UPLC–PDA–ESI–MS Analysis and TLC–DPPH[•] Activity of Wheat Varieties

I. Kowalska^{1*}, D. Jedrejek¹, K. Jonczyk² and A. Stochmal¹

¹Department of Biochemistry and Crop Quality, Institute of Soil Science and Plant Cultivation — State Research Institute, Czartoryskich 8, 24-100 Pulawy, Poland

²Department of Systems and Economics of Crop Production, Institute of Soil Science and Plant Cultivation — State Research Institute, Czartoryskich 8, 24-100 Pulawy, Poland

Received: 22 November 2017; accepted: 05 December 2017

Wheat is a major crop, an important component of the human diet and important source of animal fodder in the world. Characterization of phenolic profiles of the leading wheat cultivars is important for new opportunities for breeding and eventual commercial production of value-added cultivars rich in beneficial components. A method using ultra-performance liquid chromatography combined with photodiode-array detector–electrospray ion source–mass spectrometry (UPLC–PDA–ESI–MS) has been developed for determination of phenolic compounds contained in twelve winter and thirteen spring wheat varieties. The antioxidant activity was determined by the thin-layer chromatography–2,2-diphenyl-1-picrylhydrazyl (TLC–DPPH[•]) test with image processing by means of the ImageJ program. Based on retention time, the mass of deprotonated molecule $[M-H]^-$ and ultraviolet (UV) spectra, seven phenolic acids, and twelve flavonoids were identified and quantitated in the 80% aqueous methanol extract of the wheat varieties. The average concentrations of total researched compounds were definitely higher in spring wheat cultivars than in winter ones. Varieties Trappe and Kandela showed the most elevated values of total free phenolic acids. Kandela and Ostka Smolicka had the highest content of flavonoids, and isoorientin was detected as the main phenolic in wheat cultivars. Additionally, a correlation between radical scavenging activity and total phenolic acids content was observed. UPLC combined with PDA–ESI–MS could be applied to complete characterization of natural products (e.g., phenolics) in alcoholic extracts from wheat varieties.

Keywords: Wheat varieties, UPLC–PDA–ESI–MS analysis, phenolics, TLC–DPPH[•] test

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important crop plants worldwide, an important agricultural commodity, a component of human diet, and a significant source of animal fodder in the world. As wheat and other grains are known to provide numerous health benefits, an interest is growing in the variation of phytochemical concentrations among individual cultivars [1]. According to scientific literature, wheat grains are not only rich in phenolic phytochemicals but also they exhibit high antioxidant activity [2–4]. The beneficial effect of whole grains has been found to result from the combined action of several components such as fiber, vitamins, phenolics, carotenoids, alkylresorcinols, and other phytochemicals [5]. Natural products (secondary metabolites, e.g., phenolics) possess biological properties and play an important role in the interactions between plants and the environment. Their role is mainly to protect against microbial attacks, ultraviolet (UV) radiation, and oxidative stress [6]. Wheat cultivars with a high antioxidant activity can constitute an excellent source of antioxidants for disease prevention and health promotion in human diet [7]. However, many factors such as genotypes and growing conditions have been reported to have a significant effect on phenolic acids and antioxidant properties of wheat [8].

Due to the growing pressure to resign from pesticides, fertilizers, veterinary medicines, and growth promoters in food production systems, ecological foods are becoming more and more popular. Organic farming is of particular interest with regard to healthy, ecologically-friendly produced food due to the fact that using chemicals is not allowed [9, 10]. Two elements of agricultural technology, crop rotation and selection of varieties, are es-

sential in crop production conducted in accordance with the principles of organic farming. The emergence of the final leaf, namely, the flag leaf, is a significant phase in wheat plant development. The protection of this leaf is of high importance if high grain yields are to be obtained, keeping in mind that wheat is a major crop in organic farming [11].

There is a low number of studies on natural products in wheat aerial parts [6, 12, 13] while a comprehensive profiling of wheat phenolic compounds, especially in relation to environmental stresses, has not yet been performed [14]. Thus, the objective of study was to provide qualitative and quantitative information on the natural products of twenty five wheat varieties. Full characterization of free phenolic acids and flavonoids profiles could extend breeding and enable a commercial production of value-added cultivars that are rich in beneficial components. In this study, phenolic compounds were analyzed by a sensitive and selective ultra-performance liquid chromatography combined with photodiode-array detector–electrospray ion source–mass spectrometry (UPLC–PDA–ESI–MS) method in wheat varieties and their antioxidant properties were determined.

Experimental

Plant Material. Investigations were carried out on twenty five wheat (*T. aestivum* L.) varieties, including twelve of winter and thirteen of spring wheat varieties (Tables 2 and 3). They were grown at the experimental field of the Institute of Soil Science and Plant Cultivation — State Research Institute in Pulawy, located in Osiny (Lublin province, Poland, 51° 52' 02" N, 22° 05' 25" E) under organic system, while four cultivars of winter wheat were cultivated in conventional system, too. The plant materials

* Author for correspondence: ikowalska@iung.pulawy.pl

This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (<https://creativecommons.org/licenses/by-nc/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium for non-commercial purposes, provided the original author and source are credited, a link to the CC License is provided, and changes - if any - are indicated.

were collected from each variety at flag leaf sheath opening — BBCH 47 (Biologische Bundesanstalt, Bundessortenamt and Chemical Industry) scale. Directly after harvesting, the plant material was frozen in the laboratory freezer (−18 °C) and then lyophilized using Freeze Dryer-Gamma 2-16 LSC (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). Plant materials were powdered using Ultra Centrifugal Mill ZM 200 (Retsch, Germany), defatted in a Soxhlet extractor with chloroform, and then used for extraction.

