Journal of Applied Botany and Food Quality 84, 111 - 118 (2011)

Institute of Agronomy and Plant Breeding, Justus-Liebig-University Giessen, Germany

Effect of sprouting on the concentration of phenolic acids and antioxidative capacity in wheat cultivars (*Triticum aestivum* ssp. *aestivum* L.) in dependency of nitrogen fertilization

N. Engert, A. John, W. Henning, B. Honermeier

(Received October 26, 2010)

Summary

The study was conducted to analyze the effects of sprouting on content and composition of phenolic acids (PA) and antioxidative capacity by the Folin-Ciocalteu (total phenolic content (TPC)) and ORAC (Oxygen Radical Absorbance Capacity) assay depending on nitrogen application and cultivar. Three wheat cultivars (cv. Tommi, cv. Privileg and cv. Estevan) were treated with two different nitrogen (N) levels (N_1 without N and N_2 with 150 kg N/ha). Three fractions of the grinded wheat caryopses (free, free-insoluble and bound phenolic acids) were extracted. Mean values for total phenolic acids (TPA) ranged between 498.1 and 1726.2 µg GAE/g with significant differences for the cultivars in not sprouted samples. Nitrogen fertilization showed significant differences with lower TPA contents for not fertilized grain. Even the interaction cultivar x nitrogen was significant for not sprouted grain. The statistical analysis of sprouting time did not show effects on TPA values after 24 h and 48 h of sprouting. In the present study no significant influence of cultivar or nitrogen application was identified for TPC and ORAC values. In contrary to that, the effects of sprouting time on the antioxidative capacity by Folin-Ciocalteu and ORAC were significant. After 48 h of sprouting the values of TPC (2575.6 µg GAE/g) and ORAC (32.6 µmol TE/g) were significantly higher than for not sprouted wheat samples (TPC: 2054.8 µg GAE/g, ORAC: 28.2 µmol TE/g). The longer the sprouting time was, the higher the antioxidative capacity of free phenolic acids, indicating that more free phenolic acids were released and other phytochemicals might have been present after sprouting. Since there are only little information about the effect of sprouting time on phenolic acids and antioxidative capacity in dependence on nitrogen application further studies need to be conducted.

Introduction

Cereals, especially wheat (*Triticum aestivum* L. ssp. *aestivum*), provide an important contribution to the human diet with their basic nutrients carbohydrates, protein, dietary fiber, vitamins and minerals. Besides, they contain secondary plant metabolites such as carotinoids, flavonoids and phenolic acids that complement a balanced diet by means of their antioxidative potential. As a result of enhanced health awareness during the last years, natural antioxidants gained considerable interest due to their health beneficial functions. E. g. phenolic acids show health affecting potentials because of their antioxidativity (Serpen, 2008). Potential effects of phenolic compounds are to reduce the risk of cardiovascular diseases and cancer (Knekt et al., 2002; Peterson et al., 2003; Sesso et al., 2003; Arts and Hollman, 2005; Graf et al., 2005). Wheat phenolic acids (PA), such as ferulic (FA), *p*-cumaric (PCA), vanillic (VA), sinapic (SIA), syringic (SYA) and caffeic acid (CA) are valuable antioxidativ ingredients (Mpofu, 2006). It is reported that phenolic acids inhibit lipid oxidation by scavenging free radicals such as hydroxyl radicals (Cuppett et al., 1997). In addition, sprouts are considered to be health beneficial, even more than the cereal grain itself (Kahlil and Mansour, 1995; Prodanov et al., 1997; Yang et al., 2001, Arora et al., 2010, Tian et al., 2010). However, for some processes, like the production of bread, it is extremely necessary that wheat caryopses are of a very high quality with no degradation of their compounds. Sprouting is a result of humid conditions during and after grain development or storage. By reason of sprouting the starchy endosperm can be reduced because of enzymatic degradation processes, e.g. by α-amylase.

Little information is available about the effect of nitrogen fertilization on the content of phenolic acids. Germination of the caryopses induces a higher amylase activity in caryopses resulting in lower starch contents. During this process other components like phenolic acids might be changed, too. Thus, the aim of this study was to investigate phenolic acids, their composition and their variation in three different wheat cultivars in dependency on sprouting time and nitrogen fertilization.

Materials and methods

In 2008 winter wheat cultivars were grown in field plots of the experimental station "Rauischholzhausen" of the Institute of Agronomy and Plant Breeding (degree of longitude: 8° 52' 05" E, degree of latitude: 50° 46' 41" N, altitude: 222 m). Three cultivars (cv. Tommi, cv. Privileg, cv. Estevan) of *T. aestivum* ssp. *aestivum* L. with four randomized replications were obtained from the field experiment. Two nitrogen levels were analyzed: N_1 without N fertilization and N_2 with 150 kg N/ha (40, 40, 70 kg N/ha). To avoid lodging of wheat plants all plots were treated with the plant growth regulator chlorcholin chloride (CCC, 720 g/l) at two times. To protect the wheat against diseases fungicides were applied at two times (first: 0.8 l/ha Proline + 1.5 l/ha Pugil and second: 0.7 l/ha Diamant + 0.7 l/ha Champion). After harvesting the samples were stored in darkness at 20 °C until they were used for the sprouting experiment.

To induce sprouting 30 g wheat were steeped with 15 ml aqua dest. in petri dishes which were covered with ashless circular filters (MN 640 m, 90 mm, Macherey-Nagel). Three different sprouting times were tested at room temperature, without direct solar radiation: 0 h (no sprouting), 24 h (presprouted wheat samples) and 48 h (fully sprouted wheat samples) (Fig. 1). To stop the sprouting process, all samples were dried for 24 h at 36 °C.

In total 72 wheat samples (3 cultivars x 3 sprouting levels x 2 N fertilization doses x 4 replications) were included and ground with a 500 µm sieve on a Cyclotec 1073 Sample Mill (Foss, Germany) to get a whole wheat flour. The samples were mixed thoroughly to ensure homogeneity. Extraction followed immediately.

