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Analytical Methods

Analysis of ginsenosides in *Panax ginseng* in high pressure microwave-assisted extraction

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Abstract

High pressure microwave assisted extraction (HPMAE) was applied to extract the ginsenosides from *Panax ginseng* root. The influences of extraction solvent, extraction pressure and extraction time were individually investigated. HPMAE has been compared with other extraction methods, including Soxhlet extraction, ultrasound-assisted extraction and heat reflux extraction. The determination of ginsenosides was performed by HPLC–ESI-MS. The results indicated that the HPMAE not only took a shorter time but also afforded higher extraction yields of ginsenosides, especially ginsenoside Rb_1, Rc, Rb_2 and Rd. Furthermore, the neutral ginsenosides and malonyl ginsenosides in *Panax ginseng* root extracts by HPMAE were investigated. The malonyl ginsenoside m-Rb_1, m-Rc, m-Rb_2 and m-Rd degraded in HPMAE at 400 kPa (109–112 °C) in 70% (v/v) ethanol–water and at 600 kPa (112–115 °C) in methanol, and transformed into corresponding neutral ginsenoside Rb_1, Rc, Rb_2 and Rd. Using water as extraction solution, the neutral ginsenosides degraded under HPMAE at 400 kPa (135–140 °C), and transformed into less polarity rare ginsenosides.

Keywords: Microwave assisted extraction; *Panax ginseng*; Ginsenoside; HPLC/MS; Degradation

1. Introduction

Panax ginseng (*P. ginseng*) is widely used as traditional Chinese medicine and has been demonstrated to be against various human diseases, including increasing resistance to physical, chemical and biological stress and boosting general vitality, and it is frequently featured in traditional medicine used by cancer patients (Chang, Seo, Gyllenhaal, & Block, 2003; Kiefer & Pantuso, 2003; Xie et al., 2002). In recent years, various formulations prepared from the *P. ginseng* roots have been marketed as dietary supplements. Especially in China, *P. ginseng* is frequently used as food additives and raw materials of healthy food rather than therapeutic agents (Breemen et al., 1995; Gillis, 1997; Shen, Ren, & Chen, 2003). Ginsenosides are the main bioactive components in *P. ginseng*, including neutral ginsenosides and malonyl ginsenosides, which are frequently used as main index for ginseng product evaluation (Du, Wills, & Stuart, 2004; Fuzzati, Gabetta, Jagaker, Pace, & Peterlongo, 1999; Lau, Woo, & Koh, 2003; Ren & Chen, 1999; Zhang, Wu, & Cheng, 2003). Ginsenosides, especially malonyl ginsenosides, which were thermally unstable, can degrade during thermal extraction into corresponding neutral ginsenosides (Ren & Chen, 1999). Du et al. (2004) reported the changes of the neutral ginsenosides and malonyl ginsenosides in American ginseng roots during drying, storage and extraction. Therefore, ginsenosides extracted from *P. ginseng* are influenced by extraction method and extraction solvent. High-performance liquid chromatography (HPLC) has been applied extensively to determine the ginsenosides in extract of *P. ginseng*. In the major methods reported in the literatures normal-phase or reverse-phase column was used with water and acetonitrile mixture as solvent system in gradient elution mode and with different detection techniques (Court, Hendel, & Elmi, 1996;

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Fuzzati, 2004; Park, Park, Han, & Shin, 1996; Wang, Wang, Wu, Osinski, & Yuan, 2005). Many conventional extraction techniques, such as Soxhlet extraction (SE), reflux extraction (RE), ultrasonic extraction (UE) and supercritical fluid extraction (SFE) have been developed to extract ginsenosides from *P. ginseng* (Corbit, Ferreira, Ebbs, & Murphy, 2005; Gafner et al., 2004; Vongsangnak, Gna, Chauvatcharin, & Zhong, 2004; Wang, Chen, & Chang, 2001; Zhang, Chen, Wu, & Wang, 2006). Microwave-assisted extraction (MAE) combines microwave with traditional solvent extraction, which has been shown to enhance the extraction efficiency of interested components and used as a sample preparation technique. MAE was employed for the extraction of ginsenosides from *P. ginseng* (Kwon, Belanger, Pare, & Yaylayan, 2003; Shi, Wang, Li, Zhang, & Ding, 2007; Shu, Ko, & Shiun, 2003). High pressure microwave assisted extraction (HPMAE) is a relatively new extraction method. In HPMAE, the extraction system is closed-vessel system, and the extraction process is often carried out at high pressure and accelerated with increasing temperature of the extraction system.