Chemical Reagents. Methanol and chloroform of the analytical purity grade, acetonitrile hypergrade, and formic acid (>98%, MS-grade) for liquid chromatography (LC)–MS were purchased from J.T. Baker (Deventer, Netherlands). Ultra-pure water was prepared in-house with a Milli-Q water purification system (Millipore Co.). DPPH was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The thin-layer chromatography–2,2-diphenyl-1-picrylhydrazyl (TLC–DPPH[•]) test was performed on the surface of aluminum-backed silica gel 60 F₂₅₄ plates (Merck, Darmstadt, Germany). The standards of chlorogenic acid (5-*O*-caffeoylquinic acid, ≥95%) and rutin (≥ 94%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemical reagents were purchased from POCH S.A. (Gliwice, Poland).

Extraction and Purification of Wheat Samples. Deffated and milled wheat aerial parts (100 mg each) were extracted with 80% methanol (25 mL) at 1500 psi solvent pressure, 100 °C cell temp., flush 150%, and three static cycles, using an automated extractor, ASE 200 (Dionex, Sunnyvale, CA). The extracts (25 mL) were collected in vials, and then, the solvent was evaporated under reduced pressure using vacuum evaporator, at 40 °C. The dried extracts were suspended in Milli-Q water (10 mL) and purified by solid-phase extraction (SPE) using C18 Sep-Pak cartridges (1 cm³, 360 mg, Waters Corp., Milford, MA) to remove non-phenolic compounds. The cartridges were first washed with 4 mL of 0.5% methanol to remove carbohydrates and then washed with 4 mL of 80% methanol to elute phenolics. The phenolic fractions were evaporated to dryness using vacuum evaporator, redissolved in 1 mL of 60% methanol and stored in a freezer at −20 °C before analysis. Directly before chromatographic analyses, each fraction was sonicated for 5 min and then centrifuged at 16,000 × *g* for 10 min using Sigma 3-16KL centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). For each variety, three independent samples were prepared.

Determination of Phenolics by UPLC–PDA–ESI–MS Analysis. Quantitative analyses of free phenolic acids (caffeic acid derivatives) and flavonoids were carried out using a Waters ACQUITY UPLC system (Waters Corp., Milford, MA, USA), equipped with a PDA and a triple-quadrupole mass detector (Acquity TQD, Waters Corp.). Wheat phenolic fractions at a concentration of 100 mg of dried weight of plant material per mL (mg DW/mL) were separated on an Acquity HSS C18 (1.8 μm, 100.0 × 2.1 mm, Waters) column, which was maintained at 40 °C. The injection volume was of 2.0 μL. The elution (0.4 mL/min) was carried out with a gradient of solvent B (acetonitrile with 0.1% formic acid) in solvent A (water with 0.1% formic acid): 7% B in 4 min, 7 to 17% B from 4 to 22 min, 17 to 55% B from 22 to 29.95 min, 55 to 95% B from 29.95 to 30 min, 95% B from 30 to 32 min, 95 to 7% B from 32 to 32.05 min, and 7% B from 32.05 to 35 min. The PDA was operated in the range of 220–450 nm, with the resolution of 3.6 nm. Mass analyses were performed applying ESI(−) ionization mode, using the following conditions: capillary voltage, 2.8 kV; cone voltage, 35 V; source temp., 140 °C; desolvation temp., 350 °C; cone gas flow (nitrogen), 100 L/h; and desolvation gas flow, 800 L/h. UPLC analyses were performed in triplicate. The identification of chromatographic peaks was made on the basis of their UV spectra,

ESI–MS/MS fragmentation patterns and literature, and comparison with authentic standards (either commercially available or previously isolated compounds) [13]. The determination of individual phenolics was carried out using external standard method and was based on peak area calculated from selected UV chromatograms. Caffeic acids derivatives were quantified using 5-caffeoylquinic acid (UV detection at 320 nm), and flavonoids were quantified using rutin (UV detection at 255 nm), as a group standards. Each calibration curve was prepared from triplicate analyses of standard working solutions at seven different concentrations over the range of 1.693–270.7 μg/mL for 5-caffeoylquinic acid and 1.601–256.1 μg/mL for rutin. All data were acquired and processed using Waters MassLynx 4.1 software.

TLC–DPPH[•] Test and Image Processing. The standard of rutin (5 μL) and each wheat, and the SPE purified phenolic fractions (30 μL) were applied to silica TLC plates by means of a micropipette. As a mobile phase, we used acetonitrile–water–chloroform–formic acid 60:15:10:5 (v/v/v/v). The plates were developed at 90 mm and dried before derivatization. TLC plates were immersed for 5 s in 0.2% (w/v) DPPH[•] radical methanolic solution. After removing DPPH[•] excess, the plates were kept in the dark for 30 min and then scanned seven times, every 10 min. The test was performed in triplicate. The results of TLC–DPPH[•] test were saved in jpg documents and then processed by free ImageJ program expended by the National Institutes of Health in the USA. First, the images were turned into 8-bit ones and next denoised by median filter (radius, 5 pixels). Then, the baseline drift was removed. The images processed in the aforementioned way were then inverted. For quantitative comparisons, the areas under the common peaks were measured using the “Wand Tool” after drawing the baseline with “Straight Line Selection Tool.”

