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Generation of ginsenosides Rg3 and Rh2 from North American ginseng

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Abstract

Rg3 and Rh2 ginsenosides are primarily found in Korean red ginseng root (*Panax ginseng* C.A. Meyer) and valued for their bioactive properties. We quantified both Rh2 and Rg3 ginseng leaf and Rg3 from root extracts derived from North American ginseng (*Panax quinquefolius*). Quantification was obtained by application of HPLC with ion fragments detected using ESI-MS. Ginseng leaf contained 11.3±0.5 mg/g Rh2 and 7.5±0.9 mg/g Rg3 in concentrated extracts compared to 10.6±0.4 mg/g Rg3 in ginseng root. No detectable Rh2 was found in root extracts by HPLC, although it was detectable by ESI-MS analysis. Ginsenosides Rg3 and Rh2 were detected following hot water reflux extraction, but not from tissues extracted with 80% aqueous ethanol at room temperature. Therefore ginsenosides Rg3 and Rh2 are not naturally present in North American ginseng, but are products of a thermal process. Using ESI-MS analysis, it was found that formation of Rg3 and Rh2, among other compounds, were a function of heating time and were breakdown products of the more abundant ginsenosides Rb1 and Rc. Our findings that heat processed North American ginseng leaf is an excellent source of Rh2 ginsenoside is an important discovery considering that ginseng leaf material is obtainable throughout the entire plant cycle for recovery of valuable ginsenosides for pharmaceutical use.

Keywords: *Panax quinquefolius*; Araliaceae; North American ginseng; ginseng leaf; ginseng root; ginsenosides; Rh2; Rg3; LC/MS

1. Introduction

Ginseng root is a valuable agricultural commodity grown for use in many traditional medicinal therapies. More contemporary usages of ginseng, especially in North America, have included formulations prepared for herbal supplements or in functional foods; certain ginsenosides may also have pharmaceutical potential for drug development. Herbal product usages are based on specific health expectations that focus on immunological, anti-cancer, metabolic, neurological benefits (Kitts and Popovich, 2003) and anti-oxidant properties (Hu and Kitts, 2001). The primary active ingredients are a group of 31 triterpene saponins, also referred to as ginsenosides, and specific ginsenosides such as Rh2 (**1**) and Rg3 (**2**) have been proposed as chemotherapeutic agents. Two main sources of ginseng root are typically used, Asian ginseng (*Panax ginseng*, C.A. Meyer) and North American ginseng (*Panax quinquefolius*). Much less is known about North American ginseng leaf components as compared to the root tissue. Annual recovery of ginseng leaf could be a feasible alternative source of ginsenosides compared to the long growth cycle required for harvesting the roots for nutraceutical products. Starratt et al. (2001) reported that the leaves are a source of ginsenosides but these have not been capitalized or exploited at present. Processing conditions have been reported to effect the composition of ginsenosides. For example, Korean red ginseng is the same variety as Asian ginseng but subjected to steam preservation after harvesting (Kim et al., 2000).

Ginsenoside Rh2 (**1**) is currently sought after for its bioactive properties; Rh2 (**1**) can reduce the proliferation of a variety of cultured cancer cells and can influence apoptosis (Popovich and Kitts, 2002; Fei et al., 2002; Park et al., 1997; Nakata et al., 1998). Rg3 (**2**) has also been shown to possess anti-tumor properties and

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have an effect on drug resistant cultured cancer cells (Kim et al., 2003; Keum et al., 2003).

The purpose of this study was to test the hypothesis that North American ginseng (*Panax quinquefolius*) leaf and root are potentially a source of rare ginsenosides Rh2 (**1**) and Rg3 (**2**) following a thermal process required to specifically generate these products.

