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Comparing extraction methods to recover ginseng saponins from American ginseng (*Panax quinquefolium*), followed by purification using fast centrifugal partition chromatography with HPLC verification

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ABSTRACT

A series of experiments were carried out to compare the extraction of ginseng saponins, ginsenosides, from powdered American ginseng (*Panax quinquefolium*) using pressurized hot water and the more conventional ultrasonic-assisted extraction. Three solvents were tested, water, *n*-butanol-saturated water, and water-saturated *n*-butanol. Each resulting extract was further purified using fast centrifugal partition chromatography (FCPC) in order to better quantify the contents of the crude plant extract. The pressurized hot water system extracted a greater yield of saponins, 11.2 mg/g (extraction at 110 °C and 440 kPa), than the ultrasonic-assisted method, 7.2 mg/g (extraction at variable temperature with no external pressure). The difference in solvent system for either extraction methods was not significant, and the results gave credence for the use of water as the extraction solvent. *n*-Butanol-saturated water yielded the most saponins (10.1 mg/g), while water yielded 9.8 mg/g, and water-saturated *n*-butanol yielded 7.8 mg/g. Since water is an environmentally benign solvent, this result is quite attractive for future work.

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1. Introduction

A necessary first step in the recovery of valuable plant compounds is extraction from the plant matter. Ginsenosides are a group of triterpenoidal saponins and are the biologically active components in the *Panax* species [1–4]. American ginseng (*Panax quinquefolium*) has been used as a traditional remedy in Native American culture and *Panax ginseng* has been used for the past 200 years in Chinese medicine [5,6]. The main ginsenosides found in American ginseng are, Rb1, Re, Rc, Rd, Rg, and Rb2 [1]. Studies have shown that ginsenoside Rb1, which is the most abundant saponin in American ginseng, inhibits collagen-induced arthritis in mice [7]; blocks homocysteine (Hcy) action and, in turn, increases endothelial proliferation [8]; and has shown to have protective effects on human endothelial cells [9]. The second most abundant ginsenoside Re, has been shown to, have an antioxidant effect on heart muscle cells [10]; possess an antihyperglycemic effect on diabetic rats [11]; and reduces insulin resistance [12].

The extraction of ginseng saponins can be carried out in a variety of ways. The selected extraction method should be relatively simple, adaptable to other compounds/plant materials and safe to operate [13]. The conventional extraction of ginsenosides is performed using either Soxhlet or ultrasonic methods [14,15]. Ultrasonic-assisted extraction (UAE) allows the solvent to penetrate the cell walls, and the bubbles produced by acoustic cavitation aid in the disruption of the cell wall which then releases the ginsenosides (or other compounds of interest) [16,17]. Pressurized liquid extraction (PLE) has also been used to extract ginsenosides [6,18] and is attractive because it takes advantage of the ability of solvents (at elevated temperature and pressure) to enhance extraction efficiency [19]. Pressurized hot water extraction (PHWE) utilizes the decrease in the dielectric constant of water at higher temperature, but below the critical point – to decrease the polarity of water and permit organic compounds to be more soluble in water [20,21].

The aim of this work was to explore the ability of pressurized hot water (PHWE) in extracting ginsenosides from powdered ginseng root. As a comparison ultrasonic-assisted extraction, with the selected solvents, was also investigated. Further purification of the extracted compounds using fast centrifugal partition chromatography (FCPC) was explored to determine if single step purification

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was possible and as an aid in the identification and quantification of the ginsenosides in the crude extract.

2. Materials and methods

2.1. Reagents

Analytical grade heptane and *n*-butanol, used for FCPC separation were purchased from Alfa Aesar (Ward Hill, MA). HPLC grade methanol and acetonitrile, from EMD Chemicals (Madison, WI), were used in the HPLC analyses. The individual ginsenosides, Rg1, Re, Rb1, Rc, Rb2 and Rd, were purchased from Indofine Chemical Company (Hillsborough, NJ). Powdered 3-year old American ginseng (*P. quinquefolium*) root was purchased from Rainey Ginseng Farms (Waterford, Ontario, Canada). Water used for all experiments was filtered by a DirectQ^{TM} 5, water filtration unit (Millipore, Billerica, MA).

