ISSN 0926-6690

ELSEVIER

INDUSTRIAL CROPS AND PRODUCTS

AN INTERNATIONAL JOURNAL

Industrial Crops & Products 117 (2018) 118–127

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Subcritical water extraction of bioactive components from ginseng roots (*Panax ginseng* C.A. Mey)

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ARTICLE INFO

Keywords:
Ginseng roots
Subcritical water extraction (SWE)
Antioxidant activity
Polyphenols
Ginsenosides

ABSTRACT

The feasibility of applying subcritical water extraction (SWE), which is considered to be an environmentally friendly and efficient extraction technology for the extraction of bioactive components from ginseng roots, was evaluated by comparing it with conventional water (WE) and ethanol (EE) extraction methods SWE was conducted at different temperatures ranging from 120 to 200 °C, and WE and EE were performed by solid–liquid heating extraction methods using water or 70% (v/v) aqueous ethanol as a solvent, respectively. SWE showed significantly and markedly higher extraction yields of total sugar (TS), total protein (TPro), phenolic components (TP), and more potent antioxidant activities than WE and EE. The optimized temperature for TS, TP, and antioxidant activities was 200 °C, and that for TPro was 180 °C. Although vanillin–perchloric acid colorimetric quantification showed that SWE yielded more total ginsenosides (TG) than WE and EE, ultrafast liquid chromatography tandem mass spectroscopy (UFLC–MS/MS) analysis revealed that SWE induced extensive hydrolysis of the ginsenosides, except for Rg_2. At 160 °C, SWE yielded 9.7- and 6.2-fold more Rg_2 than WE and EE, respectively. In comparison, the extraction yields of R_1, Rb_1, Rb_2, Rb_3, Rc, Rd, Re, Rg_1, and Ro decreased significantly with the increase of SWE temperature. These findings suggested that SWE is a promising environmentally friendly and efficient technology for extracting bioactive polysaccharides, proteins, TP, and Rg_2 from ginseng roots, but it has potency to induce extensive hydrolysis of ginsenosides, such as Rb_1 and Re.

1. Introduction

Ginseng, the dry root of *Panax ginseng* C.A. Mey. (Araliaceae), is a well-known traditional Chinese medicine with numerous pharmacological effects, including antioxidant (Jung et al., 2006), anti-inflammatory (Lee et al., 2008), antidiabetic (Yun et al., 2004), antineoplastic (Baek et al., 1995), cardiovascular (Chen, 1996), immunoregulatory (Du et al., 2008), and neuroregulatory activities (Tsang et al., 1985; Rausch et al., 2006; Ru et al., 2015). These bioactivities are attributed to various bioactive components in ginseng, such as the ginsenosides, polyphenols, amino acids, and polysaccharides (Lee et al., 2015a, 2015b; Wu and Zhong, 1999). To extract these bioactive components from ginseng, various conventional extraction methods have been developed, among which the conventional solid–liquid heating extraction method using aqueous or organic solvents such as methanol, ethanol, and *n*-butanol (Jung et al., 2006) is widely used. However, most of these conventional methods are time-consuming, energy-inefficient, and involve some organic solvents that are potentially toxic to the environment or human health (Lee et al., 2014; Mlyuka et al., 2016). Therefore, there is a need for new extraction technologies with low processing costs, mild operating conditions, short processing times, and environmentally friendly solvents.

Subcritical water extraction (SWE) is considered to be an environmentally friendly extraction method due to it involving the use of water. Under ambient conditions, water acts as an extremely polar solvent and cannot be used for the extraction of moderately polar and non-polar compounds (Yan et al., 2017). However, subcritical water has a lower dielectric constant (ε) and lower viscosity but higher diffusivity, which promotes diffusion into the plant matrix and the release of moderately polar and non-polar compounds from the solid to the liquid phase (Teo et al., 2010). Therefore, SWE has been used extensively for extracting active ingredients from traditional medicinal plants, with the advantages of a short extraction time, high efficiency, and low energy consumption (Gong et al., 2015; Pavlić et al., 2016). For example, SWE has been successfully used for the extraction of total phenols and total flavonoids from the herbal dust of sage (Pavlić et al., 2016) and flower

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https://doi.org/10.1016/j.indcrop.2018.02.079

residues of marigold (Xu et al., 2015). Compared with conventional methods, SWE showed significantly higher extraction yields of total phenols and total flavonoids within a very short extraction time. In addition, SWE was used for the extraction of polysaccharides from *Grifola frondosa* (Yang et al., 2013) and triterpenoids from dry loquat leaves of *Eriobotrya japonica* (Mlyuka et al., 2016), and showed higher extraction yields than WE, conventional solid–liquid extraction, and Soxhlet extraction methods.

However, little research has been conducted on the feasibility of SWE as a rapid and efficient method for extracting bioactive components from ginseng. In a recent study conducted by Lee et al. (2014) SWE extracts of ginseng leaves and stems at 190 °C showed much higher cytotoxicity against human cancer cell lines than ethanol extract (Lee et al., 2014). However, the differences in chemical profiles of SWE and ethanol extract were not clearly elucidated in this previous study.

Therefore, the main objective of the present study was to evaluate the feasibility of using SWE for the extraction of bioactive components from ginseng roots. Considering the significant effects of temperature on the ε, viscosity, surface tension, and molecular diffusion rate of water, which would eventually influence the extraction yield of target components (Smith, 2002; Al-Farsi and Lee, 2008), SWE was conducted at different temperatures ranging from 120 to 200 °C. The extraction yields of total sugar (TS), total protein (TPro), total polyphenols (TP), total ginsenosides (TG), and ten major ginsenosides, as well as the antioxidant activities of the extracts, were evaluated and further compared to those of conventional EE and WE methods.

2. Materials and methods

2.1. Materials, reagents and standards

Ginseng samples were purchased from Beijing Tong Ren Tang (Group) Lit. Corp (Beijing, China). Dried samples were ground into powder (60 mesh) using a pulverizer, sealed and stored at −20 °C until analysis. Ten reference standards of ginsenosides, including 20(*S*)-quinquenoside R_1 (R_1), 20(*S*)-ginsenoside Rb_1 (Rb_1), 20(*S*)-ginsenoside Rb_2 (Rb_2), 20(*S*)-ginsenoside Rb_3 (Rb_3), 20(*S*)-ginsenoside Rc (Rc), 20(*S*)-ginsenoside Rd (Rd), 20(*S*)-ginsenoside Re (Re), 20(*S*)-ginsenoside Rg_1 (Rg_1), 20(*S*)-ginsenoside Rg_2 (Rg_2) and ginsenoside Ro (Ro), were obtained from National Institute for Pharmaceutical and Biological Products (Beijing, China). Coomassie Brilliant Blue G-250, bovine serum albumin (BSA), Folin-Ciocalteu's phenol reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-Azinobis(3-ethyl- benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile, methanol, and formic acid were obtained from Fisher Scientific Co. (Waltham, USA). Other chemicals and reagents (analytical grade) were purchased from Beijing Chemical Co. (Beijing, China). Water was generated by a Synergy 185 Ultrapure Water System (Millipore, MA, USA).