Statistical Analysis. The linearity of each calibration curve was determined by plotting peak area against concentration. A 1/*X* weighted linear regression analysis of data was used to calculate the slope, y-intercept, and coefficient of determination (*r*²) for both 5-caffeoylquinic acid and rutin. The limit of quantification (LOQ) of the assays was accepted as the lowest concentration of the calibration series with a precision of ±20% [15]. The experimental results were expressed as mean ± SD. The differences among cultivars were evaluated on the basis of analysis of variance and Tukey's confidence intervals for individual phenolics. Statistical significance was declared at *P* ≤ 0.05.

Results and Discussion

Tentative Identification and Quantification of Phenolic Compounds. By means of UPLC–PDA–ESI–MS/MS system (Waters Corp., USA), a number of phenolic compounds were tentatively identified and in the all researched varieties the phenolic acids and the flavonoids were quantified as 5-caffeoylquinic acid and as rutin, respectively. Quantitative analyses were carried out using a seven-point calibration curves, which showed excellent fitting to a first-order polynomial equations. The linearity of response, which was very high (*r*² > 0.999) for both 5-caffeoylquinic acid and rutin in tested ranges, and other calibration parameters are shown in Table 2. The identification of 19 compounds in resulted chromatograms was made according to our previous article [13], i.e., on the basis of their UV spectra, ESI–MS/MS fragmentation patterns and literature, and comparison with authentic standards. Thus, seven free phenolic acids and twelve flavone glycosides were identified in the wheat varieties (Table 1, Figure 1). Content of individual and total phenolics was significantly different between varieties (Tables 2 and 3). The average concentrations of total phenolic acids and flavonoids were definitely higher in spring wheat

Table 1. LC–PDA–ESI–MS/MS characteristics of phenolic compounds in wheat varieties

No.	t_R (min)	$[M-H]^-$ (m/z)	Main product ions (m/z, base peak)	UV (λ_{max} , nm)	Identification
1	2.04	353	191	325	3- <i>O</i> -caffeoylquinic acid ^a
2	3.81	353	191	325	5- <i>O</i> -caffeoylquinic acid ^b
3 4	4.71	353	173, 179, 135	325	4- <i>O</i> -caffeoylquinic acid ^a
		367	193, 134	325	3- <i>O</i> -feruoylquinic acid ^a
5	8.31	163	-	310	<i>p</i> -Coumaric acid ^b
6	8.80	609	327, 357, 411, 429	347, 269, 255	Luteolin <i>C</i> -hexoside <i>O</i> -hexoside ^a
7	9.38	367	193	325	5- <i>O</i> -feruoylquinic acid ^a
8	9.65	367	173, 193	325	4- <i>O</i> -feruoylquinic acid ^a
9	10.55	579	489, 369, 399, 459, 429, 327	350, 270, 255	Luteolin <i>C</i> -(pentosyl-hexoside) ^a
10	10.72	579	459, 399, 369, 489, 429	347, 270, 255	Isoorientin 6''- <i>O</i> - β -D-xylopyranoside ^b
11	12.07	447	357, 327, 285	347, 269, 255	Isoorientin (luteolin 6- <i>C</i> - β -glucopyranoside) ^b
12	12.71	563	443, 353, 473, 383, 503	337, 271	Apigenin <i>C</i> -hexoside <i>C</i> -pentoside ^a Isoschaftoside
13		563	443, 353, 473, 383, 503	337, 271	(apigenin 6- <i>C</i> - α -arabinopyranoside 8- <i>C</i> - β -glucopyranoside) ^b
14	12.87	593	473, 429, 357, 327	350, 269, 255	Luteolin <i>C</i> -hexoside <i>O</i> -deoxyhexoside ^a
15	14.19	771	609, 429, 651, 489, 327	335, 271	Luteolin 6- <i>C</i> -[6 _{Glc} - <i>O</i> - <i>E</i> -caffeoyl- β -D-glucopyranosyl (1'' $\rightarrow$ 2)- β -glucopyranoside] ^b
16	17.59	607	323, 443, 371, 487	350, 269, 250	Isoscoparin 2''- <i>O</i> - α -L-rhamnopyranoside ^b
17	20.72	755	429, 579, 309, 635, 173, 193	335, 271	Luteolin 6- <i>C</i> -[5 _{Rib} - <i>O</i> - <i>E</i> -feruoyl- β -D-ribofuranosyl (1'' $\rightarrow$ 2)- β -glucopyranoside] ^b
18	21.61	637	329	352, 247, 269	Tricin 7- <i>O</i> -rutinoside ^b
19	24.24	681	343	328, 270	3',4',5'- <i>O</i> -trimethyltricetin 7- <i>O</i> -[β -D-glucopyranosyl(1'' $\rightarrow$ 2)- β -D-glucopyranoside] ^b

^aTentative identification on the basis of UV spectrum and ESI–MS/MS fragmentation pattern.^bAbsolute identification on the basis of comparison with authentic standard.

cultivars than in the winter varieties. Content of total phenolic acids changed in the narrow from (332.01 ± 1.69) to (1377.96 ± 4.14) $\mu\text{g/g}$ dry weight (DW) for spring wheat varieties and from (241.96 ± 3.89) to (570.80 ± 5.18) $\mu\text{g/g}$

DW for winter wheat varieties. Analysis of the data disclosed that total phenolic acids were higher in Trappe, Kandela, and Bombona variety. The sum of 4-*O*-caffeoylquinic acid (3) and 3-*O*-feruoylquinic acid (4) was present in higher amounts

Figure 1. UPLC–UV chromatograms of wheat (*Triticum aestivum*) cv. Kandela, (A) at 320 nm for phenolic acids, (B) at 255 nm for flavonoids. Peak numbers correspond to the numbering of compounds in the Table 1

Table 2. Calibration parameters for 5-caffeoylquinic acid and rutin

Compound	t_R (min)	Calibration equation ^a	r^2	Linearity ($\mu\text{g/mL}$)	LLOQ ($\mu\text{g/mL}$)
5-Caffeoylquinic acid	3.81	$y = 614.09x - 384.66$	0.9999	1.69–270.70	1.693
Rutin	15.29	$y = 350.47x + 4.86$	0.9996	1.60–256.10	1.601

^aCalibration curve: $y = mx + b$ where y is the integrated peak area and x is the concentration in $\mu\text{g/mL}$.

