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Dynamic Changes in Phenolic Compounds and Antioxidant Activity in Oats (*Avena nuda* L.) during Steeping and Germination

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Samples from naked oat were steeped and germinated under controlled conditions in an incubator. Changes of phenolic compounds and antioxidant activity were investigated in oats during steeping and germination. Results revealed that phenolic compounds and antioxidant activity of oats varied with the difference in steeping and germination stages. Compared with raw grains, short-term steeping treatment did not show significant effects ($p > 0.05$) on phenolic content. Germination can significantly result in the decrease in bound phenolic and the increase in free and total phenolics. Main phenolic acids and avenanthramides were isolated and quantified by HPLC analysis. During steeping, phenolic acids decreased ($p < 0.05$); avenanthramide *N*-(3′,4′-dihydroxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid first decreased and then increased ($p < 0.05$), while avenanthramides *N*-(4′-hydroxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid and *N*-(4′-hydroxy-3′-methoxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid did not change significantly ($p > 0.05$). During germination, gallic and caffeic acids first increased ($p < 0.05$) and then decreased, whereas *p*-coumaric and ferulic acids and avenanthramides increased ($p < 0.05$). Nevertheless, avenanthramides did not change significantly ($p > 0.05$) during the last stage of germination. Oat extracts exhibited increasing high antioxidant activity with the steeping and germination going on, which may explain that antioxidant activity correlated ($p < 0.01$) significantly with the content of phenolic compounds.

KEYWORDS: Phenolic compounds; antioxidant activity; steeping; germination; oats (*Avena nuda* L.); DPPH; FRAP

INTRODUCTION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism in living organisms, and at low-to-moderate concentrations they are known to possess various physiological roles ranging from cellular signal transduction to defense against pathogens (*1*). However, the excessive amounts of ROS and RNS bring about degradation of cellular components such as carbohydrates, proteins, lipids, DNA and RNA, and thus lead to cell death and tissue damage, so many pathophysiological conditions are initiated by excess reactive species (*2*, *3*). Besides, ROS is also one of the major causes of spoilage of various materials, including food.

Antioxidants can delay or inhibit the oxidation of lipid or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (*3*), and they are commonly divided into chemical and natural origin antioxidants according to the material source. Lately, consumers are concerned about the security of chemical food additives. Hence antioxidants obtained from a natural source would have commercial value to both the food and personal health care product industries provided that they are concentrated and are effective in inhibiting unwanted oxidation reactions. Consequently, concentrating and isolating antioxidants from cereal seeds could present a processing opportunity to manufacture natural antioxidants or functional foods based on grain.

Oat, a cereal for human or animal consumption, although consumed in considerably lower quantities worldwide than wheat and rice, has received increased interest because of its excellent health-related properties, such as high contents of solube dietary fiber and well-balanced protein, energy in the form of carbohydrate and oil, and several vitamins and minerals (*4*). In addition, oats contained abundant antioxidant compounds that were often enriched in the outer part of the cereal grains such as tocols (*5*), phytic acid, sterols (*5*, *6*), phenolic compounds (*5*, *7*, *8*) and so on (*9*), which contribute toward protecting foods from rancidity and may also help to preserve their color and taste. Phenolic compounds exhibited a wide range of differing biological effects, such as antioxidant, anti-inflammatory, antiallergic and anticarcinogenic activities. Many of these functions have been attributed to the antioxidant activity of phenolic compounds (*10*).

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In oats, phenolic compounds were the most important antioxidants and included avenanthramides (*11*), phenolic acids (*7*, *8*), flavonoids and so on (*9*). These phenolic compounds were present as simple soluble free esters and, to a greater extent, as complex insoluble bound esters with polysaccharides, proteins, or cell walls in oat grains (*9*). Whereas the content and antioxidant activity of phenolic compounds were found to be greatly affected by variety, storage, heat treatment and different processing methods, and some processes could result in the decrease in the phenolic content of oats (*12*, *13*). In order to ensure or improve nutritional value and functionality of oat foods, some researchers (*14*, *15*) have studied in this respect and reported that germination could increase phenolic content and antioxidant activity in oats.

Cereal seeds have been germinated for centuries to soften the kernel structure, to increase nutrient compounds and decrease antinutritional compounds. Germination is initiated by steeping, when dry grains absorbed water to a moisture content of 43−45%, and the metabolic activity resumes. During the subsequent germination process, enzyme synthesis and kernel modification take place, which could result in the enhancement of intrinsic phenolic compounds and antioxidant activity (*16*). Some reported studies demonstrated that the germination of oats increased total phenolic content 3- to 4-fold (*14*, *15*) and increased antioxidant activity (*14*). Some authors (*17*, *18*) have also reported that avenanthramides also increased in concentration by steeping and germination processes. However, they did not investigate changes of phenolic content, phenolic components, and antioxidant activity of oats during steeping and germination, especially from oat cultivars (*Avena nuda* L.). From our preliminary studies, we have found that phenolic compounds and antioxidant activity in oats may be affected by the time of steeping and germination, which have not been reported up until now.