2.2. Conventional water/ethanol extraction

Comparative extraction was conducted using conventional solid–liquid heating extraction method with pure water or 70% (v/v) aqueous ethanol as a solvent, which has been extensively used in the extraction of ginseng (Jung et al., 2006). For both ethanol and water extraction, 5 g of ginseng powder was mixed with 150 mL of extraction solvents, and extracted for 3 h at 100 °C and 60 °C, respectively. Then, the slurry was filtered, and the solid residue was extracted twice under the same conditions. After extraction, all extracts were collected, emerged and evaporated using a rotary evaporator (Rotavapor® R-300, Flawil, Switzerland), respectively. Finally, resulting extracts were transferred to freeze-drying tubes and lyophilized, after which the dried samples were then weighed and stored at −20 °C until further analysis. The total extraction yield (*Ye*) was calculated by using the following equation:

$$Ye = W_1/W_0 \times 100\%$$

Where W_1 and W_0 are weights of extract and ginseng samples in gram on dried basis, respectively.

2.3. Subcritical water extraction

SWE was carried out using an SWE apparatus (Jiangsu Huaan Scientific Research Devices Co., Ltd., Nantong, Jiangsu, China) according to a previous report (Yan et al., 2017). 5 g of ginseng powder was loaded into the extraction chamber (stainless steel vessel with an inner volume of 600 mL) packed in the oven with a heating jacket. The oven was connected with pressure and temperature sensors, and the parameters were monitored and shown on digital displays. After closing the stainless steel cap, 150 mL of deionized water was injected into the chamber, and nitrogen was employed to remove dissolved oxygen before extraction. The extraction pressure was set at 6.0 MPa and the temperature at 120, 140, 160, 180, and 200 °C, respectively. All samples were extracted twice, 20 min for each time, after which all resulting extracts were filtered under vacuum, lyophilized and finally stored at −20 °C until analysis. The yield of the extract was calculated as described above.

2.4. Determination of fourier-transform infrared spectroscopy

The Fourier-transform infrared (FT-IR) spectroscopy of ginseng extracts were recorded on a Spectrum GX FT-IR spectrometer (Perkin Elmer, Inc). Briefly, ginseng extracts were incorporated with KBr powders (spectroscopic grade), and then pressed into pellets and scanned with a blank KBr as background. The FT-IR spectra were recorded using a DTGS detector in a transmittance mode over the range of 400–4000 cm^{-1} at a resolution of 4 cm^{-1} and 32 scans per sample.

2.5. Determination of total ginsenosides extraction yields

The TG content in extracts were estimated according to the method described in the Pharmacopeia of People's Republic of China (2015 Edition). Briefly, 50 mg extract was dissolved in 20 mL distilled water and extracted with 20 mL water-saturated *n*-butanol for 4 times in a separatory funnel. The upper liquid was evaporated to dryness and redissolved in 10 mL of methanol. Then, 50 μL of the extracts were transferred to glass tubes and evaporated to dryness. After which, 0.5 mL of 1% vanillin-perchloric acid solution (w/v) was added and incubated at 60 °C for 15 min after fully mixed. After incubation, the mixture was cooled down immediately in an ice bath for 2 min, and 5 mL of 77% sulfuric acid solution was added with vigorous shaking. Finally, the absorbance at 540 nm was measured using a UV spectrophotometer (TU 1810, Beijing, China). The TG content (*C*) in extracts was calculated using an external calibration curve plotted using ginsenoside Re as standard (0.01–0.2 mg/g, $r = 0.9989$), and expressed as mg Re equivalents (mg ReE)/g extract. The extraction yield (*Y*) of TG was calculated as $Y = C/Ye$ and expressed as mg ReE/g ginseng roots.

2.6. Determination of total sugar extraction yields

The TS content in freeze–dried ginseng extract was quantified by a slightly modified phenol-sulfuric acid method (Dubois et al., 1956; Lee et al., 2015a, 2015b). In brief, 1.0 mL of water solution of ginseng extracts (0.1 mg/mL) was mixed with 1.0 mL of phenol solution (6%, w/v), after which, 5.0 mL of concentrated sulfuric acid was added dropwise. Then, the mixture was fully mixed and left for reaction for 20 min. Finally, the absorbance at 490 nm was recorded against a blank using a UV spectrophotometer (TU 1810, Beijing, China) at ambient conditions. The content of TS in ginseng extracts (*C*) was calculated based on an

Table 1
Chemical constituents of *Panax ginseng* C. A. Mey and selective ion-pairs, DP, CE, and retention time of the ten ginsenosides.

No.	Name	R_1	R_2	Reference[a]	Ion-pairs I				Ion-pairs II				Rt
					Q1	Q3	DP	CE	Q1	Q3	DP	CE	(min)
Protopanaxadiol ginsenosides													
1	20*S*-quinquenoside R_1	-glc(2-1)glc(6)Ac	-glc(6-1)glc	*1,2*	933.455	753.6	96	11	933.455	423.4	96	25	4.19
2	20*S*-ginsenoside Rb_1	-glc(2-1)glc	-glc(6-1)glc	*1,2,3,4*	1109.6	325.2	150	34	1109.6	163.1	150	55	5.23
3	20*S*-ginsenoside Rb_2	-glc(2-1)glc	-glc(6-1)ara(p)	*1,2,4*	1079.5	325.2	130	32	1079.5	767.5	130	20	5.42
4	20*S*-ginsenoside Rb_3	-glc(2-1)glc	-glc(6-1)xyl	*1,2,4*	1079.5	325.2	130	32	1079.5	767.5	130	20	
5	20*S*-ginsenoside Rc	-glc(2-1)glc	-glc(6-1)ara(f)	*2,3,4*	1079.5	325.2	50	37	1079.5	407.5	50	40	5.32
6	20*S*-ginsenoside Rd	-glc(2-1)glc	-glc	*1,2,3,4*	947.463	325.2	91	25	947.463	425.3	91	25	5.68
Protopanaxatriol ginsenosides													
7	20*S*-ginsenoside Re	-glc(2-1)rha	-glc	*1,2,3,4*	947.6	423.4	100	23	947.6	309.2	100	25	4.33
8	20*S*-ginsenoside Rg_1	-glc	-glc	*1,2,3,4*	801.412	423.4	96	21	801.412	621.5	96	11	4.34
9	20*S*-ginsenoside Rg_2	-glc(2-1)rha	-H	*1,2,4*	785.386	423.4	31	19	785.386	441.4	31	13	5.48
Oleanane ginsenosides													
10	ginsenoside Ro	-glcUA(2-1)glc	-glc	*2*	974.6	439.4	80	31	974.6	795.5	80	19	5.49

[a] References: 1, Bai and Gänzle, 2015; 2, Ru et al., 2015; 3, Hsu et al., 2013; 4, Wang et al., 2016.

external calibration curve using d-glucose as standards (8–40 μg/mL, $r = 0.9998$) and expressed as mg d-glucose equivalents (mg GE)/g extract. The extraction yield (Y) of TS was calculated as C/Ye and expressed as mg GE/g ginseng roots.