This study intends to confirm the efficacy of HPMAE as a fast extraction mean of ginsenosides in *P. ginseng* root. Furthermore, degradation of malonyl ginsenosides and neutral ginsenosides in *P. ginseng* root extracts during HPMAE was investigated. The results might provide useful information for the production of high quality ginsenoside extracts.

2. Experimental

2.1. Materials and reagents

Ginsenoside Rg_1, Re, Rb_1, Rc, Rb_2 and Rd were purchased from Chinese Medical and Biological Products Institute (Beijing, China). The Chromatographic grade acetonitrile was obtained from Fisher Scientific Company (Pittsburgh, PA, USA) and pure water was from a Milli-Q water purification unit (Millipore, Mississauga, Canada). Other reagents used in this study were of analytical grade.

The raw *P. ginseng* root was obtained from Jilin province in China. The ginseng root was rinsed with water, dried at 30 °C, powdered in a mill, and passed through a 40 mesh sieve.

2.2. High pressure microwave assisted extraction

The HPMAE was performed in a WRT-C microwave preparation system with a pressure, temperature and time control system (Meicheng Technology Co., Ltd., Beijing, China). The extraction vessel consists of a vessel body (PEEK) and a liner vessel (100 mL, TFM). A sample powder of 1.00 g was accurately weighed, transferred into the liner vessel and immersed by 40 mL extraction solvent. The liner vessel was put into the vessel body. Then the closed control vessel and extraction vessels were put into the microwave preparation system. After the system was turned on, the pressure began to gradually increase. When the pressure reached preset pressure, irradiation time was counted and the extraction was carried out continuously at the preset pressure. When the extraction was completed, the extraction vessel was allowed to cool down to room temperature for about 10 min. Finally the extract was filtrated and transferred into a 50 mL volumetric flask. The liner vessel and the sediment were rinsed three times with extraction solvent. Then the rinsed solvent was also transferred into the flask and the extraction solvent was added to the mark of the flask. The sample solution was filtered through a 0.45 μm filter membrane and then analysed by HPLC.

2.3. Other extraction methods

2.3.1. Soxhlet extraction

A 1.00 g sample of *P. ginseng* root was placed in a glass Soxhlet thimble. Forty milliliters of 70% (v/v) ethanol–water were added into a 100 mL distilling flask. The flask heated in 80 °C water bath and the solvent was refluxed for 2 h. When the extraction was completed, the extract was filtrated and transferred into a 50 mL volumetric flask, and the Soxhlet flask was rinsed three times with the extraction solvent. The solvent used for rinsing was also added to the flask. Finally, the volume was made up to the mark with the extraction solvent.

2.3.2. Ultrasound-assisted extraction

Ultrasound-assisted extraction was performed with a KQ2200E Ultrasonic Cleaners (Kunshan Ultrasonic Instrument Co., Ltd., Kunshan, China). The output power is 100 W and the frequency is 40 kHz. Sample powder 1.00 g was placed into a 100 mL conical flask, into which 40 mL of 70% (v/v) ethanol–water were added. Then the flask was sonicated for 1 h in an ultrasonic water bath. When the extraction was completed, the extract was filtrated and transferred into a 50 mL volumetric flask, and the conical flask was rinsed three times with the extraction solvent. The solvent used for rinsing was also added to the flask. Finally, the volume was made up to the mark with the extraction solvent.

2.3.3. Heat reflux extraction

A sample of *P. ginseng* root 1.00 g was mixed with 40 mL of 70% (v/v) ethanol–water in a 150 mL round bottom flask fitted with a cooling condenser. The flask was heated in 80 °C water bath for 2 h. When the extraction was completed, the extract was filtrated and transferred into a 50 mL volumetric flask, round bottom flask and residue were rinsed three times with the extraction solvent. The solvent used for rinsing was also added to the flask. Finally, the volume was made up to the mark with the extraction solvent.