The main aim of this research was to investigate phenolic content, qualitative and quantitative levels of selected phenolic components, and antioxidant activity of oats (*Avena nuda* L.) at different stages of steeping and germination, as well as to characterize dynamic changes in phenolic compounds and antioxidant activity during steeping and germination. Thus, we expect to develop methods and provide some parameters which can contribute to selectively concentrate the phenolic compounds and increase antioxidant activity of oats according to the needs of various foods, and which would greatly facilitate production of nutraceuticals or food ingredients that enable consumers to gain greater access to the health benefits of oats.

MATERIALS AND METHODS

Oat Materials. Oat cultivar (*Avena nuda* L.) Baiyan II, a naked variety, was used in the study. The cultivar was grown in 2007 in bases for growing organic oat, Shanxi, China, and it is the main commercial cultivar in the local area. The harvested oat grains were dried to 10% moisture and then stored at room temperature until the time of steeping and germination, which was performed in the autumn of 2007.

Reagents. 2,4,6-Tri(2-pyridyl)-*s*-triazine (TPTZ) and ascorbic acid were purchased from Fluka (Switzerland). Sodium carbonate, sodium acetate, acetonitrile, and methanol were from Merck (Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin−Ciocalteu reagent, 2,6-di-*tert*-butyl-4-hydroxytoluene (BHT), gallic acid (GA), *p*-coumaric acid (*p*CA), caffeic acid (CA) and ferulic acid (FA) were from Sigma (USA). All other chemicals and reagent used in the experiments were of analytical grade.

Steeping and Germination. Oat grains were surface sterilized using a 1% solution of sodium hypochlorite for 30 s, and then they were washed three times with deionized water before steeping. Oat grains were steeped and germinated in deionized water under controlled conditions in an incubator (HWS-280; Hangzhou Huier Instruments, Zhejiang, China). The oat grains (500 g) were steeped with 1000 mL of deionized water at 20 °C, with aeration for 1 h every 4 h, and three samplings were carried out (S1−S3), which took place at 8, 16, and 24 h during the steeping process. After steeping, the remaining oat grains were drained and germinated for 48 h in a controlled environment at 16 °C and 95% relative humidity, and four samplings were carried out (G1−G4), which took place at 12, 24, 36, and 48 h during the germination process. The average length of oat malt at the end of germination is 2.0 mm. After sampling, samples were immediately freeze-dried (Christ Alpha1-4, Germany) and stored at −40 °C until time of analysis. All samples were milled with a micro plant grinding machine (FZ102; Tianjin Taisite Instruments, Tianjin, China) set at a fine setting of 0.5 mm. This was carried out just prior to the different analyses. Raw grains were also freeze-dried and used as reference samples in all performed analyses.

Extraction of Free Phenolic Compounds. Free phenolic compounds were extracted according to a modification of the method reported previously (*19*). Initially, milled oat sample (5.0 g dry weight (DW)) was defatted two times with 50 mL of hexane at 30 °C by an ultrasonic homogenizer (Scientz-IID, Ningbo Scientz Biotechnology Co., Ltd., Zhejiang, China). The defatted samples were blended with 25 mL of methanol and shaken with a laboratory rotary shaker (JB50-D; Shanghai Shengke Instruments, Shanghai, China) at 250 rpm for 20 min, and then the homogenates were centrifuged at 4000*g* for 15 min at 4 °C in a centrifuge (Eppendorf 5417R, Germany). After centrifugation, the methanol supernatants were removed and extraction was repeated three times at room temperature and in the absence of light. Then supernatants were pooled, vacuum-evaporated to dryness at 40 °C, and reconstituted with methanol to a final volume of 10 mL. The extracts were stored at −40 °C until used.

Extraction of Bound Phenolic Compounds. Bound phenolic compounds are mainly methanol insoluble ester-bound phenolic acids in oats and were extracted according to the method reported previously (*20*) with some modifications. After the methanol extraction, the residues were combined with 100 mL of 2 M NaOH at room temperature, dissolved O_2 was removed by flushing with nitrogen, and the residues were shaken with a laboratory rotary shaker at 250 rpm for 2 h at room temperature. The mixtures were neutralized with 6 M HCl and then extracted six times with 40 mL of ethyl acetate. The ethyl acetate fractions were evaporated to dryness at 35 °C with a nitrogen stream. Phenolic compounds were reconstituted with methanol to a volume of 10 mL, frozen and stored at −40 °C until used.

Determination of Phenolic Content. Phenolic content was determined based on the Folin−Ciocalteu colorimetric method as described by Payet et al. (*21*) with some modifications. Briefly, an aliquot (0.5 mL) of appropriately diluted extracts, 2.5 mL of deionized water and 0.5 mL of 1.0 M Folin−Ciocalteu reagent were mixed within 10 mL volumetric flasks and vortexed. After 8 min, 1.5 mL of 7.5% sodium carbonate solution was added and mixed thoroughly. The absorbance of the reaction mixtures was measured using a spectrophotometer (UV2000, Hitachi) at 765 nm wavelength after incubation for 2 h at room temperature. Methanol was used as the blank, and gallic acid (GA) was used for calibration of the standard curve (0−500 mg/L). Phenolic content was expressed as gallic acid equivalents (milligrams of GAE per gram DW).