2.7. Determination of total protein extraction yields

The TPro content in extracts was measured using the Bradford assay according to the literature (Meijer and Haeringen, 1992) with little modification. Briefly, 1.0 mL of appropriately diluted aqueous solution of the extract was mixed with 4.0 mL of working solution prepared by mixing 10 mg Coomassie Brilliant Blue-G250, 5 mL ethanol (95%, v/v) and 12 mL phosphoric acid (85%, v/v) to a final volume of 100 mL. After 5 min, the absorbance at 595 nm was recorded by a UV spectrophotometer (TU 1810, Beijing, China), and the content (C) was calculated based on a calibration curve of BSA (0.0106–0.106 μg BSA/ml, $r = 0.9995$), expressed as mg BSA equivalents (mg BSAE)/g extract. The TPro extraction yield (Y) was calculated as C/Ye, and expressed as mg BSAE/g ginseng roots.

2.8. Determination of total polyphenols extraction yields

The TP content in extracts was measured by Folin–Ciocalteu method as described in the literature (Chung et al., 2016). Briefly, 20 μL of the aqueous solution of ginseng extract was mixed with water (1.58 mL) and 100 μL of Folin–Ciocalteu reagent. 8.5 min later, 300 μL of a saturated solution of sodium carbonate was added, and then the mixture was fully shaken and incubated in dark for 2 h. Finally, the absorbance at 765 nm was recorded, and the TP content in extracts (C) was calculated using a calibration curve of gallic acid (GA) (32.1–321.0 μg BSA/ml, $r = 0.9988$). The extraction yield of TP (Y) was calculated as C/Ye and expressed as mg GA equivalents (mg GAE)/g ginseng.

2.9. Evaluation of antioxidant activities

ABTS and DPPH radicals scavenging activities of ginseng extracts were evaluated as described by Yan et al. (2017). For the DPPH assay, 0.1 mL of aqueous extract solution was mixed with freshly prepared DPPH solution (0.25 mL, 1 mM dissolved in methanol) and methanol (2.0 mL), and then the mixture was incubated in the dark for 20 min at ambient conditions before the absorbance was recorded at 517 nm. For the ABTS assay, 0.2 mL of the aqueous extract solution was mixed thoroughly with 2.8 mL of ABTS•+ working solution, and after 6–10 min in the dark at room temperature, the absorbance was read at 734 nm. In both assays, a series of concentrations of trolox were used to plot the calibration curves (10–60 μg trolox/ml, $r = 0.9950$ for the DPPH assay and 10–80 μg trolox/ml, $r = 0.9993$ for the ABTS assay) and the antioxidant activities of extracts (Ax) were expressed as mg trolox equivalent antioxidant capacity (TEAC)/g extract. The extraction yield of antioxidant capacities (Y) was calculated as Ax/Ye and expressed as mg TEAC/g ginseng roots.

2.10. Quantification of individual ginsenoside by ultrafast liquid chromatography/tandem mass spectrometry (UFLC–MS/MS)

Quantification of ten ginsenosides in extracts was performed on a SHIMADZU 20AXR UFLC System coupled with a QTRAP®6500 triple quadrupole tandem mass spectrometer (Applied Biosystems, MDS Sciex, Canada). Separation of standards was achieved on a Phenomonex F5 column (100 × 2.6 mm i.d., 2.6 μm; Dikma) with a column oven temperature of 35 °C. The mobile phase was composed of (A) water-formic acid (0.1%, v/v) and (B) acetonitrile-formic acid (0.1%, v/v). A linear gradient elution program was as follows: 0–0.5 min, 10% B; 0.5–5.4 min, 10% to 50% B; 5.4–5.5 min, 50% to 90% B; 5.5–8.0 min, 90% B; 8.0–8.1 min, 90% to 10% B; 8.1–11 min, 10% B. The flow rate was kept at 0.4 mL/min, and 10 μL of the extract dissolved in 50% (v/v) methanol was injected in each run. Multiple reaction monitoring (MRM) was used for detecting transitions in a positive ionization mode. The selective ion-pairs, DP (V), and CE (V) of the ten ginsenosides were shown in Table 1. Other operating parameters were set as follows: spray voltage, 5500 V; source temperature, 550 °C; nebulizing gas (GS1), 50 psi; heating gas (GS2), 55 psi; and curtain gas, 12 psi, respectively. External calibration standards were prepared in 50% (v/v) methanol, and the calibration curves of ten ginsenosides were established with ten concentrations ranging from 0.2 to 1000 ng/mL. The calibration curve was linear over the entire concentration range and the Pearson's correlation coefficient (r) was more than 0.99. The limit of detection (LOD) and limit of quantification (LOQ) of the ginsenosides ranged from 0.016 to 0.253 ng/mL, as shown in Table S1. Analyst 1.6.1 software (Applied Biosystems, MDS Sciex, Canada) was used to analyze and process all data collected from MS.

2.11. Statistical analysis

All extractions and tests were carried out in triplicate and the data expressed as means ± standard deviation (SD). Analysis of variance (ANOVA) and Duncan's multiple range tests were performed using IBM SPSS statistical 22.0 software (SPSS, Inc., Chicago, IL, USA). Statically significant differences were considered when $p < 0.01$. Figures were drawn with the Origin software 10.4 (OriginLab, USA). Radar chart

Table 2
The comparative results of SWE and conventional WE/EE methods.

No.	Extraction Conditions	*Ye* (%)	TG (mg ReE/g)	TS (mg GE/g)	TPro (mg BSAE/g)	TP (mg GAE/g)
1	WE 100 °C	14.71 ± 0.42^{G}	21.33 ± 1.36^{D}	15.07 ± 0.11^{D}	2.88 ± 0.19^{E}	2.00 ± 0.04^{G}
2	SWE 120 °C	14.92 ± 0.85^{F}	42.64 ± 2.38^{C}	7.03 ± 0.28^{G}	7.14 ± 0.99^{D}	6.54 ± 0.11^{E}
3	SWE 140 °C	28.33 ± 1.20^{D}	52.05 ± 2.44^{B}	10.40 ± 0.25^{F}	12.87 ± 0.42^{C}	6.94 ± 0.09^{D}
4	SWE 160 °C	52.92 ± 1.05^{C}	57.58 ± 1.04^{B}	18.58 ± 0.28^{C}	20.04 ± 0.69^{B}	11.19 ± 0.10^{C}
5	SWE 180 °C	59.33 ± 0.66^{B}	68.92 ± 2.07^{A}	42.81 ± 0.88^{B}	24.83 ± 1.16^{A}	22.90 ± 0.07^{B}
6	SWE 200 °C	63.08 ± 0.85^{A}	57.86 ± 1.79^{B}	67.87 ± 0.75^{A}	14.22 ± 0.51^{C}	24.84 ± 0.11^{A}
7	EE 60 °C	17.96 ± 0.29^{E}	65.05 ± 1.04^{A}	13.29 ± 0.21^{E}	4.50 ± 0.22^{E}	3.52 ± 0.03^{F}

All results were expressed as mean ± standard deviation (n = 3).
Different capital letters mean significantly differences with $p < 0.01$.
WE, water extraction; SWE, subcritical water extraction; EE, ethanol extraction.

analysis was performed using Microsoft Excel software 2010.