Extraction: For the analysis of phenolic acids, samples were extracted using a modified method by Krygier et al. (1982) and Adom and Liu (2002) as follows. Whole wheat flour (1 g) was extracted with 10 ml methanol/acetone/water (7:7:6, v/v/v) and divided into three fractions: (1) soluble free, (2) soluble conjugated, and (3) bound phenolic acids. The supernatant provided fraction 1 and 2, the residue fraction 3. Before all fractions were extracted with

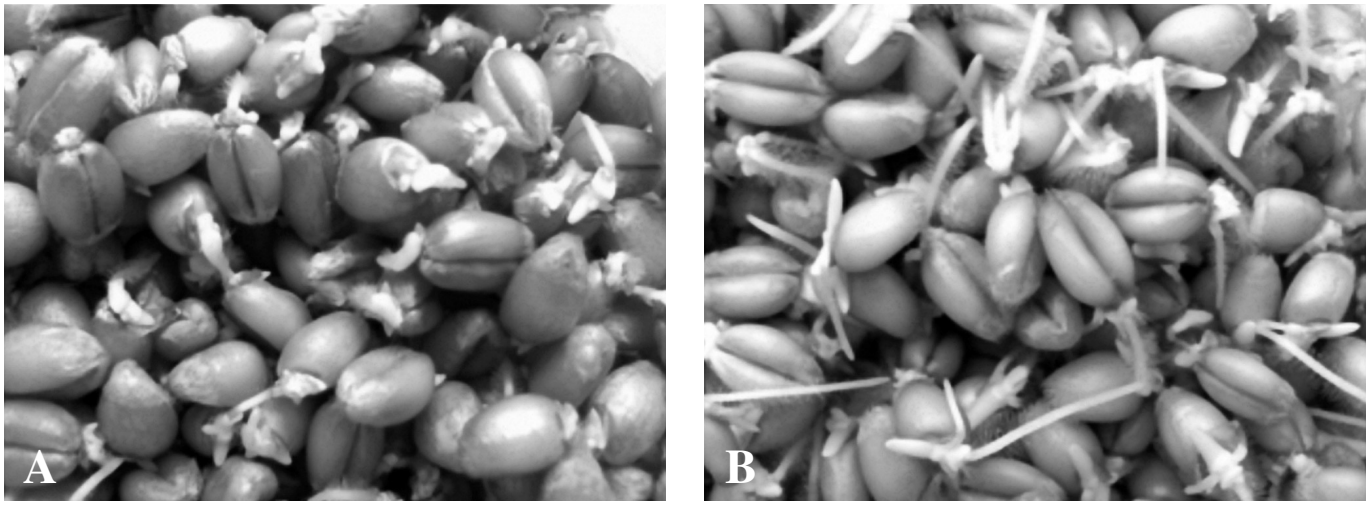

Fig. 1: Different sprouting levels of wheat: A- presprouted grain after 24 h; B- fully sprouted grain after 48 h of sprouting

ethyl acetate, bound and soluble conjugated phenolic acids had to be hydrolyzed with 5 M NaOH for three hours. The reaction was stopped with 5 M HCL and the extracts were shaken out with ethyl acetate. The organic extracts were transferred into flasks and ethyl acetate was evaporated. Referring to that, extracts were resumed in 10 ml of 10 % methanol and stored at -18 °C for all further analysis (HPLC, Folin, ORAC).

Quality Parameters: Generally, parameters concerning grain quality were measured to characterize the used wheat cultivars before starting the sprouting experiment. According to ICC and ISTA standard methods thousand grain weight (TGW), crude protein (CP), falling number (FN), gluten index and sedimentation test by Zeleny (Sedi) were analyzed. The parameter falling number was additionally used to determine the state of sprouting. That means, grain with no sprouting showed highest mean falling numbers (419 s), grain which was presprouted showed lower (104 s) and fully sprouted grain had lowest falling numbers (62 s).

HPLC: The HPLC method according to WEIDNER et al. (2000) and ZIELINSKI et al. (2001) was used with some modifications for determination of phenolic acids (PA) and total phenolic acids (TPA) as the sum of all analyzed phenolic acids. The analysis was performed on a HPLC-DAD system by Knauer on a C18 column (EC 250 x 4 Nucleodur Sphinx RP, 5 µm (MN)) at a temperature of 25 °C, a flow rate of 1 ml min^{-1} and an injection volume of 100 µl. The detection was carried out at 250 nm (hydroxybenzoic acids) and 290 nm (hydroxycinnamic acids) following the program: 0-3 min 20 % A, 3.5-7.5 min 0 % A, 8-14 min 8 % A, 14.5-32 min 30 % A, 33-39 min 95 % and 40-45 min 20 %. The gradient system consisted of (A) 95 % methanol with acetic acid (about 200 µl) adjusted to pH 4.5 and (B) water adjusted to pH 4.5 with about 5 µl acetic acid.

Folin-Ciocalteu Assay: The Folin-Ciocalteu Assay is an electron transfer based reaction assay, and measures the reducing capacity of the sample (HUANG et al., 2005). In the present analysis it is called total phenolic content (TPC) and the Folin-Ciocalteau micro method by WATERHOUSE (2001) was used. Folin-Ciocalteu reagent consisted of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids, additionally gallic acid was used as standard. To measure the absorbance spectrophotometically at 765 nm with a Specord 205 – 222A430 (Analytik Jena AG, Germany), the Folin-Ciocalteu reagent had to be reduced to blue oxides of tungsten and molybdenum. The total phenolic content was expressed as gallic acid equivalent (GAE).

ORAC Assay: ORAC (Oxygen Radical Absorbance Capacity) is a hydrogen atom transfer based reaction assay to measure the antioxidative capacity towards peroxyl radicals (HUANG et al., 2005). A previously described method by HUANG et al. (2002) was used and the analysis was operated on a 96-well plate fluorescence reader Fluoroskan (Fisher Scientific GmbH, Germany). Very briefly, fluorescein (6-hydroxy-9-(2-carboxyphenyl)-(3h)-xanthen-3-on) and the sample were mixed and incubated at 37 °C. To initiate the reaction the ROS-generator AAPH (2,2'-azobis(2-methylpropion amidine)dihydrochloride) was added to measure fluorescence at excitation wavelength 485 nm and emission wavelength 538 nm every 60 s for 90 min. As a standard Trolox® (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a vitamin E analogue, was used and the antioxidative capacity was expressed as Trolox equivalent (TE).

Statistical Analysis: One-way analysis of variance (ANOVA) was performed with PASW Statistics 18 (SPSS Inc., Chicago, IL) for all wheat cultivars (cv. Tommi, cv. Privileg, cv. Estevan) and nitrogen levels (N_1 and N_2) within all sprouting times (no sprouting, presprouted, fully sprouted) to determine the effects on the measured parameters. When significant differences occurred ($p \leq 0.05$) multiple mean comparisons were performed using the Tukey t-test. To determine the influence of sprouting time on the analyzed parameters a paired samples test (t-test) was performed. Spearman's correlation (r_s) factors (bivariate) were calculated between total phenolic acids (TPA), total phenolic content (TPC) and antioxidative capacity (ORAC). Results are reported as mean values and the significance level was at a 95 % confidence interval ($\alpha = 0.05$).

