

Spring, einkorn and emmer wheat species – potential rich sources of free ferulic acid and other phenolic compounds

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ABSTRACT

Einkorn (*Triticum monococcum* L., subsp. *monococcum*), emmer (*Triticum dicoccum* Schuebl [Schrank], subsp. *dicoccum*) and spring wheat (*Triticum aestivum* L.) may be rich in hydrophilic antioxidants, therefore being a potential food source with high nutritional properties. The aim of the present study was to assess the content of free ferulic acid (FFA) and total polyphenols (TP) beneficial for human health in wheat varieties and accessions for breeding and production. Einkorn, emmer and spring wheat varieties were assessed for TP and FFA contents in the precise two-year field experiments. The highest FFA content was determined in emmer wheat varieties and spring cv. Granny. High TP content was characteristic for emmer and spring wheat accessions, however also some einkorn ones were characterised by high levels. Year of cultivation showed a significant impact on FFA contents.

Keywords: diploid and tetraploid ancestor wheats; spring hexaploid wheat; hydrophilic antioxidants

Cereals and pseudocereals are widely consumed and are a valuable means to deliver beneficial natural antioxidants to humans (Klepacka and Fornal 2006, Bystrická et al. 2011). The antioxidant activity of wheat is caused with antioxidants, which belong to chemically different groups such as polyphenols, carotenoids, phytosterols, and selenium. Among these compounds, polyphenols were found *in vitro* to be promising agents toward cervical cancer and moreover acting as antioxidants. Wheat is the most widely grown food crop with a global production of about 600 million metric tonnes annually. A study of seven wheat varieties showed that ferulic acid (4-hydroxy-3-methoxycinnamic) is generally the predominant phenolic acid (in total 131–146 mg/kg), accounting for about 46–67% of total phenolic acids (Zhou et al. 2005). Ferulic acid is the main contributor to the antioxidant capacity, suggesting that it could be used as a marker of wheat antioxidants (Zhou et al. 2004). The antioxidant activity of wheat and its different fractions usually depends on the variety examined (Yu et al.

2002a,b), and the location where it is grown (Yu and Zhou 2004). Environmental factors (such as temperature stress, solar radiation and irrigation), and the interactions between environmental factors and genotype can modulate the antioxidant activity of wheat (Moore et al. 2006, Fernandez-Orozco et al. 2010, Shewry et al. 2010).

In order to enhance the existing knowledge of soluble hydrophilic antioxidants content in three wheat species, spring, einkorn and emmer varieties and accessions, we focused in this study on the determination of soluble total polyphenols (TP) and free ferulic acid (FFA) with the purpose to evaluate selected wheat species for breeding and production purposes. The results of Li et al. (2008) indicate that there is a genetic diversity in phenolic acid content and that it should be possible to selectively breed for lines with high contents of phenolic components. The objective of present work was therefore to explore the genetic variability of TP and FFA contents within the selected cultivated wheat species, i.e. einkorn, emmer and spring wheat varieties in order to improve the nutritional value of bread and other wheat products.

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MATERIAL AND METHODS

Plant material. Grains of spring einkorn, emmer and spring wheat varieties were obtained from the Czech Gene Bank of the Crop Research Institute in Prague-Ruzyně (CRI; GPS 50°05.165'N, 14°17.90'E, 338 m a.s.l.) from the harvests 2009 and 2010. Their major characteristics are described in detail in our previous study (Lachman et al. 2011). The wheat species were grown using a conventional production technology on an experimental field of the CRI. The field trials were performed in 3 replicates.

The tested varieties were sown at the end of March 2009 and 2010 by a plot drill. Size of the individual plots was 4.5 m². During growing season there selected traits were evaluated. A plot harvester was used for the harvest. Weather conditions are described in Table 1.

Sample preparation and total phenols (TP) assay by Folin-Ciocalteu reagent. Wheat grain samples (ca 5.0 g) finely powdered in an electric mill HR 2185 Philips (Amsterdam, the Netherlands) were weighed into 100 mL volumetric flask, filled up to the mark with ethanol, carefully mixed and left to extract for 24 h at laboratory temperature in the dark.