HPLC Analysis. The total phenolic extracts were filtered through a nylon syringe filter (0.45 μm) (Filtrex Technology, Singapore) prior to HPLC analysis and analyzed in a Waters 1525 HPLC chromatograph system (Waters, Milford, MA) equipped with Waters 2487 Diode Array Detector (DAD) (Waters, Milford, MA) and a reversed-phase C18 column (Alltech, Allsphere ODS-2, 5 μm, 150 mm × 4.6 mm) using a linear gradient from 1 to 40% acetonitrile (pH 2.8 with formic acid) as the mobile phase. The injection volume was 10 μL, the flow rate was 0.8 mL/min at room temperature, and the duration of a single run was 60 min while the monitored wavelength was 280 nm. The total phenolic extracts and standard compounds were analyzed under the same analysis conditions, and all of the above experiments were replicated three times. Identification of the main phenolic acids (gallic, caffeic, *p*-coumaric, and ferulic acids) was performed by comparisons to the retention time and UV spectra of authentic standards from Sigma, and the identification of three main avenanthramides (2c, 2p, and 2f) was performed using synthetic compounds as external standards (*22*) (**Figure 1**); then the quantitative data was calculated from their linear calibration curves under analysis conditions. Results of the analyses for the main phenolic compounds were

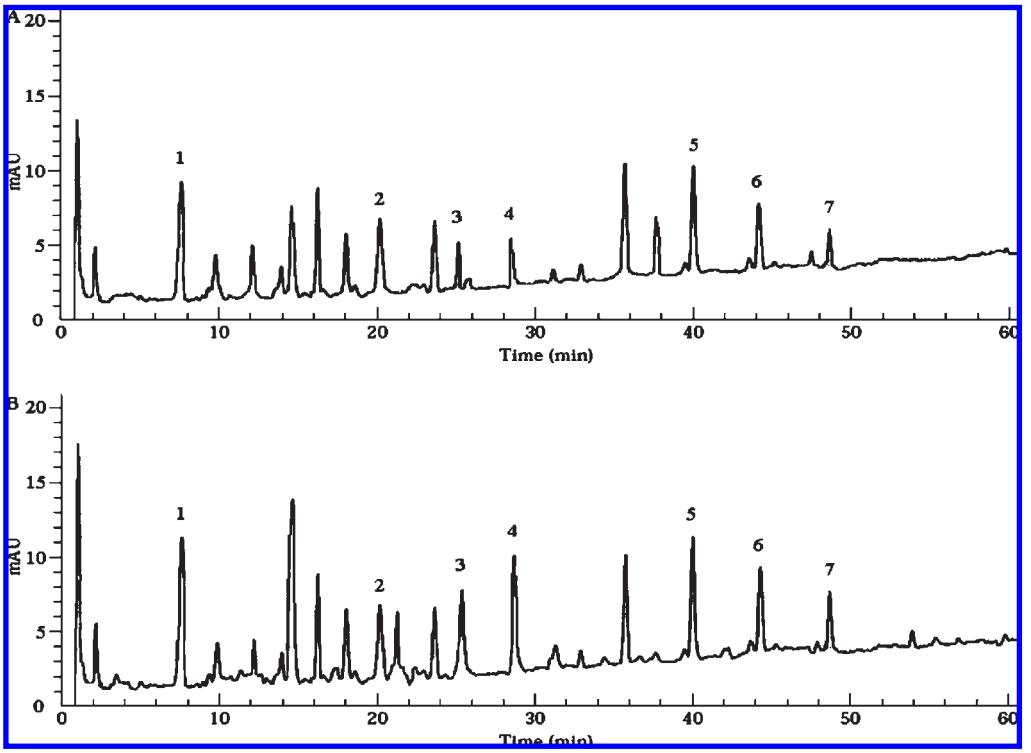

Figure 1. HPLC chromatograms of the total phenolic extracts from the naked oat at wavelength 280 nm. Raw grains (**A**) and sample after 48 h of germination (**B**) are shown. The peaks are (1) gallic acid, (2) caffeic acid, (3) *p*-coumaric acid, (4) ferulic acid, (5) *N*-(3′,4′-dihydroxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid, (6) *N*-(4′-hydroxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid, and (7) *N*-(4′-hydroxy-3′-methoxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid.

Table 1. The Retention Time and Linear Curve Features of Individual Compounds

compounds	RT,[a] min	LR,[b] μg/mL	R^{2} [c]
gallic acid	7.7	1.0−20.0	0.9985
caffeic acid	20.1	1.0−10.0	0.9963
p-coumaric acid	25.2	1.0−10.0	0.9992
ferulic acid	28.8	1.0−20.0	0.9976
2c[d]	40.1	1.0−20.0	0.9928
2p	44.5	1.0−20.0	0.9935
2f	49.0	1.0−20.0	0.9952

[a] Retention time at 280 nm. [b] Linearity range. [c] Correlation coefficient values. [d] Abbreviations of avenanthramides: 2c, *N*-(3′,4′-dihydroxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid; 2f, *N*-(4′-hydroxy-3′-methoxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid; 2p, *N*-(4′-hydroxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid).

expressed as micrograms per gram DW. The linearity range and correlation coefficient (R^2) of curves and retention time of individual compounds are given in **Table 1**.

