Effects of Extraction Solvent Mixtures on Antioxidant Activity Evaluation and Their Extraction Capacity and Selectivity for Free Phenolic Compounds in Barley (*Hordeum vulgare* L.)

HAIFENG ZHAO,[†] JIANJUN DONG,[§] JIAN LU,*,[†,#] JIAN CHEN,[†,#] YIN LI,[#]
LIANJU SHAN,[§] YAN LIN,[§] WEI FAN,[§] AND GUOXIAN GU[#]

Key Laboratory of Industrial Biotechnology, Ministry of Education, Southern Yangtze University,
Wuxi 214036, People's Republic of China; Tsingtao Brewery Company Ltd., Qingdao 266061,
People's Republic of China; and School of Biotechnology, Southern Yangtze University,
Wuxi 214036, People's Republic of China

Four kinds of solvent extracts from three Chinese barley varieties (Ken-3, KA4B, and Gan-3) were used to examine the effects of extraction solvent mixtures on antioxidant activity evaluation and their extraction capacity and selectivity for free phenolic compounds in barley through free radical scavenging activity, reducing power and metal chelating activity, and individual and total phenolic contents. Results showed that extraction solvent mixtures had significant impacts on antioxidant activity estimation, as well as different extraction capacity and selectivity for free phenolic compounds in barley. The highest DPPH$^•$ and ABTS$^{•+}$ scavenging activities and reducing power were found in 80% acetone extracts, whereas the strongest $^•$OH scavenging activity, O$_2$$^{•-}$ scavenging activity, and metal chelating activity were found in 80% ethanol, 80% methanol, and water extracts, respectively. Additionally, 80% acetone showed the highest extraction capacity for (+)-catechin and ferulic, caffeic, vanillic, and *p*-coumaric acids, 80% methanol for (−)-epicatechin and syringic acid, and water for protocatechuic and gallic acids. Furthermore, correlations analysis revealed that TPC, reducing power, DPPH$^•$ and ABTS$^{•+}$ scavenging activities were well positively correlated with each other ($p < 0.01$). Thus, for routine screening of barley varieties with higher antioxidant activity, 80% acetone was recommended to extract free phenolic compounds from barley. DPPH$^•$ scavenging activity and ABTS$^{•+}$ scavenging activity or reducing power could be used to assess barley antioxidant activity.

KEYWORDS: Barley; antioxidant activity; extraction solvent; free phenolic compounds; TPC

INTRODUCTION

Beer flavor stability has become one of the most important topics in brewing science over the past decades. It is widely recognized that the main reasons responsible for beer flavor instability are oxygen content of the bottled beer and oxidation during malting and brewing (*1*). Oxidation is involved in off-flavor formation, occurrence of haze, modification of bitterness and astringency, and sometimes color changes (*2, 3*). With modern filling equipment, the attainable levels of total in-pack oxygen might be as low as 0.1 mg/L, but the deterioration of beer flavor still occurs. Thus, attention is now increasingly paid to the protection and development of intrinsic antioxidant potential during malting and brewing (*4, 5*).

Phenolic compounds, Maillard reaction products, and sulfite are usually considered to be three major endogenous antioxidants

of beer. Of these antioxidants, phenolic compounds are of particular interest to brewers because they play a key role in the brewing process by delaying, retarding, or preventing oxidation processes (*6*). Beer is known to contain a wide variety of phenolic compounds, most of which originate from the raw materials of brewing, that is, barley malt and hop. About 80% of phenolic compounds in beer originate from barley malt, and the remaining 20% come from hop (*7, 8*). Those phenolic compounds in barley include phenolic acids (benzoic and cinnamic acid derivatives), flavonoids, proanthocyanidins, tannins, and amino phenolic compounds (*9−11*), all of which are known to possess antioxidant and antiradical properties. Therefore, to inhibit the oxidative deterioration of beer, natural antioxidants of barley need to be protected, thus helping to improve the flavor stability and avoiding the use of exogenous antioxidant compounds.

Several studies on barley phenolic compounds and their antioxidant activities have been reported (*12−15*), and various aqueous solutions of acetone, methanol, and ethanol have also been used to extract the free phenolic compounds from barley (*16*). However, it is difficult to compare data within the

* Corresponding author (telephone +86-510-85864691; fax +86-510-85805219; e-mail jlu@sytu.edu.cn).
† Key Laboratory of Industrial Biotechnology, Southern Yangtze University.
§ Tsingtao Brewery Co. Ltd.
# School of Biotechnology, Southern Yangtze University.

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literature, owing to the different antioxidant activity evaluation methods and extraction solvents used by various researchers. Moreover, antioxidant compounds present in barley extracts are complex, and their activities and mechanisms would largely depend on the composition and conditions of the test system. Many authors had stressed the need to perform more than one type of antioxidant activity measurement to evaluate the antioxidant activity of plant (*17, 18*). In this study, DPPH radical scavenging activity, ABTS radical cation scavenging activity, superoxide anion radical scavenging activity, hydroxyl radical scavenging activity, reducing power, and metal chelating activity were used to evaluate antioxidant activities of different solvent extracts prepared from barley. Of them, the DPPH radical and ABTS radical cation scavenging activities have been widely used to evaluate the antiradical activities of various samples (*19–21*). Both hydroxyl radicals and superoxide anion can lead to the formation of hydroperoxides by causing the autoxidation of unsaturated fatty acids. The hydroperoxides are further degraded to aldehydes, which have been generally recognized as contributing to a stale flavor in beer (*22*). Thus, it is important to choose hydroxyl radical and superoxide anion radical scavenging activities as antioxidant activity evaluation indices of barley extracts. Moreover, some previous studies have reported that the reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (*23*), and some phenolic compounds exhibit antioxidant activity through the chelation of metal ions (*24*). Therefore, it appears to be necessary to determine the reducing power and metal chelating activity of barley to better understand antioxidant mechanisms. Additionally, total and individual phenolic contents were also determined in this study because phenolic compounds were considered to be a major group of compounds that contributed to the antioxidant activity of barley. Currently, there are very few reports on the relationship between different solvent extracts of barley and antioxidant activity evaluated by different methods. Furthermore, the extraction capacity and selectivity of different solvent mixtures for individual phenolic compounds of barley have not been studied thoroughly. Correlations among barley antioxidant activity evaluation indices and individual and total phenolic contents have also not been fully elucidated.

Therefore, the first objective of this study was to ascertain whether various extraction solvent mixtures significantly influenced barley antioxidant activity evaluation including free radical scavenging activities against DPPH radical, ABTS radical cation, and superoxide anion radical as well as hydroxyl radical, reducing power, and metal chelating activity. The second objective was not only to investigate and compare the extraction capacity and selectivity of different solvent mixtures for free phenolic compounds in barley but also to reveal the correlations among barley antioxidant activity evaluation indices and individual and total phenolic contents. The last objective was to screen for more accurate and efficient solvents for barley antioxidants extraction as well as a method for its antioxidant activity evaluation. Results from this preliminary study will provide a better understanding of endogenous antioxidant activity for beer production optimization and allow the screening of barley varieties with higher antioxidant activity to produce beer with good flavor stability.