2. Results and discussion

We have shown for the first time that using a simple hot water extraction of North American ginseng leaf is a valuable source of rare ginsenosides. Table 1 lists the ginsenoside fingerprint information identified by HPLC. Root samples extracted by hot water reflux contained greater amounts of ginsenosides Rg1, Re, Rb1, Rc and Rd compared to leaf samples. Ginsenoside Rg3 (**2**) was detected in both leaf and root. However, ginsenoside Rh2 (**1**), an otherwise rare ginsenoside was detected only in the leaf and not in the root, by HPLC, when employing hot water reflux extraction and preparation process. The main source of both ginsenosides Rg3 (**2**) and Rh2 (**1**) have been reported to be exclusively Korean red ginseng root (Shin et al., 2000; Park et al., 2002; Kim et al., 2000). Preparation of Korean red ginseng relies on steam processing to ensure preservation which may alter the ginsenoside composition. Steaming ginseng has been shown to produce ginsenosides that are not present in raw ginseng (Kim et al., 2000). We compared the effect of heat produced from a hot water reflux extraction procedure, to a room temperature extraction using 80% aqueous ethanol to determine if Rg3 (**2**) and Rh2 (**1**) were indeed formed as a result of the addition of thermal energy. Table 2 shows the major ion fragments detected by ESI-MS analysis of the water reflux extraction of ginseng root and leaf and the 80% aqueous ethanol extraction at room temperature. Ginseng root and leaf hot water reflux extract were found to have 23 and 19 different ion fragments, respectively, whereas, the 80% aqueous ethanol extract contained 14 fragments. Furthermore, the 80% aqueous ethanol extract contained a malonyl ginsenoside that was not present in the hot water reflux extract. Malonyl ginsenosides are heat-labile and readily demalonylate (Yamaguchi et al., 1988). Ion fragments of ginsenosides Rg3 (**2**) and Rh2 (**1**), among others, were not recovered in the 80% aqueous ethanol extraction, but were found following the hot water extraction procedure. HPLC analysis of the leaf extracts (Table 1) revealed the presence of both Rg3 (**2**) (7.5 ± 0.9 mg/g) and Rh2 (**1**) (11.3 ± 0.5 mg/g) in the hot water reflux extract, but not in the 80% aqueous ethanol extraction. Conformational isomers of ginsenosides Rg3 (**2**) and Rh2 (**1**) [20(*S*) and 20(*R*)] were detected by comparing retention order and ion fragments produced during ESI-MS analysis. Isomers of Rg3 (**2**), Rg2 (**1**) and Rh1 ginsenosides have been also reported in 'Shenmai' derived from red ginseng and ophiopogon (Haijiang et al., 2003; Bae et al., 2002).

We provide evidence that Rg3 (**2**) and Rh2 (**1**) are formed during thermal processing of North American ginseng, and that these breakdown products are not naturally present in leaf or root materials. The second set of experiments were designed to identify the heating times required to form Rh2 (**1**) after hot water reflux and to identify the compounds that contributed to the formation of the rare ginsenoside Rh2 (**1**). Table 3 shows the ESI-MS analysis of the hot water reflux of ginseng leaf that was sampled between 10 and 60 min intervals. Fragments corresponding to malonyl ginsenosides disappeared after 30 min of heating. After 10 min of water reflux, ginsenosides Rg3 (**2**) and Rh2 (**1**) were not detected, however, both Rg3 (**2**) and Rh2 (**1**) began to appear after 20 min. The compounds needed for Rg3 (**2**) and Rh2 (**1**) formation were studied and Table 4 shows the ion fragments of protopanaxadiol ginsenosides Rc and Rb1 produced after hot-water reflux. Both ginsenosides Rg3 (**2**) and Rh2 (**1**) were breakdown components from both ginsenosides Rc and Rb1 after a hot water reflux. Breakdown products or artifacts generated from heat processing of saponins from *Medicago sativa* L have recently been reported by Tava et al. (2003). They showed that after hot water reflux of plant material, artifacts were produced which contained decreased polarity or higher retention time compared to original compounds when measured by TLC and HPLC. Furthermore, ginsenosides Rb1 and Rc were classified in the same category as Rg3 (**2**) and Rh2 (**1**), the 20(S)-protopanaxadiol group of ginsenosides.

