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EXTRACTION OF GINSENOSIDES FROM AMERICAN GINSENG (PANAX QUINQUEFOLIUM L.) ROOT WITH DIFFERENT EXTRACTION METHODS AND CHROMATOGRAPHIC ANALYSIS OF THE EXTRACTS

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Abstract:
Microwave-assisted extraction (MAE) was compared with room temperature extraction (RTE) and reflux temperature extraction (RFX) on the extraction of ginsenosides from fresh American ginseng root. Extraction times of 5, 10, 30 and 60 min were investigated. An 86 – 300% increase in extraction rate was observed by raised temperature. The use of microwave energy instead of hotplate heating in the extraction resulted in a 31 – 96% increase in extraction rate with the exception of ginsenoside Re. Visual analysis of the

chromatographs of extracts helps choose conditions for selectively obtaining specific ginsenosides enriched extracts.

Keywords: Microwave-assisted extraction; American ginseng; Ginsenosides; Extraction methods; Chromatographic analysis

Introduction:

American ginseng (Panax quinquefolium L.) is one of the most important medicinal herbs in North America. Extracts from American ginseng root were reported to have many biological and pharmaceutical properties e.g. anti-anxiety, anti-tumor, antioxidant properties, immune system enhancing, benefit to the central nervous system, and slowing aging processes (Duda, et al., 2001; Hu and Kitts, 2001; Ni, et al., 2001; Wang, et al., 1999; Yuan, et al., 2001; Zhou and kitts, 2002). Many of its biological and pharmaceutical properties were believed to be due to the activity of a group of compounds called dammarane saponins, generally referred to as ginsenosides (Ni, et al., 2001; Yuan, et al., 2001; Zhou and Kitts, 2002; Dou, et al., 2001; Li, et al., 1996; Liu, et al., 2001). Major ginsenosides include Rb1, Rb2, Rc, Rd, Re, Rg1, mRb1, mRb2, mRc and mRd (Wang, et al., 1999; Ren and Chen, 1999). Among all ginsenosides, Rb1 and Re contribute 70 – 80% to the total for ginseng grown in British Columbia, Canada (Li, et al., 1996). HPLC chromatographs of extracts of ginseng root from Jilin, P.R. China indicate that ginsenosides Re, mRb1 and Rb1 contribute to more than 90% of total ginsenosides (Ren and Chen, 1999).

The majority of American ginseng is consumed in East Asia in the form of dried whole root, while a smaller portion is processed into extracts. Due to its many biological and pharmaceutical properties, the American ginseng root extract is processed into medicinal or nutraceutical products or in combination with extracts from other herbs. Some extracts are also used in cosmetic, food and beverages (Berg, 2002; Zou, et al., 2001; Meybeck, et al., 1999). In an extraction process, solid matrices are immersed in a proper solvent, into which the constituents of interest are diffused; subsequently the solvent is evaporated to obtain the extracts. Many factors can influence the extraction rate; among all the factors, increasing temperature is a common method to achieve higher extraction rate. For ginseng roots, reports showed that even at increased temperature, the extraction process was still a slow one (Ryu, et al., 1979; Sung and Yang, 1986). Microwave-assisted extraction (MAE), an extraction method using microwave energy as the heating source, was reported to greatly enhance the extraction rate and increase the extraction yield (Hao, et al., 2002; Pan, et al., 2003; Pan, et al., 2002; Paré and Bélanger, 1994). This study compared the effectiveness of MAE with two conventional extraction methods in extracting various ginsenosides from fresh ginseng roots.

Material and Methods

Fresh American Ginseng Roots. Roots of four year old American ginseng (P. quinquefolium L.) were provided by Agriculture and Agri-food Canada (ON, Canada). The fresh ginseng roots were stored in refrigerated storage at 4 ^{o}C. Before extraction, samples were washed, carefully peeled and cut into approx. 3mm cubes.

Ginsenosides Content of the American Ginseng Root. 5 g of the above mentioned fresh ginseng root was homogenized with a small coffee bean grinder. 2 g of the paste was transferred into a conical flask followed by the addition of 20 mL methanol. The extraction was carried out under magnetic stirring for 2 hrs. After removing the supernatant from the flask, the residue was further extracted twice with 20 mL methanol stirring 2 hrs each. Finally, the residue was washed with 20 mL methanol, the resulting solution together with the three supernatants were transferred into a 100 mL volumetric flask. After making the solution to set volume by adding methanol, the solution was filtered through a 0.22 μm syringe filter (Cameo brand, Fisher Scientific, Montreal, Canada) before injecting into the HPLC for ginsenosides analysis of the fresh sample. The extraction was repeated to get three replicates.