^bLLOQ, lower limit of quantification.

(Table 3). Besides this, *p*-coumaric acid (5) represented 9.9–29.1% of total phenolic acids content in spring wheat and 19.3–53.6% in winter wheat varieties. The sum of 4-*O*-caffeoylquinic acid (3) and 3-*O*-feruoylquinic acid (4) constituted 28.9–45.6% in spring wheat and 9.6–38.9% in winter wheat varieties. Concentrations of other determined phenolic acids were considerably lower.

Content of total flavonoids was higher than phenolic acids and ranging from (4698.6 ± 33.5) to (13753.7 ± 72.1) $\mu\text{g/g}$ for spring and from (2612.8 ± 22.2) to (6451.8 ± 10.2) $\mu\text{g/g}$ DW for winter wheat varieties (Table 4). There were large differences in the flavonoid content among the tested wheat cultivars. Higher total flavonoids concentration (13753.7 ± 72.1 $\mu\text{g/g}$, DW) was recorded in Kandela variety followed by Ostka Smolicka (12484.1 ± 80.7 $\mu\text{g/g}$, DW) and Łagwa (9201.3 ± 29.1 $\mu\text{g/g}$, DW), respectively. Isoorientin (11) and luteolin C-hexoside O-deoxyhexoside (14) were found to be the main flavonoids in researched varieties. Isoorientin (11) represented 16.4–33.5% of total flavonoids content in spring wheat and 5.0–27.4% in winter wheat cultivars. Luteolin and its glycosides might be used also as cancer chemopreventive agents [16]. Moheb et al. [6] demonstrated that isoorientin was the major constituent (42% of total flavonoids) of wheat leaves (in leaf development stage), too. These authors suggest that the relatively high abundance of isoorientin in wheat leaves attests to its use as a potential source of active natural health-promoting

compounds. Besides this, mainly in North America, wheat leaves are used as a source of juice (wheatgrass juice) and elsewhere as a beneficial healthy supplement (<http://www.wheatgrass.ca/>). Flavonoids, which have been accumulated in the plants in lower amounts, were also characterized by a smaller variability within the group of the analyzed cultivars. Among the examined wheat cultivars, the smallest total content of the tested compounds was recorded for Arkadia, which was the most infected by fungal pathogens occurring on the leaves. According to Winkel-Shirley [17], flavonoids and other phenolic derivatives play important roles in plant's interaction with its environment, as defense against pathogens and pests, as signaling with microorganisms.

Our research on the influence of cultivars and production system on phenolic concentrations showed large differences in the content of phenolics in the tested cultivars of winter wheat (Table 5). The analysis showed a significant variability of their amount among individual cultivars. Despite the lack of meaningful differences in the compared production systems, there was a tendency to accumulate more phenolic acids in the organic system and more flavonoids in the conventional system. Flavonoids are also known to accumulate when plants are exposed to synthetic compounds such as diphenyl ether herbicides [18]. The level of only one phenolic acid (4-*O*-feruoylquinic acid, 8) was higher in varieties cultivated in conventional system. Besides this, 3-*O*-caffeoylquinic acid (1) and 5-*O*-feruoylquinic acid (7) were below limit of quantification. Zuchowski et al. [10] confirmed the thesis that organically produced wheat grains contain higher levels of phenolic acids. They reported also that comparing the ferulic and *p*-coumaric acid, as well as total phenolic acids content, varieties grown in the organic system, had usually their higher level. In other previous studies, it was demonstrated that the concentration of defence-related natural products in conventionally produced crop are often thought to be lower than in crops grown by organic methods [19, 20].

Table 3. Content of phenolic acids ($\mu\text{g/g} \pm \text{SD}$, DW, as 5-*O*-caffeoylquinic acid) and antioxidant activity (in relation to rutin's activity = 1, $\pm \text{SD}$) in wheat varieties grown in organic farming system