Table 1
Ginsenoside profile of North American ginseng root and leaf quantified by HPLC[a]

Fraction	Rg1	Re	Rb1	Rc	Rb2	Rd	Rg3 (**2**)	Rh2 (**1**)
Root (hot water)[b]	47.0 (0.6)	151.1 (2)	157.7 (2)	27.3 (5)	7.8 (0.3)	21.8 (0.5)	10.6 (0.4)	ND
Leaf (hot water)[b]	8.0 (0.3)	50.3 (4)	12.0 (1)	6.5 (4)	10.8 (3)	6.4 (0.9)	7.5 (0.9)	11.3 (0.5)
Leaf (aq. 80% ETOH)[c]	5.1 (0.4)	30.8 (0.1)	36.0 (0.4)	1.9 (0.03)	26.7 (3)	10.2 (0.03)	ND	ND

[a] Data is expressed as mean ±(S.D.) measured in triplicate (mg/g) dry weight).
[b] 90 min hot water reflux extraction.
[c] Room temperature aq. ethanol (80%) extraction. ND: not detected.

Table 2
ESI-MS ion fragments of North American ginseng root and leaf extraction

	Main fragmentation ions, *m/z*			Others			
	[M−H]−	[M + Cl]−	[M + Aco]−				
Root (hot water) retention order							
Rg1	799.4		859.5	1200			
Re	945.5		1005.5		1036.5	1159.4	
Rb1	1107.5				1121.3	1321.4	
Rc	1077.5			1140.6	1199.5	1291.5	
Rb2	1077.5			1080.1	1134.4	1191.4	
Rg2*	783.4		843.4	897.4	1149.4		
Rg2 iso[a]	783.4		843.4	881.3	897.4	1149.4	
Rd	945.5			1008.4	1159.5		
Rd iso	945.5			1005.5	1067.4	1159.3	
Rd2[b]	915.5		975.4	1037.5	1129.4	1371.2	
Rd2iso[b]	915.5			987.4	1031.4	1120.3	
Rg6[a]	765.4			843.4	883.4		
Rg6 iso[a]	765.4		825.5	887.4			
Not reported	712.4						
Rg3 (**2**)	783.4						
Rg3 iso[b]	783.4						
Not reported	793.3						
Rs3[a]	825.4			853.3	1007.3		
Rs3 iso[a]	825.4			853.3	1107.3		
Rg5[a]	764.4			828.3	979.3		
Rg5 iso	764.4			979.3			
Rh2 (**1**)	621.4		681.4	1147.4	1191.5	1243.8	
Rh2 iso[b]	621.4		681.4	1147.4	1191.5		
Leaf (hot water)							
Rg1	799.5		895.5	1137.5			
Re	945.5	981.4		1035.4			
Rb1	1107.5			1165.8	1121.5	1259.1	1322.6
Rc	1077.5						
Rb2	1077.5			1140.4	1291.4		
Rg2 iso[a]	783.4		843.4	905.4	997.3		
Rd	945.5		1005.5	1159.4			
Rs1/Rs2[b]	1119.5			1124.3	1140.2	1217.2	
Rd2	915.4	975.4	978.5	1029.5	1129.7	1374.2	1374.2
Rg6[a]	765.4	825.4					
Rk3/Rh4[a]	619.5	656.2	680.5	1241			
Rg3 (**2**)	783.4	819.4		905.4	997.5		
Rg3 iso[b]	783.4	819.3		905.4	997.5		
Not reported	675.3	711.3		773.2	885.5		
Not reported	793.3			825.4	956.4	1108.3	1136.8
Rg5[a]	765.4			838.4			
Rg5 iso[a]	765.4	801.2					
Rh2 (1)	621.4		681.4	743.4	1243.8	1245.7	
Rh2 iso[b]	621.4		681.4	743.4	1243.8	1245.7	
Leaf (80% EtOH)							
Rg1	799.4		859.4				
Re	954.4	981.4		1035.4	1067.5		
Not reported	977.5	1013.3					
Not reported	959.5			1091.5	1151.6	1195.4	1271.8
Rb1	1107.6			1110.5	1173.5	1125.4	1272.7
Rc	1077.5						
Rb2	1077.5			1140.6	1291.4		
Rs1/Rs2[b]	1119.5			1233.5	1242.6	1333.5	
Rd	945.5			1059.4	1159.4		
Malonyl-Rb2/Rc[a]	1163.4			1261.6			
Not reported	955.4						
Rd2[b]	915.5	975.4		1037.4	1374.3		
Not reported	843.6						
Not reported	793.4			875.2			