For the TP determination 5 mL aliquot of sample extract was transferred into a 50 mL volumetric flask and diluted with approximately 5 mL of distilled water. Then, 2.5 mL of Folin-Ciocalteu reagent (Penta, Chrudim, Czech Republic) and 7.5 mL of 20% (w/w) Na₂CO₃ (Lach-Ner, Ltd., Neratovice, Czech Republic) were added, adjusted with distilled water to 50 mL, agitated and left to stand for 2 h. Absorbance of the sample was measured using an UV-VIS spectrophotometer Spectronic Helios γ (Thermo Spectronic, Cambridge, UK) at λ = 765 nm against a blank prepared with distilled water. Gallic acid (G.R. purity, Merck KGaA, Darmstadt,

Germany) was used for calibration. The results were expressed as gallic acid equivalents (GAE) in mg/kg dry matter (DM).

Defatting. Approximately 4 g of wheat grain powder were weighed into a test tube with ground glass. 20 mL hexane (Lach-Ner p.a., Ltd., Neratovice, Czech Republic) was added. The mixture was stirred for 1 min on Vortex and then left in an ultrasonic bath for 10 min. Subsequently, the mixture was again stirred for 1 min and mixed using Vortex IKA MS 3 basic (IKA[®]-Werke GmbH & Co. KG, Staufen, Germany). Then it was left for 5 min to decant in order to minimise matrix received from the tube during following filtration through filter paper. The defatting operation was repeated with further 20 mL of hexane. The entire contents of the tube was transferred to filter paper, defatted sample was washed with further portion of 10 mL of hexane and let to dry thoroughly.

Extraction. 1 g powdered defatted wheat grain was weighed into a test tube with ground glass and 10 mL of 64% aqueous ethanol was added. The tube was plugged and the mixture was stirred for 1 min using Vortex and then given for 25 min to the ultrasound bath. Subsequently, the mixture was again stirred for 1 min using Vortex. The liquid was poured into centrifuge tubes and centrifuged for 10 min (5000 g, 4°C). Aliquots 1 mL were pipetted from the centrifuge tube and filtered through a PVDF microfilter (0.45 μ) into glass vials.

Determination of FFA by high performance liquid chromatography – electrospray tandem mass spectrometry (HPLC-ESI-MS/MS). Analysis was carried out using a High Performance Liquid Chromatograph Ultimate 3000 RS (Dionex, Sunnyvale, USA) with a binary pump, refrigerated autosampler and column heater. An analytical column Pinnacle DB C18 (30 × 2.1 mm; 1.9 μm) (Restek, Bellefonte, USA) was used. Flow rate was 0.3 mL/min with a gradient elution of solvent A:

Table 1. Weather conditions during the vegetation period in 2009 and 2010 and comparison with long term period 1971–2001

Year		February	March	April	May	June	July	August	Vegetation period
2009	R	19.8	27.7	29.0	63.4	66.9	67.8	61.8	336
	T	0.1	4.6	13.6	14.4	15.7	19.3	20.3	12.6
2010	R	11.5	15.0	35.0	94.4	83.2	123	118	479
	T	-0.9	4.3	9.6	12.3	17.5	21.4	18.3	11.8
Mean 1971–2001	R	16.8	37.6	24.2	109	69.0	79.0	20.8	357
	T	-0.2	3.8	7.9	13.3	16.2	18.1	18.1	11.0

R – sum of rainfalls (mm); T – average temperature (°C)

0.1% formic acid (super gradient, min. 99.9%, Sigma-Aldrich GmbH, Sternheim, Germany) in Milli-Q water and solvent B: 0.1% formic acid in methanol (Lach-ner, Ltd. Neratovice, Czech Republic), 90:10 (v/v). Injected volume was 1 µL and column temperature was 40°C. The HPLC instrument was coupled to a 3200 Qtrap hybrid triple quadrupole-linear ion trap mass spectrometer (AB Sciex, Foster City, USA) with an electro-spray ionisation source. All data were acquired and processed using Analyst 1.4 Software (AB Sciex, Foster City, USA).

The limit of detection (LOD = 0.005 µg/mL) was defined as the lowest concentration with a signal-to-noise (S/N) ratio of 3, and the limit of quantification (LOQ = 0.015 µg/mL) was defined as the concentration with S/N ratio of 9.

All the extractions and HPLC analysis were repeated three times for all the analysed wheat samples.

Statistical analysis. Statistical analyses were performed using the software Statistica 7.0 (StatSoft Prague, Czech Republic) on the basis of para-

metrical and non-parametrical tests at the level of significance $P \leq 0.05$. Further ANOVA multiple factorial analysis, one-way and two-way factorial ANOVA Tukey's Post Hoc HSD test and t -test were used for statistical evaluation. The least significant difference test was applied to determine differences among means $P < 0.05$.