2.2. Ultrasonic and pressurized hot water extraction

Ultrasonic-assisted extraction (UAE) was performed in an ultrasound-cleaning bath (Model 75T, VWR International, West Chester, PA). In total 5 g of dried, powdered ginseng root and 120 mL of solvent were divided equally between 12 tubes, with each tube containing 0.42 g of ginseng and 10 mL of solvent. The three solvents studied were water, *n*-butanol-saturated water and water-saturated *n*-butanol. The choice of extraction solvents was influenced by the low environmental impact of water as a solvent, and one of the selected solvents was pure water. Water-saturated *n*-butanol was chosen since it was found to be the best extraction solvent for ginsenosides by Wu et al. [16]. The third solvent, *n*-butanol-saturated water, combined the extraction properties of *n*-butanol with water as a benign solvent.

The tubes were distributed in a wire rack and then immersed in an ultrasound-cleaning bath, at a frequency of 40 kHz, for 2 h. The liquid level in the tubes was maintained at the water level in the bath. The protocol followed was similar to that developed by Wu et al. [16], except that continuous shaking during extraction was not possible so the tubes were manually shaken every half-hour during the extraction period. The extraction method used by Wu et al. [16] consisted of adding 200 mg of sample to 15 mL of solvent in a polypropylene centrifuge tube which was then placed in a continuously shaken water bath initially at 25 °C and ending at 38 °C after the 2 h extraction. For this extraction, the temperature in the bath increased from 22 °C to 55 °C during the 2-h extraction period. The differences in the extraction conditions were the lack of continuous shaking, because it was not available, and a greater rise in temperature during the extraction period.

After extraction, the tubes were removed and the liquid portion was filtered using a Büchner funnel and Whatman Number 1 filter paper (Whatman, Florham Park, NJ). The liquid portion, except for the water-saturated *n*-butanol, was partitioned with 50 mL of *n*-butanol and allowed to sit overnight. *n*-Butanol was used for partitioning since it has been shown that ginsenosides partition well into *n*-butanol [22,23]. The *n*-butanol portion was then evaporated from 50 mL down to approximately 6 mL using a Yamato RE 200 vacuum rotary evaporator (Akashi, Japan). The remaining 6 mL was evaporated to dryness, sans heat, under nitrogen.

Pressurized hot water extraction (PHWE) was performed in a Parr reactor (Parr Instrument Company Moline, IL No. 452HC3) with 5 g of the ginseng root powder material and 120 mL of solvent. All three solvents were also studied in the PHWE Parr reactor. The root powder and solvent were added to the reactor, which was then sealed tightly and pressurized with nitrogen. Ginsenoside extraction, for the water and the *n*-butanol-saturated water, occurred at 110 °C ($T_{c,\,water}$ = 373.9 °C), 440 kPa ($P_{c,\,water}$ = 22,060 kPa) and an agitation rate of 150 rpm, modified from a method by Duan et al. [24]. For the water-saturated butanol extraction, the temperature was increased to 120 °C ($T_{c,\,n\text{-butanol}}$ = 289.9 °C, $T_{B,\,n\text{-butanol}}$ = 117.7 °C), but the pressure ($P_{c,\,n\text{-butanol}}$ = 4708 kPa) and the agitation rate remained the same. Timing for the extraction began when the temperature attained 110 °C, and ran for 30 min; the time to reach extraction temperature was approximately 5 min. After extraction, the liquid portion was filtered and handled in the same manner as the ultrasonic extracts.

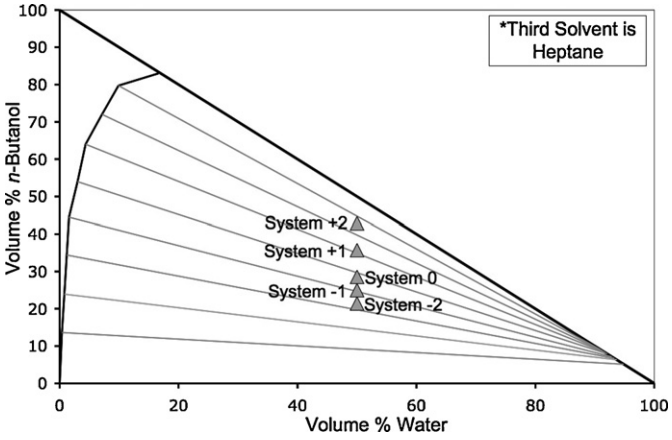

Fig. 1. Ternary phase diagram of the heptane/*n*-butanol/water system displayed on two-axes as molar percentage of *n*-butanol and water only. The solvent systems tested for FCPC separation are shown as triangles on the diagram. The '−2' system is the least polar while the '+2' system is the most polar.