[a] Identified from Haijiang et al. (2003).
[b] Modecular weight match, iso-isomer

Table 3
ESI-MS ion fragments of North American ginseng leaf sampled during hot water reflux extraction

Heating time	Identification retention order	Main fragmentation ions, *m/z*			Others			
		[M−H]−	[M + Cl]−	[M + Aco]				
10 min								
	Rg1	799.4			984.5	1071		
	Re	945.5	981.4		1130.6	1213.1	1263.6	
	Not reported	1053.4			1069.4	1123.4	1197.4	1289.6
	Not reported	1053.4			921.5	977.5		
	Rg1 iso[b]	799.4		859.4	1067.5	1160.3		
	Rd	945.4	981.4		1234.5			
	Not reported	1063.5			1037.5	1129.4		
	Rd2[b]	915.5		975.4	905.4	997.4	1374	
	F2[b]	783.5		843.5	797.1			
	Not reported	675.2	711.7					
20 min								
	Rg1	799.4		859.5	860.1			
	Re	945.5	981.4		1006.4	1067.5		
	Not reported	651.1			799.4			
	Not reported	1053.4			1123.5	1199.6	1275.2	
	Rb2[a]	1077.5			1199.5	1292.4	1140.3	
	Not reported	703.5			905.5			
	Rd	945.4			1108.4	1063.5	1067.5	
	Rs1/Rs2[b]	1119			1163.4			
	Malonyl-Rb2/Rb3/Rc	1063.5						
	Rd2[b]	915.5		975.4	1037.5	1129.5	1374.3	
	Not reported	957.5		1017.4	979.5	1079.5	1080.5	1121.6
	Not reported	905.4			933.5	1175.5		
	Rg3 (**2**)	783.5		843.5	905.4	997.4		
	Rg3 iso[b]	783.4						
	Not reported	957.4			1001.5			
	Rh2 (**1**)	621.4		681.3	682.3			
	Rh2 iso[b]	621.4		681.3	682.3			
30 min								
	Rg1	799.4						
	Re	945.5	981.4		1067.4			
	Not reported	651.1			799.4			
	Not reported	1053.4						
	Not reported	1053.4						
	Rg1 iso[b]	799.4			862.3	921.3	991.5	
	Rc/Rb2	1077.5	1113.6		1199.6	1291.3	1350.4	
	Rg2[a]	783.4			906.5			
	Rd	945.4	981.3		1017	1067.4		
	Malonyl-Rb2/Rb3/Rc	1063.5			1158.6			
	Rd2[b]	915.4	951.5	975.4	1037.5	1129	1374.7	
	Not reported	957.3		1017.3	1079.5			
	Rg3 (**2**)	783.4		843.5	905.4	997.4	1176.7	
	Rg3 iso[b]	783.4						
	Not reported	675.3			1304.2			
	Rh2 (**1**)	621.2		681.4	682.4			
60 min								
	Rg1	799.4			862.4			
	Re	945.5	981.4					
	Not reported	931.4		991.4	1053.4			
	Not reported	931.4		991.4	1053.4	1056.4	1137.2	1214.2
	Rg1 iso[b]	799.4			921.4	991.4		
	Rc/Rb2[a]	1077.4			1199.5	1249	1291.3	
	Rg2[a]	783.4			906.5			
	Rd	945.4	981.3		1067.4			
	Rs1/Rs2[b]	1119.4			1224			