2.4. HPLC–ESI-MS

An Agilent 1100 liquid chromatograph (Palo Alto, CA, USA) equipped with a quaternary pump, a heated column compartment and a UV detector was used. The analytes were separated by a Zorbax Eclipse XDB-C18 (250 mm × 4.6 mm i.d., 5 μm) column (Agilent, USA). The detection wavelength was set at 203 nm. The flow rate was kept at 1.0 mL/min and temperature of column was controlled at 25 °C. An ABI Q-Trap Mass Spectrometer (Applied Biosystems Sciex, Foster City, USA) equipped with electrospray ionization source was used for ESI-MS analysis. Nitrogen (99.999%) was used for nebulizer gas and curtain gas. The ion polarity was set to positive mode. User controlled voltages, gas pressures, and source temperature were optimized for the detection of ginsenosides. The source temperature was set to 400 °C. The curtain gas and nebulizer gas were 25, 35 psi, respectively. The ion spray voltage was 5000 V. The declustering potential and collision energy were 160 V and 10 eV respectively. The binary gradient elution solvent consists of acetonitrile (A) and water (B). 0-30 min, 20% A, 80% B; 30–40 min, 20–31% A, 80–69% B; 40–60 min, 31–39% A, 69–61% B; 60–70 min, 39–50% A, 61–50% B; 70–85 min, 50–85% A, 50–15% B. Sample injection volume was 10 μl. The ginsenoside standards were prepared in HPLC grade methanol. Concentrations of ginsenosides were determined by standard curves prepared by injecting different concentrations of ginsenoside standards.

3. Results and discussion

3.1. Determination of ginsenosides

The validation of the proposed method has been performed for determination of ginsenosides. The relationships between the analyte concentration (C) and measured absorbance (A) for six kinds of ginsenosides were established. The linearity of the calibration curves was verified by the correlation coefficients. The injection precision (repeatability) was found to be within the range 1.02–2.88% ($n = 6$). Ginsenoside Rg_1, Re, Rb_1, Rc, Rb_2 and Rd in the extract of sample were identified by comparison of the retention times with that obtained from the chromatograms of mixed ginsenoside standards. The identification of other ginsenosides depends on ESI-MS ion fragments and those reported in previously studies (Popovich & Kitts, 2004; Zhang et al., 2003).

3.2. The effect of solvent

Extraction solvent is one of important conditions for extraction. Generally, extraction efficiency depends on the solubility of the analytes in extraction solvent. In HPMAE, the rate of system temperature and pressure increase seemed to be related to the solvent's ability to absorb the microwave energy, its specific heat and its heat of vaporization. In this paper, the effects of different extraction solvents including methanol, 70% (v/v) ethanol–water and water were studied. The highest extraction yields of ginsenosides were obtained by three kinds of solvents under 600, 400 and 300 kPa, respectively. However, contents of ginsenosides in water extract observably decreased with the increase of pressure from 400 to 500 kPa. The results also showed that the high extraction yields were obtained by 70% (v/v) ethanol–water and the lowest extraction yields were obtained by water.

3.3. The effect of pressure

When 70% (v/v) ethanol–water was used as the solvent and the extraction time was fixed to 10 min, tests were conducted under these pressures: 100, 200, 300, 400 and 500 kPa, respectively. Fig. 1 shows the plot of ginsenosides yield as function of the extraction pressure. It shows that the contents of six neutral ginsenosides in *P. ginseng* root increase with pressure and the increase rates of the contents of ginsenosides are different. The contents of ginsenoside Rg_1 and Re increase obviously from 100 to 300 kPa but tardily from 300 to 500 kPa. The contents of ginsenoside Rb_1, Rc, Rb_2 and Rd increase slowly from 100 to 300 kPa but increased greatly from 300 to 400 kPa, and the change is slight from 400 to 500 kPa.

3.4. The effect of extraction time

The effect of microwave irradiation time on HPMAE extraction yields of ginsenosides was investigated at 400 kPa when 70% (v/v) ethanol–water was used as extraction solvent. The irradiation time was 2, 5, 10, 15 and 30 min, respectively. The results in Fig. 2 indicate that the change of the contents of the six neutral ginsenosides is very significant from 2 to 10 min. However the change from 10 to 30 min is not obvious.