DPPH Radical Scavenging Capacity. The antioxidant activity of the free, bound, and total phenolic compounds were assessed by measuring the capacity of bleaching a purple colored methanol solution of DPPH radicals as described by Bratt et al. (*22*) with some modifications. Briefly, extracts of different samples were serially diluted to various concentrations in methanol, respectively, and then 0.50 mL of methanolic extracts was mixed with 4.5 mL of 60 μM DPPH dissolved in methanol. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a methanol blank. The scavenging rate on DPPH radicals was calculated according to the formula, scavenging rate (%) = $[1 - (A_1 - A_s)/A_o] \times 100$, where A_o is the absorbance of the control solution (0.5 mL of methanol in 4.5 mL of DPPH solution), A_1 is the absorbance in the presence of the plant extract in DPPH solution, and A_s, which is used for error correction arising from unequal color of the sample solutions, is the absorbance of the sample extract solution without DPPH. The scavenging capacity of the phenolic compounds on DPPH radicals was determined by the IC_{50} value. The IC_{50} value is the effective concentration at which DPPH radicals are scavenged by 50% and was obtained by interpolation from nonlinear regression analysis. Effectiveness of antioxidant activity inversely correlates with IC_{50} value. Ascorbic acid and BHT were used as a positive control. Finally, the unit of IC_{50} was expressed as micrograms of gallic acid equivalents (μg of GAE) per milliliter.

Ferric Reducing Antioxidant Power (FRAP) Assay. The reducing ability was determined by using the FRAP assay described by Corral-Aguayo et al. (*23*) with some modifications. Briefly, the FRAP reagent was freshly prepared from 300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3$ solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37 °C in a water bath prior to use. Then 0.1 mL of the tested sample solution was mixed with 1.8 mL of FRAP reagent and 3.1 mL of ultrapure water. The absorption of the reaction mixture was measured at 593 nm after incubation for 30 min at 37 °C. Ascorbic acid and BHT were used as a positive control, and a standard curve was constructed using $FeSO_4$ solution (100−1000 μM). FRAP value was expressed as micromoles of Fe(II) per gram DW.

Statistical Analysis. All experiments were conducted three times independently, and the experimental data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Duncan's multiple range test were carried out to determine significant differences ($p < 0.05$) between the means by SPSS (version 13.0). Correlation coefficient and regression analyzes were determined by DPS (version 3.01) EXCEL program.

RESULTS AND DISCUSSION

Changes of Phenolic Content during Steeping and Germination. The levels of the free, bound and total phenolics from raw grains and samples were measured using Folin−Ciocalteu's colorimetric method, which was incoordinately influenced by the steeping and germination process (**Table 2**). Compared with raw grains, the free and total phenolic contents were gradually increased and the bound phenolic content was gradually decreased with the processing of steeping and germination. The free phenolic content increased ($p > 0.05$) approximately 30% during the first 16 h

Table 2. Changes of Phenolic Content (mg of GAE/g DW) in Oat Grains during Steeping (20 °C Treatment) and Germination (16 °C Treatment)[a]

samples	free phenolic	bound phenolic	total phenolic
raw grains	0.43 ± 0.04 e	1.23 ± 0.11 a	1.65 ± 0.05 e
S1[b]	0.51 ± 0.09 e	1.19 ± 0.10 ab	1.70 ± 0.03 de
S2	0.56 ± 0.08 de	1.15 ± 0.12 abc	1.72 ± 0.07 de
S3	0.70 ± 0.06 cd	1.05 ± 0.07 bc	1.76 ± 0.09 cd
G1[c]	0.84 ± 0.07 c	1.03 ± 0.09 cd	1.90 ± 0.06 c
G2	1.29 ± 0.09 b	0.87 ± 0.06 d	2.16 ± 0.12 b
G3	1.68 ± 0.10 a	0.89 ± 0.05 d	2.58 ± 0.14 a
G4	1.74 ± 0.13 a	0.87 ± 0.08 d	2.62 ± 0.10 a

[a] Numbers represent mean values of three independent replicates ± SD. Different letters indicate statistically significant differences between the means ($p < 0.05$) for free, bound, and total phenolic contents. [b] S1−S3 refer to the different steeping stages. [c] G1−G4 refer to the different germination stages.

Table 3. Changes of Main Phenolic Acids Content (μg/g DW) in Oat Grains during Steeping (20 °C Treatment) and Germination (16 °C Treatment)[a]

	phenolic acids			
samples	gallic acid	caffeic acid	*p*-coumaric acid	ferulic acid
raw grains	113.3 ± 2.6 b	14.3 ± 0.5 b	23.8 ± 0.7 c	187.5 ± 3.0 d
S1[b]	104.3 ± 2.1 c	8.8 ± 0.4 c	15.0 ± 0.3 e	161.6 ± 2.7 e
S2	98.8 ± 1.8 d	6.2 ± 0.4 d	12.6 ± 0.4 f	138.7 ± 2.0 f
S3	84.2 ± 1.5 f	4.0 ± 0.1 e	11.9 ± 0.2 f	109.3 ± 1.9 g
G1[c]	93.6 ± 1.1 e	6.4 ± 0.3 d	20.3 ± 0.4 d	164.9 ± 2.1 e
G2	127.8 ± 2.6 a	15.1 ± 0.5 a	20.1 ± 0.3 d	265.2 ± 3.5 c
G3	115.7 ± 2.4 b	14.8 ± 0.4 ab	29.4 ± 0.6 b	286.4 ± 3.4 b
G4	97.8 ± 1.3 d	14.5 ± 0.3 ab	42.2 ± 0.8 a	313.0 ± 3.9 a