Extraction Procedures for Comparing Different Extraction Methods. (a) Room Temperature Extraction (RTE): 2 g of the ground ginseng was placed in a 100 mL conical flask, followed by the addition of 40 mL of methanol. The extraction was carried out at room temperature under magnetic stirring for 5, 10, 30 and 60 min. After the extraction, the supernatant was filtered through the 0.22 μm syringe filter and injected into the HPLC for analysis. (b) Reflux Extraction (RFX). The procedure was almost the same as that of RTE, except that it was carried out under reflux condition using a hotplate for heating. No stirring was necessary because the system was kept at a boiling state. (c) Microwave-assisted Extraction (MAE). 2 g of sample was placed in the quartz extraction vessel of the Prolabo Synthewave 402 (focused microwave-assisted extraction/synthesis equipment at atmospheric pressure, Fontenay-Sous-Bois, Cesex, France) followed by the addition of 40 mL of methanol. The extraction was carried out using 90 W fixed power continuously for the period of 5, 10, 30, 60 min.

HPLC Analysis. Varian ProStar liquid chromatograph (Walnut Creek, CA, USA) was equipped with a ProStar 410 autosampler, a ProStar 220 pumping system and ProStar 330 photodiode array/UV detector. Separation method was modified from Ren and Chen (10). Separations were carried out using a reverse phase MicroSorb-MV^{TM} 5 μm C_{18} column (25 x 4.6 mm). Mobile phases were: (A) deionized water, (B) phosphate buffer at PH 5.82, and (C) acetonitrile (HPLC grade, Fisher Scientific, Montreal Canada) using the following gradient: 0-6 min, 0%A, 73-66% B, 27-34% C; 6-9 min, 0%A, 66% B, 34% C; 9-12 min, 0% A, 66-60% B, 34-40% C; 12-17 min, 0% A, 60-40% B, 40-60% C; 17-21 min, 0% A, 40-15% B, 60-85% C; 21-28 min, 0% A,15% B, 85% C. The flow rate was 1.0 ml/min until 17 min and 1.3 ml/min until 28 min. The chromatographs were obtained at 203 nm.

Calibration with Standards. Standard ginsenosides Re and Rb1 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Both ginsenosides Re and Rb1 were made into one mixed stock solution containing 1 mg/mL of each ginsenosides in it. The stock solution was further diluted into 0.5, 0.2, 0.1, 0.05, 0.02 mg/mL. Calibration curves in the concentration range of 0.02 – 0.5 mg/mL were obtained for Re and Rb1 based on triplicate injection of each solution. The location of mRb1 peak was found by comparing with Ren and Chen (1999) and the amount of mRb1 was calculated based on the calibration result for Rb1.

Statistical Analysis. Single-factor ANOVA was carried out using Microsoft Excel for each pair of methods under the same extraction time. Linear regression for lnA – t relationship shown in the results and discussion section was obtained using Microsoft Excel.

Results and Discussion:

Extraction of ginsenosides with three extraction methods. The results of extracting ginsenoside Re, mRb1 and Rb1 using three extraction methods MAE, RTE and RFX are presented in Figures 1 to 3. As far as the individual extraction time is concerned, MAE is observed to be generally more effective than RTE especially at longer extraction times. However, at 5 min, MAE is only more effective than RTE for the extraction of Re. As far as MAE and RFX are concerned, no significant increase is observed for all three ginsenosides at most extraction times except for 10 min for Re, 30 min for mRb1 and 30 min for Rb1.