Results

Quality Parameters: Thousand grain weight (TGW) ranged from 40.1 to 45.0 g with significant differences (≤ 0.001) between all three cultivars (Tab. 1). Cv. Privileg could be characterized as the cultivar with largest grain (45.0 g) followed by cv. Tommi (42.8 g) and cv. Estevan (40.1 g). Analyzed falling numbers (FN) ranged from 323 to 388 s significantly affected by the cultivars ($p \leq 0.001$) and N fertilization ($p \leq 0.001$). Cv. Tommi with a mean value of

388 s showed the highest falling number which was significantly higher than cv. Estevan with 323 s. Cv. Tommi and cv. Privileg (377 s) were at the same level, which indicates a very low amylase activity in the caryopses. Nitrogen fertilized samples had higher FN (381 s) than not fertilized (345 s) ones. Sedimentation volumes (Fig. 2) differed between 33 ml and 58 ml with significantly ($p=0.003$) interaction effects for cultivar and nitrogen treatment. Nitrogen application to cv. Estevan (58 ml) and cv. Tommi (55 ml) induced significantly higher sedimentation volumes than nitrogen application to cv. Privileg (44 ml). Gluten Indices (GI) ranged between 81 and 92 indicating a high gluten quality. N fertilization ($p \leq 0.001$) and cultivar ($p \leq 0.001$) were significant with regard to GI. Fertilized wheat samples had significantly lower GI than not fertilized wheat samples. The cultivar Estevan (92) reached significantly higher GI than cv. Tommi (81) and cv. Privileg (84) which were at the same level. Crude protein (CP) ranged between 12.5 and 12.9 %. As to expect, nitrogen treatment (13.6 %) led to higher contents of crude protein ($p \leq 0.001$) in wheat caryopses than in unfertilized (11.6 %) ones, whereas between cultivars no significant CP differences were observed.

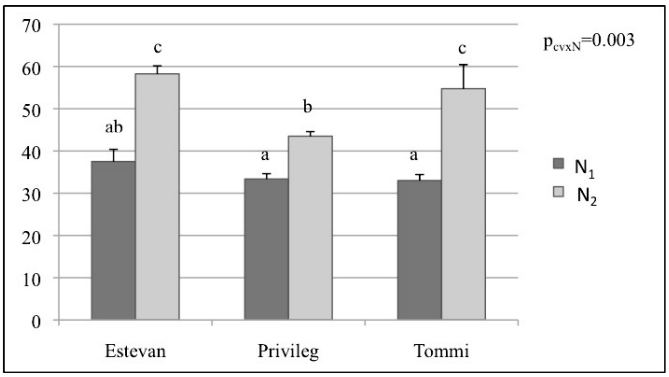

Fig. 2: Interaction effect of N fertilization and cultivar on sedimentation by Zeleny in wheat samples (mean ± SD, α=0.05)

Phenolic Acids (PA): The analyzed total phenolic acids (TPA) consisted of vanillic acid (VA), syringic acid (SYA), caffeic acid (CA), p-coumaric acid (PCA) and ferulic acid (FA), measured by the HPLC method. Results were expressed as mean values in sum of the measured phenolic acids, ranging between 498.1 and 1726.2 µg GAE/g. Cultivars which were not sprouted showed significantly differences ($p \leq 0.001$) in their TPA contents. However, cv. Privileg (1066.1 µg GAE/g) and cv. Estevan (1154.3 µg GAE/g) had significantly lower values than cv. Tommi (1726.2 µg GAE/g). TPA values of non-fertilized wheat (N_1=1085.7 µg GAE/g) were significantly lower than of fertilized ones (N_2=1545.3 µg GAE/g). Moreover, significantly ($p \leq 0.001$) interaction effects between cultivar and nitrogen occurred for not sprouted wheat samples mainly resulting from cv. Tommi x N_2 which was about twice as high than the other cultivar x nitrogen interactions. After a sprouting time of 24 h an interaction effect between cultivar and nitrogen fertilization ($p=0.044$) was observed. The combination of cv. Tommi x N_2 showed once more the highest TPA value (1669.0 µg GAE/g) which significantly differed only from cv. Tommi x N_1 (760.5 µg GAE/g). After a sprouting time of 48 h a significant cultivar effect on TPA was noticeable (Tab. 2). The cultivars cv. Tommi (1642.2 µg GAE/g) and cv. Estevan (1554.0 µg GAE/g) had the same level of TPA contents which was significantly higher than the TPA of cv. Privileg (498.1 µg GAE/g). Nitrogen application had no significant effects on TPA contents of the analyzed wheat samples but a tendency of higher TPA contents of N_2 fertilized samples in comparison with the N_1 fertilization level was observed.

Looking at the means of TPA values of the three sprouting treatments, which varied from 1315.5 µg (0 h) to 1099.2 µg (24 h) and 1231.4 µg GAE/g (48 h), no significant differences were detectable.

Total phenolic acids (TPA) were most concentrated in fraction 3 (TPA3) for all three sprouting times (Fig. 3). Unsprouted wheat samples contained 86 % (1128.4 µg GAE/g) in fraction 3 followed by fraction 1 (TPA1, 77.9 µg GAE/g) and fraction 2 (TPA2, 109.3 µg GAE/g) with less than 10 %. While sprouting for 24 h the relative and absolute values did not change much. After 48 h TPA1 increased to 28 % (346.3 µg GAE/g) and TPA3 provided 59 % (722.3 µg GAE/g). TPA2 rose up to 13 % (162.8 µg GAE/g).

Looking at the composition of the five analyzed phenolic acids, the predominant phenolic acid was ferulic acid. During sprouting the proportion of each analyzed phenolic acid shifted. Nevertheless, ferulic acid (FA) was the main phenolic acid with 67 % (881.8 µg GAE/g) for not sprouted, 74 % (809.3 µg GAE/g) for presprouted and 54 % (669.9 µg GAE/g) for fully sprouted grain (Fig. 4).

Tab. 1: Effect of N fertilization and cultivar on thousand grain weight (TGW, [g]), falling number (FN, [s]), gluten index (GI) and crude protein (CP, [% DM]) in wheat samples

Treatment (cultivar/nitrogen)	TGW g	FN s	GI	CP % DM
Main effect cv.				
Estevan	40.1 c	323 b	92 a	12.9 n.s.
Privileg	45.0 a	377 a	84 b	12.5 n.s.
Tommi	42.8 b	388 a	81 b	12.5 n.s.
Main effect N				
N_1	42.7 n.s.	345 a	93 a	11.6 a
N_2	42.6 n.s.	381 b	78 b	13.6 b
p cultivar (cv.)	≤ 0.001	≤ 0.001	≤ 0.001	n.s.
p nitrogen (N)	n.s.	≤ 0.001	≤ 0.001	≤ 0.001
p cv. x N	n.s.	n.s.	n.s.	n.s.