[a] Numbers represent mean values of three independent replicates ± SD. Different letters indicate statistically significant differences between the means ($p < 0.05$) for gallic, caffeic, *p*-coumaric, and ferulic acid contents. [b] S1−S3 refer to the different steeping stages. [c] G1−G4 refer to the different germination stages.

of steeping (S1−S2). Then a significant increase ($p < 0.05$) in the free phenolic content was detected during the remainder of the steeping and germination process (S3−G4), and its content increased by from 86% to 304%. Nevertheless, the content of free phenolics did not change significantly ($p > 0.05$) and only increased 3.6% between stages G3 and G4. For bound phenolics, the trend of changes in the content was the direct opposite of the free phenolics. There was an insignificant decrease ($p > 0.05$) in phenolic content and it decreased approximately 6.5% during the first 16 h of steeping (S1−S2), then bound phenolic content significantly decreased ($p < 0.05$) approximately 29% by the end of germination as compared to raw grains. However, the bound phenolic content did not change significantly ($p > 0.05$) during the whole germination process (G1−G4) and decreased approximately 3.6%, and even remained unchanged at the G2, G3, and G4 stages. Total phenolics is mainly made up of the free and bound phenolics in oats, so its content was influenced by the free and bound phenolics. Changes of the total phenolic content had a more similar trend to the free phenolics (**Table 2**), but the variation range of the total phenolic content was different from that of the free phenolic content. Compared with raw grains, the total phenolic content only increased 4.2% ($p > 0.05$) at the S2 stage, and then its content increased significantly ($p < 0.05$) and increased by from 6.8% to 59% during the remainder of the process (S3−G4). Similar to free phenolics, the content of total phenolics did not change significantly ($p > 0.05$) and only increased 1.5% between stages G3 and G4. Consequently, free phenolics contributed significantly to the increase of the total phenolic content during the steeping and germination process. The relative contributions of free and bound phenolics to the total phenolics levels are also shown in **Table 2**. These data showed that most phenolics in oat raw grains were in the bound form, which was in agreement with previous reports (*7*, *19*) that the majority of phenolic compounds of cereals like wheat, barley, rice, oats, and corn, especially low molecular weight phenolic compounds, are bound or attached through a covalent association with cell wall polysaccharides. Steeping and germination of cereals can produce enzymes to break down cell walls surrounding compounds, and the free phenolics and bound phenolics may be released, which can result in the increase of total phenolics (*16*). Our results confirm this conclusion and were also in agreement with previous reports (*14*, *18*) that the germinated oat grains display higher total phenolic concentration than untreated oat grains. Nevertheless, along with the processing of steeping and germination, we found that the rise of free phenolic content coincided with a decline in bound phenolic content, although not to the same extent, even free phenolic content was higher than content of bound phenolics, and it became the predominant phenolic compound model during germination (G2−G4), indicating that the increased free phenolics might mainly come from the liberation of the intrinsic free phenolics, and that degradation of bound phenolics during germination might entirely or partly contribute to a rise in free phenolics (*18*).

However, when the total phenolic content reached higher levels (2.58 ± 0.14 mg of GAE/g DW) after 36 h of germination, with the prolong of germination time, total phenolic content had no significant increase ($p < 0.05$) during the subsequent germination process and only increased 1.5%. These results showed that short-term steeping had no obvious effects on different phenolic content, and that germination can increase the free, total phenolic content and decrease the bound phenolic content in oat grains, and also indicate that the positive influence created by the germination on phenolic content is still limited in oat grains. Furthermore, we found that oat raw grains used in the experiment contained much higher amounts of phenolics than oat cultivars Ogle, Dane, Gem, and Belle (*8*). One possible explanation for this discrepancy is difference in oat cultivars and growing environment.

HPLC Analysis of Phenolic Components during Steeping and Germination. HPLC is the traditional technique for the analysis of phenolic compounds, such as phenolic acids, and polyphenols, such as flavonoids. In order to intuitively compare phenolic changes, HPLC chromatogram of extracts for raw grains and sample after 48 h of germination are shown in **Figure 1**. It can be seen from the figure that some compounds such as gallic, caffeic, *p*-coumaric, and ferulic acids and three main avenanthramides (2c, 2p, 2f) were detected, and some peaks have not been identified so far, and we found there were some new arisen substances while some intrinsic phenolic compounds increased or decreased, and even disappeared after germination. This result indicates that phenolic compounds in oat grains may be changed during the steeping and germination. The RP-HPLC quantitative analytical results of the phenolic compounds extracted from oats are shown in **Table 3** and **Table 4**. The levels of the main phenolic acids and avenanthramides varied significantly ($p < 0.05$) among various stages of steeping and germination. The most abundant phenolic acids were ferulic and gallic acids, followed by *p*-coumaric and caffeic acids in raw grains, as well as in samples (**Table 3**). Four phenolic acid contents decreased significantly ($p < 0.05$) during steeping. Thereafter, during germination gallic and caffeic acid content first increased ($p < 0.05$) and then decreased, and *p*-coumaric and ferulic acid content significantly increased by 255% and 186%, respectively. However, these facts did not mean that phenolic acid contents of samples during germination were higher than those of raw grains (**Table 3**).