(continued on next page)

Table 3 (*continued*)

Heating time	Identification retention order	Main fragmentation ions, *m/z*			Others		
		[M−H]−	[M + Cl]−	[M + Aco]			
	Not reported	1035.4					
	Rd2[b]	915.4		975.4	1063.5	1129.4	1379.4
	Not reported	1107.5			1110.4		
	Not reported	783.5		843.5	1176.2		
	Rg3 (**2**)	783.4					
	Rg3 iso[b]	783.4			849.5	775.5	
	Not reported	675.2	711.3	681.4	797.3		
	Not reported	653.3	689.3	681.4	751.2		
	Rh2 (**1**)	621.3			682.2		
	Rh2 iso[b]	621.3			682.6		

[a] Identified from Haijiang et al. (2003).
[b] Molecular weight match, iso-isomer.

Table 4
ESI-MS ion fragments of breakdown products of ginsenosides Rc and Rb1 after hot water reflux extraction

Identification retention order	Main fragmentation ions, *m/z*			Others			
	[M−H]−	[M + Cl]−	[M + Aco]				
Rc + heat							
Rc	1077.5			1140.4	1291.4		
Rd	945.4			983.4	1043.3	1077.4	1159.3
Rd2[b]	915.4		975.4	976.6	1037.5	1130.3	1373.9
Not reported	843.4			905.4	928.4	979.4	
Rg3 (**2**)	783.4	819.4		881.3	997.3		
Rg3 iso[b]	783.4	819.4		881.3	1001.4		
Not reported	793.4						
Rg5/Rk1[a]	765.4	802.3		887.4	979.2	1087.8	
Rg5/Rk1 iso[a]	765.4	801.3		855.3	887.4	979.2	1043.5
Rh2 (**1**)	621.5		681.3	682.4	743.6		
Rh2 iso[b]	621.4		681.4	682.4	743.6		
Rb1 + heat							
Rb1	1107.5			1129.6	1321.5		
Rd	945.4		1005.4	1043.5	1131.4		
Not reported	671.3						
Rg3 (**2**)	783.4		843.3				
Rg3 iso[b]	783.4	819.2		901.5	997.4		
Not reported	793.3						
Not reported	793.3						
Rh2 (**1**)	622.3		681.4	682.3	724.4		

[a] Identified from Haijiang et al. (2003).
[b] Molecular weight match, iso-isomer.

These compounds are organized according to the attachment of sugar moieties to position c-3 of the triterpene ring structure (Fig. 1). Conversions of ginsenosides Rb1, Rc and Rd have been reported and treatment with a specific β-glucosidase, resulted in the production of Rh2 (**1**) (Bae et al., 2002; Zhang et al., 2001). Future studies are needed to generate quantitative data on the conversion of Rh2 (**1**) and Rg3 (**2**) from Rb1 and Rc precursors. A comprehensive time course analysis of Rh2 (**1**) and Rg3 (**2**) generation from Rb1 and Rc ginsenosides during optimized thermal process for conversion is required.

We have identified an additional source of the rare ginsenosides Rh2 (**1**) and Rg3 (**2**), which are valued for potential anti-cancer properties. In cultured cancer cell experiments, Rh2 (**1**) has been shown to reduce proliferation. We previously found that the LC_{50} for Rh2 (**1**) standard was 15 μg/ml in THP-1 human leukemia cells (Popovich and Kitts, 2002) and 53–55 μg/ml in intestinal cell lines (Popovich and Kitts, 2003). Furthermore, Rh2 (**1**) induced apoptosis in a variety of cell lines (Popovich and Kitts, 2002; Fei et al., 2002; Park et al., 1997; Nakata et al., 1998). Rg3 (**2**) was effective in reducing proliferation of prostate cancer