RESULTS AND DISCUSSION

Total polyphenol (TP) content in wheat accessions. The highest TP content was found for all analysed emmer varieties and accessions in both years 2009 and 2010 (Table 2, Figure 1). The most distinctive varieties were Kahler Emmer, Krajova-Horny Tisovnik (Malov) and Rudico with average amounts 668 ± 32.3 , 646 ± 101.1 , and 645 ± 21.5 mg GAE/kg DM, respectively. Also some spring wheat and einkorn varieties were rich in the TP. High TP content was typical for Granny, Postoloprtská přesívka 6, SW Kadrilj and Jara varieties (620 ± 19.0 , 615 ± 125 , 603 ± 88.1 and

Table 2. Content of total phenolics (TP) in wheat accessions (expressed as gallic acid equivalents (GAE) in mg/kg dry matter) from the harvests 2009 and 2010 and average 2009 + 2010

Variety	2009	2010	Average 2009 + 2010
SW Kadrilj***	534 ± 24.0 ^{abcd}	696 ± 9.09 ^{ef}	603 ± 88.1 ^{abcd}
Granny***	623 ± 25.8 ^{fg}	617 ± 7.69 ^{bcd}	620 ± 19.0 ^{bcd}
Jara***	533 ± 10.4 ^{abcd}	692 ± 13.8 ^{def}	601 ± 85.7 ^{abcd}
Kärntner Früher***	502 ± 15.5 ^a	687 ± 50.8 ^{cdef}	581 ± 103.8 ^{abcd}
Postoloprtská přesívka 6***	516 ± 21.1 ^{ab}	746 ± 23.0 ^f	615 ± 125 ^{bcd}
Spring wheat varieties – average	542 ± 47.0 ^a	688 ± 48.0 ^b	604 ± 87.0 ^b
Escana*	523 ± 42.8 ^{abc}	507 ± 25.5 ^a	516 ± 34.8 ^a
Schwedisches Einkorn*	591 ± 24.6 ^{defg}	673 ± 31.3 ^{cdef}	626 ± 50.2 ^{bcd}
<i>T. monococcum</i> 2101/01C0204039*	608 ± 15.4 ^{efg}	661 ± 16.9 ^{cde}	631 ± 31.8 ^{cd}
<i>T. monococcum</i> 2102/ 01C0204040*	507 ± 12.7 ^a	562 ± 5.17 ^{ab}	530 ± 31.1 ^{ab}
<i>T. monococcum</i> 2103/01C0204044*	550 ± 8.31 ^{abcde}	516 ± 24.6 ^a	536 ± 23.7 ^{abc}
Einkorn wheat varieties – average	556 ± 45.2 ^a	584 ± 75.4 ^a	568 ± 60.7 ^a
Rudico**	652 ± 5.81 ^{gh}	635 ± 32.9 ^{bcd}	645 ± 21.5 ^d
Kahler Emmer**	686 ± 27.5 ^h	643 ± 21.3 ^{cde}	668 ± 32.3 ^d
<i>T. dicoccon</i> (Tapioszele)**	577 ± 26.8 ^{cdef}	612 ± 16.6 ^{bc}	592 ± 28.4 ^{abcd}
Krajova-Horny Tisovnik (Malov)**	569 ± 29.6 ^{bcd}	748 ± 42.6 ^f	646 ± 101.1 ^d
<i>T. dicoccon</i> No. 8909**	623 ± 35.0 ^{fg}	661 ± 16.9 ^{cde}	639 ± 33.5 ^d
Emmer wheat varieties – average	621 ± 51.3 ^b	660 ± 54.0 ^b	638 ± 55.2 ^b

*einkorn varieties; **emmer varieties; ***spring wheat varieties; values marked with different superscript letters in individual columns are significantly different at $P \leq 0.05$

