 mM (*10*, *11*). The saponin content of each macerate was measured by HPLC on a weekly basis for 6 weeks (**Figures 3** and **4**). The results for the glycerin macerate indicated an increase in the amounts of ginsenoside Rb_1 and gypenoside XVII by 22.7 and 54.5%, respectively, over the whole 6 weeks. The addition of $CuSO_4$ to the extraction solvent gave a larger increase of ginsenoside Rb_1 (54.2%), while the amount of gypenoside XVII increased only by 28.9%. These data provide evidence of an enzymatic cleavage of ginsenoside Rb_1 in the glycerin extract. This means that the large amount of gypenoside XVII is at least in part due to a hydrolysis of ginsenoside Rb_1 and that the enzyme responsible for the degradation may be membrane bound, as degradation stops as soon as the solid root parts are filtered off. Similar enzymatic reactions may take place with ginsenosides Rc and Rd as well, as the increase in levels of these two compounds over the 6 weeks maceration time was much lower in the 65% aqueous glycerin macerate than in the sample extracted with 65% aqueous glycerin containing $CuSO_4$.

Despite the fact that the ethanol extracts gave the highest yields of triterpene saponins, the study shows that the ethanol/glycerin/water mixture or the 65% aqueous glycerin extracts contain high amounts of the saponins as well. It remains to be clarified if the differences in the amount of individual saponins lead to an important change in the efficacy, especially as the ginsenosides have demonstrated a distinct bioactivity depending on the sugar moieties attached. Eventually, the presence of

gypenoside XVII and ginsenoside F_2 may lead to an increased bioavailability of an extract, as it has been shown in rats that the major metabolites of ginsenosides Rb1 and Rd are formed via gypenoside XVII and/or ginsenoside F_2 (*12*).

ACKNOWLEDGMENT

We are thankful to the Biochemistry NMR facility at Oregon State University for the NMR spectrometer time and to Elizabeth R. Dumas for her help with the manuscript.

LITERATURE CITED

(1) Yuan, C. S.; Attele, A. S.; Wu, J. A.; Liu, D. Modulation of American ginseng on brainstem GABAergic effects in rats. *J. Ethnopharmacol.* **1998**, *62*, 215−222.

(2) Li, Z.; Guo, Y. Y.; Wu, C. F.; Li, X.; Wang, J. H. Protective effects of pseudoginsenoside-F11 on scopolamine-induced memory impairment in mice and rats. *J. Pharm. Pharmacol.* **1999**, *51*, 435−440.

(3) Vuksan, V.; Stavro, M. P.; Sievenpiper, J. L.; Koo, V. Y.; Wong, E.; Beljan-Zdravkovic, U.; Francis, T.; Jenkins, A. L.; Leiter, L. A.; Josse, R. G.; Xu, Z. American ginseng improves glycemia in individuals with normal glucose tolerance: Effect of dose and time escalation. *J. Am. Coll. Nutr*. **2000**, *19*, 738−744.

(4) Assinewe, V. A.; Arnason, J. T.; Aubry, A.; Mullin, J.; Lemaire, I. Extractable polysaccharides of *Panax quinquefolius* L. (North American ginseng) root stimulate TNF-alpha production by alveolar macrophages. *Phytomedicine* **2002**, *9*, 398−404.

(5) Yahara, S.; Tanaka, O.; Komori, T. Saponins of the leaves of *Panax ginseng* C. A. Meyer. *Chem. Pharm. Bull.* **1976**, *24*, 2204−2208.

(6) Takemoto, T.; Arihara, S.; Nakajima, T.; Okuhira, M. Studies on the constituents of *Gymnostema pentaphyllum* Makino. II. Structures of gypenosides XV−XXI. *Yakugaku Zasshi* **1983**, *103*, 1015−1023.

(7) Li, W.; Fitzloff, J. F. A validated method for quantitative determination of saponins in notoginseng (*Panax notoginseng*) using high-performance liquid chromatography with evaporative light-scattering detection. *J. Pharm. Pharmacol.* **2001**, *53*, 1637−1643.

(8) Ackloo, S. Z.; Smith, R. W.; Terlouw, J. K.; McCarry, B. E. Characterization of ginseng saponins using electrospray mass spectrometry and collision-induced dissociation experiments of metal-attachment ions. *Analyst* **2000**, *125*, 591−597.

(9) Nüsslein, B.; Kurzmann, M.; Bauer, R.; Kreis, W. Enzymatic degradation of cichoric acid in *Echinacea purpurea* preparations. *J. Nat. Prod*, **2000**, *63*, 1615−1618.

(10) Zhang, C. Z.; Yu, H. S.; Bao, Y. M.; An, L. J.; Jin, F. X. Purification and characterization of ginsenoside-α-arabinofuranase hydrolyzing ginsenoside Rc into Rd from the fresh root of *Panax ginseng*. *Proc. Biochem.* **2002**, *37*, 793−798.

(11) Zhang, C. Z.; Yu, H. S.; Bao, Y. M.; An, L. J.; Jin, F. X. Purification and characterization of ginsenoside-*β*-glucosidase from ginseng. *Chem. Pharm. Bull.* **2001**, *49*, 795−798.

(12) Karikura, M.; Miyase, T.; Tanizawa, H.; Taniyama, T.; Takino, Y. Studies on absorption, distribution, excretion, and metabolism of ginseng saponins. VII. Comparison of the decomposition modes of ginsenoside Rb1 and − Rb2 in the digestive tract of rats. *Chem. Pharm. Bull.* **1991**, *39*, 2357−2361.

Received for review November 7, 2003. Revised manuscript received January 13, 2004. Accepted January 14, 2004. This work has been financially supported by the National Institute of Health (grant GM63554).

JF0307503