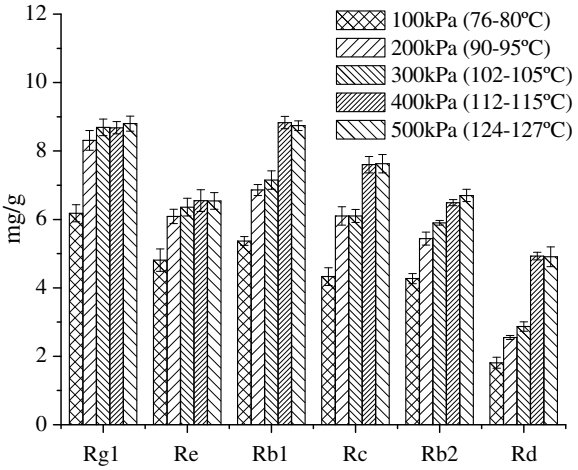

Fig. 1. The influence of extraction pressure on the extraction yields of ginsenosides.

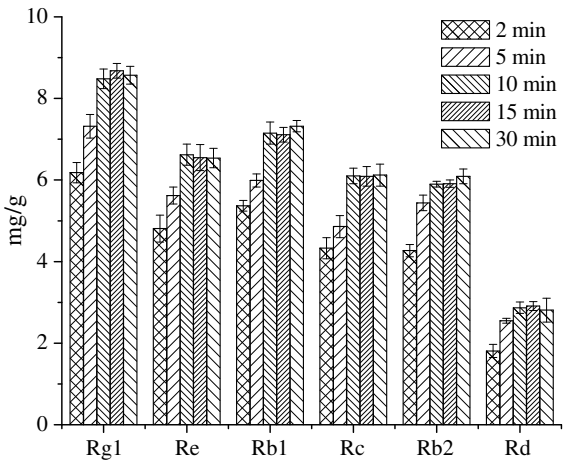

Fig. 2. The influence of microwave irradiation time on the extraction yields of ginsenosides.

3.5. Comparision of different extraction methods

HPMAE has been compared with other extraction methods, including Soxhlet extraction, ultrasound-assisted extraction and heat reflux extraction. In this experiment, 70% (v/v) ethanol–water was used as extraction solvent. Four methods were tested for extraction of six neutral ginsenoside Rg_1, Re, Rb_1, Rc, Rb_2 and Rd from *P. ginseng* root. From Table 1 it can be seen that the extraction yields of ginsenosides, especially ginsenoside Rb_1, Rc, Rb_2 and Rd, obtained by HPMAE for 10 min were much higher than those by Soxhlet extraction for 2 h, ultrasonic extraction for 1 h and heat reflux extraction for 2 h. Therefore, HPMAE is more efficient compared with the other three methods. The chromatograms of HPLC–UV of *P. ginseng* root extract obtained by Soxhlet extraction and HPMAE are shown in Fig. 3A and B respectively. It can be seen that ginsenoside m-Rb_1, m-Rc, m-Rb_2 and m-Rd exist in the extract obtained by Soxhlet extraction, but hardly can be found in extract obtained by HPMAE. Therefore, the yields of the neutral ginsenosides and malonyl ginsenosides extracted from *P. ginseng* were influenced by extraction method. For the six major ginsenoside Rg_1, Re, Rb_1, Rc, Rb_2 and Rd, HPMAE is better, but it is disadvantageous for extracting malonyl ginsenosides due to its loss during HPMAE.

3.6. Degradation of ginsenosides

The malonyl ginsenoside m-Rb_1, m-Rc, m-Rb_2 and m-Rd in *P. ginseng* root also are abundant and thermally unstable. During thermal extraction, the malonyl ginsenosides were much less stable than the corresponding neutral ginsenosides and degraded into corresponding neutral ginsenosides. Most studies have been done on the degradation of malonyl ginsenosides applying various methods. However, very little work has been carried out on the degradation of malonyl ginsenosides in HPMAE. In our study, HPLC–ESI-MS has been employed for the analysis of ginsenosides in *P. ginseng* root extract obtained by HPMAE. The results indicated that the malonyl ginsenosides disappeared after 400 kPa while the corresponding neutral ginsenosides increased. Table 2 shows the major ion fragments detected by ESI-MS in HPMAE at 100 and 500 kPa. It can be seen from Fig. 1 that the contents of ginsenosides Rg_1 and Re do not change with the increase of pressure from 300 to 400 kPa. The contents of ginsenoside Rb_1, Rc, Rb_2 and Rd increase greatly from 300 to 400 kPa, because malonyl ginsenoside m-Rb_1, m-Rc, m-Rb_2 and m-Rd degrade at 400 kPa and transform into corresponding neutral ginsenoside Rb_1, Rc, Rb_2 and Rd, when system temperature is 109–112 °C. In methanol extract, malonyl ginsenosides degrade at 600 kPa, when temperature is 112–115 °C. The degradation of malonyl ginsenosides does not occur in Soxhlet extraction, ultrasound-assisted extraction and heat reflux extraction where the temperature used for the extraction systems is less than 80 °C. On one hand, the pressure or temperature can accelerate the extraction process, but on the other hand the chemical change of some compounds may occur in too high pressure or under very high temperature. In our work, it is hard to decide which one plays the key role. Zhang et al. (2006) reported the extraction of ginsenosides from American ginseng root applying ultrahigh pressure extraction (UPE) at room temperature. In their work at 500 kPa, the degradation of ginsenosides could not be seen. So we may deduce that degradation of malonyl ginsenosides may be related to high temperature not pressure.