In preparation of the extracts for FCPC separation, the dry extracts were combined with 4 mL of each phase of the solvent system to be used for FCPC separation. FCPC is a technique that employs two immiscible solvents in order to separate the desired compounds; one phase is stationary within the ducts of the instruments' rotor while the other phase flows through its tortuous ducts and channels. Separation of the compounds in the sample occurs when the phases are in contact with one another, which is why the partition coefficient is so important to the solvent system selection for the FCPC separation and is discussed at length in the following section. The sample was then filtered with a Whatman Puradisc 25TF filter (10 μm PFTE membrane, 25 mm diameter, Florham Park, NJ) and placed in a centrifuge at 2500 rpm for 20 min in order to (1) remove any large particles that may otherwise obstruct the flow channels in the FCPC rotor and (2) break up any emulsions formed prior to FCPC injection.

For each extraction method and solvent combination, two replicates were made from the beginning of the extraction to the final quantification of the ginsenosides recovered. The data points presented in the results are the means of the duplicates.

2.3. Solvent system preparation

The FCPC separation was performed with a two-phase solvent system composed of heptane/*n*-butanol/water (3:4:7, v/v/v), and was based on a solvent system proposed by Du et al. [25]. The appropriateness of the solvent system was verified by calculating the partition coefficient, *K*, of the six ginsenosides most likely to be present in the root extract. The partition coefficient was determined using the shake flask method as described by Wang-Fan et al. [26]. The shake flask procedure was performed on five variations of the chosen system as shown in Fig. 1. The ternary phase diagram was prepared from the phase equilibria data published by Sørenson and Arlt [27], converted from mole% to vol.%. The solvent systems tested were two less polar systems and two more polar systems; in

Table 1
Volume ratios of solvents used to determine the partition coefficient in the different solvent systems for the six ginsenosides.

Solvent system designation	Heptane	*n*-Butanol	Water
−2	4	3	7
−1	3	3	6
0	3	4	7
1	2	5	7
2	1	6	7

total, five systems ranging from designations −2 (less polar) to +2 (more polar) with 0 designating the original system. The shake flask procedure began by combining the desired volume ratios, as shown in Table 1, of the solvents and mixing them on a stir plate for 6 h. The solvents were allowed to settle for 10 h, then 1.5 mL of each phase was removed and added to a vial containing one of the ginsenosides (0.4 mg each), totaling 30 vials. Each vial was then mixed well and allowed to settle for 3 h. Then, 1 mL of each phase was removed and placed in a clean glass tube and dried using a speedvac (Savant Model AES1010, Ramsey, MN) on the lowest heat setting. Once dry, the tubes were reconstituted with 0.5 mL of methanol and analyzed by HPLC. The area under the corresponding peak for each ginsenoside was used to calculate K and then $\log K_{SF}$. K is the area of the peak for the upper phase divided by the area of the peak for the lower phase and $\log K_{SF}$ is the log of K.

Fig. 2 displays the results of the shake flask method with $\log K_{SF}$ as the ordinate and the solvent systems tested, designated by the vol.% of *n*-butanol in the system, on the abscissa. To determine which of the systems would best separate the ginsenosides, the partition coefficient and the separation factor were used. The separation factor, S, equals K_i/K_j when $K_j > K$, where K_i is the partition coefficient for component i and K_j is the partition coefficient for component j. For an acceptable separation, S should be greater than 1.5 and K should fall between 0.5 and 2.5 [28]. Table 2 displays the partition coefficient calculated from the peak areas from HPLC analysis of the six ginsenosides and Table 3 displays the separation factor for each system tested. The chosen system, 0, is circled in Fig. 2.

When used for FCPC separation, the solvent system was prepared in a separatory funnel, allowing for full mixing and equilibration. The solvents were separated just prior to use in the FCPC. For separation, the FCPC was operated in descending mode, which used the organic upper phase as the stationary phase and the aqueous lower phase as the mobile phase.

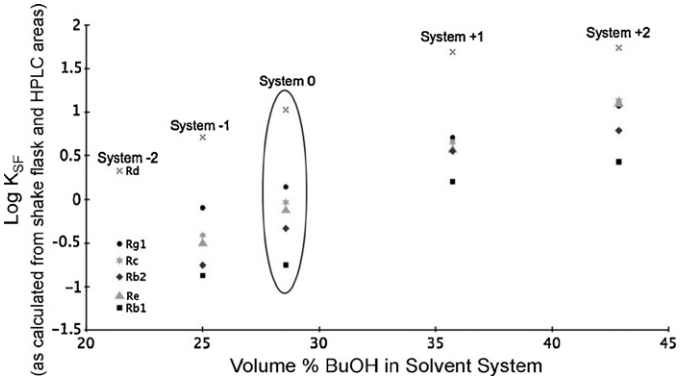

Fig. 2. Partition coefficients, $\log K_{SF}$, for the solvent systems tested for FCPC separation. System 0 was determined to be the best option to separate the ginsenosides because the partition coefficients fell within the acceptable range and had the greatest difference between the coefficients, which indicated that they would give better separation.