Variety	Compound						Total	Antioxidant activity
	1	2	3 + 4	5	7	8		
Spring wheats								
Bombona	<LQD	104.0 ± 0.5 ^f	283.0 ± 2.9 ⁱ	98.9 ± 2.3 ^d	61.8 ± 4.3 ^d	65.5 ± 6.0 ^j	726.69 ± 6.94	4.10 ± 0.06
Brawura	<LQD	33.0 ± 1.0 ^b	193.3 ± 1.3 ^e	89.6 ± 0.9 ^b	24.7 ± 1.6 ^{ab}	32.6 ± 0.9 ^{cd}	493.86 ± 8.58	4.29 ± 0.10
Hewilla	<LQD	70.9 ± 0.3 ^e	233.1 ± 1.0 ^g	158.4 ± 0.8 ^g	33.4 ± 0.7 ^c	39.2 ± 0.4 ^{ef}	628.36 ± 4.23	3.56 ± 0.23
Kandela	<LQD	112.8 ± 2.8 ^g	401.1 ± 4.9 ^j	99.6 ± 0.7 ^d	70.0 ± 0.3 ^e	86.8 ± 0.6 ^k	1004.93 ± 13.24	4.69 ± 0.19
Katoda	<LQD	54.3 ± 0.5 ^d	201.9 ± 2.3 ^f	93.3 ± 1.2 ^{bc}	25.5 ± 0.9 ^{ab}	36.9 ± 0.9 ^{de}	616.35 ± 3.81	4.38 ± 0.12
Łagwa	<LQD	44.4 ± 0.6 ^c	232.5 ± 0.9 ^g	167.4 ± 2.1 ^h	28.8 ± 3.4 ^{bc}	50.0 ± 1.6 ^{gh}	669.33 ± 7.64	4.45 ± 0.22
Monsun	<LQD	<LQD	124.3 ± 1.5 ^a	110.1 ± 1.2 ^c	21.5 ± 0.9 ^a	25.0 ± 0.6 ^{ab}	395.74 ± 5.39	1.98 ± 0.09
Ostka Smol.	<LQD	26.3 ± 0.2 ^a	145.2 ± 0.7 ^b	71.1 ± 1.8 ^a	28.8 ± 0.9 ^{bc}	50.9 ± 0.7 ⁱ	502.63 ± 1.09	2.84 ± 0.07
Parabola	<LQD	26.6 ± 0.5 ^a	157.1 ± 1.0 ^c	131.6 ± 2.2 ^f	25.7 ± 1.2 ^{ab}	26.9 ± 0.7 ^{abc}	471.47 ± 4.69	2.70 ± 0.08
Trappe	36.8 ± 0.4	354.2 ± 2.6 ^h	452.2 ± 1.4 ^k	207.0 ± 1.3 ⁱ	76.9 ± 2.8 ^c	44.8 ± 1.0 ^{fg}	1377.96 ± 4.14	4.89 ± 0.11
Tybalt	<LQD	<LQD	125.0 ± 1.3 ^a	96.6 ± 2.4 ^{cd}	<LQD	21.9 ± 1.0 ^a	332.01 ± 1.69	3.33 ± 0.12
Werbena	<LQD	<LQD	166.0 ± 2.7 ^d	105.5 ± 0.8 ^e	27.3 ± 4.1 ^{abc}	29.4 ± 2.2 ^{bc}	432.74 ± 10.00	3.36 ± 0.14
Żura	<LQD	34.7 ± 0.9 ^b	274.5 ± 0.7 ^h	97.6 ± 1.0 ^{cd}	33.8 ± 3.0 ^c	45.3 ± 1.0 ^{gh}	602.40 ± 7.84	4.32 ± 0.15
Winter wheats								
Arkadia	<LQD	<LQD	31.3 ± 0.3 ^b	127.8 ± 2.5 ^f	ND	<LQD	267.75 ± 3.23	1.71 ± 0.09
Bamberka	<LQD	20.8 ± 0.4 ^b	134.0 ± 1.2 ^j	110.1 ± 0.8 ^c	<LQD	26.9 ± 1.1 ^c	570.80 ± 5.18	3.79 ± 0.18
Banderola	<LQD	18.6 ± 0.3 ^a	105.5 ± 1.5 ^h	98.4 ± 1.2 ^b	<LQD	23.9 ± 0.5 ^b	470.95 ± 4.39	3.33 ± 0.12
Jantarka	<LQD	<LQD	130.4 ± 2.5 ^j	89.8 ± 0.9 ^a	<LQD	28.3 ± 0.7 ^c	335.45 ± 1.11	3.50 ± 0.16
Julius	<LQD	<LQD	75.6 ± 0.4 ^f	174.9 ± 1.8 ⁱ	<LQD	20.9 ± 0.1 ^a	433.38 ± 0.96	3.46 ± 0.16
KWS Ozon	<LQD	23.0 ± 0.5 ^c	116.9 ± 3.0 ⁱ	158.9 ± 1.8 ^h	<LQD	24.0 ± 0.5 ^b	450.03 ± 5.22	2.92 ± 0.07
Muszelka	<LQD	<LQD	63.2 ± 0.5 ^d	118.3 ± 0.5 ^{de}	<LQD	20.7 ± 1.0 ^a	375.03 ± 3.71	2.44 ± 0.04
Ostroga	<LQD	<LQD	70.0 ± 0.6 ^c	171.6 ± 3.0 ⁱ	<LQD	<LQD	373.73 ± 3.15	2.18 ± 0.07
Rokosz	<LQD	ND	36.2 ± 0.3 ^c	134.8 ± 1.2 ^g	ND	<LQD	251.32 ± 3.37	2.54 ± 0.06
Sailor	<LQD	<LQD	64.7 ± 0.2 ^d	114.3 ± 1.2 ^{cd}	<LQD	<LQD	241.96 ± 3.89	2.64 ± 0.08
Skagen	<LQD	<LQD	83.0 ± 0.5 ^g	194.0 ± 0.6 ^j	<LQD	22.5 ± 0.3 ^{ab}	438.08 ± 1.50	3.73 ± 0.10
Smuga	<LQD	ND	25.6 ± 0.3 ^a	121.7 ± 0.9 ^e	ND	<LQD	266.88 ± 0.60	1.91 ± 0.06

Values expressed as mean $\pm$ SD.

Mean values with the same letter within each column are not significantly different ($P \leq 0.05$).

<LQD indicates below limit of quantitative determination; ND, not detected.