MATERIALS AND METHODS

Materials. Ken-3, KA4B, and Gan-3 barley were the representative malting barley varieties in China and obtained from northeastern (Heilongjiang province), eastern (Jiangsu province), and northwestern (Gansu province) China, respectively. 1,1-Diphenyl-2-picrylhydrazyl

(DPPH) and 6-hydroxy-2,5,7,8-tetramethylchromancarboxylic acid (Trolox) were purchased from Sigma-Aldrich (Steinheim, Germany). 2-Deoxy-D-ribose, xanthine oxidase (XOD), (+)-catechin, (−)-epicatechin, vanillic acid, protocatechuic acid, syringic acid, *p*-coumaric acid, *trans*-ferulic acid, gallic acid, caffeic acid, and Folin–Ciocalteu's phenol reagent were obtained from Sigma-Aldrich (St. Louis, MO). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from Wako (Osaka, Japan). 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid monosodium salt (ferrozine) and nitrotetrazolium blue chloride (NBT) were purchased from Fluka (Buchs, Switzerland). All other chemicals and solvents were of the highest commercial grade and obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Preparation of Extracts from Barley. Barley was finely ground in a laboratory mill from Bühler-Miag (Braunschweig, Germany). Five grams of ground samples was sonicated (40 kHz, 120 W) for 1 h with 50 mL of 80% acetone (v/v), 80% methanol (v/v), 80% ethanol (v/v), or water under nitrogen at 20 °C, respectively. After centrifugation (10000g, 10 min), the supernatant was collected and evaporated to dryness under vacuum at 35 °C. Each residue was redissolved in 50 mL of methanol for DPPH$^\bullet$ scavenging activity, ABTS$^{\bullet+}$ scavenging activity, reducing power, and TPC determinations or in 50 mL of deionized water for $^\bullet$OH scavenging, $O_2^{\bullet-}$ scavenging, and metal chelating activity assays. For HPLC analysis, each residue was redissolved in 2 mL of methanol (HPLC grade) and then was filtered through a 0.45 μm membrane (Sartorius, Goettingen, Germany). The filtrates were analyzed by HPLC.

DPPH Radical Scavenging Activity. DPPH radical scavenging activity of barley extracts was determined according to the method of Gaulejac et al. (*25*) with minor changes. Every barley extract (0.1 mL) was added to 2.9 mL of 6×10^{-5} M methanolic solution of DPPH. The absorbance at 517 nm was measured after the solution had been allowed to stand in the dark for 60 min. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. DPPH radical scavenging activity was calculated using the formula

$$\text{DPPH}^\bullet \text{ scavenging activity (\%)} = [1 - (S - S_B)/(C - C_B)] \times 100 \tag{1}$$

where S, S_B, C, and C_B are the absorbances of the sample, the blank sample, the control, and the blank control, respectively.

ABTS Radical Cation Scavenging Activity. The radical scavenging activity of barley extracts against ABTS radical cation was measured using the method of Re et al. (*26*) with some modifications. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The ABTS$^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C. An aliquot of each barley extract (0.1 mL) was mixed with 2.9 mL of diluted ABTS$^{\bullet+}$ solution. After reaction at 30 °C for 20 min, the absorbance at 734 nm was measured. The Trolox calibration curve was plotted as a function of the percentage of ABTS$^{\bullet+}$ scavenging activity. The final results were expressed as micromoles of Trolox equivalents (TE) per gram of dry barley (μmol of TE/g of db).

Hydroxyl Radical Scavenging Activity. The scavenging activity of barley extracts toward hydroxyl radical was determined by using the deoxyribose method with some modifications (*24*). FeCl$_3$·6H$_2$O and ascorbic acid were prepared in degassed deionized water prior to use. The reaction tube contained 100 μL of barley extract, 100 μL of 1 mM EDTA, 100 μL of 1 mM FeCl$_3$·6H$_2$O, 100 μL of 36 mM 2-deoxy-D-ribose, 100 μL of 10 mM H$_2$O$_2$, and 100 μL of 1 mM l-ascorbic acid in 25 mM phosphate buffer (pH 7.4), and the total volume was made up to 1.0 mL with the same phosphate buffer. After incubation at 37 °C for 1 h, the reaction was stopped by adding 1.0 mL of 10% TCA (w/v) and 1.0 mL of 1.0% TBA (w/v) in 0.05 M NaOH. The mixture was heated in a boiling water bath for 15 min. Once samples were cooled, the final volume was adjusted to 5.0 mL with deionized water, and the absorbance was read at 532 nm. The capability to scavenge the $^\bullet$OH was calculated using the equation

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$$\bullet\text{OH scavenging activity (\%)} = [1 - (S - S_B)/(C - C_B)] \times 100 \tag{2}$$

where S, S_B, C, and C_B are the absorbances of the sample, the blank sample, the control, and the blank control, respectively.

Superoxide Anion Radical Scavenging Activity. $O_2^{\bullet-}$ scavenging activity of barley extracts was performed using an HPX/XOD system following a procedure described by Lee et al. (*27*) with some modifications. Briefly, NBT, EDTA, HPX, and XOD solution were prepared with 0.05 M phosphate buffer (pH 7.4), respectively. Each barley extract (100 μL) was added to the reaction solution containing 100 μL of 30 mM EDTA, 100 μL of 3 mM HPX, and 200 μL of 1.42 mM NBT. After the solution had been preincubated at room temperature for 3 min, 100 μL of 0.75 unit/mL XOD was added to the mixture, and the volume was brought up to 3 mL with 0.05 M phosphate buffer (pH 7.4). Then, the solution was incubated at room temperature for 40 min, and the absorbance was measured at 560 nm. The $O_2^{\bullet-}$ scavenging activity was calculated by using the formula

$$O_2^{\bullet-} \text{ scavenging activity (\%)} = [1 - (S - S_B)/(C - C_B)] \times 100 \tag{3}$$

where S, S_B, C, and C_B are the absorbances of the sample, the blank sample, the control, and the blank control, respectively.

Reducing Power. The determination was carried out as described by Oktay et al. (*28*). Briefly, 1 mL of barley extract was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and $K_3Fe(CN)_6$ (2.5 mL, 1%, w/v). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of TCA (10%, w/v) was added to the mixture, which was then centrifuged at 10000g for 10 min. The upper layer of solution (2.5 mL) was mixed with deionized water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%, w/v), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Metal Chelating Activity. The chelating activity of the barley extracts for ferrous ions was measured following the ferrozine method with minor modifications (*29*). The reaction mixture contained 0.1 mL of barley extract and 0.05 mL of $FeCl_2$ (2 mM). After 5 min, the reaction was initiated by the addition of 5 mM ferrozine (0.1 mL), and the total volume was adjusted to 3 mL with deionized water. Then, the mixture was shaken vigorously and incubated at room temperature for 10 min. Absorbance of the solution was measured at 562 nm. The metal chelating activity of the barley extracts was calculated as

$$\text{metal chelating activity (\%)} = [1 - (S - S_B)/(C - C_B)] \times 100 \tag{4}$$

where S, S_B, C, and C_B are the absorbances of the sample, the blank sample, the control, and the blank control, respectively.