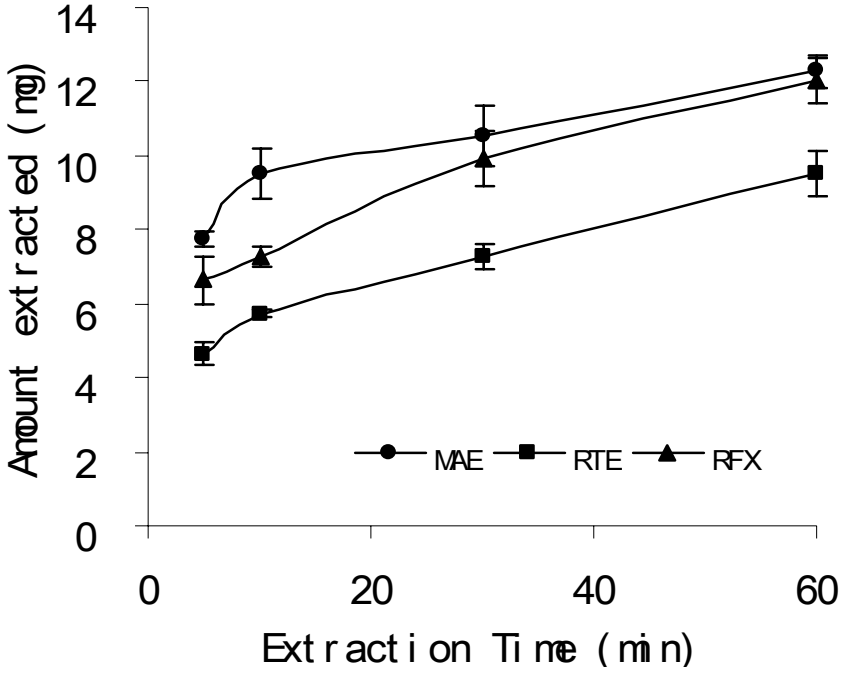

Figure 1: Extraction of ginsenoside Re by three extraction methods.

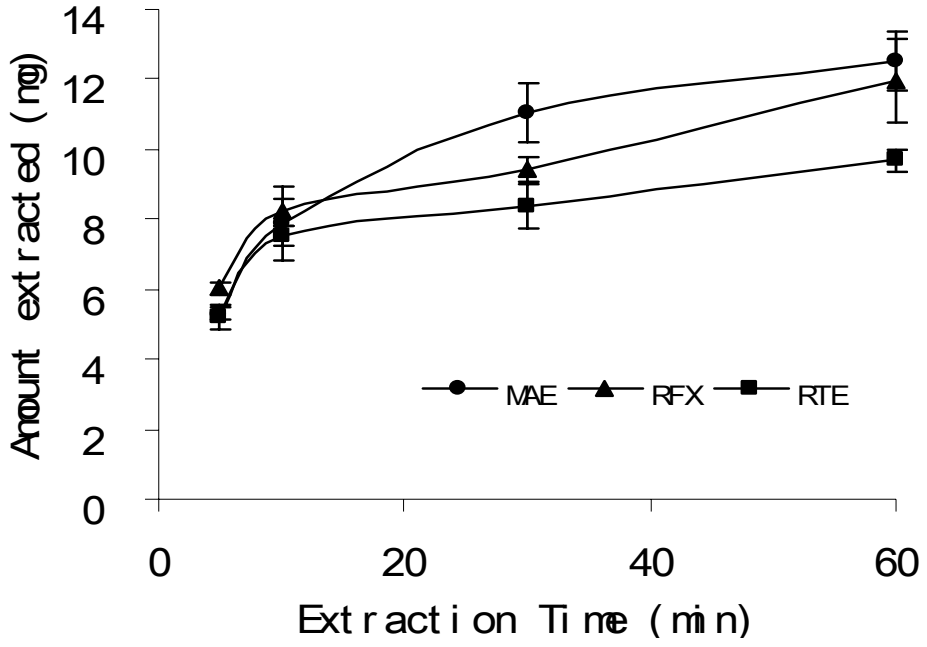

Figure 2: Extraction of ginsenoside mRb1 by three extraction methods.