3. Results and discussion

3.1. Extraction yields of different methods

The *Ye* values of SWE at different temperatures and conventional water/ethanol solid–liquid heating extraction methods are listed in Table 2. For SWE, *Ye* increased significantly with temperature, and peaked at 63.1% at 200 °C. The 70% ethanol extraction (EE) method showed higher *Ye* than WE and SWE at 120 °C. However, the *Ye* of SWE showed much higher values when the temperature was over 140 °C. These findings indicate that the diffusivity of water increased dramatically with increasing temperature, which favors the diffusion of water into the ginseng matrix and the release of components during the solid to liquid mass transfer.

3.2. FT-IR spectroscopy analysis

FT-IR spectroscopy is widely used in qualitative and quantitative analyses of either pure compounds or complex mixtures. In this study, this approach was used as a rapid and simple method to obtain a general view of chemical bonds and functional groups in the complex extracts from ginseng. All SWE samples showed spectral profiles similar to those of the WE and EE samples, and variations were reflected in the absorption intensity and characteristics at corresponding wavelengths (Fig. 1). A broad absorption peak at 3388–3401 cm^{-1} was recorded for all samples, which could be assigned to the stretching vibrations of an O—H bond, indicating the presence of an intramolecular or intermolecular hydrogen bond (Xie et al., 2013; Li et al., 2017). The weak absorption bands at 2928–2932 cm^{-1} and 1636 cm^{-1} were attributed to C—H stretching vibrations and hydrogen bonding between cellulose and water molecules, respectively (Li et al., 2017; Wen et al., 2017). The absorption bands in the range of 1636–1658 cm^{-1} as well as 1411–1414 cm^{-1} were ascribed to C=O asymmetric and symmetric stretching vibrations (Li et al., 2017). The N—H bending vibration was also present in this range, indicating the probability of the presence of a protein component (Xie et al., 2013). Characteristic absorption in the range of 1154–1024 cm^{-1} represented stretching vibrations of C—O, which was similar to the absorption peak of ginseng polysaccharide, demonstrating the existence of a polysaccharide component (Wan et al., 2012; Wen et al., 2017). The absorption at 1078 cm^{-1} was assigned to the asymmetric stretching vibration of the C—O—C bond, the specific spectral signal of dextran corresponding to the absorption peak around 1411–1414 cm^{-1}, which confirmed the existence of an oligosaccharide component. In addition, the weak bands at about 939–924 cm^{-1} and 760–762 cm^{-1} were typical absorptions for d-glucose in pyranose form (Wan et al., 2012).

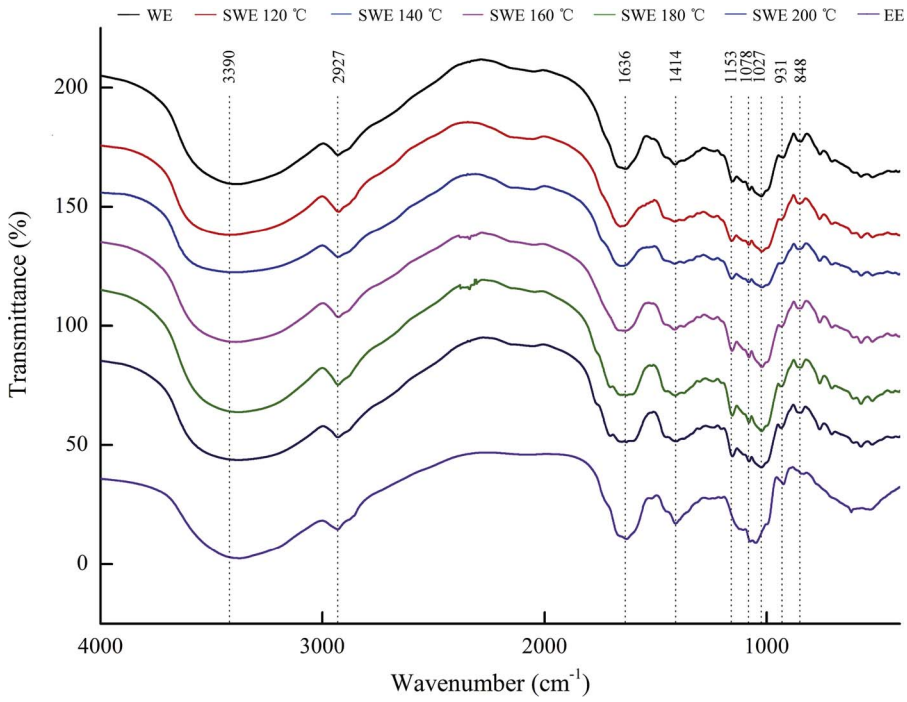

Fig. 1. Fourier-transform infrared spectra of ginseng extracts obtained by subcritical water extraction (SWE) and conventional water (WE)/ethanol extraction (EE).

The identification and quantitative analysis of ginseng extracts conducted using the conventional infrared spectra were insufficient, due to the superimposed peaks and low resolution. To distinguish the overlapping absorption peaks and enhance the characteristics of the spectrum with improved apparent resolution, a transition of classical spectra to second-derivative spectra was performed, as shown in Fig. S1. Characteristic absorptions in the range of 1154–1024 cm^{-1}, which represent the polysaccharide composition, were significantly strengthened in SWE extracts, and the intensities increased from 120 to 200 °C, indicating the improved extraction efficiency of TS. Similar phenomena were also observed at about 1078, 924, and 762 cm^{-1}, demonstrating the increased yields of the polysaccharide and protein components in SWE extracts. The absorption bands in the ranges of 1337–1322 cm^{-1} and 1231–1200 cm^{-1} were ascribed to O—H stretching vibration and bending vibration of C—O (Sun et al., 2010); the enhanced intensities in SWE extracts could indicate higher yields of phenolic components (TP).

These observations from FT-IR analysis indicated that all of the ginseng extracts were composed of several kinds of components, which may include polysaccharides, proteins, and polyphenols. The absorption intensities of characteristic absorption bands also indicated the different contents of these components in different extracts. Therefore, the yields of TS, TPro, TP, as well as TG and individual ginsenosides, the characteristic components of ginseng, were further compared to reveal the differences of these extraction methods.