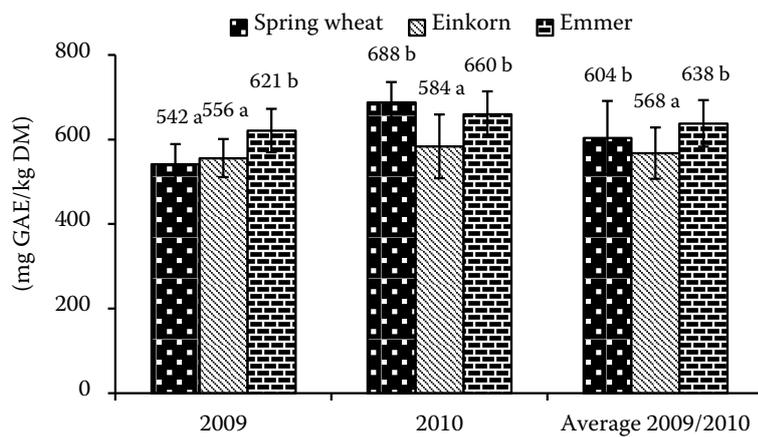


Figure 1. Comparison of total polyphenol content (TP) in spring, einkorn and emmer wheat species in 2009, 2010 and average of both years (mg/kg dry matter(DM)); values marked with different letters are significantly different at $P \leq 0.05$; GAE – gallic acid equivalents

601 ± 85.7 mg GAE/kg DM, respectively. Among einkorn varieties and accessions, *T. monococcum* 2101/01C0204039 and Schwedisches Einkorn were distinguished with higher TP content (631 ± 31.8 and 626 ± 50.2 mg GAE/kg DM, respectively). Moreover, our results also show that total phenolic content is closely associated with wheat genotype (Yu et al. 2003). The total phenolic content was found to be the highest (1258–3157 mg/kg) in bran layer, followed by that of grains (168–459 mg/kg) and

the lowest it was in flour (44–140 mg/kg) (Vaher et al. 2010). The results of Adom et al. (2003) in eleven wheat varieties showed total phenolic content (1208–1463 mg GAE/kg) not significantly different, however with significant differences in total ferulic acid content ($P < 0.05$).

Free ferulic acid (FFA) content in wheat accessions. The highest FFA content was found to be typical for emmer wheat varieties (in average 2.36 ± 0.18 mg FFA/kg DM, Table 3, Figure 2)

Table 3. Content of free ferulic acid (FFA) in wheat accessions (mg/kg dry matter) from the harvests 2009 and 2010 and average 2009 + 2010

Variety	2009	2010	Average 2009 + 2010
SW Kadrilj***	2.44 ± 0.17 ^{abcd}	1.41 ± 0.02 ^{ab}	1.92 ± 0.10 ^{abc}
Granny***	3.45 ± 0.04 ^e	1.62 ± 0.04 ^{abcd}	2.54 ± 0.04 ^{cd}
Jara***	2.58 ± 0.09 ^{ab}	1.66 ± 0.11 ^{abcde}	2.12 ± 0.10 ^{abcd}
Kärntner Früher***	1.83 ± 0.02 ^a	1.46 ± 0.14 ^{abc}	1.65 ± 0.08 ^a
Postoloprtská přesívka 6***	2.32 ± 0.13 ^{abc}	1.63 ± 0.36 ^{abcd}	1.98 ± 0.25 ^{abc}
Spring wheat varieties – average	2.52 ± 0.09 ^{gh}	1.55 ± 0.13 ^h	2.04 ± 0.11 ^g
Escana*	2.43 ± 0.05 ^{abcd}	1.75 ± 0.29 ^{bcdef}	2.09 ± 0.17 ^{abcd}
Schwedisches Einkorn*	2.36 ± 0.23 ^{abc}	2.34 ± 0.11 ^{ef}	2.35 ± 0.17 ^{bcd}
<i>T. monococcum</i> 2101/01C0204039*	2.14 ± 0.18 ^{ab}	1.96 ± 0.07 ^{bcdef}	2.05 ± 0.13 ^{abcd}
<i>T. monococcum</i> 2102/ 01C0204040*	1.96 ± 0.09 ^{ab}	2.02 ± 0.19 ^{bcdef}	1.99 ± 0.14 ^{abc}
<i>T. monococcum</i> 2103/01C0204044*	2.52 ± 0.01 ^{abcd}	2.38 ± 0.09 ^f	2.45 ± 0.05 ^{cd}
Einkorn wheat varieties – average	2.28 ± 0.11 ^g	2.09 ± 0.15 ^g	2.19 ± 0.13 ^{gh}
Rudico**	3.24 ± 0.07 ^{de}	2.21 ± 0.07 ^{def}	2.72 ± 0.07 ^d
Kahler Emmer**	2.82 ± 0.12 ^{bcde}	2.10 ± 0.58 ^{cdef}	2.46 ± 0.35 ^{cd}
<i>T. dicoccon</i> (Tapioszele)**	3.08 ± 0.16 ^{cde}	2.10 ± 0.34 ^{cdef}	2.59 ± 0.25 ^{cd}
Krajova-Horny Tisovnik (Malov)**	2.36 ± 0.02 ^{abcd}	2.00 ± 0.08 ^{bcdef}	2.18 ± 0.05 ^{abcd}
<i>T. dicoccon</i> No. 8909**	2.64 ± 0.24 ^{abcde}	1.03 ± 0.13 ^a	1.68 ± 0.18 ^{ab}
Emmer wheat varieties – average	2.84 ± 0.12 ^h	1.89 ± 0.24 ^g	2.35 ± 0.18 ^h