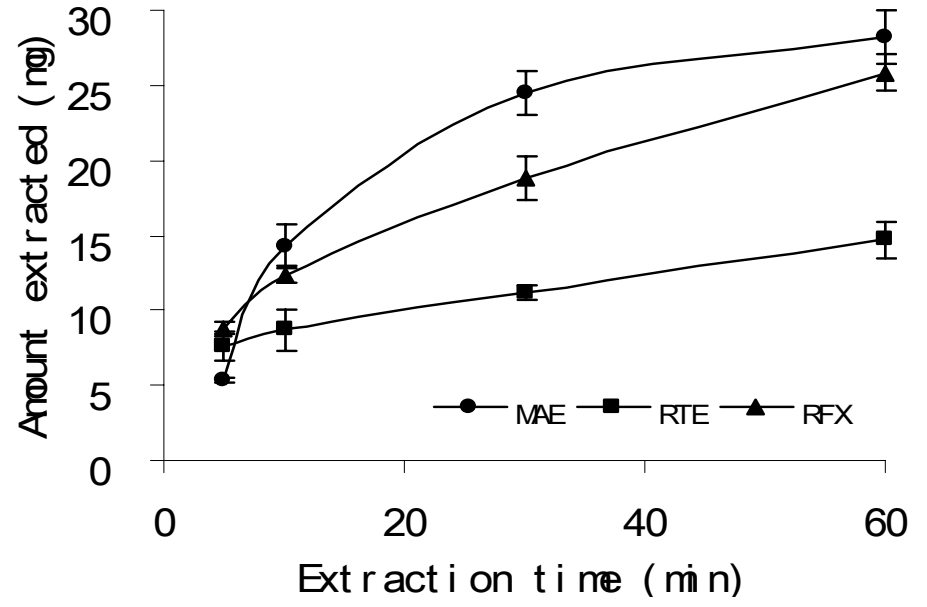

Figure 3: Extraction of ginsenoside Rb1 by three extraction methods.

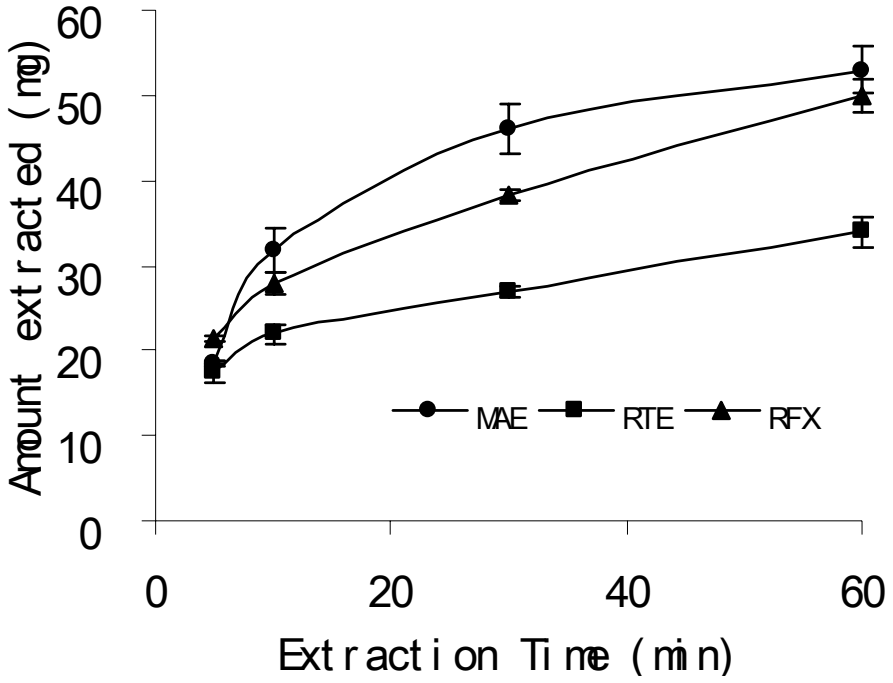

Figure 4: Extraction of total ginsenosides represented the Re, mRb1 and Rb1 by three extraction methods

The three ginsenosides Re, mRb1 and Rb1 are reported to contribute to more than 90 percent of the total ginsenosides (Li, et al., 1996). The chromatographs of one-hour RFX or MAE extracts agree with this report (See Figure 9). Therefore the sum of three major ginsenosides is used here to represent the total ginsenosides. The extraction of total ginsenosides by the three methods is presented in Figure 4. Similar to the three individual ginsenosides, MAE is more effective than RTE at longer extraction time but significant increase in the extract amount is only observed at 30 min extraction when compared with RFX for extracting total ginsenosides.