Table 2
Partition coefficients for each of the six ginsenosides for each solvent system tested. The partition coefficient is calculated from the areas on the HPLC chromatogram resulting from the shake flask procedure. For good separation, the partition coefficient should fall between 0.5 and 2.5.

Solvent system	Ginsenoside					
	Rg1	Re	Rb1	Re	Rb2	Rd
−2	0.31	0.08	0.06	0.2	0.13	2.12
−1	0.81	0.32	0.13	0.39	0.18	5.13
0	1.39	0.75	0.18	0.93	0.47	10.64
1	5.09	3.67	1.59	4.49	3.54	49.39
2	11.77	12.48	2.68	13.61	6.13	55.15

2.4. FCPC separation

FCPC separation was performed using a bench scale fast centrifugal partition chromatograph, FCPC (Kromaton, Angers, France). The solvents were pumped into the FCPC with a Waters 510 pump (Waters Milford, MA). The 200 mL column was first filled with stationary phase at 18 mL/min with the rotor spinning at 200 rpm. Once the rotor was fully loaded with stationary phase, the mobile phase was introduced at 3.1 mL/min and the rotation rate increased to 1100 rpm until the column attained equilibrium of both phases. The sample was then suctioned into the 10 mL sample loop and injected onto the column. The eluent was monitored with a UV detector (VUV24 Reflect Scientific, Orem, UT) equipped with a preparatory flow cell at 203 nm. Fractions were collected in a Waters Fraction Collector III (Milford, MA).

2.5. HPLC analysis

The HPLC system consisted of a Waters Alliance 2695 system equipped with a Waters 2996 photodiode array detector, controlled by Empower chromatographic software (Milford, MA). The detection wavelength was set to 203 nm. The ginsenosides were separated using a Symmetry (Waters, Milford, MA) C18 pre-column placed in series with a Symmetry (Waters, Milford, MA) C18 column (150 mm × 4.6 mm, 5 mm), set at 25 °C. A 20 μL sample volume was injected. Solvent A (100% water) and solvent B (100% acetonitrile) followed a linear gradient, which was modified from a study by Corbit et al. [29], to become: 0–15 min, 79% A; 16–38 min, 70% A; 39–44 min, 58% A; 45–49 min, 79% A and remained at 79% A for

Table 3
Separation factor for the three best solvent systems tested using the shake flask method. The separation factor is calculated from the partition coefficient by diving the larger of the two partition coefficients by the smaller of the two. A desirable separation factor is anything greater than 1.5, the values less than this are shown as bolded and italicized in the table. Solvent systems '+1' and '+2' were not included in this table since their partitions coefficients were not within the acceptable range and therefore did not require further examination.

K_i:K_j	Solvent system		
	−2	−1	0
Rg1:Re	3.96	2.55	1.84
Rg1:Rb1	5.44	6.02	7.82
Rg1:Rc	1.56	2.06	***1.49***
Rg1:Rb2	2.41	4.57	2.94
Rd:Rg1	6.81	6.38	7.68
Re:Rb1	***1.37***	2.36	4.24
Rc:Re	2.53	***1.24***	***1.23***
Re:Rb2	1.64	1.79	1.6
Re:Rd	26.97	16.28	14.15
Rb1:Rc	3.48	2.92	5.24
Rb1:Rb2	2.26	***1.32***	2.66
Rb1:Rd	37.08	38.42	60.04
Rc:Rb2	1.54	2.22	1.97
Rc:Rd	10.65	13.16	11.46
Rb2:Rd	16.44	29.16	22.6