Total Phenolic Content. The total phenolic content of barley extracts was determined according to the Folin−Ciocalteu spectrophotometric method (*30*) with some modifications. Briefly, 0.5 mL of barley extract was mixed with 2.5 mL of 10-fold diluted Folin−Ciocalteu's phenol reagent and allowed to react for 5 min. Then, 2 mL of 7.5% Na_2CO_3 solution (w/v) was added, and the final volume was made up to 10 mL with deionized water. After 1 h of reaction at room temperature, the absorbance at 760 nm was determined. The measurement was compared to a standard curve of prepared gallic acid (GA) solution, and the total phenolic content was expressed as milligrams of gallic acid equivalents per gram of dry barley (mg of GAE/g of db).

Analysis of Individual Phenolic Compounds by Analytical HPLC. HPLC analyses were performed using a Waters 1525 pump (Waters, Milford, MA) equipped with a Waters 717 plus autosampler coupled with a Waters 2478 dual λ absorbance detector at 280 and 254 nm. Separation was performed with Zobax 300 SB-C18 (5 μm, 4.6 mm × 250 mm) column (Agilent, Palo Alto, CA) at room temperature. Elution was carried out by using a gradient procedure with a mobile phase containing solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in methanol) as follows: 0 min, 5% B; 15 min, 20% B; 35 min, 40% B; 42 min, 65% B; 50 min, 80% B; 52 min, 5% B; 60 min, 5% B. Run time was 60 min, the solvent flow rate was 1.0 mL/min, and the injection volume was 10 μL. The concentrations of individual
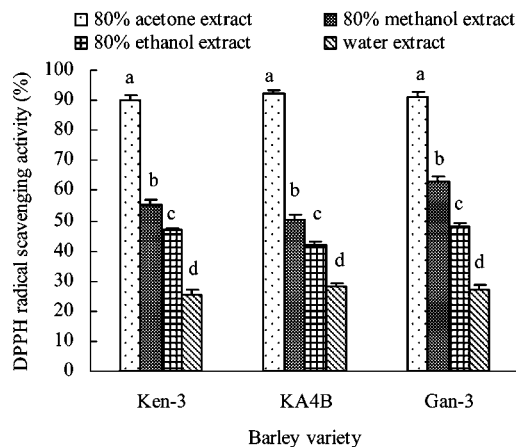

Figure 1. DPPH radical scavenging activities (percent) of different solvent extracts from three barley varieties. The concentrations of all tested extracts are on the same dry weight basis. Vertical bars represent the standard deviation for each data point. Locations for each barley variety marked by the different letters are significantly different ($p < 0.05$).

phenolic compounds in barley extracts were calculated using standard curves. Results were expressed in micrograms per gram of dry barley (μg/g of db).

Statistical Analysis. Each solvent extract from the same barley variety was prepared in triplicate, and triplicate determinations were made for each measurement. The results are given as means ± SD. Analysis of variance and significant differences among means were tested by one-way ANOVA, using SPSS (version 13.0 for Windows, SPSS Inc., Chicago, IL). The Pearson correlation test was employed to determine the correlations among means.

RESULTS AND DISCUSSION

DPPH Radical Scavenging Activity. The DPPH$^\bullet$ scavenging activities of different solvent extracts from the three barley varieties are shown in **Figure 1**. For Ken-3 barley, the values of DPPH$^\bullet$ scavenging activity ranged from 25.6% to 90.2%, indicating that extraction solvent had a significant ($p < 0.05$) influence on DPPH$^\bullet$ scavenging activity evaluation. Among three barley variety extracts analyzed, 80% acetone extract exhibited the highest DPPH$^\bullet$ scavenging activity, followed by 80% methanol, 80% ethanol, and water extracts, respectively. These results are partly in accordance with the report that the aqueous acetone extract from barley had the highest DPPH$^\bullet$ scavenging activity, followed by ethanol and methanol extracts (*10*). The contrary order of DPPH$^\bullet$ scavenging activity for ethanol and methanol extracts in two studies could be explained by the difference of extraction time and reaction time. In the present study, the 80% acetone extract was found to contain the highest levels of (+)-catechin and ferulic, caffeic, vanillic, and *p*-coumaric acids, whereas the 80% methanol extract contained the highest (−)-epicatechin and syringic acid contents and the water extract had the highest levels of protocatechuic and gallic acids (discussed later and see **Table 1**). Bonoli et al. (*16*) found that aqueous acetone selectively enhanced the catechin and proanthocyanidin extraction yield. However, the ethanol and methanol extractions enabled the recovery of considerable amounts of catechins and proanthocyanidins as well as hydrolyzable tannins. Although high levels of hydrolyzable tannins were found in ethanol and methanol extracts, this class of phenolic compounds did not contribute to antioxidant activity measured by the DPPH$^\bullet$ method. The high amount of catechins and proanthocyanidins in acetone extract exhibited the highest antioxidant activity. Moreover, all of these phenolic compounds

Table 1. Contents of Individual Phenolic Compounds in Different Solvent Extracts from Three Barley Varieties[a]

barley variety	extract	(+)-catechin	(−)-epicatechin	syringic acid	ferulic acid	protocatechuic acid	caffeic acid	vanillic acid	gallic acid	*p*-coumaric acid	SPC
						μg/g of dry barley					
Ken-3	AE	56.15 ± 0.09a	12.45 ± 0.24b	10.29 ± 0.32b	12.05 ± 0.08a	ND	7.88 ± 0.26a	3.62 ± 0.04a	2.71 ± 0.13b	1.78 ± 0.16a	106.93
	ME	45.94 ± 0.12b	15.13 ± 0.16a	12.01 ± 0.12a	11.22 ± 0.08b	ND	5.09 ± 0.19b	3.51 ± 0.25a	1.87 ± 0.16c	1.63 ± 0.13a	96.41
	EE	41.10 ± 1.25c	4.12 ± 0.06c	3.57 ± 0.08c	5.64 ± 0.05c	0.37 ± 0.05b	2.48 ± 0.09c	1.61 ± 0.09c	1.62 ± 0.23c	0.27 ± 0.06c	61.53
	WE	27.81 ± 1.93d	2.64 ± 0.11d	1.53 ± 0.05d	3.87 ± 0.10d	1.58 ± 0.15a	1.33 ± 0.13d	2.02 ± 0.10b	14.19 ± 0.84a	0.58 ± 0.10b	55.55
KA4B	AE	59.10 ± 2.11a	8.70 ± 0.20b	7.78 ± 0.22b	7.62 ± 0.21a	ND	6.69 ± 0.07a	4.50 ± 0.07a	2.32 ± 0.07b	1.67 ± 0.16a	98.38
	ME	42.64 ± 1.03b	10.94 ± 0.19a	9.97 ± 0.18a	7.14 ± 0.16b	0.68 ± 0.06b	4.81 ± 0.21b	3.57 ± 0.08b	2.07 ± 0.06c	0.90 ± 0.08b	82.72
	EE	31.92 ± 1.21c	4.09 ± 0.09c	3.34 ± 0.06c	4.72 ± 0.22c	0.41 ± 0.05c	2.02 ± 0.13c	2.19 ± 0.09c	1.24 ± 0.11d	0.53 ± 0.05c	50.46
	WE	24.61 ± 2.21d	2.19 ± 0.06d	1.23 ± 0.09d	2.91 ± 0.05d	2.78 ± 0.08a	1.72 ± 0.07d	2.13 ± 0.05c	10.92 ± 0.26a	0.32 ± 0.08d	48.81
Gan-3	AE	58.03 ± 1.36a	12.65 ± 0.13b	7.79 ± 0.15b	9.38 ± 0.06a	ND	6.32 ± 0.14a	3.87 ± 0.08a	2.64 ± 0.08b	1.43 ± 0.07a	102.11
	ME	43.38 ± 0.72b	14.92 ± 0.28a	10.56 ± 0.23a	6.24 ± 0.11b	0.19 ± 0.05b	5.70 ± 0.26b	3.33 ± 0.05b	2.16 ± 0.13c	0.88 ± 0.12b	87.36
	EE	34.00 ± 0.92c	8.65 ± 0.10c	4.54 ± 0.05c	5.55 ± 0.13c	ND	2.63 ± 0.11c	2.70 ± 0.08c	1.99 ± 0.18c	0.99 ± 0.02b	61.04
	WE	20.94 ± 0.87d	1.82 ± 0.07d	1.26 ± 0.06d	2.51 ± 0.09d	1.74 ± 0.12a	1.00 ± 0.09d	2.62 ± 0.31c	12.40 ± 0.33a	0.54 ± 0.18c	44.83