Comparison of the extraction rates. The predominant mechanism in an extraction process is diffusion, through which constituents of interest are diffused into the solvents. The driving force of the diffusion process is the concentration gradient between the sample particles and the solution. The extraction rate can be described as:

$$dA/dt = k\Delta c \tag{1}$$

Where: A is the amount of the component of interest, t is the extraction time, k is a constant, and Δc is the concentration gradient.

$$\Delta c = c_s - c_{sol} \tag{2}$$

Where c_s is the concentration in the sample particles, and c_{sol} is the concentration in the solution.

$$c_s = A / V_{sample} \tag{3}$$

$$c_{sol} = (A_o - A) / V_{sol} \tag{4}$$

Where: V_{sample} is the volume of solvent that enters the free space of sample, V_{sol} is the volume of solution, A_o is the original amount of the target component in the sample. When plenty of solvent is used in an extraction, the volume that enters the sample is negligible compared with that in the solution. Therefore c_{sol} is negligible. Equation 1 can be written as:

$$\begin{aligned} dA/dt &= k'(A/V_{sample}) \\ &= kA \end{aligned} \tag{5}$$

Where $k = k'/V_{sample}$ is a constant. Integration of equation 5 results in:

$$\ln A = kt + c \tag{6}$$

Where A is the amount of the target component left in the sample. It can be calculated from the original amount and the amount extracted:

$$A = A_o - A_{extr} \tag{7}$$

In this study, the original amounts of ginsenosides Re, Rb1 and mRb1 were obtained as described in a previous section “Ginsenosides Content of the Ginseng Root” and are: 14.15 +/- 1.08, 13.40 +/- 0.85, 31.29 +/- 1.84 mg respectively. The amount of ginsenosides extracted at different extraction times is presented in Figures 1 through 3. Therefore, the amount residing in the sample can be obtained. The lnA – extraction time (t) relationship for ginsenosides Re, Rb1, mRb1 and the total ginsenosides vs. time are presented in Figs. 5 – 8. The linear regression results are shown in Table 1. Very good linearity was observed as indicated by their R square values.

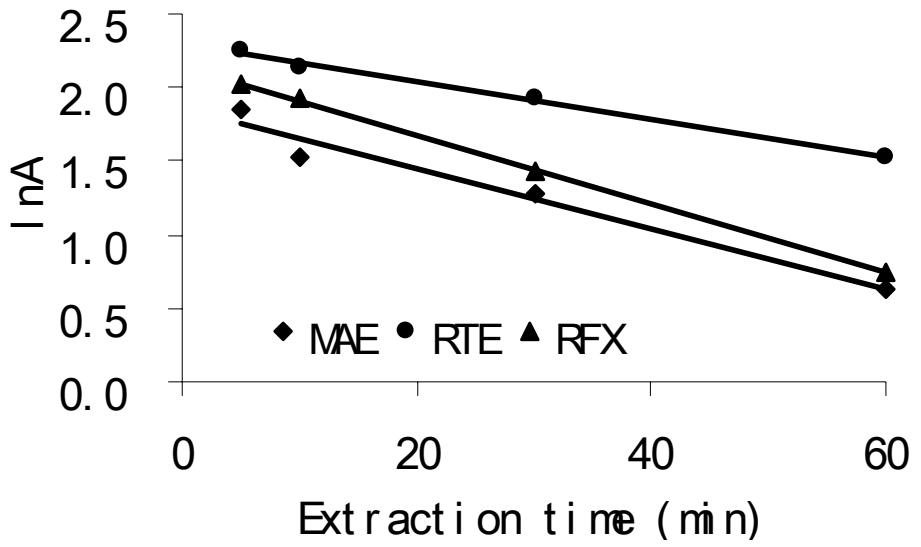

Figure 5: Linear regression of the natural log of residue amount of ginsenoside Re in the sample vs. extraction time for MAE, RTE and RFX extraction methods