Table 4. Flavonoids concentration ($\mu\text{g/g}$ DW, as rutin) of wheat varieties, grown in organic farming system

Variety	Compound											Total
	6	9	10	11	12 + 13	14	15	16	17	18	19	
Spring wheats												
Bombona	146.5 ^d	504.2 ⁱ	647.7 ^g	1753.7 ^f	1365.1 ^k	1237.0 ^g	271.3 ^g	104.4 ^c	68.9 ^c	84.1 ^{abc}	25.8 ^a	9145.4 ± 75.2
Brawura	145.7 ^d	319.6 ^c	340.2 ^b	1765.4 ^f	1199.0 ^j	1185.8 ^f	210.2 ^f	117.7 ^{de}	99.0 ^c	77.2 ^a	50.4 ^c	8357.7 ± 92.3
Hewilla	114.8 ^c	205.9 ^b	274.3 ^a	1523.8 ^c	655.3 ^b	923.7 ^d	204.2 ^f	69.0 ^a	71.6 ^c	40.8 ^a	23.2 ^a	6528.9 ± 28.0
Kandela	398.3 ^h	539.4 ^j	859.4 ⁱ	4613.1 ^j	1086.9 ^h	1933.8 ^j	467.2 ^h	277.8 ^j	<LQD	137.4 ^{bcd}	ND	13753.7 ± 72.1
Katoda	228.9 ^f	448.5 ^g	515.5 ^d	2723.4 ^h	859.2 ^d	1323.7 ^h	521.3 ⁱ	113.5 ^d	ND	38.4 ^a	36.9 ^b	9158.6 ± 67.3
Łagwa	175.9 ^c	335.4 ^f	415.3 ^c	2171.6 ^g	949.9 ^f	1044.5 ^c	262.2 ^g	150.6 ^h	157.3 ^f	95.2 ^{abc}	68.2 ^e	9201.3 ± 29.1
Monsun	69.7 ^a	178.8 ^a	293.9 ^a	979.1 ^b	653.7 ^b	513.7 ^a	46.8 ^b	125.6 ^{ef}	53.3 ^b	95.8 ^{abc}	<LQD	4872.8 ± 21.5
Ostka Smol.	285.3 ^g	617.3 ^k	703.2 ^h	3663.7 ⁱ	1132.0 ⁱ	1712.1 ⁱ	598.3 ^j	231.0 ⁱ	317.4 ^h	187.9 ^c	25.7 ^a	12484.1 ± 80.7
Parabola	69.3 ^a	254.1 ^c	359.4 ^b	771.8 ^a	689.3 ^c	516.1 ^a	62.3 ^c	93.2 ^b	38.2 ^a	77.5 ^a	60.9 ^d	4698.6 ± 33.5
Trappe	165.1 ^c	305.8 ^d	278.5 ^a	1786.8 ^f	658.2 ^c	1043.7 ^c	209.1 ^f	87.7 ^b	ND	48.1 ^a	49.4 ^c	6433.4 ± 14.1
Tybal	90.1 ^b	515.0 ⁱ	594.5 ^c	1268.9 ^c	984.1 ^g	771.6 ^c	27.7 ^a	158.4 ^h	104.1 ^f	81.8 ^{ab}	34.0 ^b	6521.8 ± 16.4
Werbena	119.0 ^c	334.5 ^f	400.9 ^c	1389.7 ^d	585.6 ^a	707.5 ^b	77.1 ^d	126.2 ^f	82.5 ^d	143.0 ^{cd}	34.7 ^b	6657.4 ± 88.4
Żura	137.4 ^d	465.1 ^h	617.7 ^f	1392.3 ^d	902.8 ^e	1166.3 ^f	159.0 ^c	142.6 ^g	102.8 ^f	77.3 ^a	<LQD	7416.1 ± 31.3
Winter wheats												
Arkadia	15.6 ^a	27.9 ^a	42.9 ^a	130.1 ^a	259.8 ^a	59.5 ^a	29.5 ^a	<LQD	<LQD	83.1 ^b	ND	2612.8 ± 22.2
Bamberka	225.2 ^h	176.4 ^g	257.8 ⁱ	1431.4 ⁱ	461.1 ^f	555.5 ^j	235.2 ^j	54.1 ^c	37.3 ^c	128.5 ^e	ND	5446.6 ± 34.1
Banderola	204.0 ^g	160.7 ^b	236.1 ^h	1347.5 ^h	436.8 ^{de}	492.0 ^h	216.8 ⁱ	36.4 ^b	35.4 ^c	116.9 ^{de}	ND	5040.5 ± 51.4
Jantarka	182.1 ^f	221.3 ^k	256.4 ⁱ	1103.4 ^g	441.7 ^e	527.6 ⁱ	137.9 ^f	70.6 ^{de}	61.9 ^g	94.5 ^{bc}	<LQD	5523.9 ± 59.7
Julius	113.4 ^d	183.1 ^h	239.3 ^h	970.5 ^f	786.0 ^g	799.6 ^j	182.0 ^h	77.1 ^c	44.4 ^f	105.8 ^{cd}	39.1 ^b	5635.1 ± 35.3
KWS Ozon	80.9 ^c	147.4 ^e	217.6 ^g	768.2 ^c	434.8 ^{de}	353.8 ^f	161.4 ^g	37.6 ^b	31.9 ^d	104.1 ^{cd}	ND	3945.2 ± 41.1
Muszelka	119.1 ^d	133.7 ^d	154.1 ^d	648.1 ^d	438.9 ^{de}	466.9 ^g	107.8 ^d	57.2 ^c	24.2 ^c	82.9 ^b	26.8 ^a	4224.9 ± 47.6
Ostroga	54.3 ^b	95.2 ^c	108.6 ^b	380.0 ^b	391.9 ^c	339.2 ^c	69.5 ^b	42.4 ^b	12.5 ^a	61.6 ^a	45.3 ^c	3444.7 ± 27.8
Rokosz	87.9 ^c	204.8 ^j	172.6 ^f	467.7 ^c	393.8 ^c	265.5 ^c	116.1 ^c	65.8 ^d	43.0 ^f	107.2 ^{cd}	ND	4287.2 ± 36.1
Sailor	78.9 ^c	156.5 ^f	162.0 ^e	634.0 ^d	432.8 ^{de}	324.1 ^d	73.3 ^{bc}	36.8 ^b	20.2 ^b	104.1 ^{cd}	<LQD	3973.7 ± 42.9
Skagen	163.0 ^c	197.0 ⁱ	390.8 ^j	1769.3 ^j	427.8 ^d	599.9 ^k	333.8 ^k	41.9 ^b	72.0 ^h	129.0 ^c	ND	6451.8 ± 10.2
Smuga	61.1 ^b	81.6 ^b	114.7 ^c	483.9 ^c	320.6 ^b	217.0 ^b	78.1 ^c	21.7 ^a	17.6 ^b	51.3 ^a	41.7 ^{bc}	3412.4 ± 31.6