Tab. 2: Effect of sprouting time on total phenolic acids (TPA) [µg GAE/g] in wheat samples depending on N fertilization and cultivar

Treatment (cultivar/nitrogen)	Sprouting time [h]		
	0	24	48
Main effect cv.			
Estevan	1154.3 a	1148.6 n.s.	1554.0 a
Privileg	1066.1 a	934.4 n.s.	498.1 b
Tommi	1726.2 b	1214.8 n.s.	1642.2 a
Main effect N			
N_1	1085.7 a	1048.5 n.s	1151.7 n.s.
N_2	1545.3 b	1149.9 n.s	1311.2 n.s.
Interaction effect			
Estevan x N_1	1136.9 a	1500.5 ab	1542.7 n.s.
Estevan x N_2	1171.8 a	796.7 ab	1565.3 n.s.
Privileg x N_1	1057.3 a	884.5 ab	359.1 n.s.
Privileg x N_2	1074.9 a	984.2 ab	637.1 n.s.
Tommi x N_1	1063.0 a	760.5 a	1553.3 n.s.
Tommi x N_2	2389.3 b	1669.0 b	1731.1 n.s.
Mean	1315.5 n.s.	1099.2 n.s.	1231.4 n.s.
p cultivar (cv.)	≤ 0.001	n.s.	0.002
p nitrogen (N)	≤ 0.001	n.s.	n.s.
p cv. x N	≤ 0.001	0.044	n.s.

Total Phenolic content (TPC): The total phenolic content (TPC) analyzed by the Folin-Ciocalteu assay ranged between 1523.8 and 2836.7 µg GAE/g (Tab. 3). At the beginning of the experiment not sprouted wheat samples were characterized by a small variability of TPC values. Neither main effects (cultivars, nitrogen) nor interaction effects (cv. x N) could be observed. Contrary to that after 24 h of sprouting there was a significant nitrogen effect of TPC expressed by lower TPC for N_1 (1464.6 µg GAE/g) in comparison to N_2 (2038.6 µg GAE/g). After 48 h of sprouting the treatments cultivar (p=0.008) and fertilization (p=0.011) showed significantly effects, however, the interaction between both parameters is not significant. Cv. Estevan (2836.7 µg GAE/g) and cv. Tommi (2684.9 µg GAE/g) had significantly higher TPC values than cv. Privileg (2205.2 µg GAE/g). TPC values for fertilized wheat (N_2=2790.1 µg GAE/g) were significantly higher than for non-fertilized samples (N_1=2361.0 µg GAE/g).

A sprouting time of 48 h significantly (p≤0.001) influenced the TPC. After 48 h (2575.6 µg GAE/g) of sprouting the TPC were higher than after 24 h (1751.6 µg GAE/g) and for unsprouted wheat samples (2054.8 µg GAE/g) (Tab. 3). The highest TPC provided fraction 1 (TPC1) for all three sprouting times. Unsprouted wheat samples showed a total phenolic antioxidative capacity of 46 % (940.2 µg GAE/g for TPC1, followed by fraction 3 (TPC3) with 28 % (574.7 µg GAE/g) and fraction 2 (TPC2) 26 % (539.7 µg GAE/g) (Fig. 5). During 24 h of sprouting, TPC1 (38 %, 678.1 µg GAE/g) lost some of its antioxidative capacity while TPC3 (35 %, 574.2 µg GAE/g) gained. After 48 h TPC1 (46 %, 1194.3 µg GAE/g) and TPC2 (31 %, 799.8 µg GAE/g) values increased whereas TPC3 (23 %, 581.5 µg GAE/g) decreased (Fig. 5).

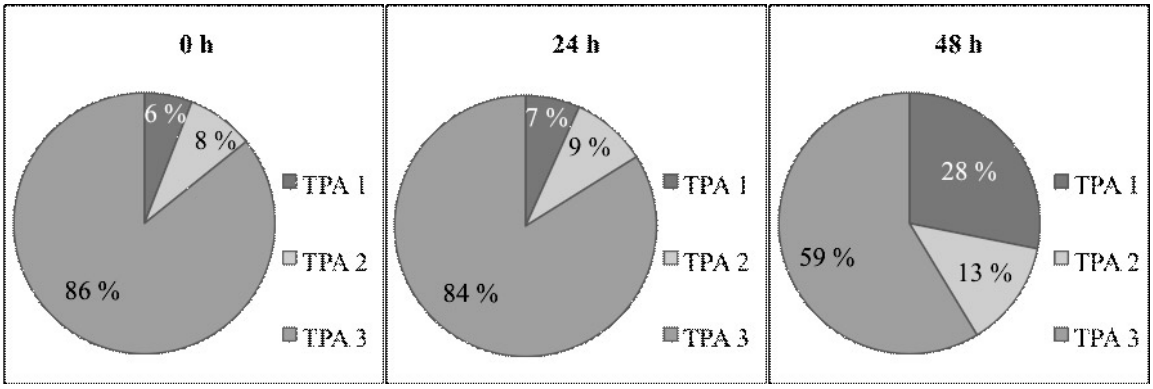

Fig. 3: Effect of sprouting time on the composition of the extracted fractions (TPA1, TPA2, TPA3) of total phenolic acids (TPA) in wheat samples

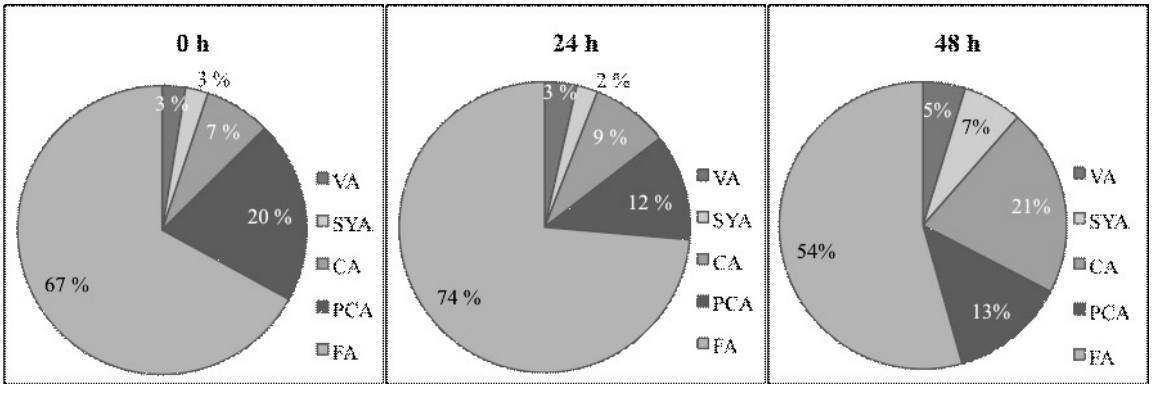

Fig. 4: Effect of sprouting time on the composition of phenolic acids (TPA) in wheat grain

Tab. 3: Effect of sprouting time on the total phenolic content (TPC) [µg GAE/g] in wheat samples depending on N fertilization and cultivar

Treatment (cultivar/nitrogen)	Sprouting time [h]		
	0	24	48
Main effect cv.			
Estevan	2012.0 n.s.	1523.8 n.s.	2836.7 a
Privileg	2170.5 n.s.	1646.7 n.s.	2205.2 b
Tommi	1981.9 n.s.	2084.4 n.s.	2684.9 a
Main effect N			
N_1	1970.6 n.s.	1464.6 a	2361.0 a
N_2	2139.0 n.s.	2038.6 b	2790.1 b
Interaction effect			
Estevan x N_1	2090.9 n.s.	1031.6 n.s.	2634.9 n.s.
Estevan x N_2	1933.1 n.s.	2015.9 n.s.	3038.5 n.s.
Privileg x N_1	2005.9 n.s.	1656.9 n.s.	2151.2 n.s.
Privileg x N_2	2335.2 n.s.	1636.6 n.s.	2259.1 n.s.
Tommi x N_1	1815.1 n.s.	1705.4 n.s.	2297.0 n.s.
Tommi x N_2	2148.7 n.s.	2463.5 n.s.	3072.7 n.s.
Mean	2054.8 a	1751.6 a	2575.6 b
p cultivar (cv.)	n.s.	n.s.	0.008
p nitrogen (N)	n.s.	0.029	0.011
p cv. x N	n.s.	n.s.	n.s.