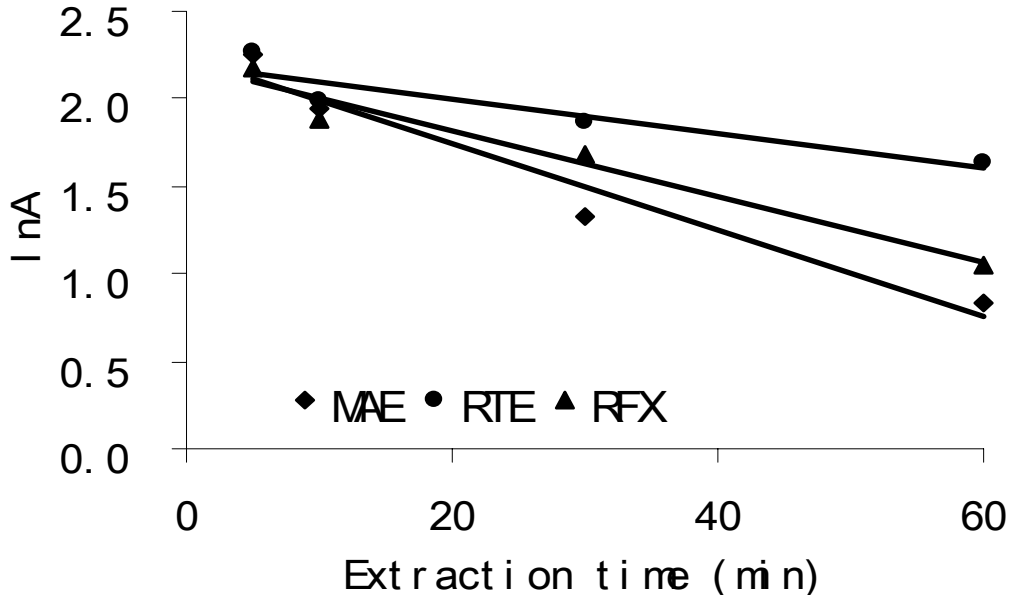

Figure 6: Linear regression of ln*A* vs. extraction time for ginsenoside mRb1 using the three extraction methods.

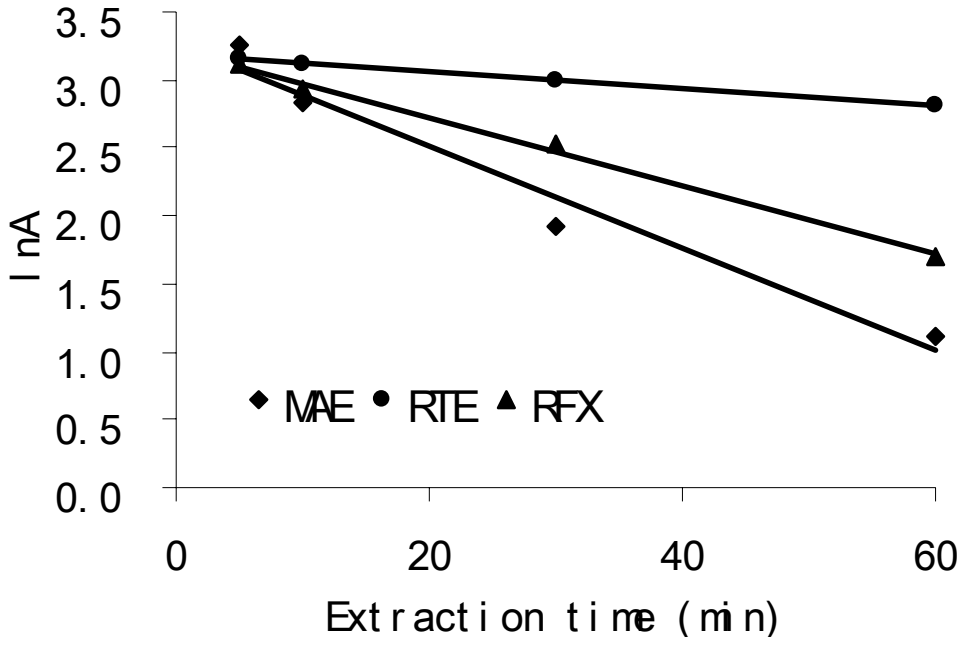

Figure 7: Linear regression of ln*A* vs. extraction time for ginsenoside Rb1using the three extraction methods.