The increased levels of phenolic acids found in the present study may be due to liberation of some bound forms because some enzymes such as amylolytic, proteolytic, and lipolytic enzymes are synthesized to degrade storage macromolecules,

Table 4. Changes of Main Avenanthramide Content (μg/g DW) in Oat Grains during Steeping (20 °C Treatment) and Germination (16 °C Treatment)[a]

	avenanthramides			
samples	2c[b]	2p[b]	2f[b]	TA[c]
raw grains	58.9 ± 0.2 d	35.9 ± 0.1 e	26.7 ± 0.2 d	121.8 ± 0.6 e
S1[d]	53.5 ± 0.1 f	36.2 ± 0.2 e	26.7 ± 0.1 d	116.3 ± 0.5 f
S2	57.2 ± 0.2 e	37.5 ± 0.3 e	27.2 ± 0.2 d	122.2 ± 0.6 e
S3	59.4 ± 0.3 d	39.4 ± 0.2 de	27.4 ± 0.2 d	126.5 ± 0.5 d
G1[e]	61.2 ± 0.2 c	47.3 ± 0.3 c	37.3 ± 0.3 c	145.8 ± 0.8 c
G2	63.5 ± 0.4 b	52.4 ± 0.4 b	44.8 ± 0.2 b	160.3 ± 1.0 b
G3	64.3 ± 0.3 ab	64.1 ± 0.3 a	50.0 ± 0.4 a	178.8 ± 1.2 a
G4	64.7 ± 0.4 a	64.4 ± 0.2 a	50.6 ± 0.4 a	180.4 ± 1.2 a

[a] Numbers represent mean values of three independent replicates ± SD. Different letters indicate statistically significant differences between the means ($p < 0.05$) for 2c, 2p, 2f and total avenanthramide contents. [b] Abbreviations of avenanthramides: 2c, *N*-(3′,4′-dihydroxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid; 2f, *N*-(4′-hydroxy-3′-methoxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid; 2p, *N*-(4′-hydroxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid. [c] Total avenanthramides (2c, 2p, and 2f). [d] S1−S3 refer to the different steeping stages. [e] G1−G4 refer to the different germination stages.

which lead to softening of the kernel structure during germination of cereals. On the other hand, phenoloxidase and peroxidase enzymes were resumed during germination and they can catalyze oxidation of phenolic substrates (*16*), which lead to the decrease in phenolic acids. Besides, the content of phenolic acids decreased during the steeping and germination, indicating that they were metabolized in some way. For example, caffeic and *p*-coumaric acids may have been used in the biosynthesis of avenanthramides (*17*, *18*). Consequently, the content of different phenolic acids presented various changes during steeping and germination because of difference in structure and properties. In addition, unexpectedly, we found high gallic acid level (113.3 ± 2.6 μg/g DW) in phenolic compounds from the raw grains in our study, which was little reported in previous studies (*7*, *8*, *10*). The result indicates that the high amount of gallic acid may be characteristic in oats (*Avena nuda* L.) from China.

Avenanthramides, a group of phenolic antioxidants, are unique to oats among the cultivated cereals (*11*, *22*). They may act as antioxidants in vitro (*9*, *22*) and in vivo (*24*). The most common avenanthramides are esters of 5-hydroxyanthranilic acid with *p*-coumaric (2p), caffeic (2c), or ferulic (2f) acid (*5*, *9*, *12*, *17*, *22*). The levels of avenanthramides 2c, 2p and 2f were also influenced by the germination process and are shown in **Table 4**. There was a significant decrease ($p < 0.05$) in the total content of the avenanthramides (2c, 2p, and 2f) during the first hours of steeping, and then a subsequent increase ($p < 0.05$) in the total avenanthramide content was detected during the remainder of steeping and germination process (S1−G4). Compared with raw grains, avenanthramides increased by 3.85% and 48% at the end of steeping and germination respectively. However, the total avenanthramide content did not change significantly ($p > 0.05$) between stages G3 and G4. Similar to the change trends of the total avenanthramide, the content of avenanthramide 2c first decreased and then increased with the processing of steeping and germination, and increased less than 10% by the end of germination. The levels of avenanthramides 2p and 2f increased continuously along with the processing of steeping and germination. However, the increase resulting from steeping was not significantly different while the germination resulted in a significant increase ($p < 0.05$) in the content of avenanthramides 2p and 2f as compared to raw grains, and at the end of germination they increased by 79% and 89%, respectively. Nevertheless, the content of avenanthramides 2p and 2f did not significantly change ($p > 0.05$) between stages G3 and G4 (**Table 4**). Unlike changes of phenolic acids, the increase in avenanthramide content seems to have reached a plateau at the later stage of germination, which indicates that longer germination might not have a significant influence on the increase of avenanthramide content.

The three main avenanthramides (2c, 2p, and 2f) were present in raw grain extracts, and the avenanthramide 2c comprised the largest fraction (**Table 4**), which is in agreement with previous reports (*11*). However, it is also known that the content and composition of avenanthramides vary greatly with processing (*12*), genotype, growing environment and conditions (*27*). In the present study, steeping and germination increased the avenanthramide contents, which is in agreement with other reports (*17*, *18*). Obviously, the avenanthramides 2p and 2f contributed significantly to the increase of the total avenanthramide during the germination process and the proportion of all three avenanthramides from the last germination time showed little difference, which is in agreement with the research results of Skoglund et al. (*18*). However, changes of avenanthramides and some phenolic acids at some stages of the steeping and germination in the present study were in disagreement with their results that the levels of both caffeic and *p*-coumaric acids decreased to an almost undetectable level after germination, and that there was a significant increase in the total avenanthramide content during the first hours of steeping. This is probably due to the discrepancy in extraction technology, germination conditions, oat cultivars, sampling time and so on.