ORAC: The antioxidative capacity by the ORAC assay ranged from 24.4 to 35.1 µmol TE/g whole wheat flour (Tab. 4). In unsprouted wheat samples no significant effects could be observed, neither for main effects nor for the interaction of both treatments (cv x N). After 24 h of sprouting the factor cultivar ($p \leq 0.001$) and the interaction cv. x N had significant influence ($p=0.006$) on the antioxidative capacity. Cv. Privileg x N_1 (28.3 µmol TE/g), cv. Privileg x N_2 (22.6 µmol TE/g) and cv. Tommi x N_2 (36.5 µmol TE/g) showed significant differences whereas cv. Estevan x N_1 (24.1 µmol TE/g), cv. Estevan x N_2 (24.6 µmol TE/g) and cv. Tommi x N_1 (28.2 µmol TE/g) were at the same level. After 48 h of sprouting no significant differences neither for main effects nor for the interaction effect could be observed. The results showed a tendency that wheat samples fertilized with 150 kg N/ha (N_2) had higher ORAC values than N_1 ones. Furthermore, Tab. 4 reveals a significant influence of sprouting time on the antioxidative capacity. The values were significantly higher after 48 h (32.6 µmol TE/g) of sprouting than after 24 h (27.4 µmol TE/g) or with no sprouting (28.2 µmol TE/g).

The highest antioxidative capacity contributed fraction 3 (ORAC3) for not sprouted wheat samples (39 %, 10.9 µmol TE/g) and pre-sprouted cultivars (40 %, 10.8 µmol TE/g). After a sprouting time of 48 h the values of ORAC1 increased to 48 % (15.7 µmol TE/g) whereas ORAC3 (26 %, 8.6 µmol TE/g) decreased (Fig. 6).

Tab. 4: Effect of sprouting time on the antioxidative capacity by the ORAC assay [µmol TE/g] in wheat samples depending on N fertilization and cultivar

Treatment (cultivar/nitrogen)	Sprouting time [h]		
	0	24	48
Main effect cv.			
Estevan	27.9 n.s.	24.4 a	35.1 n.s.
Privileg	26.4 n.s.	25.4 a	29.5 n.s.
Tommi	30.2 n.s.	32.3 b	33.3 n.s.
Main effect N			
N_1	28.0 n.s.	26.9 n.s.	30.8 n.s.
N_2	28.3 n.s.	27.9 n.s.	34.5 n.s.
Interaction effect			
Estevan x N_1	28.4 n.s.	24.1 ab	35.1 n.s.
Estevan x N_2	27.3 n.s.	24.6 ab	35.1 n.s.
Privileg x N_1	27.6 n.s.	28.3 a	29.3 n.s.
Privileg x N_2	25.2 n.s.	22.6 b	29.8 n.s.
Tommi x N_1	28.1 n.s.	28.2 ab	27.9 n.s.
Tommi x N_2	32.4 n.s.	36.5 c	38.7 n.s.
Mean	28.2 a	27.4 a	32.6 b
p cultivar (cv.)	n.s.	≤ 0.001	n.s.
p nitrogen (N)	n.s.	n.s.	n.s.
p cv. x N	n.s.	0.006	n.s.

Discussion

The present study focused on the effects of sprouting time on phenolic acids and antioxidative capacity of whole wheat samples

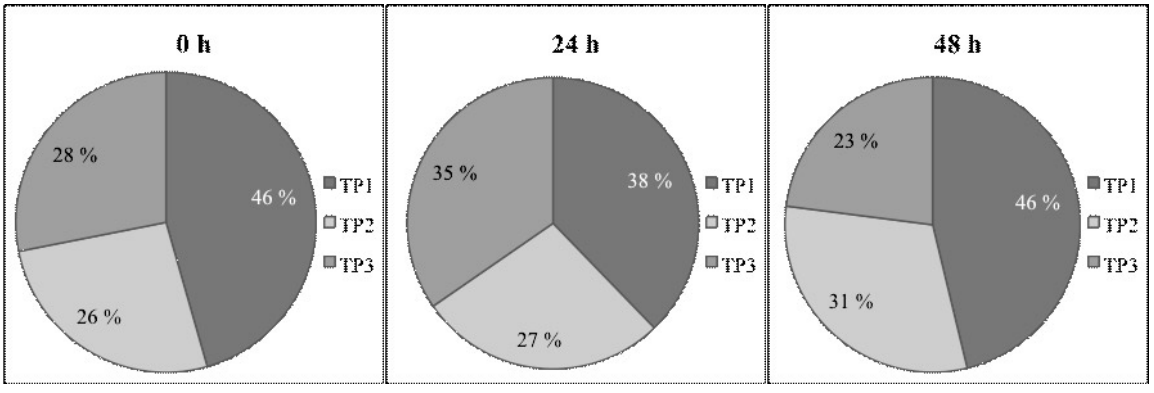

Fig. 5: Effect of sprouting time on the composition of the extracted fractions (TPC1, TPC2, TPC3) of total phenolic contents (TPC) in wheat samples