When water was used as a solvent, the contents of six neutral ginsenosides were the highest when the pressure was up to 300 kPa, but decreased with the increase of pressure from 400 to 600 kPa. Six neutral ginsenosides also

Table 1
Comparison of ginsenoside contents in 70% (v/v) ethanol extracts from *P. ginseng* root by different extraction methods

Method	Extraction time	Content of ginsenosides (mg/g[a] dry plant material)						Total content (mg/g)
		Rg_1	Re	Rb_1	Rc	Rb_2	Rd	
HPMAE[b]	10 min	8.80 ± 0.19	6.54 ± 0.24	8.74 ± 0.21	7.63 ± 0.19	6.70 ± 0.41	4.91 ± 0.26	43.32 ± 1.50
Soxhlet	2 h	8.55 ± 0.41	6.61 ± 0.41	6.62 ± 0.27	5.87 ± 0.33	5.66 ± 0.29	3.82 ± 0.25	37.13 ± 1.96
Ultrasonic-assisted	1 h	8.31 ± 0.23	6.21 ± 0.31	6.46 ± 0.19	5.72 ± 0.51	5.41 ± 0.48	3.64 ± 0.14	35.85 ± 1.63
Heat reflux	2 h	8.58 ± 0.31	6.36 ± 0.22	7.15 ± 0.34	6.10 ± 0.13	5.90 ± 0.26	2.87 ± 0.08	37.89 ± 1.34

[a] Mean value ± standard deviation ($n = 3$).
[b] The pressure of HPMAE is 400 kPa.