The significant increase in the total avenanthramide content during steeping and germination might be owing to biosynthesis of avenanthramides where avenanthramide-synthesizing enzyme (HHT) utilized existing free precursors, like caffeic and *p*-coumaric acids and so on (*28*). Alternatively, the increase might be owing to a release of avenanthramides that were bound to the various cell structures. The decrease in the total avenanthramide content that occurred during the steeping process might be explained by shortage of substrates, avenanthramide degradation and further metabolism of the avenanthramides (*18*, *29*).

DPPH Radicals Scavenging Capacity during Steeping and Germination. In order to evaluate variation in antioxidant activity of the extracts, a method based on the reduction of DPPH was performed. The scavenging ability assayed herein on DPPH radicals is shown in **Table 5**, and results were expressed as IC_{50} values (μg of GAE/mL) for comparison. A significant increase ($p < 0.05$) in the DPPH radical-scavenging activity of phenolic extracts from samples was detected during steeping and germination. Oksman-Caldentey et al. (*14*) also reported relatively higher levels of antioxidant activity during germination in cereal seeds. However, the DPPH radical-scavenging activity showed no obvious changes ($p < 0.05$) during the latter part of the germination (G2−G4), which indicates that the longer time of germination does not mean the higher antioxidant activity. According to present results (**Table 5**), in this test system, though the DPPH radical-scavenging activity of phenolic extracts from treatments increased and even exhibited a higher antioxidant activity than of BHT (IC_{50} was 64.66 μg/mL), they were much lower than of ascorbic acid (IC_{50} was 18.58 μg/mL).

Regression analysis was performed to determine individual, free, bound and total phenolic compounds that would explain the greatest amount of variation in the antioxidant activity of total phenolic extracts (**Table 6**). The contents for free, bound, and total phenolics were highly correlated ($p < 0.01$) to the antioxidant activity. Also free and bound phenolics correlated positively ($p < 0.01$) with total phenolic content. Similar to free and bound phenolics, ferulic acid and the three avenanthramides (2c, 2p, and 2f) were also highly correlated ($p < 0.01$) to the antioxidant activity and total phenolic content. Conversely, gallic

Table 5. Changes of DPPH Radical Scavenging Capacity and FRAP from Different Extracts during Steeping (20 °C Treatment) and Germination (16 °C Treatment)[a]

	DPPH IC_{50} value (μg of GAE/mL)			FRAP value (μmol of Fe(II)/g DW)		
samples	free	bound	total	free	bound	total
raw grains	59.5 ± 1.2 a	106.7 ± 1.3 a	82.2 ± 1.1 a	3.37 ± 0.03 h	11.18 ± 0.08 a	14.67 ± 0.07 f
S1[b]	56.1 ± 1.2 b	105.2 ± 1.4 a	78.9 ± 0.9 b	4.08 ± 0.02 g	10.31 ± 0.06 b	14.24 ± 0.04 g
S2	57.3 ± 1.0 b	87.2 ± 1.2 b	72.4 ± 0.7 c	4.39 ± 0.05 f	10.32 ± 0.05 b	15.08 ± 0.06 e
S3	53.6 ± 0.8 c	72.8 ± 1.1 c	68.5 ± 0.7 d	5.11 ± 0.04 e	9.13 ± 0.05 c	14.16 ± 0.08 g
G1[c]	49.8 ± 0.7 d	56.3 ± 0.8 d	53.6 ± 0.6 e	7.79 ± 0.07 d	9.21 ± 0.03 c	17.11 ± 0.10 d
G2	42.3 ± 0.4 e	52.2 ± 0.5 e	45.8 ± 0.4 f	12.49 ± 0.06 c	8.25 ± 0.04 e	21.21 ± 0.14 c
G3	42.4 ± 0.6 e	52.5 ± 0.6 e	45.4 ± 0.5 f	15.44 ± 0.08 b	8.21 ± 0.05 e	24.06 ± 0.18 b
G4	42.7 ± 0.5 e	51.6 ± 0.4 e	44.6 ± 0.6 f	18.35 ± 0.11 a	8.37 ± 0.03 d	26.2 ± 0.21 a

[a] Numbers represent mean values of three independent replicates ± SD. Different letters indicate statistically significant differences between the means ($p < 0.05$) for free, bound, and total phenolic extracts. [b] S1-S3 refer to the different steeping stages. [c] G1-G4 refer to the different germination stages.