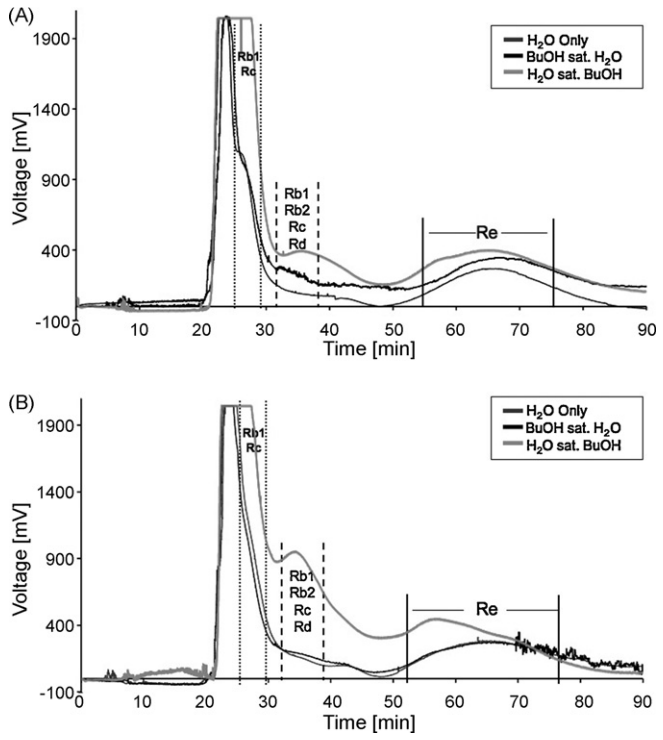

Fig. 3. Averages of duplicates of each solvent system separated by CPC for each of the differing extraction methods. The FCPC method was the same for each separation, but the solvent used to extract the ginsenosides differed as shown in the legend. The two methods used for extraction were: (A) ultrasonic-assisted extraction and (B) pressurized hot water extraction. Each solvent/method combination was run in duplicate. Fractions were collected and combined and are labeled to indicate the elution time and fraction contents.

minute 50. The flow rate of solvent was set to 1.3 mL/min. A standard curve was made for each of the six ginsenosides using the described HPLC method.

2.6. Statistical analysis

Statistical analysis was performed using the JMP software program. Analysis of variance was performed using the full factorial ANOVA procedure. Significant differences between extraction parameters were determined using the Student's *t*-test.

3. Results and discussion

3.1. FCPC results

The FCPC was operated using a heptane/*n*-butanol/water (3:4:7, v/v/v) solvent system in descending mode. The FCPC solvent system was modified from the Du et al. [25] system by substituting heptane for hexane, since it was shown that the solvents are interchangeable in liquid–liquid separations [30]. The FCPC separation ran from 0 min to 90 min with fractions collected every minute starting with minute 20. The fractions were dried down via speedvac on the medium heat setting. For the first extract separated on the FCPC, all 70 fractions were dried, reconstituted with 0.5 mL methanol and individually analyzed on HPLC. This analysis gave a 'fingerprint' for subsequent FCPC separations of ginsenoside extracts. Essentially, the first run was a test to see which fractions could be combined depending on the contents of the fraction. Fig. 3 displays the FCPC chromatograms from the ginsenoside extracts. Fig. 3A displays the averages of each of the solvents tested for the UAE method and Fig. 3B displays the averages of each of the solvents tested for the PHWE method. Each of the three solvents on each method was