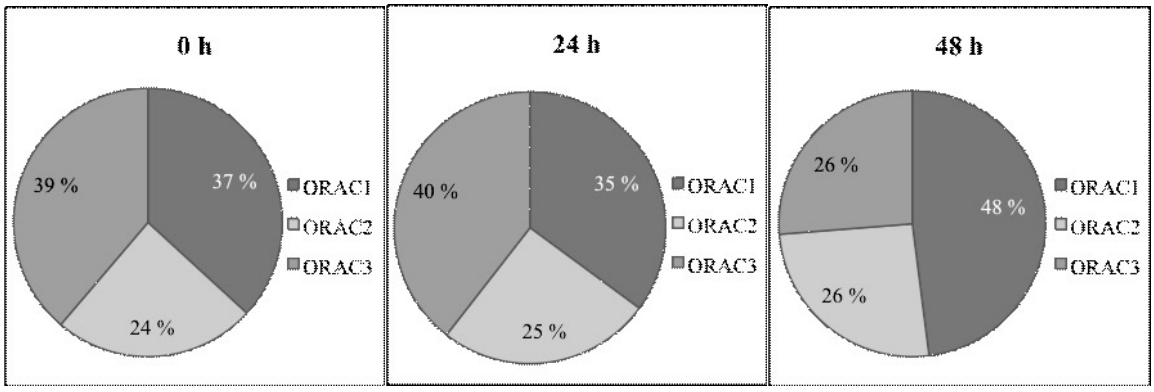

Fig. 6: Effect of sprouting time on the composition of the extracted fractions (ORAC1, ORAC2, ORAC3) of the antioxidative capacity by the ORAC assay in wheat samples

depending on cultivar and nitrogen fertilization. Sprouting is the first important stage during biogenesis of wheat. Enzymatic processes, like starch degradation by α-amylase, take place to provide the embryo with nutrients. Normally sprouting occurs after a certain time of dormancy when the caryopsis has come to full ripeness (HAUMANN and DIETZSCH, 2000). If it starts too early, because of humid conditions during ripening, it is a negative parameter for grain quality, e.g. for flour processing. Nevertheless it is necessary to know, if deliberately induced sprouting has an influence on phenolic acids and their antioxidative capacity with regard to their health beneficial effects (KAHLIL and MANSOUR, 1995; PRODANOV et al., 1997; YANG et al., 2001; TIAN et al., 2004; ARORA et al., 2010; TIAN et al., 2010). In the conducted study analysis of phenolic acids (TPA) and antioxidative capacity by the Folin-Ciocalteu and ORAC assay was carried out in whole wheat flour because of highest concentrations of phenolic acids in the bran and aleurone layer of caryopses (PUSSAYANAWIN et al., 1988; ZHOU et al., 2004; ADOM et al., 2005; ANSON et al., 2008).

The analyzed phenolic acids (TPA) didn't show a significantly influence for the different sprouting times, indicating that enzymatic processes during sprouting did not affect phenolic acids while differences for the analyzed cultivars were visible. After 48 h of sprouting in the current study cultivar Estevan (1554.0 µg GAE/g) showed higher TPA values than after 0 h of sprouting (1154.3 µg GAE/g), whereas cv. Privileg and cv. Tommi showed lower values (Tab. 2). The cultivar seems to have an influence whether the TPA concentration is higher in sprouted wheat samples than in not sprouted ones or not. Until now it is known that genotype and environment influence the content of phenolic acids (ABDEL-AAL et al., 2001; MPOFU et al., 2006; MOORE, 2006). The current study presents higher results than in literature which could be due to different extraction methods. STRACKE et al. (2009) reported concentrations between 282 µg/g and 1262 µg/g in soft wheat samples whereas ZHOU et al. (2007) only reported concentrations between 219.7 µg/g and 389.1 µg/g in eleven soft red winter wheat grain cultivars. MOORE et al. (2005) analyzed eight soft red winter wheat genotypes with TPA contents of 486.6 µg/g - 656.0 µg/g and MPOFU et al. (2006) conducted a study with six western Canadian wheat genotypes where TPA values ranged between 580.2 µg/g and 724.7 µg/g. Different cultivars were used in these studies which displayed a variation in TPA values between the used cultivars. Even though only three cultivars were analyzed in the current study and a genetic determination was found for not sprouted cultivars, as well. N fertilization was significantly influencing TPA contents for not sprouted grain resulting in higher values after application. MARGNA (1977) traced this effect back to the sufficiently available amino acid phenylalanine which is primarily used for protein biosynthesis and which can be released by protein molecules. Since there is only little information about the correlation between N application and phenolic acid content, especially for wheat caryopses, further studies need to be conducted, to get a greater knowledge about the influence of nitrogen on phenolic acids in wheat caryopses.

Fraction 3 (TPA 3) represented cell wall bound phenolic acids and provided the biggest proportion of all three fractions, being well in line with further studies (STRACKE et al., 2009; ABDEL-AAL et al., 2001). According to other authors free phenolic acids (fraction 1) contributed the smallest part to total phenolic acids ranging between 1 % and 2 %. This is in agreement with the detected results for not sprouted grain but not for fully sprouted samples (LI et al., 2008; ABDEL-AAL et al., 2001). Generally, phenolic acids are bound to hydrolysable tannins, lignins, cellulose and proteins which are mainly structural components of bran, building a protective layer to inner parts of the caryopsis. Furthermore, important functions of phenolics, including phenolic acids, are defending mechanisms against pathogens, parasites and predators in plants (GRAF, 1992; LIU, 2004; PARR and BOLWELL, 2000). The highest ratio in all sprouting times was observed for TPA 3 (Fig. 3). Nevertheless, it is noticeable that after 48 h of sprouting TPA 3 decreases from 86 % (not sprouted) to 59 % for fully sprouted grain and TPA 1 increases from 6 % (not sprouted) to 28 %. It can be assumed that conjugated phenolic acids are released from the breakdown of cell walls, maybe to protect the inner parts of the caryopsis which is still needed to support the developing germ. A study by CHENG et al. (2006) suggests, that the conjugation of polyphenolics, e.g. tannins, are broken and simple phenolics are released.

It is reported that the main phenolic acid in wheat is ferulic acid (FA) which is proposed to enhance wall extensibility during cell elongation (GRAF, 1992). Fig. 4 shows that the composition of the phenolic acids shifted from 67 % FA and 7 % caffeic acid (CA) for not sprouted wheat samples to 54 % FA and 21 % CA for fully sprouted samples. Since CA is a precursor of FA it suggests itself that breakdown processes of cellular constituents are in progress during sprouting, e.g. when the coleoptile breaks through. Another assumption is that FA and CA, as intermediates in the biosynthesis of lignin, are released to provide substrates for the lignification. For human nutrition the use of fully sprouted grain could provide positive effects because of the higher amounts of free phenolic acids. In most findings the bioavailability and bioaccessibility of free phenolic acids is higher than of bound forms, which leads to a better resorption, e.g. of ferulic acid, in the small intestine. Bound phenolic acids firstly have to be released by bacterial hydrolytic enzymes during fermentation in the large intestine (KROON et al., 1997; ZHAO et al. 2003; ANSON et al., 2009).

In contrary to TPA values the antioxidative capacity by the Folin-

Ciocalteu (TPC) and by the ORAC assay showed significant differences for sprouting times. After 48 h of sprouting the TPC was significantly higher (2575.6 µg GAE/g) than for not sprouted wheat samples. The same was visible for ORAC values, which were higher for fully sprouted samples (32.6 µmol TE/g) than for not sprouted samples (28.2 µmol TE/g).