[a] Each value is the mean ± standard deviation of triplicate determinations. Means with different letters in the column for each barley variety are significantly different ($p < 0.05$). AE, 80% acetone extract; ME, 80% methanol extract; EE, 80% ethanol extract; WE, water extract; SPC, sum of individual phenolic contents; ND, not detected.
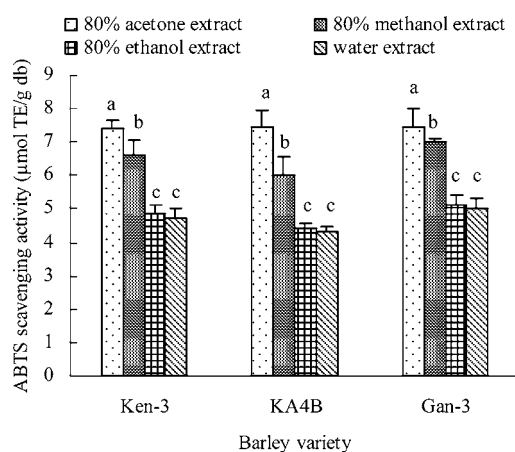

Figure 2. ABTS radical cation scavenging activities (micromoles of TE per gram of dry barley) of different solvent extracts from three barley varieties. The concentrations of all tested extracts are on the same dry weight basis. Vertical bars represent the standard deviation for each data point. Locations for each barley variety marked by the different letters are significantly different ($p < 0.05$).
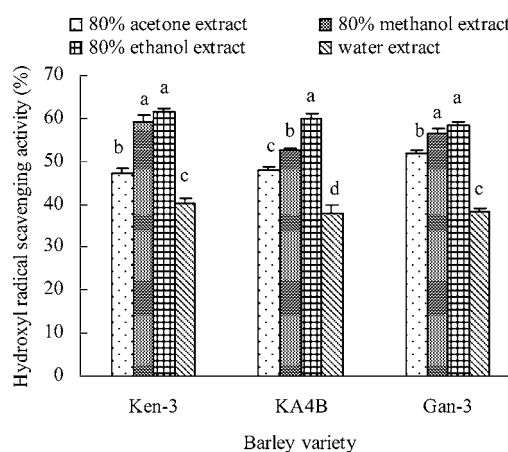

Figure 3. Hydroxyl radical scavenging activities (percent) of different solvent extracts from three barley varieties. The concentrations of all tested extracts are on the same dry weight basis. Vertical bars represent the standard deviation for each data point. Locations for each barley variety marked by the different letters are significantly different ($p < 0.05$).

found in barley extracts had various DPPH• scavenging activities because of different chemical structures. A relationship between structure and activity of different compounds by the DPPH• method has been established. Generally, monophenols are less efficient as antioxidants than polyphenols. Methoxy substitution also can increase the antioxidant activity of monophenols, but for phenolic acids, this fact is less important than the addition of a hydroxyl group (*31*). Therefore, the differences in DPPH• scavenging activities of the four kinds of solvent extracts from barley might be due to the difference in solvent selectivity for extracting certain phenolic groups with diverse DPPH• scavenging activities (*20*). All of these data indicated that 80% acetone might be a better antioxidant extraction solvent from barley against DPPH• scavenging activity evaluation.

ABTS Radical Cation Scavenging Activity. The ABTS•+ scavenging activities of barley extracts are expressed as micromoles of Trolox equivalents per gram of dry barley (μmol of TE/g of db) and are presented in **Figure 2**. ANOVA showed that different solvent extracts from the same barley variety exhibited significantly ($p < 0.05$) different ABTS•+ scavenging activities under the experimental conditions, except 80% ethanol and water extracts. Similar to the DPPH• scavenging activity

mentioned above (**Figure 1**), the 80% acetone extract from the same barley variety showed greater ABTS•+ scavenging activity than the 80% methanol or 80% ethanol extract. The lowest ABTS•+ scavenging activity was found in the water extract of barley. Although ABTS•+ scavenging activities of 80% ethanol extracts from three barley varieties were found to be higher than those of corresponding water extracts, the differences were not statistically significant ($p > 0.05$). This finding was in agreement with the report that a 50% acetone extract from wheat bran had the greatest ABTS•+ scavenging activity among all bran extracts (*20*). These data also verified considerable effects of extraction solvent mixtures on ABTS•+ scavenging activity evaluation. Thus, 80% acetone might be the appropriate solvent for extracting antioxidants with higher ABTS•+ scavenging activity.

Hydroxyl Radical Scavenging Activity. Figure 3 shows that the 80% ethanol extract from each barley variety exhibited the strongest •OH scavenging activity, followed by 80% methanol, 80% acetone, and water extract, respectively. It should be noted that there were no significant ($p > 0.05$) differences in •OH scavenging activities between 80% ethanol and 80% methanol extracts prepared from both Ken-3 and Gan-3 barley. Moreover, the 80% ethanol extract showed higher •OH scavenging activity than the 80% methanol or 80% acetone extracts from the KA4B
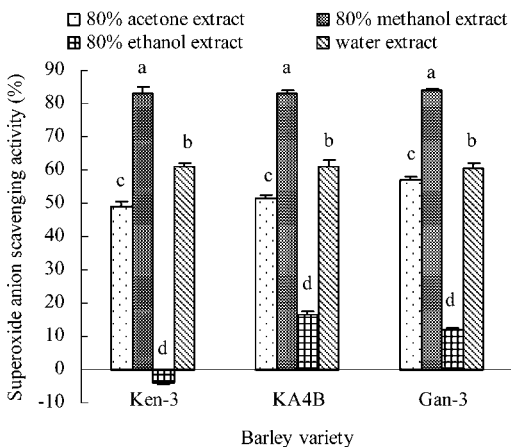
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Figure 4. Superoxide anion radical scavenging activities (percent) of different solvent extracts from three barley varieties. The concentrations of all tested extracts are on the same dry weight basis. Vertical bars represent the standard deviation for each data point. Locations for each barley variety marked by the different letters are significantly different ($p < 0.05$).