3.3. Total sugar and protein extraction yields

As shown in Table 2, the TS yields were much lower than those of WE and EE when the SWE temperature was lower than 160 °C ($p < 0.01$), which could be attributable to the shorter extraction time in SWE treatment. However, when the SWE temperature was above 160 °C, the TS yields were much higher than those of WE and EE, even though the SWE extraction time was much shorter. During the SWE process, the TS yields increased constantly and significantly from 7.03 mg GE/g to 67.87 mg GE/g with the rise of temperature from 120 to 200 °C. Similar results were observed in the SWE of polysaccharides from *Grifola frondosa*, where the yields of polysaccharides increased significantly as the temperature increased from 100 to 230 °C (Yang et al., 2013), because higher temperature could induce enhanced permeability and biodegradation of cell wall materials to facilitate the release of polysaccharides (Yang et al., 2013; Gong et al., 2015).

Owing to the increase of permeability and biodegradation of cell wall materials under SWE conditions, the TPro yields were increased significantly with the rise of temperature, and reached a maximum value of 24.83 BSAE/g at SWE 180 °C. A decline in the TPro yield was observed when the SWE temperature went above 180 °C, which could be attributed to the Maillard reaction (Plaza et al., 2010a, 2010b). However, the TPro yields of SWE at all temperatures were dramatically higher than those of conventional WE and EE methods, which were only 2.88 and 4.50 mg BSAE/g, respectively.

3.4. Total ginsenoside extraction yields

Ginsenosides, as unique bioactive components in ginseng, accounted for most of the pharmacological effects (Attele et al., 1999; Ru et al., 2015). In this study, the SWE yields of TG (Table 2) increased initially with increasing temperature until reaching a maximum value of 68.92 mg ReE/g at 180 °C, and then decreased to 57.86 mg ReE/g at 200 °C. All SWE yields of TG were significantly higher than those of WE ($p < 0.01$), but not higher than those of EE, even though the maximum yield of TG at SWE 180 °C was almost the same as that of EE. Therefore, the extraction efficiency of TG by SWE was much higher than that of WE, but lower than that of EE. In addition, SWE showed higher TG yield than other extraction techniques, such as microwave-assisted extraction (MAE, 53.1 mg/g) (Kwon et al., 2003), ultrasonic-assisted extraction (UAE, 47.5 mg/g) (Wu et al., 2001), and high pressure microwave-assisted extraction (43.32 mg/g) (Wang et al., 2008).

3.5. Total polyphenol extraction yields

Under SWE conditions, the viscosity and surface tension of water decrease, which results in a decrease of ε (Fan et al., 2016). When the SWE temperature is elevated from 120 to 200 °C, the ε of subcritical water decreases significantly from 52 to 34, which is close to that of methanol ($\varepsilon = 33$) (Fernández et al., 1997; He et al., 2012). Applying SWE at a high temperature could extensively damage the linkage between polyphenols and other substances in plant tissue, such as the ester linkages between proteins or polysaccharides and polyphenols. However, SWE is capable of extracting free polyphenols with almost the same yield as organic solvents, as well as extract-bound polyphenols, which are not extractable by organic solvents (Kim and Mazza, 2006; Gong et al., 2015; Xu et al., 2015). The TP yields of different extraction methods are presented in Table 2, which illustrates that SWE at temperatures from 120 to 200 °C exhibited much higher TP yields than WE and EE ($p < 0.01$). The highest TP yield reached 24.84 mg GAE/g at SWE 200 °C, which was 12.4- and 7.1-fold higher than those of WE and EE, respectively. Similar results were reported by Fan et al. (2016), who demonstrated that the TP extraction yield from *Glycyrrhiza uralensis* Fisch increased with the rise of temperature and reached its maximum of 4.43 g GAE/100 g at 200 °C. Pavlić et al. also reported that SWE at high temperature showed about 65% higher yield of TP from *Salvia officinalis* L. than maceration extraction under ambient conditions (Pavlić et al., 2016).

3.6. Antioxidant activities of ginseng extracts

The antioxidant activities of ginseng extracts obtained by SWE and conventional WE/EE methods were assessed by ABTS and DPPH radical scavenging assays. The radical scavenging capabilities were greatly affected by temperature in SWE ($p < 0.01$), as shown in Fig. 2. As the temperature increased, total antioxidant activities (TAA) of the extracts improved accordingly. The highest levels of ABTS and DPPH antioxidant properties were both obtained at 200 °C. In addition, the TAA of SWE samples was significantly higher than that of conventional methods at all temperatures ($p < 0.01$), which was in agreement with the findings of a previous study (Xu et al., 2015).

The antioxidant activities of plant extracts are usually correlated with the levels of TP (Lu and Foo, 2000; Zeković et al., 2014; Gong et al., 2015). In the present study, similar trends between TP content and TAA were observed, and their r values were 0.8845 (ABTS) and 0.9572 (DPPH). These results indicate that SWE extracts exhibited much stronger antioxidant capacities than conventional methods, and the antioxidant properties of ginseng extracts were highly correlated to the content of polyphenols (Pavlić et al., 2016).

3.7. Identification and quantification of major ginsenosides by UFLC–MS/MS

Ginsenosides, also known as saponins, are considered to be the major bioactive constituents of ginseng. According to the positioning of sugar moieties at carbon (C)-3 and −6, ginsenosides can be divided into the protopanaxadiol type (I-1 type) and the protopanaxatriol type (I-2 type), and further differentiated into the 20(S) and 20(R) types based on the different substitutes of chiral carbon at the C-20 position. To date, more than 70 ginsenosides have been isolated from ginseng, among which ginsenosides Rb_1, Rb_2, Rc, Rd (I-1 type), Re, Rg_1, and Rg_2 (I-2 type) are major constituents.

In the present study, the extraction yields of ten ginsenosides, namely, R_1, Rb_1, Rb_2, Rb_3, Rc, Rd, Re, Rg_1, Rg_2, and Ro, were quantified by ultrafast liquid chromatography/tandem mass spectrometry (UFLC–MS/MS). Peaks were identified by comparison with the retention time and MS/MS patterns of reference standards (as shown in

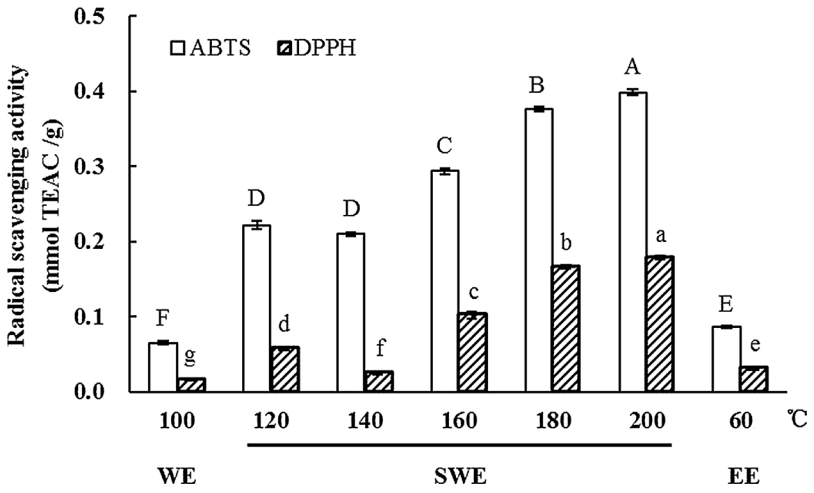

Fig. 2. ABTS and DPPH radical scavenging activities of the ginseng extracts obtained by different extraction methods. SWE, subcritical water extraction; WE, water extraction; EE, ethanol extraction. Different letters indicate a significant difference ($p < 0.05$), calculated by Duncan's multiple range tests.