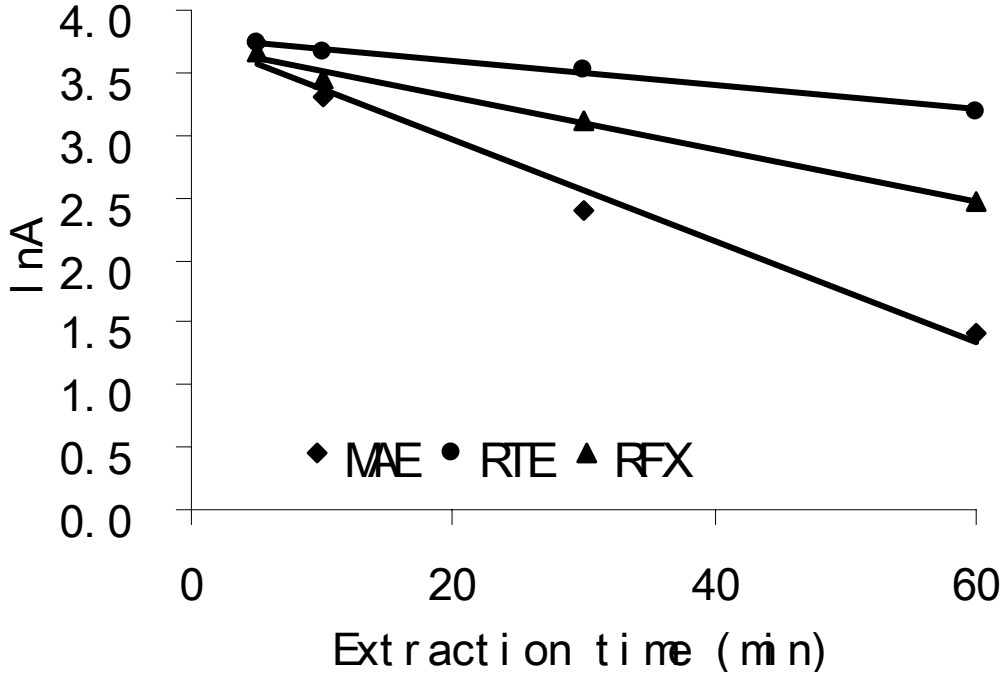

Figure 8: Linear regression of ln*A* vs. extraction time for total ginsenosides using the three extraction methods.

Equation 5 shows that the extraction rate is proportional to the residue amount of ginsenosides in the sample and its proportionality constant is k. Therefore, when the

amount of ginsenosides left in the sample is the same, the larger the absolute value of the k, the higher the extraction rate. The linear regression results (Table 1) shows that RFX and MAE has generally higher extraction rate than RTE suggesting that extraction rate can be increased by raising the temperature. For a diffusion-based extraction process, increased temperature causes a faster diffusion rate between concentration gradients.

Both MAE and RFX work at the reflux temperature of the solvent, in this case, methanol. The difference between them is the source of heating with MAE using microwave energy and RFX using conventional hotplate. However the comparison of MAE and RFX shows that MAE has generally larger k value than RFX except for ginsenoside Re. As discussed earlier, for the sample having the same amount of ginsenosides content, the extraction rate is proportional to the k value. The ratio of the k values for different extraction methods, therefore, represents the acceleration ratio between methods. Table 2 shows that increased temperature using hotplate leads to a 86 – 300% increase in extraction rate. However, both at raised temperature, 31 – 96% increase was observed for MAE as compared to RFX with the only exception of Re. Especially for total ginsenosides, the acceleration rate of MAE/RFX is comparable with that of RFX/RTE. This indicates that the acceleration in extraction rate for the extraction process using microwave energy is beyond just a temperature factor.

Table 1: Linear regression results of ln*A* – t relationships

		C	|k|	R^2
Re	MAE	1.86	0.0205	0.9685
	RTE	2.29	0.0126	0.9932
	RFX	2.15	0.0234	0.9994
Rb1	MAE	3.27	0.0375	0.9642
	RTE	3.19	0.0063	0.9986
	RFX	3.23	0.0252	0.9964
mRb1	MAE	2.24	0.0248	0.9541
	RTE	2.19	0.0097	0.8612
	RFX	2.20	0.0189	0.9675
Total ginsenosides	MAE	3.78	0.0408	0.9921
	RTE	3.78	0.01	0.9943
	RFX	3.71	0.0208	0.9908

MAE method is a solvent extraction method using microwave energy as heating source. Microwave energy differs from the conventional hotplate heating in its heating mechanism and heating behavior. Microwave heating comes from the interaction of polar molecules with the alternating electromagnetic field. This heating mechanism leads to the selectivity and volumetric behaviors of microwave heating. The special microwave-molecule interaction may have caused the acceleration in extraction rate of MAE over RFX, both having similar process temperatures. The microwave energy has double roles; to increase the temperature thus increasing the diffusion rate and to create localized super heating effect causing an additional increase in extraction rate. The latter reason was suggested by Paré and Bélanger (1994) to be the dominant mechanism

when extracting peppermint using nonpolar solvents causing a dramatic increase in extraction rate.

Table 2: The extraction rate enhancement factor RFX vs. RTE and MAE vs. RFX.