Figure 5. Reducing power (absorbance at 700 nm) of different solvent extracts from three barley varieties. The concentrations of all tested extracts are on the same dry weight basis. Vertical bars represent the standard deviation for each data point. Locations for each barley variety marked by the different letters are significantly different ($p < 0.05$).

barley, but its ability to scavenge DPPH[•] (**Figure 1**) and ABTS[•+] (**Figure 2**) was weaker than that of both of the other solvent extracts. This could be because the phenolic compounds in different solvent extracts of barley had different contributions to various free radical scavenging activity evaluations. For example, using the DPPH[•] method, *p*-coumaric acid appeared to be less antioxidant than caffeic acid. In contrast, *p*-coumaric acid was a more efficient ABTS[•+] scavenger than caffeic acid (*26, 31*). The different radical scavenging activities of phenolic compounds depend on the number of hydroxyl groups attached to the aromatic ring and methoxy substitution as well as the reaction system (*31*). These data demonstrated that 80% ethanol might be a more suitable solvent for extracting antioxidants from barley with higher [•]OH scavenging activity, but not for extracting DPPH[•] and ABTS[•+] scavenging agents. On the other hand, the ability of barley to scavenge [•]OH was important because hydroxyl radicals could cause lipid peroxidation through the Fenton reaction, resulting in the formation of stale aldehydes in beer (*22*).

Superoxide Anion Radical Scavenging Activity. As seen from **Figure 4**, 80% acetone, 80% methanol, 80% ethanol, and water extracts differed considerably in their $O_2^{•-}$ scavenging activities ($p < 0.05$), indicating that extraction solvents had an obvious impact on $O_2^{•-}$ scavenging activity evaluation. Of all solvent extracts from the same barley variety, the 80% methanol extract possessed the highest $O_2^{•-}$ scavenging activity, followed by water, 80% acetone, and 80% ethanol extracts, respectively. Interestingly, only the 80% ethanol extract from Ken-3 barley showed prooxidant activity in $O_2^{•-}$ scavenging activity evaluation. Indeed, the observation was reported by Gaulejac et al. (*25*) that some of the phenolic compounds became prooxidizing in a range of concentrations. Prooxidant activity of the barley extracts might be attributed to, for example, flavonoids, procyanidins, and phenolic acids. Almost all phenolic compounds tested exhibited some prooxidant behavior at low concentrations (*32*). In the present study, the prooxidant activity of the ethanol extract from Ken-3 barley might be due to the different contents of (+)-catechin and vanillic and *p*-coumaric acids in extracts because (+)-catechin content in the ethanol extract from Ken-3 barley was found to be significantly higher than those in ethanol extracts from KA4B and Gan-3 barley, whereas the vanillic and *p*-coumaric acid contents were much lower than those in the
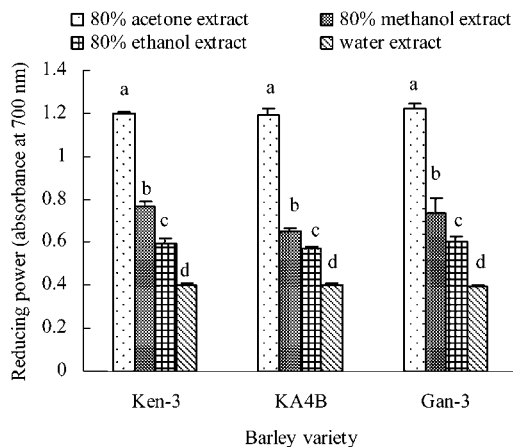
other two ethanol extracts (**Table 1**). This also could be explained by the fact that barley extracts are very complex mixtures of many different phenolic compounds with distinct polarity as well as antioxidant and prooxidant properties, sometimes showing synergic actions by comparison with individual compounds (*33*). All of the above data suggest that 80% methanol might be effective for extracting $O_2^{•-}$ scavenging agents from barley. It was also important for barley to have higher $O_2^{•-}$ scavenging activity, because it could reduce the production of many free radicals, which improved beer flavor stability by protecting beer components from free radical attack.

Reducing Power. Figure 5 illustrates the reducing power of different solvent extracts from the three barley varieties. Various solvent extracts from the same barley variety showed significant ($p < 0.05$) differences in their reducing power, indicating that extraction solvent also influenced significantly barley reducing power evaluation. Regardless of barley variety, the reducing power of different solvent extracts from barley decreased in the following order: 80% acetone extract > 80% methanol extract > 80% ethanol extract > water extract. This was similar to the observation for DPPH[•] and ABTS[•+] scavenging activities. These observations suggested that 80% acetone was more efficient solvent than other solvent mixtures in barley reducing power evaluation. The reducing power of barley might be due to the presence of flavanoids and phenolic acids, which had been found to contribute to antioxidant activity by various reaction mechanisms (*13, 14*). Indeed, some phenolic compounds such as flavanoids and phenolic acids exhibited antioxidant activity through their reductive capacity in a $Fe^{3+}-Fe^{2+}$ system. Caffeic acid was found to exhibit a much stronger reducing power than synthetic antioxidants such as BHA and BHT (*34*). Pascoe et al. (*5*) also reported that the increase in the level of antioxidant activity measured by ferric reducing antioxidant power assay during mashing was due to the increased levels of catechin and ferulic, vanillic, and *p*-coumaric acids, suggesting that all of these phenolic compounds may be responsible for this property. In the current study, all of these phenolic compounds were also found in different barley extracts and made contributions to the reducing power. However, the different levels and varieties of phenolic compounds resulted in the differences of reducing power for different solvent extracts from barley.
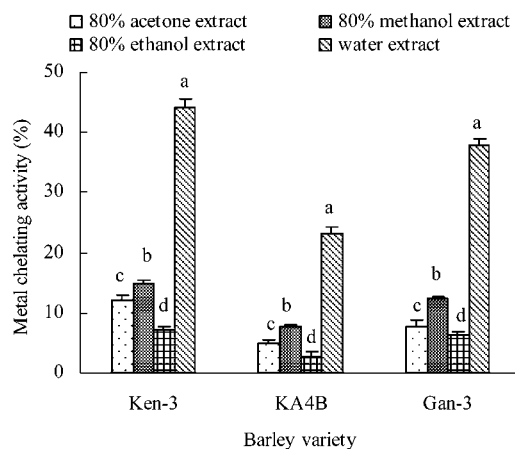
Figure 6. Metal chelating activities (percent) of different solvent extracts from three barley varieties. The concentrations of all tested extracts are on the same dry weight basis. Vertical bars represent the standard deviation for each data point. Locations for each barley variety marked by the different letters are significantly different ($p < 0.05$).
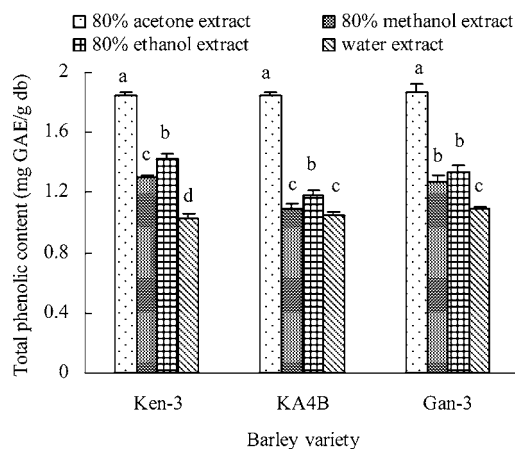