Table 1). The yields of ten ginsenosides were quantified based on the calibration curves, established with ten concentrations ranging from 0.2 to 1000 ng/mL. The extracted ion chromatogram (EIC) of standard ginsenosides and ginseng extracts are shown in Fig. 3, and the extraction yields of each ginsenoside with the different extraction methods are shown in Table 3. The ginsenoside profiles in extracts obtained by SWE at different temperatures and WE/EE were noticeably different, as displayed in both Fig. 3 and Table 3. The highest total yield of the ten ginsenosides (TYs) was obtained by EE, with a value of 20,158.6 μg/g, which was about 2.78- and 2.70-fold higher than those of WE and SWE 120 °C, respectively. Unexpectedly, the TYs obtained by SWE decreased dramatically with the rise of temperature, and reached 126.1 μg/g at SWE 200 °C, which was only about 1.7% of that at SWE 120 °C.

In the case of SWE, the extraction yield of all ginsenosides in I-1 type decreased with the rise of temperature until reaching the lowest value at SWE 180 °C, and then increased slightly at SWE 200 °C. Similar tendencies of the extraction yields were observed for ginsenosides Re, Rg_1 (I-2 type), and Ro. However, ginsenoside Rg_2 showed different trends from all the others. At SWE 120 °C, its extraction yield was 892.0 μg/g, which was 4.2- and 2.7-fold higher than those of WE and EE, respectively. Moreover, the yield of Rg_2 increased with the rise of temperature and reached a peak of 2069.5 μg/g at SWE 160 °C, which was 9.7-, 6.2-, and 2.3-fold higher than those of WE, EE, and SWE 120 °C, respectively. However, when the temperature was above 180 °C, the yield of Rg_2 decreased significantly.

As reported previously, ginsenosides with high molecular weight (*Mw*) are easily hydrolyzed at low pH or high temperature to form hydrolysates with low *Mw* (Kim et al., 2000). For example, Rg_2 is one of the major hydrolysates of Re, which was one of the most abundant ginsenosides in EE and WE (Zhang et al., 2007; Park et al., 2017). Therefore, the increased extraction yields of Rg_2 and decreased yields of Re could probably be attributed to the hydrolysis of Re and the production of Rg_2 at high temperature. However, when the temperature was raised above 180 °C, Rg_2 was further hydrolyzed. Additionally, upon increasing the temperature, the polarity and ε decreased, which did not promote the solubility of high-polarity ginsenosides. It has been reported that Rg2 exhibited neuro-protective effect in β-amyloid or glutamate induced PC12 cells (Cui et al., 2017; Li et al., 2007), antidepressant-like effect in chronic mild stress model of depression (Ren et al., 2017), protective effect on memory impairment (Zhang et al., 2008) and Alzheimer's disease (Li et al., 2016). Therefore, the increased extraction yields of Rg_2 in SWE is of great significance.

The findings also showed that the methods of evaluation have a remarkable impact on the results. The total contents of ten ginsenosides determined by UFLC–MS/MS analysis were dramatically lower than the TG yields obtained using a colorimetric method, although it was reported that the total contents of Re, Rg_1, Rb_1, Rb_2, Rc, and Rd accounted for about 90% of the total saponins in ginseng (Park et al., 2017), which involved in ten ginsenosides detected using UFLC–MS/MS method. This significant difference was putatively considered to be due to the differences in accuracy and specificity of the quantification methods. The vanillin–perchloric acid colorimetric method is based on the reactions that occur between the hydroxyl groups at the C-3 and/or C-12 positions of saponins and the aldehyde groups in vanillin, and the absorption at 540 nm reflects the formation of acetal in new conjugate products. The accuracy of this method is usually affected by the structures of saponins and the completeness of hydrolysis. It is also easily disturbed by other substances, such as carbohydrates, proteins, volatile oil, and especially components containing a hydroxyl group, for example, hydrolyzed sugar chains and TP. By comparison, the UFLC–MS/MS analysis method is more specific and accurate, and the yields of the ten ginsenosides were precisely determined and calculated. However, extensive hydrolysis of R_1, Rb_1, Rb_2, Rb_3, Rc, Rd, and Rg_1 occurred at high temperature (Park et al., 2017); many potential hydrolysates were neglected in the present UFLC–MS/MS analysis, which led to an underestimation of the total ginsenosides in the extracts. Therefore, further studies are needed to elucidate the potential hydrolysates during the SWE treatment at high temperature.

3.8. Radar chart comparison of active ingredients among different extraction methods

The extraction yields of bioactive components among SWE and WE/EE methods were further compared using a radar chart (also known as a spider chart), which is commonly used to describe multidimensional data in a two-dimensional plane via a data visualization approach (Zhang et al., 2015). As shown in Fig. 4, the *Ye*, the extraction yields of TG, TS, TPro, and TP, as well as DPPH and ABTS antioxidant activities, were regarded as target variables for drawing the radar charts. Circles were equally divided into seven circular sectors based on the number of variables, with each index axis representing one variable. A closed polygon was obtained by connecting the data points in the different index axes with lines. Several geometric features were then extracted to provide a general view of the extraction efficiencies of different bioactive components. Here, significant differences of the closed polygon were easily observed among samples obtained from the different extraction methods. As one of the most widely used conventional methods, WE exhibited the lowest extraction efficiency of all kinds of bioactive components and showed the weakest antioxidant activities. By comparison, SWE showed much better performance than WE and EE