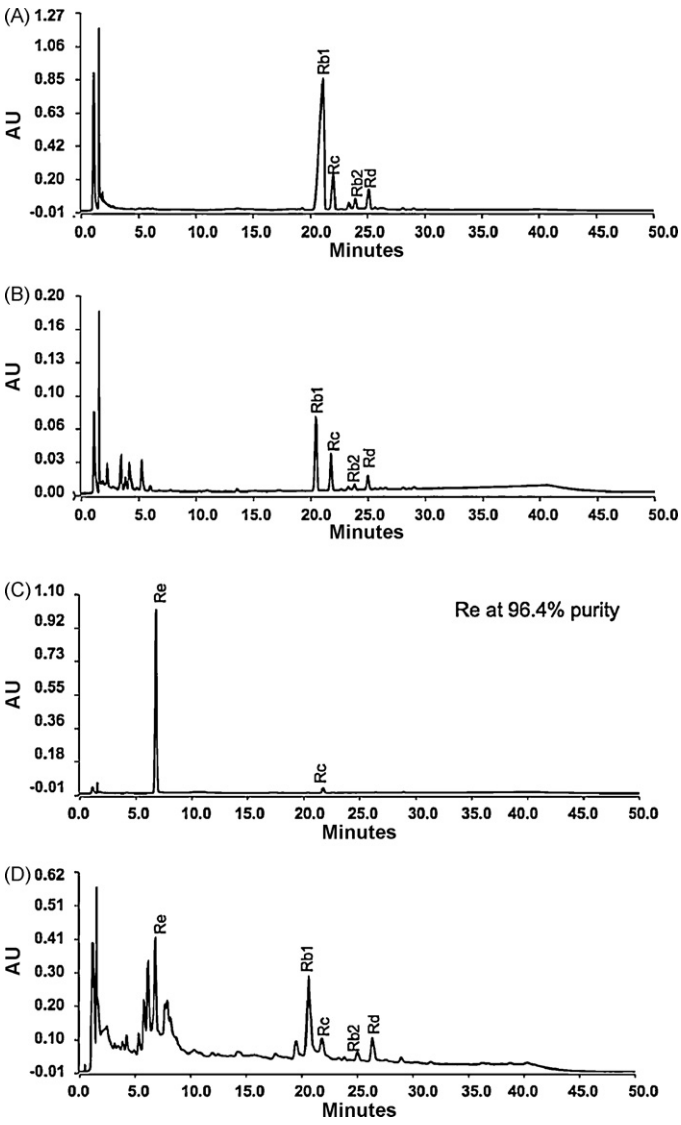

Fig. 4. HPLC chromatograms of the combined fractions from the FCPC separation. (A) Combination 1, containing mostly Rb1 and Rc; (B) combination 2, containing Rb1, Rc, Rb2 and Rd; (C) combination 3 containing Re at 96.4% purity; and (D) crude extract prior to CPC separation. All chromatograms are from PHWE using *n*-butanol-saturated water. These are examples of the combinations since it would be space consuming to display all chromatograms. The chromatograms of the combinations from the other experiments are very similar.

investigated in duplicate. The fractions for all runs were combined as follows: minutes 24–28, containing mostly Rb1 and Rc; minutes 33–38, containing mostly Rb1, Rc, Rb2 and Rd; and minutes 56–76 (50–70 for the PHWE, water-saturated *n*-butanol since the peak shifted to the left as shown in Fig. 3B), containing mostly Re. The three fraction combinations will henceforth be referred to as combination 1 (minutes 24–28), combination 2 (minutes 33–38) and combination 3 (minutes 56–76 or 50–70). The elution times of the combined fractions are indicated in Fig. 3.

The combined fractions were analyzed on HPLC, and Fig. 4 displays an indicative chromatogram for each of the three combinations and of a crude extract prior to CPC purification (D). A portion of each crude extract was analyzed by HPLC prior to CPC purification. This was prepared by removing 100 μL from each phase of the sample that had been reconstituted with the CPC solvent system, combining to obtain 200 μL, and then drying and reconstituting the 200 μL with 1.0 mL of methanol for HPLC analysis. Fig. 4A and B, combinations 1 and 2, respectively, show that these fractions did