Figure 7. Total phenolic contents (milligrams of GAE per gram of dry barley) of different solvent extracts from three barley varieties. The concentrations of all tested extracts are on the same dry weight basis. Vertical bars represent the standard deviation for each data point. Locations for each barley variety marked by the different letters are significantly different ($p < 0.05$).

Metal Chelating Activity. As shown in **Figure 6**, although values of metal chelating activity in all solvent extracts from barley were all <50% under our assay conditions, there were significant ($p < 0.05$) differences in metal chelating activities of four kinds of solvent extracts from the same barley variety. Among them, the water extract exhibited the strongest metal chelating activity for all barley varieties, followed by 80% methanol and 80% acetone extracts. The lowest activity was found in 80% ethanol extract. These data revealed that water appeared to be the most appropriate solvent for extracting metal chelators from barley. Although the water extract from barley showed the highest metal chelating activity, its reducing power (**Figure 5**) and abilities to scavenge DPPH• (**Figure 1**), ABTS•+ (**Figure 2**), and •OH (**Figure 3**) were the lowest in this study. All of these differences in metal chelating activities were due to diverse compounds in solvent extracts from barley, because the chelating activity of compound was related with their structure−function configuration (*24*). Thus, the barley extracts in the present study potentially contained weak-chelating phenolic compounds. Indeed, there were numerous flavonoids, such as the prenylated and nonprenylated chalcones and flavanones found in beer and hops, that did not chelate copper ions in vitro (*35*).

Total Phenolic Content (TPC). The total phenolic contents of barley extracts were expressed as milligrams of gallic acid equivalents per gram of dry barley (mg of GAE/g of db) and are presented in **Figure 7**. Different solvent extracts showed significant ($p < 0.05$) differences in their TPC, with the exception of between 80% methanol and water extracts from KA4B barley, as well as 80% methanol and 80% ethanol extracts from Gan-3 barley. For the same barley variety, TPC followed the order 80% acetone extract > 80% ethanol extract > 80% methanol extract > water extract. The values of TPC varied from 1.03 (water extract from Ken-3 barley) to 1.87 mg of GAE/g of db (80% acetone extract from Gan-3 barley). All of these data demonstrated that various solvent mixtures had significantly different extraction capacities for barley free phenolic compounds and that 80% acetone might be a suitable solvent for extracting free phenolic compounds in barley when TPC was used as evaluation index. Compared with the 80% methanol extract, it should be emphasized that the 80% ethanol extract showed higher TPC and •OH scavenging activity (**Figure 3**), whereas its reducing power (**Figure 5**), metal chelating

activity (**Figure 6**), and abilities to scavenge $O_2^{•−}$ (**Figure 4**), DPPH• (**Figure 1**), and ABTS•+ (**Figure 2**) were weaker.

Individual Phenolic Compounds in Barley. A typical HPLC chromatogram of barley extract is shown in **Figure 8**, and the contents of individual phenolic compounds in barley extract are summarized in **Table 1**. Nine phenolic compounds including (+)-catechin, (−)-epicatechin, and syringic, ferulic, protocatechuic, caffeic, vanillic, gallic, and *p*-coumaric acids were identified and quantitated in different solvent extracts from these three barley varieties. The results indicated that (+)-catechin was the major free phenolic compound that existed in barley under our experimental conditions, which accounted for ≈50% of total identified phenolic compounds in all barley extracts. For the extraction capacity and selectivity of solvent mixtures, 80% acetone showed the highest extraction capacity for (+)-catechin and ferulic, caffeic, vanillic, and *p*-coumaric acids, whereas 80% methanol had the strongest extraction capacity for (−)-epicatechin and syringic acid and water exhibited the highest extracting ability for protocatechuic and gallic acids. Moreover, (+)-catechin was also efficiently extracted by 80% methanol and 80% ethanol, ferulic and *p*-coumaric acids by 80% methanol, and vanillic acid by 80% methanol and water. Four kinds of solvent extracts from barley were significantly ($p < 0.05$) different in their (+)-catechin, (−)-epicatechin, and syringic, ferulic, and caffeic acids contents. As seen by a comparison of 80% methanol extracts with 80% ethanol extracts from Ken-3 and Gan-3 barley, there were no significant ($p > 0.05$) differences in gallic acid contents. The vanillic acid contents in 80% ethanol and water extracts for KA4B and Gan-3 barley and in 80% acetone and 80% methanol extracts for Ken-3 barley were not significantly different ($p > 0.05$). Furthermore, there were also no significant ($p > 0.05$) differences in *p*-coumaric acid contents between 80% methanol and 80% acetone extracts from Ken-3 barley, as well as 80% methanol and 80% ethanol extracts from Gan-3 barley. It also should be noted that protocatechuic acid was not be detected in 80% acetone extracts from all three barley varieties. The sum of individual phenolic contents varied considerably, which ranged from 44.83 (water extract from Gan-3 barley) to 106.93 μg/g of dry barley (80% acetone extract from Ken-3 barley). It should be emphasized that our results are related to practical beer production, because all of these phenolic compounds found in
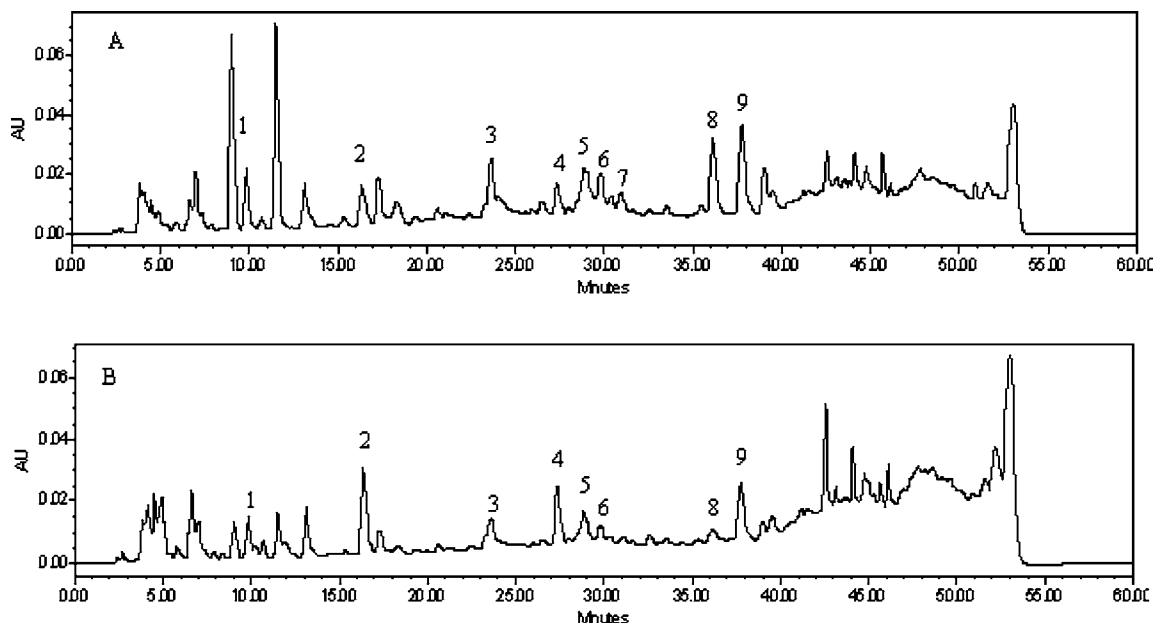
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Figure 8. HPLC chromatogram of phenolic compounds in barley extracts: (**A**) absorbance at 280 nm; (**B**) absorbance at 254 nm. Peaks: 1, gallic acid; 2, protocatechuic acid; 3, (+)-catechin; 4, vanillic acid; 5, caffeic acid; 6, syringic acid; 7, (−)-epicatechin; 8, *p*-coumaric acid; 9, ferulic acid.