*einkorn varieties; **emmer varieties; ***spring wheat varieties; values marked with different superscript letters in individual columns are significantly different at $P \leq 0.05$

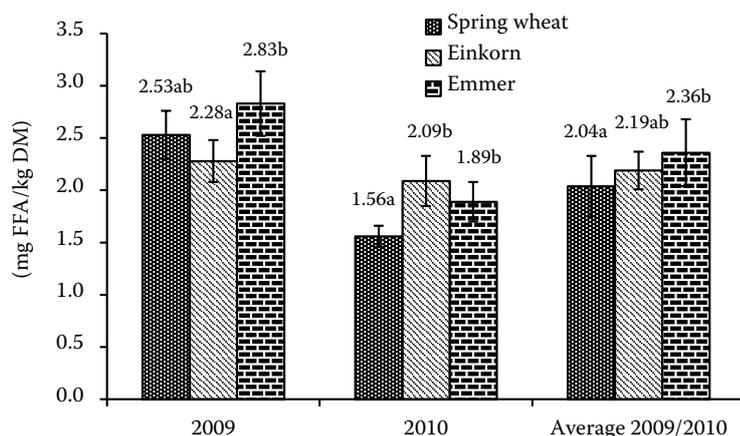


Figure 2. Comparison of free ferulic acid content (FFA) in spring, einkorn and emmer wheat species in 2009, 2010 and average of both years (mg/kg dry matter (DM)); values marked with different letters are significantly different at $P \leq 0.05$

with especially high concentrations in Rudico, *T. dicoccon* (Tapioszele) and Kahler Emmer varieties (2.72 ± 0.07 , 2.59 ± 0.25 and 2.46 ± 0.35 mg FFA/kg DM, respectively). However, unlike emmer varieties, only one spring wheat variety – Granny – showed high FFA content in grains (2.54 ± 0.04 mg FFA/kg DM) and nearly the same was observed in the einkorn *T. monococcum* 2103/01C0204044 accession (2.45 ± 0.05 mg FFA/kg DM). Significant differences between wheat species and individual varieties and accessions correspond with the recent findings reporting highly significant genotypic differences in total phenolic acid concentrations (Irmak et al. 2008) and differences of ferulic acid found in waxy and non-waxy wheat (Jonnala et al. 2010).

In 64% aqueous ethanol extract of defatted powdered wheat grain we found FFA as a basic component. Hung et al. (2011) evaluated the proportion of ferulic acid from the total detected phenolic compounds as 71.0–72.3%. Thus, the phenolic acids profile and the concentration of individual phenolic acids are dependent on the wheat varieties and growing location. As Adom and Liu (2002) demonstrated, there exists a close correlation between bound phenolic and ferulic acid content and the total antioxidant activity ($r^2 = 0.991$, $P < 0.01$). We found a close correlation between TP and FFA contents, especially for spring wheat (in 2009 $r = 0.655$ for all wheat species, $P < 0.01$ and $r = 0.969$, $P < 0.01$ for spring wheat varieties, respectively). Correlation coefficient between TP and antioxidant activity (Lachman et al. 2012) of spring wheat was $r = 0.600$ and that between FFA and antioxidant activity of emmer wheat $r = 0.532$.

In wheat bran fraction, ferulic acid is prevalent in the group of phenolic acids derived from cinnamic acid, but small quantities of other phenolic acids are also present (Irmak et al. 2008). In cereal grains, ferulic acid serves as a bridge between lignin and