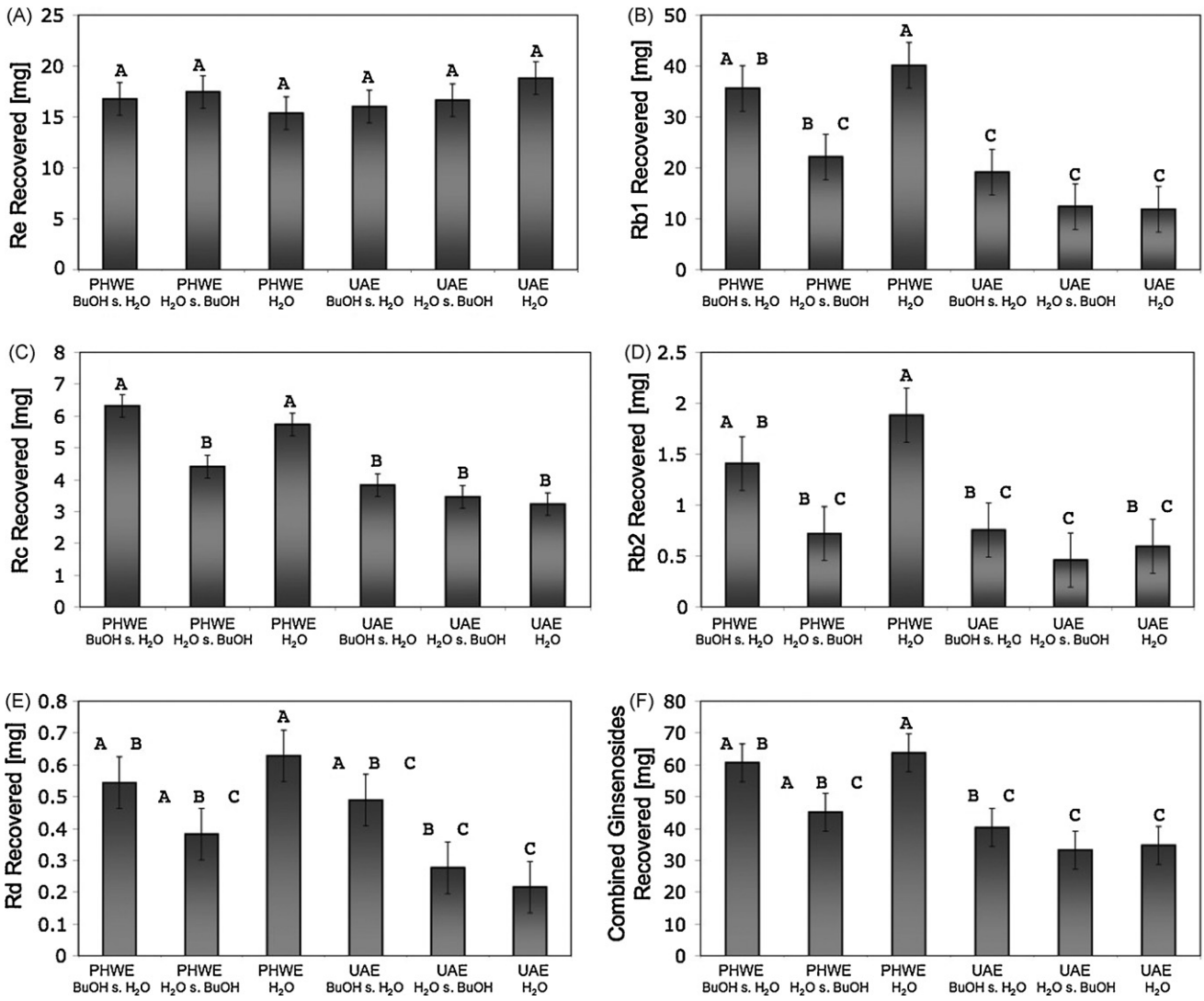

Fig. 5. Results of full factorial ANOVA on individual ginsenosides recovered and the combined ginsenosides recovered. Letters above columns indicate significant statistical differences in means. Error bars shown are standard error for each set.

not afford high-quality separation of the ginsenosides, but Fig. 4C shows that Re was purified – all reproductions of combination 3 afforded Re at a purity of 93% or greater. This discrepancy in purification could be attributed to the high concentration of Rb1 and Rc and the lower concentration of Rb2 and Rd, which can make separation more difficult when the elution times are close. Essentially, the results presented in Fig. 4 show that the solvent system proposed by Du et al. is effective in separating Rb1 and Re from the crude extract. It should be noted that ginsenoside Rg1 was not mentioned in the combinations since it was found that the powdered root only contained trace amounts of Rg1.

3.2. Comparison of extraction methods and solvents

The combined fractions were used in a statistical analysis in order to distinguish if the method, the solvent or both variables had an effect on the quantity of ginsenoside extracted. The amount of each ginsenoside in the combined fractions was calculated from the standard curve prepared using the HPLC method. The individual amounts extracted, as well as the combined or total amount extracted, were investigated. Separation using the FCPC proved very useful for this comparison because when the crude extract was analyzed by HPLC, the chromatogram was muddled and difficult to interpret as shown in Fig. 4D. Crude extracts contain more than just the compounds of interest and further separation prior to quantification should afford for more reliable results.

A full factorial ANOVA was performed on the individual compounds extracted and on the summation of the compounds. Fig. 5 displays each of the five individual compounds (Fig. 5A–E) and the combined compounds in Fig. 5F. A Student's *t*-test was performed and the resulting differences (or lack of difference) are displayed above the columns. As noted, in Fig. 5A there is no difference in the amount of Re extracted due to either the solvent used or the method of extraction. However, as noted in Fig. 5B, for the amount of Rb1 recovered, PHWE with water alone, was not significantly different from the *n*-butanol-saturated water PHWE, but was significantly different from water-saturated *n*-butanol PHWE and all three of the solvents on UAE. The *n*-butanol-saturated water PHWE was not significantly different from the water-saturated *n*-butanol PHWE, but was significantly different from the three UAEs. Wu et al. also reported the effects of extraction method and solvent used for Rb1 at a yield of 0.342 wt%, obtained with UAE using water-saturated *n*-butanol for a 2 h extraction time [16]. With respect to Rc, Rb2 and Rd, PHWE with *n*-butanol-saturated water and water extraction conditions resulted in the highest extracted yields, as shown in Fig. 5C–E.