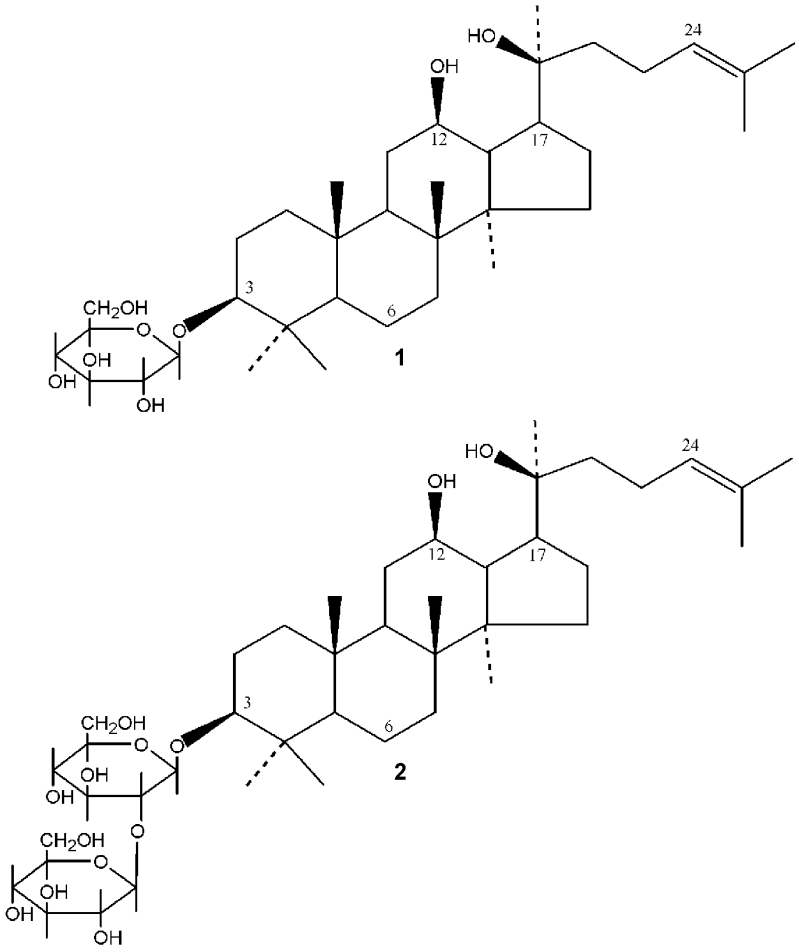

Fig. 1. The structures of 20(*S*)-ginsenoside Rh2 and Rg3.

cells (Liu et al., 2000) and cisplatin resistance cells (Keum et al., 2003).

With the addition of heat in the extraction process for recovery of ginsenosides from ginseng leaf, we have shown that bioactive potentials of subsequent extracts are enhanced by generating Rh2 (**1**) and Rg3 (**2**). Further studies are required to quantitative the conversion of Rg3 (**2**) and Rh2 (**1**) from Rc and Rb1 during thermal processing of North American ginseng leaf materials and to assess the leaf extract ability to induce biological responses in human clinical studies which are critical for the development and use this novel source of ginsenosides for potential pharmaceutical usage.

3. Experimental

3.1. Experiment 1. The effect of heat on ginseng extract preparation

Two extraction techniques were used to determine the effect of heat on the ginsenoside leaf composition. The first technique consisted of a hot water extraction of ginseng under reflux and the second technique consisted of room temperature extraction using 80% aqueous ethanol and constant stirring. These techniques will be referred herein as hot water reflux and 80% aqueous ethanol extraction respectively.