Mean values with the same letter within each column are not significantly different ($P \leq 0.05$).

<LQD indicates below limit of quantitative determination; ND, not detected.

Table 5. Influence of cultivars and production system on phenolic acids ($\mu\text{g/g}$ DW, as 5-*O*-caffeoylquinic acid) and flavonoids ($\mu\text{g/g}$ DW, as rutin) concentrations in four winter wheats grown in conventional farming system

Factor	Compound				Total p.a. ^a	Compound											Total flav. ^b
	2	3 + 4	5	8		6	9	10	11	12 + 13	14	15	16	17	18	19	
Variety																	
Arkadia	0.0	38.6	109.4	10.0	252.4	41.3	78.1	77.1	332.6	301.6	225.8	62.9	25.7	9.2	100.2	0.0	3431.0
Bamberka	10.4	89.2	82.4	23.0	388.7	199.9	173.2	226.3	1262.1	448.5	572.5	226.7	53.2	37.2	123.5	0.0	5261.9
Jantarka	0.0	117.5	76.9	29.2	321.5	186.5	251.9	286.5	1230.2	435.9	661.6	155.4	86.8	79.4	106.4	4.9	6123.1
Sailor	0.0	55.5	111.4	0.0	263.1	104.7	179.7	168.2	736.5	440.9	430.2	98.7	53.8	28.4	119.9	0.0	4721.1
LSD _{0.05} ^c	0.3	2.5	2.5	1.0	6.7	8.9	3.1	3.6	14.1	6.9	8.1	4.7	3.5	1.6	8.1	0.7	86.9
System																	
Conventional	0.0	60.2	79.6	17.3	258.9	140.7	195.9	199.2	956.0	414.6	578.4	152.9	69.4	47.3	122.5	2.4	5379.2
Organic	5.2	90.1	110.5	13.8	354.0	125.4	145.5	179.8	824.7	398.9	366.7	119.0	40.4	29.9	102.6	0.0	4389.3
LSD _{0.05}	0.1	1.3	1.3	0.5	3.5	4.6	1.6	1.9	7.4	3.6	4.2	2.5	1.8	0.8	4.2	0.4	45.5

^ap.a., phenolic acids.^bflav., flavonoids.^cLSD, least significant difference based on Tukey's test ($P \leq 0.05$).

DPPH[•] Scavenging Activity. In order to determine antioxidant activity of wheat samples, TLC–DPPH[•] test was performed. According to our previous studies, which focused on *Triticum* species, the analyzed wheat varieties contain phenolic acids, flavone glycosides, or acylated flavone glycosides [13]. The activity of the acylated glycosides also differed depending on the amount of sugar moieties attached to aglycone, as well as on the sugar type [21]. In our study, the activity of the separated compounds has been expressed for the first time for each variety as the ratio of area under common peak to the area under rutin's peak. Then, the sum of active compounds for each variety was determined. Quantitative data obtained for 25 wheat varieties were presented in Table 3. The results of TLC–DPPH[•] test (Figure 2) indicated that spring wheat varieties are generally a richer source of compounds with direct antiradical properties than winter varieties, the average activity being 3.76 and 2.85, respectively. Average antioxidant activity for the spring wheat cultivars was in a range from 1.98 to 4.89, in relation to rutin's activity (1.00). Comparing the activity of individual wheat varieties, the relationships between the content and the type occurring in the phenolics and the level of antioxidant activity

were observed. A significant ($P \leq 0.05$) correlation between antiradical capacity and total phenolic acids content was identified. Interestingly, Trappe, Kandela and Bombona, which exhibited the highest total phenolic acids, mainly 5-*O*-caffeoylquinic acid (2), *p*-coumaric acid (5), and the sum of 4-*O*-caffeoylquinic acid (3) and 3-*O*-feruoylquinic acid (4), registered the highest DPPH radical scavenging activity. According to a review by Rice-Evans et al. [22], hydroxycinnamic acids were esterified with quinic acid, with free hydroxyl groups exhibiting strong antioxidant properties. Moreover, these varieties were found to be rich in luteolin glycosides, isoorientin (11) and luteolin C-hexoside O-deoxyhexoside (14). Furthermore, the presence of acylated flavone glycosides no. 15 and 17 increases activity of variety. According to Ciesla et al. [23], flavonoid glycosides acylated with hydroxycinnamic acids are stronger free radical scavengers in comparison with the corresponding non-acylated polyphenols. Comparing the antioxidant activity of four winter wheat cultivars (Arkadia, Bamberka, Jantarka, Sailor) grown in both farming systems, we have shown that the analyzed varieties had higher average activity in conventional crops: 2.86, 4.08, 4.99 and 3.50, respectively.