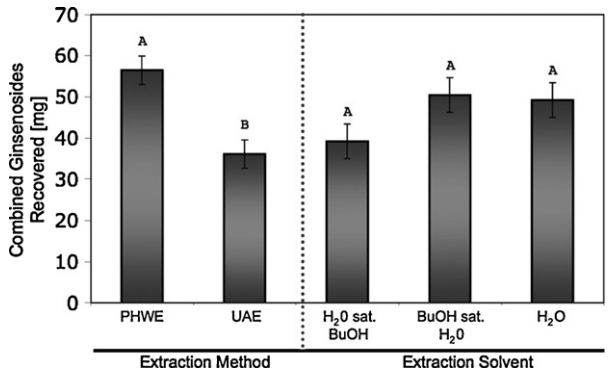

Fig. 6. Comparison of extraction method and extraction solvent for the combined ginsenosides recovered. Letters above indicate significant differences in the means of the total compound extracted. Error bars are standard error for each set.

A summation of the amount of compounds recovered is displayed in Fig. 5F, which shows that all three solvents in the PHWE were not significantly different from the *n*-butanol-saturated water UAE, but are significantly different from the water-saturated *n*-butanol and the water only UAE. A condensation of the results in Fig. 5 is shown in Fig. 6, which displays the total amount of ginsenosides extracted compared by extraction method, on the left, and by extraction solvent on the right. Fig. 6 illustrates that the extraction method does play a role in the amount of ginsenoside extracted as noted by the different letters above the columns. Pressurized hot water is able to extract more ginsenosides than ultrasonic extraction, by a significant margin. The solvent used during extraction is not as significant, as noted by the same letter above each of the solvent columns in Fig. 6. Kim et al. showed that the extraction of adventitious ginseng-powdered roots with 80 °C water was as effective as 80 °C, 70% ethanol [13]. Our results combined with those of Kim et al. show that hot water can extract ginsenosides and in amounts comparable to traditional extraction techniques (i.e. microwave and ultrasonic-assisted). While Wu et al. found that water-saturated *n*-butanol was able to extract more ginsenosides than other solvents tested during ultrasonic-assisted extraction [16], this study found that *n*-butanol-saturated water and water alone were able to extract more ginsenosides than water-saturated *n*-butanol, though not to a significant degree.

Qu et al. found that 3-year old ginseng root contains about 35 mg/g, which is comparable with the 11.2 mg/g quantified from the PHWE [4]. The discrepancy in the amount of ginsenosides extracted can partially be attributed to the loss of the compound during FCPC separation. Since not all fractions were analyzed, some of the compound may not have been included when the fractions were combined. Additionally, the methods used were not optimized for ginsenoside extraction. It should also be noted that the amount of ginsenosides initially present in the plant varies with age, location and cultivation practices [2].

The extraction temperature may be a significant parameter to consider when devising effective water extraction protocols. In this work, 110 °C water was as effective as *n*-butanol-saturated water and water-saturated *n*-butanol. This result is compatible with the findings of Kim et al., which stated that by increasing the water temperature from 40 °C to 80 °C the total amount of ginseng extracted also increased [13]. The two extraction methods explored for this study were performed at different temperatures, which may have been a major factor in obtaining increased extraction capability with PHWE. The other factor acknowledged for increasing the extractive effectiveness of PHWE, is the increased solubility of the water in the sub-critical state [21].

4. Conclusion

Ginseng saponins from a powdered root extract were found to be best extracted using the described pressurized hot water method. The use of water as an extraction solvent makes the recovery much more attractive since water is an environmentally benign solvent. Another advantage of using water as the extraction solvent is that the extract can be lyophilized, which may aid in inhibiting the production of degradation compounds formed by further exposing the extract to heat [16]. Scale-up of the pressurized water method is more feasible than scale-up of ultrasonic extraction, which has not been applied industrially [17]. The use of FCPC as the purification technique illustrated that purification of compound Re was possible in a single step. By adjusting the solvent system used, other ginsenosides could also be purified, but this would require more than one FCPC separation. With PHWE as the best-case method for the extraction of ginsenoside saponins, the door has been opened for further study for the extraction of valuable saponins from other plant matters using water alone as the extraction solvent.

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