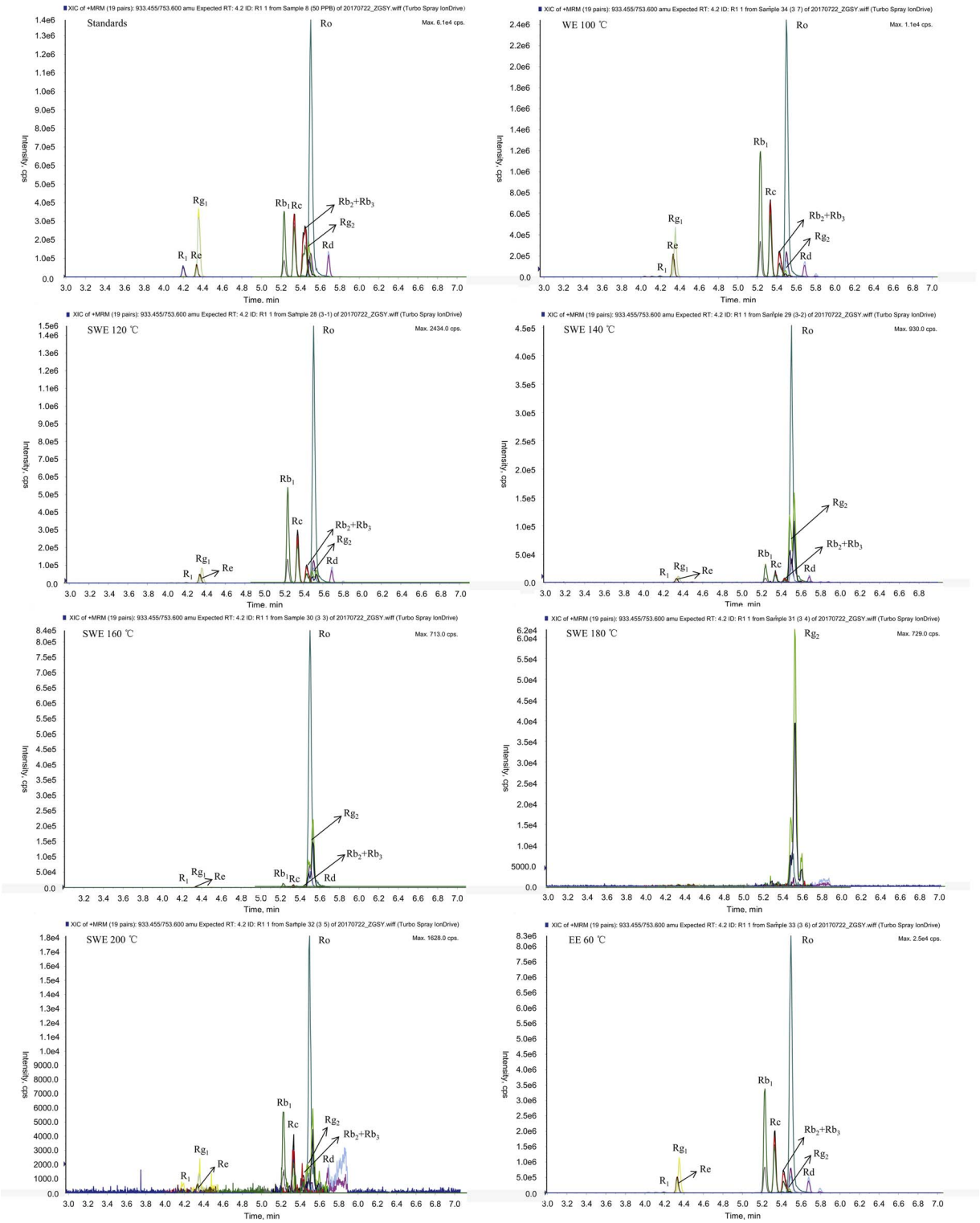

Fig. 3. Ultrafast liquid chromatography/tandem mass spectrometry-extracted ion chromatogram of ten major ginsenosides in reference standards and ginseng extracts.

regarding the extraction yields of TS, TP, and TPro, and DPPH and ABTS radical scavenging activities. Specifically, the highest TPro yield was achieved at SWE 180 °C, while the highest yields of TS and TP were obtained at SWE 200 °C, at which the strongest DPPH and ABTS radical scavenging activities were also observed. The extraction yields of TG, determined based on the colorimetric method, varied markedly among the different extraction methods, and the highest value was obtained at SWE 180 °C, followed by EE and SWE 200 °C. Another set of radar charts, which reflect the efficacy of extraction of ten major ginsenosides by different methods, are shown in Fig. 5. Circles were equally divided into ten sectors with a central angle of 36° based on the types of detected ginsenosides, including TYs, R_1, Rb_1, Rb_2 + Rb_3, Rc, Rd, Re, Rg_1, Rg_2, and Ro, with each index axis representing one variable. By connecting the data points in different index axes with lines, a closed polygon can be obtained. The conventional EE method showed the highest values of TYs and the best extraction yields of all ginsenosides except Rg_2. However, the highest value of the Rg_2 axis was observed at SWE 160 °C.

Table 3
The amounts of ten major ginsenosides in extracts obtained by SWE and conventional WE/EE methods.

Ginsenosides	Content (μg/g)						
	WE 100 °C	SWE 120 °C	SWE 140 °C	SWE 160 °C	SWE 180 °C	SWE 200 °C	EE 60 °C
20*S*-quinquenoside R_1	63.8 ± 0.4	42.7 ± 0.4	3.4 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	165.2 ± 0.6
20*S*-ginsenoside Rb_1	1825.9 ± 1.3	1858.3 ± 1.5	110.3 ± 0.0	47.0 ± 0.0	2.2 ± 0.0	16.0 ± 0.0	5048.4 ± 1.9
20*S*-ginsenoside Rb_2 + Rb_3	358.1 ± 0.6	370.2 ± 1.3	24.4 ± 0.4	7.6 ± 0.0	0.6 ± 0.0	3.2 ± 0.0	1035.3 ± 1.0
20*S*-ginsenoside Rc	1054.9 ± 1.4	1018.5 ± 0.8	63.8 ± 0.1	28.7 ± 0.0	1.3 ± 0.0	11.0 ± 0.0	2853.7 ± 1.3
20*S*-ginsenoside Rd	524.0 ± 1.2	772.0 ± 1.4	107.7 ± 1.4	25.4 ± 0.9	2.5 ± 0.0	15.7 ± 0.0	1903.9 ± 1.2
20*S*-ginsenoside Re	1736.6 ± 0.8	1011.4 ± 1.1	137.6 ± 0.4	29.0 ± 0.0	1.2 ± 0.0	11.5 ± 0.0	4238.8 ± 1.4
20*S*-ginsenoside Rg_1	592.3 ± 0.9	285.8 ± 0.9	38.9 ± 0.1	9.2 ± 0.0	1.0 ± 0.0	5.6 ± 0.0	1476.7 ± 1.2
20*S*-ginsenoside Rg_2	213.7 ± 1.0	892.0 ± 1.3	1764.4 ± 1.3	2069.5 ± 0.6	486.4 ± 0.6	48.5 ± 0.0	335.1 ± 0.9
ginsenoside Ro	886.9 ± 0.9	1216.8 ± 1.3	382.1 ± 1.2	752.2 ± 0.9	8.1 ± 0.0	14.5 ± 0.1	3101.7 ± 1.4
Total yields	7256.3 ± 4.2	7467.8 ± 2.5	2632.5 ± 2.1	2968.9 ± 0.6	503.3 ± 0.6	126.1 ± 0.1	20158.6 ± 2.7

The results were expressed as mean ± standard deviation (n = 3).
WE, water extraction; SWE, subcritical water extraction; EE, ethanol extraction.

In practice, steam processing has been commonly used for the preparation of ginseng to obtain red ginseng and black ginseng (Jin et al., 2015). In recent decades, many studies reported that the steaming process resulted in a significant increase in reducing sugar, acidic polysaccharide and phenolic compounds content (Jin et al., 2015). In steamed American ginseng berries, the total ginsenoside content and ginsenoside Rg_1, Re, Rb_1, Rc, Rb_2, Rb_3 and Rd content decrease, while some trace ginsenosides such as Rh_1, Rg_2, Rg_3, and Rh_2 increase (Wang et al., 2006). These results are very similar to those observed in this study, indicating that SWE has a similar effect on the chemical components of ginseng to steaming process. Therefore, much better pharmacological activities of SWE extract are expected, since the antioxidant (Jin et al., 2015) and anticancer (Wang et al., 2006) activities were significantly augmented after steaming process.