	Re	Rb1	mRb1	Total
RFX vs. RTE	86%	300%	95%	106%
MAE vs. RFX	-13%	49%	31%	96%

Note: the enhancement factor for RFX vs. RTE = (k_{RFX} - k_{RTE})/ k_{RFX} * 100%; and MAE vs. RFX = (k_{MAE} - k_{RFX})/k_{MAE} * 100%; k_{MAE}, k_{RTE}, and k_{RFX} are extraction constants for MAE, RTE, and RFX respectively;

Chromatographic Analysis. Chromatographs of extracts obtained with different extraction methods at different extraction times provide a method to visualize the progress of the extraction process and how different components are extracted (see Fig. 9). Chromatographs presented here are not using the same scales; the purpose is to show the relative content of different components in the extracts as indicated by the size of the peaks. On the chromatograph, the small peaks other than the three major ginsenosides are believed to be other ginsenosides as compared with the chromatographs provided by Ren and Chen (1999). The areas of peaks on the chromatograph correspond to the quantity of this ginsenoside in the extract. The relative size of the peak to that of Rb1 corresponds to the relative content of each individual ginsenoside to that of Rb1. After 5 min of extraction, the chromatographs showed that the various minor ginsenosides have fair content in the extracts, especially for RFX and MAE. With the increase of extraction time, the relative contents of the minor ginsenosides decrease rapidly. At 60 min of extraction, the minor peaks on the chromatographs are negligible as compared with Rb1.

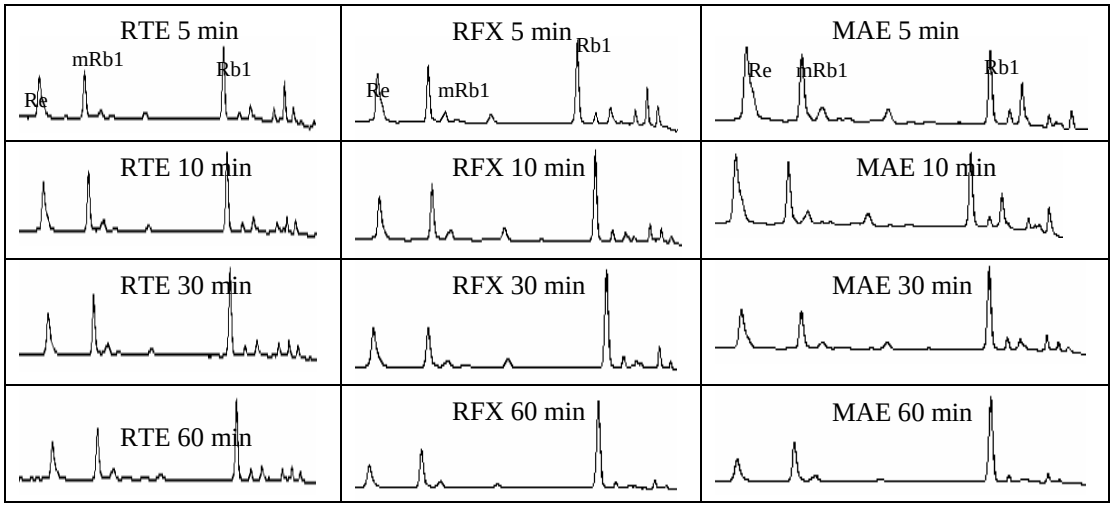

Figure 9: Chromatographs of ginseng root extracts obtained using different extraction methods and extraction times

In the early stage of the extraction process, all components are diffused into the solvent. With the progress of the extraction process, due to the relatively lower amount of the minor ginsenosides, the diffusion process ends within five to ten minutes with the

establishment of an equilibrium. While for the major ginsenosides, this equilibrium stage takes longer time resulting in the drop of the relative content of minor ginsenosides in the extracts with the progress of extraction. Similar trend was also observed within the three major ginsenosides. Relative contents of Re and mRb1 also drop with the progress of the extraction process. Comparison of RTE with RFX and MAE indicates that at room temperature the equilibrium stage comes much later than at the raised temperature.

The visual analysis of the chromatographs provides a method for obtaining specific ginsenosides enriched extracts. At raised temperature and short extraction times, the extract obtained contains relatively higher content of minor ginsenosides. The residue can than be further extracted to obtain extracts containing mainly three major ginsenosides.

Conclusion:

MAE was compared with two conventional extraction methods RTE and RFX for the extraction of ginsenosides from fresh American ginseng root. MAE and RFX have higher extraction rate than RTE suggesting that extraction rate can be accelerated by increasing temperature. MAE has higher extraction rate than RFX due to a factor other than just temperature. Specific ginsenosides enriched extract can be obtained by controlling the extraction time.

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