Table 6. Correlation Analysis of Phenolics and Antioxidant Activity

	total phenolic extracts		
compounds	DPPH	FRAP	TPC[a]
gallic acid	0.0584[b]	0.1216	0.0754
caffeic acid	0.1834	0.4808	0.3912
p-coumaric acid	0.3652	0.7400**[c]	0.6859*
ferulic acid	0.5725*	0.8987**	0.8215**
2c[d]	0.7989**	0.7401**	0.7113**
2p	0.8541**	0.9598**	0.9827**
2f	0.8988**	0.9567**	0.9380**
free phenolic	0.8481**	0.9633**	0.9851**
bound phenolic	0.9486**	0.7821**	0.7899**
total phenolic	0.7752**	0.9711**	

[a] Total phenolic content. [b] Correlation coefficient R^2. [c] Significantly different: ** $p < 0.01$, * $p < 0.05$. [d] Abbreviations of avenanthramides: 2c, *N*-(3′,4′-dihydroxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid; 2f, *N*-(4′-hydroxy-3′-methoxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid; 2p, *N*-(4′-hydroxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid.

and caffeic acids were low correlated ($p > 0.05$) to the antioxidant activity and total phenolic content. *p*-Coumaric acid significantly correlated ($p < 0.05$) to the total phenolic content, but there was no correlation ($p > 0.05$) to the antioxidant activity. However, the correlation between the antioxidant activity and bound phenolics is significantly negative, which may imply that some high reactive antioxidative substances might be produced during steeping and germination. From the regression analysis it can be concluded that changes of DPPH radical-scavenging activity of phenolic extracts from oats during steeping and germination can be attributed to changes of phenolic contents, especially changes of contents of some phenolic components that are associated highly with antioxidant activity, such as ferulic acid, avenanthramides (2c, 2p, and 2f) and so on.

FRAP during Steeping and Germination. The antioxidant activity is influenced by many factors, and there are more commonly used methods that have their advantages and disadvantages of each for measuring antioxidant activity, which cannot be fully described with one single method (*28*). Therefore, it is necessary to perform more than one type of antioxidant activity measurement to take into account the various mechanisms of antioxidant action. In this assay, the antioxidant activity is measured on the basis of the ability to reduce ferric(III) ions to ferrous(II) ions. The results of ferric(III) ion reduction are shown in **Table 5**. The antioxidant activities of the free, bound, and total phenolic extracts of each sample were different, and there was a relationship that the total phenolic extracts were the sum of free and bound phenolic extracts in antioxidant activity determined by FRAP assay. There was a significant increase ($p < 0.05$) in the antioxidant activity of the free, and total phenolic extracts during steeping and germination, which was similar to the results determined by DPPH assay. At the same time the antioxidant activity of bound phenolic extracts significantly decreased ($p < 0.05$) during the whole processing, which was just the opposite of the results determined by DPPH assay because of different chemistries and reaction conditions (*28*, *29*). From **Table 5**, during the steeping and germination process, the antioxidant activity of bound phenolics decreased approximately 25%, on the contrary, the antioxidant activity of free, and total phenolics nearly increased 5.4-fold and 1.8-fold, respectively. However, the antioxidant activities of raw grains and samples were much lower than of ascorbic acid (1.12×10^4 μmol of Fe(II)/g) and BHT (1.04×10^4 μmol of Fe(II)/g) in this test system.

To further investigate the influence of different phenolic compounds on the antioxidant activity of total phenolic extracts, the correlation between the antioxidant activity determined by FRAP assay and phenolic contents of samples was established, and correlation coefficients (R^2) are shown in **Table 6**. Gallic and caffeic acids were low correlated ($p > 0.05$) to the antioxidant activity and total phenolic content. On the contrary, other phenolic compounds correlated ($p < 0.01$) significantly with the antioxidant activity and total phenolic content. These results are supported by the previous study (*23*, *30*) that the phenolic compounds may be responsible for a large proportion of the antioxidant activity determined by FRAP assay in some plants. Therefore, phenolic contents could be used as an important indicator of antioxidant activity of steeped or germinated oat grains and used as natural sources of antioxidant functional foods.

Conclusively, these data suggested that phenolic compounds and antioxidant activity in oats were affected by steeping and germination. Phenolic components, phenolic content and antioxidant activity of oats were different at different stages of steeping and germination. The optimum treatment showed greater phenolic content and antioxidant activity while prolonged treatments did not have significant influence on the phenolic content and antioxidant activity. Therefore, the choice of time might be of great importance because the differences in the response to the steeped and germination treatment varied with the time. Besides, the physiological and biochemical reaction resulting from steeping and germination of cereal seeds is an extremely complex process, which is affected by several factors such as the time, temperature, aeration, humidity and so on. Moreover, some new substances appeared during germination, which were not identified due to shortage of standards in the present study. Nevertheless, these new substances might also have a critical part for action in antioxidant activity. Consequently, further studies need to be performed on the steeping and germination process to optimize the increase of the phenolic compounds and antioxidant activity. In addition, some unknown

antioxidative substances also have to be identified in oat raw grains and oats during steeping and germination.

ABBREVIATIONS USED

2c, *N*-(3′,4′-dihydroxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid; 2f, *N*-(4′-hydroxy-3′-methoxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid; 2p, *N*-(4′-hydroxy)-(*E*)-cinnamoyl-5-hydroxyanthranilic acid; GA, gallic acid; CA, caffeic acid; *p*CA, *p*-coumaric acid; FA, ferulic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; BHT, 2,6-di-*tert*-butyl-4-hydroxytoluene; GAE, gallic acid equivalents; TPTZ, 2,4,6-tri(2-pyridyl)-*s*-triazine; DW, dry weight of sample; HPLC, high-performance liquid chromatography; FRAP, ferric reducing antioxidant power.

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