4. Conclusion

By comparison with conventional WE/EE methods, SWE showed noticeably higher extraction yields of TS, TPro, and TP, as well as the best antioxidant activities from ginseng roots. Both the extraction yields and the antioxidant activities of these bioactive components in SWE were significantly influenced by temperature, and the best performance was observed at a temperature of 180 or 200 °C. However, SWE showed significantly lower extraction yields of the major ginsenosides, including R_1, Rb_1, Rb_2 + Rb_3, Rc, Rd, Re, Rg_1, and Ro, than the conventional EE method, indicating that extensive hydrolysis occurred in SWE, especially at high temperature. Exceptionally, SWE at 140 and 160 °C showed much higher extraction yields of Rg_2 than the other methods. Thus, as an environmentally friendly extraction technology, SWE is more feasible and efficient than the conventional solid–liquid heating extraction method for the extraction of sugars, antioxidant TP, proteins, and ginsenoside Rg_2. However, further studies should be carried out to assess the feasibility of applying SWE to extract ginsenosides from ginseng roots, as the extraction yields of major ginsenosides using SWE were much lower than those with the EE method and extensive hydrolysis of ginsenosides may occur during SWE.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The study was financially supported by the Special Fund of Public Welfare of State Forestry Administration of the People' Republic of China (201404718 and 201504606), and National Natural Science

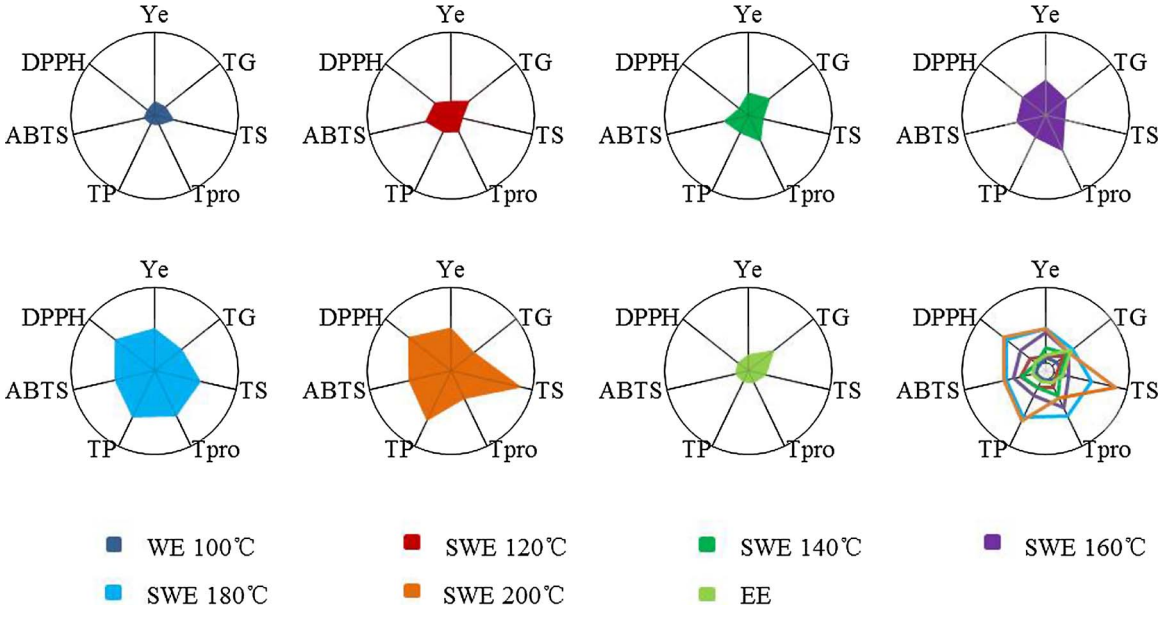

Fig. 4. Radar chart comparison of SWE at different temperatures, and WE/EE regarding the yields of extracts (Ye), total sugar (TS), total protein (TPro), total polyphenols (TP), and DPPH and ABTS antioxidant activities.

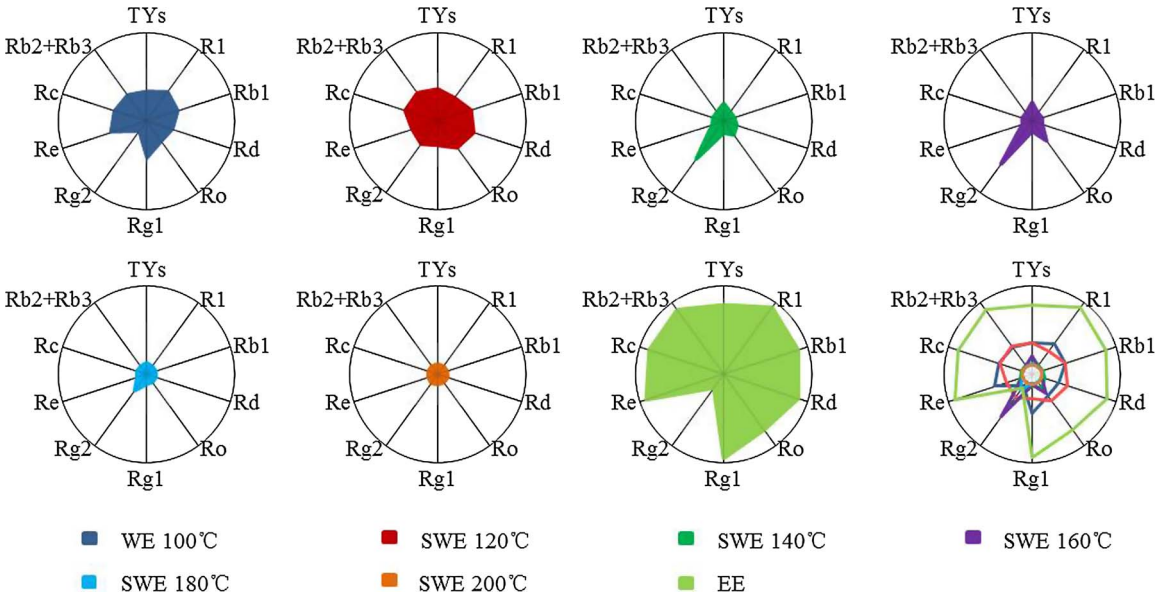

Fig. 5. Radar chart comparison of SWE at different temperatures, and WE/EE regarding the total yields of ten ginsenosides and individual ginsenosides, R_1, Rb_1, Rb_2, Rb_3, Rc, Rd, Re, Rg_1, Rg_2, and Ro.

Foundation of China (31770614).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.indcrop.2018.02.079.

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