North American ginseng root and plant components were collected from two-year old ginseng (Panax Q farms, Vernon BC). Ginseng root and leaf were separated, washed, carefully dried and lyophilized 48 h after harvesting. Samples were subsequently vacuum packaged and stored at −18 °C until analysis. The hot water reflux extraction procedure consisted of freeze-dried leaf tissue (2 g) that were powdered and blended separately in of distilled water for 5 min (500 ml). Samples were refluxed for 1.5 h at 100 °C. The extracts were vacuum filtered through Watman no. 4 filter paper. The hot water reflux extraction was applied to a polymeric absorbent Amberlite XAD-4 (Sigma St. Louis, MO) column (with an average pore diameter of 40 Å, bed volume of 60 cm^3 and flow rate of 8 ml/min) and washed with distilled water (1 L). The extract was eluted from the column using absolute ethanol (500 ml), which was subsequently reduced under heat and a stream of nitrogen. The 80% aqueous ethanol extract preparation differed from the hot water reflux extraction by the

absence of heat. Freeze-dried leaves (2g) were powdered and blended separately in 80% aqueous ethanol (500 ml) for 5 min and extracted using a magnetic stirrer for 24 h at room temperature. The extract was vacuum filtered through Watman no. 4 filter paper, concentrated under a stream of nitrogen to a volume of 100 ml and combined with distilled water (400 ml) and applied to an Amberlite XAD-4 column as described above. The ethanol was evaporated under a stream of nitrogen (12 h). The extract was lyophilized and stored at −18 °C until analyzed.

3.2. HPLC-ESI-MS

A Hewlett-Packard (HP) series 1100 high-performance liquid chromatograph was used with a diode array detector coupled to a HP Vectra computer running Chem Station for LC 3D (Agilent Technologies). The column used was an HP Zorbax C18 (4.0×150 mm, 3.5 μm). An Agilent 1100 MSD mass spectrometer with electrospray ionization was used for ESI-MS analysis. The MS conditions used for analysis were negative ion mode, drying gas 10.0 l/min, temperature 300 °C, nebulizer pressure 60 psig, and a scan range of 400–1400 μ. Solvent systems consisted of acetonitrile (HPLC grade) and nano-pure water (Barnstead Dubuque, IA) with a flow rate of 1 ml/min. The solvent gradient program used for HPLC quantification was H_2O (A) and acetonitrile (B) (91:9) at time 0 changing to A:B (88:12) 5 min, A:B (75:25) at 15 min, A:B (65:35) at 35 min, A:B (30:70) at 60 min and finally (B) 100% by 70 min. Test samples and ginsenoside reference standards were prepared in HPLC grade methanol and injected in a 10 μl volume monitored at a wavelength of 203 ηm and measured in triplicate. Concentrations of ginsenosides were determined by standard curves prepared by injecting different concentrations of ginsenoside standards (Rg1, Re, Rb1, Rc, Rb2, Rd, Rg3, Rh2).

3.3. Experiment 2. Thermal generation of Rg3 (2) and Rh2 (1) from abundant ginsenosides

Freeze-dried leaf tissues (2 g) were powdered and blended separately in of distilled water (500 ml) for 5 min and refluxed for 60 min at 100 °C. Samples of heated extract were removed at timed intervals of 10, 20, 30 and 60 min and filtered through Watman no. 4 filter paper. The extracts were applied separately to a fresh polymeric absorbent Amberlite XAD-4 (Sigma St. Louis, MO) column (with an average pore diameter of 40 Å, bed volume of 16 cm^3 and flow rate of 8 ml/min) and washed with distilled water (100 ml). The timed extracts were eluted from the column separately using absolute ethanol (100 ml) and subsequently reduced under heat and a stream of nitrogen and analyzed by ESI-MS.

3.4. Ginsenoside breakdown products

Ginsenoside standards Rb1 and Rc were initially dissolved separately in 70% aqueous ethanol (100 μl) and subsequently heated to reflux of nano-pure water (20 ml) (Barnstead Dubuque, IA) for 1.5 h at 100 °C. The samples were concentrated under heat, analyzed by ESI-MS and compared to unheated reference standards.

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