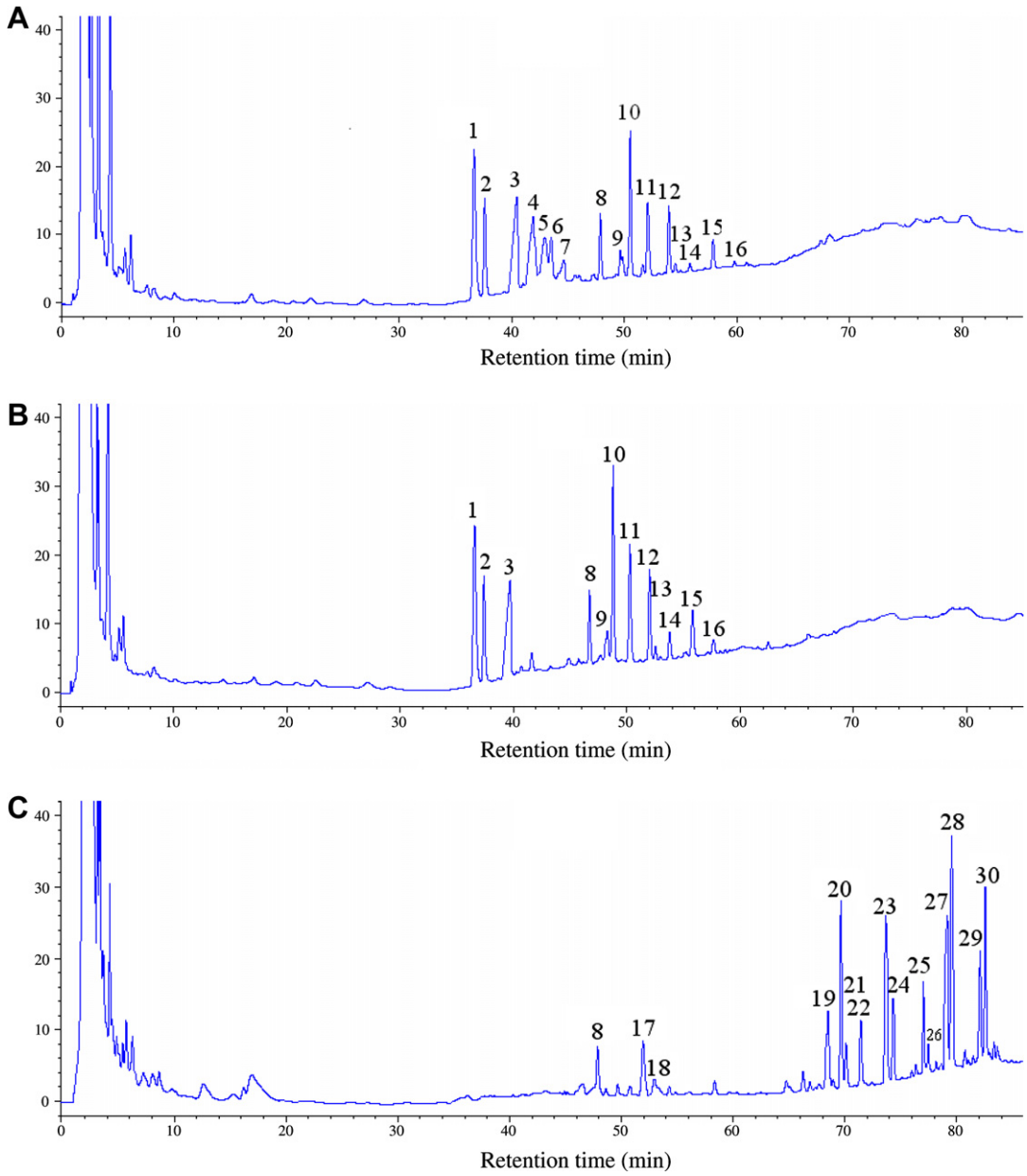

Fig. 3. Typical chromatograms of *P. ginseng* root extracts obtained by: (A) Soxhlet extraction using 70% (v/v) ethanol; (B) HPMAE using 70% (v/v) ethanol; (C) HPME using water. 1. Rg_1, 2. Re, 3. Ro, 4. m-Rb_1, 5. m-Rc, 6. m-Rb_2, 7. m-Rd, 8. Rf, 9. Ra_1/Ra_2, 10. Rb_1, 11. Rc, 12. Rb_2, 13. Rb_3, 14. No report, 15. Rd, 16. Rs_1/Rs_2, 17. Rh1, 18. No report, 19. Rg2 iso, 20. Rg2 iso, 21. Rg6 iso, 22. Not report, 23. Rg3, 24. Rg3 iso, 25. Rs3 iso, 26. Rs3 iso, 27. Rk1/Rk5, 28. Rk1/Rk5, 29. Rs4/Rs5, 30. Rs4/Rs5.

degraded in water extract under 400 kPa, when the temperature of extraction was 135–140 °C. The chromatograms of water extract of root by HPMAE at 600 kPa are shown in Fig. 3C, which show that peaks of neutral ginsenosides and malonyl ginsenosides almost disappeared and a number of less polarity constituents appeared in the retention time ranging from 68 to 85 min. Table 3 shows the ESI-MS analysis results of extract obtained by HPMAE at 600 kPa using water as extraction solvent. In the water extract, 15 kinds of ginsenosides were identified and there were rare ginsenosides: Rh1, Rg3, Rg2, Rg5, Rk1 etc. These ginsenosides, valued for pharmaceutical use (Lee et al., 2003; Yue et al., 2006), are not naturally present in *P. ginseng* but in the degradation products of ginsenosides. When 70% (v/v) ethanol was used as solvent, pressure was set at 1000 kPa and temperature was 150–155 °C, degradation of neutral ginsenoside was not observed. So the degradation of neutral ginsenosides may be related to the kind of solvent employed. Processing conditions have been reported to affect the composition of ginsenosides. For example, Popovich and Kitts (2004) reported that Rg3 and Rh2 from North American ginseng were detected following hot water reflux extraction, but not from tissues extracted with 80% aqueous ethanol at room temperature.