Table 2. Average Range of Extraction of Each Phenolic Compound in the Different Barley Extracts[a]

					μg/g of dry barley					
extract	(+)-catechin	(−)-epicatechin	syringic acid	ferulic acid	protocatechuic acid	caffeic acid	vanillic acid	gallic acid	*p*-coumaric acid	SPC
AE	56.15–59.10	8.70–12.65	7.78–10.29	7.62–12.05	ND	6.32–7.88	3.62–4.50	2.32–2.71	1.43–1.78	98.38–106.93
ME	42.64–45.94	10.94–15.13	9.97–12.01	6.24–11.22	ND–0.68	4.81–5.70	3.33–3.57	1.87–2.16	0.88–1.63	82.72–96.41
EE	31.92–41.10	4.09–8.65	3.34–4.54	4.72–5.64	ND–0.41	2.02–2.63	1.61–2.70	1.24–1.99	0.27–0.99	50.46–61.53
WE	20.94–27.81	1.82–2.64	1.23–1.53	2.51–3.87	1.58–2.78	1.00–1.72	2.02–2.62	10.92–14.19	0.32–0.58	44.83–55.55

[a] AE, 80% acetone extract; ME, 80% methanol extract; EE, 80% ethanol extract; WE, water extract; SPC, sum of individual phenolic contents; ND, not detected.

barley were also found in beer, which indicated that the levels of phenolic compounds in beer could be increased through screening of barley varieties with higher phenolic compounds levels.

Table 2 shows the average range of extraction of each phenolic compound in the different barley extracts. Both individual and total phenolic contents in different solvent extracts from barley were significantly different. The ranges of (+)-catechin for 80% acetone, 80% methanol, 80% ethanol, and water extracts were 56.15−59.10, 42.64−45.94, 31.92−41.10, and 20.97−27.81 μg/g of dry barley, respectively. This range was similar to the report of a range of 30−95 μg/g with a mean of 51μg/g when acetone/water was used as extraction solvent (*36*). The levels of (−)-epicatechin and syringic, ferulic, and caffeic acids in 80% acetone and 80% methanol extracts were significantly higher than those in 80% ethanol and water extracts, whereas the range of gallic acid in water extract was 10.92−14.19 μg/g of dry barley, which was 5 times higher than that in other solvent extracts. Compared with other phenolic compounds, the contents of vanillic, protocatechuic, and *p*-coumaric acids were relatively low in all barley extracts. It should be noted that the range of (−)-epicatechin and *p*-coumaric acid contents in 80% ethanol extracts for three barley varieties varied considerably; the values ranged from 4.09 to 8.65 μg/g of dry barley and from 0.27 to 0.99 μg/g of dry barley, respectively. The ranges of the sums of individual phenolic compounds (SPC) for 80% acetone, 80% methanol, 80% ethanol, and water extracts were 98.38−106.93, 82.72−96.41,

50.46−61.53, and 44.83−55.55 μg/g of dry barley, respectively, which indicated that the extraction capacities of different solvent mixtures were significantly different.

The results from **Tables 1** and **2** reveal that different solvent mixtures had significantly different extraction capacity and selectivity for free phenolic compounds in barley. Compared with the results from Yu et al. (*37*), some differences were found in contents and varieties of phenolic compounds in barley. They reported only two phenolic acids in the hot water extract from barley without hydrolysis; however, after acid and α-amylase hydrolysis, six phenolic acids with higher levels were found. These discrepancies could be explained by the differences of extraction method, solvent, and barley variety. Sonication effects in this study made some barley cell walls break down, leading to the release of some bound phenolic compounds (*38*). Therefore, these bound phenolic compounds were also detected in barley extracts, but low contents were measured due to lack of further hydrolysis.

Correlations among Barley Antioxidant Activity Evaluation Indices and Individual and Total Phenolic Contents. To make further understanding of the interrelationship between barley antioxidant activity evaluation index and phenolic compounds content, all solvent extracts prepared from three barley varieties were used to analyze the correlations among free radical scavenging activity, metal chelating activity, reducing power, and individual and total phenolic contents. **Table 3** shows that (+)-catechin, (−)-epicatechin, and syringic, ferulic, caffeic, vanillic, and *p*-coumaric acids contents gave strong

Table 3. Correlations among Barley Antioxidant Activity Evaluation Indices and Individual and Total Phenolic Contents[a]

	DSA	ASA	HSA	SSA	RP	MCA	TPC	CC	ECC	SA	FA	PA	CA	VA	GA	PCA	SPC
DSA	1	0.913**	0.256	0.077	0.992**	−0.597*	0.940**	0.963**	0.700*	0.694*	0.792**	−0.826*	0.936**	0.816**	−0.627*	0.867**	0.903**
ASA		1	0.154	0.429	0.902**	−0.384	0.775**	0.902**	0.837**	0.838**	0.811**	−0.549	0.958**	0.911**	−0.495	0.852**	0.962**
HSA			1	−0.377	0.202	−0.762**	0.194	0.357	0.475	0.447	0.376	−0.912**	0.229	0.045	−0.866**	0.315	0.254
SSA				1	0.087	0.335	−0.168	0.112	0.449	0.483	0.221	0.256	0.333	0.512	0.235	0.094	0.384
RP					1	−0.545	0.949**	0.958**	0.669*	0.668*	0.814**	−0.863*	0.923**	0.812**	−0.574	0.883**	0.903**
MCA						1	−0.517	−0.617*	−0.497	−0.508	−0.476	0.662	−0.526	−0.378	0.948**	−0.463	−0.459
TPC							1	0.885**	0.488	0.465	0.703*	−0.696	0.790**	0.642*	−0.519	0.843**	0.765**
CC								1	0.746**	0.771**	0.853**	−0.813*	0.939**	0.788**	−0.679**	0.905**	0.943**
ECC									1	0.961**	0.832**	−0.626	0.841**	0.744**	−0.664*	0.725**	0.876**
SA										1	0.860**	−0.659	0.870**	0.770**	−0.678*	0.756**	0.894**
FA											1	−0.802*	0.872**	0.687*	−0.623*	0.919**	0.917**
PA												1	−0.614	−0.271	0.836**	−0.799*	−0.616
CA													1	0.862**	−0.607*	0.863**	0.976**
VA														1	−0.432	0.750**	0.858**
GA															1	−0.586*	−0.575
PCA																1	0.904**
SPC																	1