In the present study significant influences of the cultivar on TPC or on ORAC in not sprouted samples were not observed. According to other authors, there should be a significant variation in ORAC and TPC values of different cultivars due to genetic variability (Moore et al., 2005; Moore et al., 2006; Mpofu et al., 2006; Zhou et al., 2007). One reason for the differing results might be the small number of treatments (three cultivars) analyzed in the present study. The effect of N fertilization on the antioxidative capacity by Folin-Ciocalteu and ORAC assay was not significant for not sprouted grain. However, there was the tendency of higher TPC and ORAC values after nitrogen application which needs to be analyzed further to get more information about the influence of nitrogen application on the antioxidative capacity.

These two tests of the antioxidative capacity are working differently with the Folin-Ciocalteu assay being an electron transfer based reaction assay and ORAC a hydrogen atom transfer based reaction assay (Huang et al., 2005). Nevertheless, they both are testing the antioxidative activity which is the reason for the same tendency. Moore et al. (2006) and Okarter et al. (2010) reported a correlation between TPC and ORAC values. In the current study TPC were correlating strongly with ORAC values after 48 h of sprouting (r_s=0.714, $p \leq 0.001$). For not sprouted samples it is at least a positively tendency (r_s=0.327). Highest TPC were present for free phenolic acids with 46 % in not sprouted grain and fully sprouted grain, even though fraction 3 supplied the highest amount of phenolic acids. The same was visible for the ORAC values where ORAC 1 and ORAC 3 were about at the same level in unsprouted grain. After 48 h of sprouting ORAC 1 was the predominant fraction in terms of antioxidative capacity by the ORAC assay. These results indicate that sprouting alters the phenolic acids and other free phytochemicals might be present, leading to an improved antioxidative capacity in fully sprouted grain. The presence of tocopherols in wheat grain including the vitamers α-tocopherol, γ-tocopeherol and δ-tocopherol and carotenoids including β-carotene, zeaxanthin and lutein was reported by several studies (Zhou et al., 2004; Moore et al., 2005; Okarter et al., 2010). During the biosynthetic pathway the different tocopherol isomers can be transformed into α-tocopherol which has the highest vitamin E activity (Bramley et al., 2000). The antioxidative capacity of the tocopherol isomers seems to differ, too. It is possible that tocopherols and carotenoids are released to a greater extent from the aleurone fraction and improved the antioxidative capacity after 48 h of sprouting. Zhou et al. (2004) reported a correlation between δ-tocopherol and TPC, with the strongest antioxidative capacity among the tocopherols. Those phytochemicals, including phenolic acids could be the reason for a health benefit of whole wheat products or sprouted products, which is in line with the assumption of Okarter et al. (2010).

In conclusion, the current study showed that the TPA composition was comparable with the literature while TPA values were higher than reported. Phenolic acids contribute together with other phytochemicals in wheat, especially in sprouted wheat, a high potential health benefit by scavenging free radicals in caryopses as well as in the human organism. The composition of free, free-insoluble and bound phenolic acids changed during the process of sprouting for the benefit of free phenolic acids after 48 h. Furthermore, the antioxidative capacity by the Folin-Ciocalteu (TPC) and ORAC assay was increased after the caryopses were fully sprouted. The composition of the three extracted fractions changed for the benefit of the antioxidative capacity of free phenolic acids. This indicated that the more free phenolic acids and other free phytochemicals were present, the higher was the antioxidative capacity. Sprouted grain is a negative quality parameter with regard to standard backing processes. Nevertheless, it should be taken in consideration for human diets, e.g. in bread with whole cereals or in special breads named “Essener bread” with up to 100 % spouted grain flour (Beck et al., 2010). It is assumed that bioavailability along with bioaccessibility is upgraded because of free phytochemicals. Further studies need to be conducted with more than three cultivars to show whether the interaction between cultivar and fertilization has an effect on phenolic acids and antioxidative capacity of sprouted grain.

References

Abdel-Aal, E.-S. M., Hucl, P., Sosulski, F.W., Graf, R., Gillott, C., Pietrzak, L., 2001: Screening spring wheat for midge resistance in relation to ferulic acid content. J. Agric. Food Chem. 49, 3559-3566.

Adom, K., Liu, R., 2002: Antioxidant activity of grains. J. Agric. Food Chem. 50, 6182-6187.

Adom, K., Sorrells, M.E., Liu, R.H., 2005: Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. J. Agric. Food Chem. 53, 2297-2306.

Anson, N.M., Berg, van den, R., Havenaar, R., Bast, A., Haenen, G.R.M.M., 2008: Ferulic acid from aleurone determines the antioxidant potency of wheat grain (*Triticum aestivum* L.). J. Agric. Food Chem. 56, 5589-5594.

Anson, N.M., Berg, van den, R., Havenaar, R., Bast, A., Haenen, G.R.M.M., 2009: Bioavailability of ferulic acid is determined by its bioaccessibility. J. Cereal Sci. 49, 296-300.

Arora, S., Jood, S., Khetarpaul, N., 2010: Effect of germination and probiotic fermentation on nutrient composition of barley based food mixtures. Food Chem. 119, 779-784.

Arts, I.C., Hollman, P.C., 2005: Polyphenols and disease risk in epidemiologic studies. Am. J. Clin. Nutr. 81, 317-325.

Arts, I.C., Hollman, P.C., Bas Bueno De Mesquita, H., Feskens, E.J., Kromhout, D., 2001: Dietary Catechin and Epithelial Cancer Incidence: The Zutphen Elderly Study. 92, 298-302.

Bramley, P.M., Elmadfa, I., Kafatos, A., Kelly, F.J., Manios, Y., Roxborough, H.E., Schuch, W., Sheehy, P.J.A., Wagner, K.-H., 2000: Review Vitamin E. J. Sci. Food Agric. 80, 913-938.

Beck, A., Busch, K.G., Erik, D., Deinert, C., Dylla, R., Gruhn, A., 2010: Essener Brot – Herstellung und Verwendung von Keimlingen in der Bäckerei. 1-24, Fibl Deutschland e.V., Hrsg., Frankfurt/Main.

Cheng, Z., Su, L., Moore, J., Zhou, K., Luther, M., Yin, J.-J., 2006: Effects of post harvest treatment and heat stress on availability of wheat antioxidants. J. Agric. Food Chem. 54, 5623-5629.

Cuppett, S., Schnepf, M., Hall, C., 1997: Natural Antioxidants – Are they a reality?. In: Shahidi, F., 1997: Natural antioxidants: chemistry, health effects, and applications. AOCS Press, Champaign, USA.

Graf, E., 1992: Antioxidant potential of ferulic acid. Free Radical Bio. Med. 13, 435-448.

Graf, B.A., Milbury, P.E., Blumberg, J.B., 2005: Flavonols, flavones, flavanones, and human health. Epidemiological Evidence. 8, 281-290.

Haumann, G., Dietzsch, H., 2000: Winter- und Sommerweizen. In: Lütke Entrup, N., Oehmichen, J. (eds.), Lehrbuch des Pflanzenbaus 2, 258-324, Th. Mann, Gelsenkirchen.