Table 2
ESI-MS ion fragments of ginsenosides in 70% (v/v) ethanol extracts obtained by HPMAE at 100 kPa and 500 kPa

Peak identification		t_R (min)	Main fragment ions (m/z)		Others (m/z)		
			$M+N_a^+$	$M+K^+$			
100 kPa							
1	Rg_1	36.5	823.5		643.7	789.7	
2	Re	37.6	969.5		643.7	789.7	
3	Ro	40.4	979.5	995.3	1033.6		
4	Malony-Rb_1	41.7	1218.2	1233.5	1187.8	1203.7	875.7
5	Malony-Rc	42.4	1187.5	1203.4	1319.6	1335.4	
6	Malony-Rb_2	43.3	1187.5	1203.4	875.9	891.7	1149.9
7	Malony-Rd	44.4	1055.5	1071.5	875.3	891.3	
8	Rf	47.4	823.6	839.3	1191.3		
9	Rb_1	50.5	1129.7		1233.7	796.3	
10	Rg2	51.7	807.5	823.4	405.5	423.4	
11	Rc	52.4	1102.0	1117.4	1233.7	1249.4	
12	Rb_2	54.2	1101.6	1117.6	789.8	805.6	
13	Rb_3	54.7	1101.6	1117.6	789.4		
14	No report	55.8	1173.7	1189.6	831.7	847.6	
15	Rd	58.0	969.6	985.5	1143.4	1159.6	789.7
16	Rs_1/Rs_2	59.9	1143.7	1159.7	831.7	847.8	
500 kPa							
1	Rg_1	36.5	823.7	839.6	643.8		
2	Re	37.6	969.7	985.6	643.8	789.8	
3	Ro	40.4	979.7	995.7	817.6	641.6	
8	Rf	47.4	823.5	839.5			
9	Rb_1	50.5	1131.7	1147.6	789.6		
10	Rg2	51.7	807.5	823.4	405.5	423.4	
11	Rc	52.4	1101.7	1117.6	789.4		
12	Rb_2	54.2	1101.6	1117.6	789.8	805.6	
13	Rb_3	54.7	1101.6	1117.7	789.4		
14	No report	55.8	1173.7	1189.6	831.7	847.6	
15	Rd	58.0	969.7	985.7	789.7	831.6	1143.6
16	Rs_1/Rs_2	59.9	1143.7	1159.7	831.7	847.8	

Table 3
ESI-MS ion fragments of ginsenosides in water extract obtained by HPMAE at 600 kPa

Peak identification		t_R (min)	Main fragment ions (m/z)		Others (m/z)	
			$M+N_a^+$	$M+K^+$		
8	Rf	47.5	823.6	839.3	1191.3	
17	Rh1	51.9	661.9	683.6	807.6	829.7
18	No report	52.8	805.7	827.8		
19	Rg2 iso	68.4	807.7	823.6		
20	Rg2 iso	69.4	807.7		789.6	805.7
21	Rg6 iso	69.9	789.8	811.8	792.4	
22	No report	71.7	849.8	865.7	789.9	
23	Rg3	73.8	807.7	823.6	845.6	789.6
24	Rg3 iso	74.4	807.6	823.6		
25	Rs3 iso	76.9	849.8	865.7		
26	Rs3 iso	77.3	849.9	865.7		
27	Rk1/Rk5	79.1	789.7	791.5	805.7	
28	Rk1/Rk5	79.8	789.7		805.6	
29	Rs4/Rs5	81.8	831.6	847.6		
30	Rs4/Rs5	82.6	831.6	847.6		

4. Conclusions

The HPMAE, as a relatively new extraction method, was used to extract ginsenosides from *P. ginseng* root. Comparing with other extraction methods, HPMAE had excellent advantages, such as shorter extraction time, and a higher yield. In the kind of solvent employed, extraction pressure and extraction time, the optimization conditions of HPMAE for six neutral ginsenosides in *P. ginseng* root were: 70% (v/v) ethanol–water solution, extraction pressure of 400 kPa, and extraction time of 10 min. The malonyl ginsenoside m-Rb_1, m-Rc, m-Rb_2 and m-Rd degraded under HPMAE and transformed into the corresponding neutral ginsenoside Rb_1, Rc, Rb_2 and Rd. Therefore, extraction yields of ginsenoside Rb_1, Rc, Rb_2 and Rd obtained by HPMAE was much higher than those obtained by other extraction methods. When water was used as a solvent, six neutral ginsenosides extracted from *P. ginseng* root by HPMAE also degraded. Some rare ginsenosides were also found in the extract.

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