[a] DSA, DPPH radical scavenging activity; ASA, ABTS radical cation scavenging activity; HSA, hydroxyl radical scavenging activity; SSA, superoxide anion radical scavenging activity; RP, reducing power; MCA, metal chelating activity; TPC, total phenolic content; CC, (+)-catechin; ECC, (−)-epicatechin; SA, syringic acid; FA, ferulic acid; PA, protocatechuic acid; CA, caffeic acid; VA, vanillic acid; GA, gallic acid; PCA, *p*-coumaric acid; SPC, sum of individual phenolic contents; *, significant at $p < 0.05$; **, significant at $p < 0.01$.

positive correlations with DPPH• and ABTS•+ scavenging activities and reducing power (ranging from 0.668 to 0.963, $p < 0.05$), whereas negative correlations with metal chelating activity [ranging from −0.617 to −0.378, $p > 0.05$, except for (+)-catechin] were obtained, indicating that these phenolic compounds were mainly responsible for the DPPH• and ABTS•+ scavenging activities and reducing power of barley. On the contrary, protocatechuic and gallic acids contents exhibited significant positive correlations with metal chelating activity (0.662 and 0.948, respectively) and negative correlations with DPPH• and ABTS•+ scavenging activities and reducing power (ranging from −0.912 to −0.574, $p < 0.05$, except for the case of gallic acid for reducing power assay), suggesting that both phenolic acids made considerable contributions to the metal chelating activity of barley. In addition, (+)-catechin and ferulic, caffeic, vanillic, and *p*-coumaric acids contents also showed striking positive correlations with TPC and SPC (ranging from 0.642 to 976, $p < 0.05$), which indicated that they were the main free phenolic compounds in barley under the experimental conditions. Significant negative correlations between •OH scavenging activity and protocatechuic as well as gallic acids contents also were found in this study (−0.912 and −0.866, respectively, $p < 0.01$). Moreover, there were significant positive correlations among (+)-catechin, (−)-epicatechin, and syringic, ferulic, caffeic, vanillic, and *p*-coumaric acids (ranging from 0.687 to 0.961, $p < 0.05$), but all of these acids negatively correlated with gallic and protocatechuic acids (ranging from −0.813 to −0.432).

DPPH• and ABTS•+ scavenging activities, reducing power, TPC, and SPC were also well positively correlated with each other (ranging from 0.765 to 0.992, $p < 0.01$) and all negatively correlated with metal chelating activity (ranging from −0.597 to −0.384), especially DPPH• scavenging activity, which had a significant negative correlation with metal chelating activity (−0.597, $p < 0.05$). Furthermore, •OH scavenging activity also showed a more negative correlation coefficient (−0.762) with metal chelating activity than with O_2•− scavenging activity (−0.377). A well positive correlation (0.765, $p < 0.01$) between TPC and SPC suggested that both methods could provide accurate information on total phenolic contents of barley. Finally, it should be pointed out that the different antioxidant responses and levels of phenolic compounds in different solvent

extracts were the result of negative correlations among barley antioxidant activity evaluation. Although all of these methods could be used to evaluate the antioxidant activity of barley, the evaluation results were different, because different methods involve distinct reaction mechanisms. Thus, the correlations observed in antioxidant activity evaluation and phenolic compounds contents provide a good means for brewers to select appropriate methods for barley free phenolic compounds extraction and antioxidant activity evaluation. Obtaining the maximum recovery for phenolic compounds from barley gave a basis for accurate estimation of the antioxidant activity of barley, although some compounds such as carotenoids and tocopherols also made contributions to barley antioxidant activity. In the present study, the aqueous acetone extract exhibited the highest total phenolic content determined by both Folin−Ciocalteu and HPLC methods; thus, it was a more appropriate choice for barley phenolic compounds extraction than other solvents used in the test. Additionally, DPPH radical scavenging activity, ABTS radical cation scavenging activity, or reducing power was recommended to evaluate the antioxidant activity of barley because of their high correlations with total phenolic content and some individual phenolic contents, especially with the amount of (+)-catechin and caffeic acid ($r > 0.9$). Furthermore, good correlations between phenolic contents and antioxidant activities of barley extracts also indicated that the antioxidant activity of beer could be increased through developing and protecting the phenolic compounds in beer itself and raw materials, that is, barley, which was of importance for practical beer brewing.

In conclusion, the present study has clearly demonstrated that extraction solvent mixtures greatly affected barley antioxidant activity evaluation including DPPH radical, ABTS radical cation, superoxide anion radical, and hydroxyl radical scavenging activities and reducing power as well as metal chelating activity. Different extraction solvent mixtures had significant differences in their extraction capacity and selectivity for free phenolic compounds in barley. Different phenolic compounds with different levels in barley extracts also made distinct contributions to different barley antioxidant activity evaluations. (+)-Catechin is the predominant phenolic compound in three Chinese barley varieties and accounts for ≈50% of the total free phenolic compounds on a weight basis. In addition, TPC, SPC, reducing power, and DPPH• and ABTS•+ scavenging activities were well

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positively correlated with each other (p < 0.01), but all negatively correlated with metal chelating activity. Therefore, 80% acetone might be a better solvent for extracting free phenolic compounds in barley. It might also be a better choice for barley antioxidant activity evaluation with DPPH radical scavenging activity, ABTS radical cation scavenging activity, or reducing power. Moreover, this research was part of our continuous efforts to improve beer flavor stability by protecting endogenous antioxidants in raw materials and beer. On the basis of results obtained from this study, further works on screening malting barley varieties and optimizing brewing processes are in progress to improve the flavor stability of beer.

ABBREVIATIONS USED

DPPH•, 1,1-diphenyl-2-picrylhydrazyl radical; ABTS•+, 2.2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation; •OH, hydroxyl radical; O_2•−, superoxide anion radical; TPC, total phenolic content; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromancarboxylic acid; NBT, nitrotetrazolium blue chloride; XOD, xanthine oxidase; HPX, hypoxanthine; EDTA, disodium ethylenediaminetetraacetate; TCA, trichloroacetic acid; TBA, thiobarbituric acid; HPLC, high-performance liquid chromatography; TE, Trolox equivalents; GAE, gallic acid equivalents; SPC, sum of individual phenolic contents.

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