Huang, D., Ou, B., Hampsch-Woodhill, M., Flanagan, J.A., Prior, R.L., 2002: High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J. Agric. Food Chem. 50, 4437-4444.

Huang, D., Ou, B., Prior, R.L., 2005: The chemistry behind antioxidant capacity assays. J. Agric. Food Chem. 53, 1841-1856.

ISTA – International Seed Testing Association, 1999: Internationale

Vorschriften für die Prüfung von Saatgut. Vorschriften. Seed Sci. Technol. 27, Supplement.

KHALIL, A.H., MANSOUR, E.H., 1995: The effect of cooking, autoclaving and germination on the nutritional quality of faba beans. Food Chemistry 54, 177-182.

KNEKT, P., KUMPULAINEN, J., JÄRVINEN, R., RISSANEN, H., HELIÖVAARA, M., REUNANEN, A., 2002: Flavonoid intake and risk of chronic diseases. Am. J. Clin. Nutr. 76, 560-568.

KROON, P.A., FAULDS, C.B., RYDEN, P., ROBERTSON, J.A., WILLIAMSON, G., 1997: Release of covalently bound ferulic acid from fiber in the human colon. J. Agric. Food Chem. 45, 661-667.

KRYGIER, K., SOSULSKI, F., HOGGE, L., 1982: Free, esterified, and insoluble-bound phenolic acids. 1. Extraction and Purification Procedure. J. Agric. Food Chem. 30, 330-334.

LI, L., SHEWRY, P.R., WARD, J.L., 2008: Phenolic acids in wheat varieties in the HEALTHGRAIN diversity screen. J. Agric. Food Chem. 56, 9732-9739.

LIU, R.H., 2004: Potential synergy of phytochemicals in cancer prevention: mechanism of action. J. Nutr. 134, 3479-3485.

LIYANA-PATHIRANA, C.M., SHAHIDI, F., 2006: Importance of insoluble-bound phenolics to antioxidant properties of wheat. J. Agric. Food Chem. 54, 1256-1264.

MARGNA, U., 1977: Control at the level of substrate supply – an alternative in the regulation of phenylpropanoid accumulation in plant cells. Phytochemistry 16, 419-426.

MOORE, J., HAO, Z., ZHOU, K., LUTHER, M., COSTA, J., YU, L., 2005: Carotenoid, tocopherol, phenolic acid, and antioxidant properties of Maryland-grown soft wheat. J. Agric. Food Chem. 53, 6649-6657.

MPOFU, A., SAPIRSTEIN, H.D., BETA, T., 2006: Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. J. Agric. Food Chem. 54, 1265-1270.

ORKATER, N., LIU, C.-S., SORRELLS, M.E., LIU, R.H., 2010: Phytochemical content and antioxidant activity of six diverse varieties. Food Chem. 119, 249-257.

PARR, A.J., BOLWELL, G.P., 2000: Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. J. Sci. Food Agric. 80, 985-1012.

PETERSON, J., LAGIOU, P., SAMOLI, E., LAGIOU, A., KATSOUYANNI, K., LA VECCHIA, C., 2003: Flavonoid intake and breast cancer risk: a case-control study in Greece. Br. J. Cancer 89, 1255-1259.

PRODANOV, M., SIERRA, I., VIDAL-VALVERDE, C., 1997: Effect of germination on the thiamine, riboflavin and niacin contents in legumes. Z. Lebensmittelunters. Forsch. 205, 48-52.

PUSSAYANAWIN, V., WETZEL, D.L., FULCHER, R.G., 1988: Fluorescence detection and measurement of ferulic acid in wheat milling fractions by microscopy and HPLC. J. Agric. Food Chem. 36, 515-520.

SERPEN, A., GÖKMEN, V., KARAGÖZ, A., KÖKSEL, H., 2008: Phytochemical quantification and total antioxidant capacities of emmer (*Triticum dicoccon* Schrank) and einkorn (*Triticum monococcum* L.) wheat landraces. J. Agric. Food Chem. 56, 7285-7292.

SESSO, H.D., GAZIANO, J.M., LIU, S., BURING, J.E., 2003: Flavonoid intake and the risk of cariovascular disease in women. Am. J. Clin. Nutr. 77, 1400-1408.

SOSULSKI, F., KRYGIER, K., HOGGE, L., 1982: Free, esterified, and insoluble-bound phenolic acids. 3. Composition of phenolic acids in cereal and potato flours. J. Agric. Food Chem. 30, 337-340.

STRACKE, B., EITEL, J., WATZL, B., MÄDER, P., RÜFER, C.E., 2009: Influence of the production method on phytochemical concentrations in whole wheat (*Triticum aestivum* L.): a comparative study. J. Agric. Food Chem. 57, 10116-10121.

TIAN, S., NAKAMURA, K., KAYAHARA, H., 2004: Analysis of phenolic compounds in white rice, brown rice, and germinated brown rice. J. Agric. Food Chem. 52, 4808-4813.

TIAN, B., XIE, B., SHI, J., WU, J., CAI, Y., TUOMING, X., XUE, S., DENG, Q., 2010: Phytochemical changes of oat seeds during germination. Food Chem. 119, 1195-1200.

WATERHOUSE, A.L., 2001: Folin-Ciocalteau micro method for total phenol in wine. Current Protocols in Food Analytical Chemistry, I1.1.1-I1.1.8, Wrolstad, R.E., Wiley.

WEIDNER, S., AMAROWICZ, R., KARAMAĆ, M., FRŠCZEK, E., 2000: Changes in endogenous phenolic acids during development of *Secale cereale* caryopses and after dehydration treatment of unripe rye. Plant Physiol. Biochem. 38, 595-602.

YANG, F., BASU, T.K., OORAIKUL, B., 2001: Studies on germination conditions and antioxidant contents of wheat grain. Int. J. Food Sci. Nutr. 52, 319-330.

ZIELINSKI, H., KOZLOWSKA, H., LEWCZUK, B., 2001: Bioactive compounds in the cereal grains before and after hydrothermal processing. Innov. Food Sci. Emerging Technol. 2, 159-169.

ZHAO, Z., EGASHIRA, Y., SANADA, H., 2003: Digestion and absorption of ferulic acid sugar esters in rat gastrointestinal tract. J. Agric. Food Chem. 51, 5534-5539.

ZHOU, K., SU, L., YU, L.L., 2004: Phytochemicals and antioxidant properties in wheat bran. J. Agric. Food Chem. 52, 6108-6114.

ZHOU, K., HAO, J., GRIFFEY, C., O'KEEFE, S.F., CHEN, J., HOGAN, S., 2007: Antioxidant properties of *fusarium* head blight-resistant and – susceptible soft red winter wheat grains grown in Virginia. J. Agric. Food Chem. 55, 3729-3736.

Address of the authors:
Institute of Agronomy and Plant Breeding, Ludwigstrasse 23, 35390 Giessen, Germany
Corresponding author: bernd.honermeier@agrar.uni-giessen.de