arabinoxylans via ether and ester bonds (Buranov and Mazza 2008). Gasztonyi et al. (2011) after quantification of soluble conjugated and bound forms of ferulic acid reported that the unavailable bound form was present up to 85–90% of total content. This is why we focused on the content of FFA in our study because its good bioavailability was reported previously (Anson et al. 2009). Their results showed that bioaccessibility of ferulic acid appeared to be determined by the percentage of FFA. Several studies have reported highly variable results on ferulic acid bioavailability. For instance, in consumed cereal products, particularly bran, bound ferulic acid had a low bioavailability: 3% in humans (Kern et al. 2003) and 2.5–5% in rat (Adam et al. 2002). The binding of ferulic acid to polysaccharides in the cereal matrix limits its bioavailability. In wheat grain, most of ferulic acid is bound to arabinoxylans and other indigestible polysaccharides restricting its release in the small intestine. Ferulic acid is linked by ester bonds with hemicellulose chains and it also polymerises with lignin through ether bonds and forms specific complexes with proteins through bonds with amino acids (Klepacka and Fornal 2006). Absorption of soluble free ferulic acid occurred mostly from the small intestine (Kern et al. 2003). However, ferulic acid found in the insoluble-bound fraction, did not show any cellular antioxidant activity (Okarter 2012). Digestion partially hydrolyses the hydrolysable phenolics and thereby increases the antioxidant capacity of cereal products (Liyana-Pathirana and Shahidi 2005). In a study of Arranz and Calixto (2010) it was confirmed that for phenolic antioxidant intake free extractable polyphenols are especially important; and non-extractable polyphenols cannot be used in the diet without acidic hydrolysis. The urinary excretion in rats suggests the following order for absorbability of dietary ferulic acid: FFA > feruloyl mono- and

disaccharides > feruloyl polysaccharides (Zhao et al. 2003a,b). Therefore, the form of bound ferulic acid determines the degree of its absorbability. This is why the determined higher FFA contents in emmer varieties and accessions are very significant; although the share of ferulic acid in the bound form is significantly higher, the bound form is very little available in human nutrition.

Effect of year on free ferulic acid content in wheat species. Environmental factors, such as light intensity, precipitation and temperature, may have important implications on the year-to-year variability of phytochemical levels. Our FFA results from 2009 and 2010 are in agreement with those of Gasztonyi et al. (2011) and Stracke et al. (2009) that the year (weather conditions) influences significantly the soluble conjugated ferulic acid content in wheat varieties. The differences in ferulic acid content in wheat, and therefore overall phenolic content are thought to have a genetic basis but with strong environmental influences (Moore et al. 2006). Statistically significant year-to-year differences – higher FFA amounts in 2009 in comparison with 2010 in emmer and spring wheat species (Figure 2) – may be affected by lower sum of precipitation and higher average temperatures in 2009 (Table 1). Shewry et al. (2010) reported for free phenolic acids $r = 0.899$, $P = 0.015$ for average temperature and $r = -0.706$, $P = 0.0117$ for precipitation heading to harvest. The respective data for conjugated phenolic acids were $r = 0.753$, $P = 0.084$ for average temperature and $r = -0.744$, $P = 0.090$ for precipitation heading to harvest. Values of the means across the 26 genotypes ranged from 11 to 18 mg/kg DM and showed a high relative standard deviation ($RSD = 22-54\%$). The variance of individual genotypes due to year of cultivation ranged between 15 and 61%, which was higher than for other phenolic classes and masked any genotypic effects (Fernandez-Orozco et al. 2010). TP year-to-year differences were significant only for spring wheat; however, TP levels were higher in 2010 and may be influenced in addition also by other factors such as length of sunshine exposure, etc. Ward et al. (2008) in agreement with our results reported the distribution of total phenolics in wheat species as emmer > spring wheat > einkorn, free phenolics einkorn > spring wheat = emmer, conjugated phenolics spring wheat > einkorn > emmer and bound phenolics emmer > spring wheat > einkorn.

In conclusion, emmer wheat varieties Rudico, *T. dicoccon* (Tapioszele) and Kahler Emmer and also spring wheat cv. Granny had the highest FFA

content; however, also einkorn wheat *T. monococcum* 2103/01C0204044 and Schwedisches Einkorn differed from other varieties by high FFA levels. High TP content was characteristic for emmer and spring varieties and accessions. The highest TP contents were found in emmer varieties with the exception of *T. dicoccon* (Tapioszele). Among spring wheat varieties, higher values showed Granny and Postoloprtská přesívka 6, and among einkorn wheat, *T. monococcum* 2101/01C0204039 and Schwedisches Einkorn cultivars. These results indicate the benefit of emmer, spring and einkorn wheat varieties/accessions characterised by high levels of TP and FFA with high antioxidant potential. According to easy bioavailability of FFA and other polyphenols it may lead to new opportunities for wheat breeders and eventually commercial wheat growers to promote the production of wheat with enhanced levels of health beneficial compounds.

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