Figure 2. The results obtained after processing TLC video scans with ImageJ program, including: (A) TLC plate image for 13 spring wheat varieties and rutin, after processing; (B) line profile plot obtained for one (cv. Trappe) of the variety; (C) and line profile plot obtained for rutin (standard)

Conclusion

In this study, a detailed phenolic profiles and antioxidant activity of Polish spring and winter wheat were reported. Individual free phenolic acids and flavonoids and the total content of these compounds were significantly different among varieties. The average concentrations of total researched compounds were definitely higher in spring wheat cultivars than in winter ones. Content of total phenolic acids was higher in Trappe variety ($1377.96 \pm 4.14 \mu\text{g/g}$) and Kandela ($1004.93 \pm 13.24 \mu\text{g/g}$). Among studied flavonoids, isoorientin was detected as a main phenolic in researched varieties. Kandela and Ostka Smolicka exhibited the highest content of flavonoids (13753.7 ± 72.1 and $12484.1 \pm 80.7 \mu\text{g/g}$, respectively). The obtained results could broaden our understanding of the roles of various types and amounts of wheat compounds in the environment, especially of a variety and systems of farming selection. These results suggest that the high concentration of phenolics with antioxidant activity in wheat aerial parts can play a crucial role in forming plant's resistance against pests and protect against diseases in the beginning of heading.

Acknowledgments. The study was supported by the National Science Centre of Poland (project number 2011/01/D/NZ9/04684).

References

- Adom, K. K.; Sorrells, M. E.; Liu, R. H. *J. Agric. Food Chem.* **2005**, *53*, 2297.
- Liyana-Pathirana, C. M.; Shahidi, F. *Food Chem.* **2007**, *101*, 1151.
- Okarter, N.; Li, C. S.; Sorrell, M. E.; Liu, R. H. *Food Chem.* **2010**, *119*, 249.
- Blandino, M.; Sovrani, V.; Marinaccio, F.; Reyneri, A.; Rolle, L.; Giacosa, S.; Locatelli, M.; Bordiga, M.; Travaglia, F.; Coisson, J. D.; Arlorio, M. *Food Chem.* **2013**, *141*, 2549.
- Luthria, D. L.; Lu, Y.; John, K. M. *J. Funct. Foods* **2015**, *18*, 910.
- Moheb, A.; Ibrahim, R. K.; Roy, R.; Sarhan, F. *Phytochem.* **2011**, *72*, 2294.
- Abozed, S. S.; El-kalyoubi, M.; Abdelrashid, A.; Salama, M. F. *Ann. Agric. Sci.* **2014**, *59*, 63.
- Moore, J.; Hao, Z.; Zhou, K.; Luther, M.; Costa, J.; Yu, L. L. *J. Agric. Food Chem.* **2005**, *53*, 6649.
- Nelson, L.; Giles, J.; Macilwain, C.; Gewin, V. *Nature*, **2004**, *428*, 796.
- Zuchowski, J.; Jonczyk, K.; Pecio, L.; Oleszek, W. *J. Sci. Food Agric.* **2011**, *91*, 1089.
- Mäder, P.; Hahn, D.; Dubois, D.; Gunst, L.; Alföldi, T.; Bergmann, H.; Oehme, M.; Amadò, R.; Schneider, H.; Graf, U.; Velimirov, A.; Fliebach, A.; Niggli, U. *J. Sci. Food Agric.* **2007**, *8*, 1826.
- Penuelas, J.; Estiarte, M.; Kimball, B. A. *Photosynthetica* **1999**, *37*, 615.
- Kowalska, I.; Pecio, L.; Ciesla, L.; Oleszek, W.; Stochmal, A. *J. Agric. Food Chem.* **2014**, *62*, 11200.
- Ma, D.; Sun, D.; Wang, C.; Li, Y.; Guo, T. *Plant Physiol. Biochem.* **2014**, *80*, 60.
- Khan, T. Waters Corporation, Manchester, UK. <http://www.waters.com/webassets/cms/library/docs/720000521en.pdf>, **2003**.
- Lopez-Lazaro, M. *Mini Rev. Med. Chem.* **2009**, *9*, 31.
- Winkel-Shirley, B. *Plant Physiol.* **2001**, *126*, 485.
- Landini, S.; Graham, M. Y.; Graham, T. L. *Phytochem.* **2003**, *62*, 865.
- Brandt, K.; Mølgaard, J. P. *J. Sci. Food Agric.* **2001**, *81*, 924.
- Rembalkowska, E. *J. Sci. Food Agric.* **2007**, *87*, 2757.
- Olech, M.; Komsta, L.; Nowak, R.; Ciesla, L.; Waksmundzka-Hajnos, M. *Food Chem.* **2012**, *132*, 549.
- Rice-Evans, C. A.; Miller, N. J.; Paganga, G. *Trends Plant Sci.* **1996**, *20*, 933.
- Ciesla, L.; Kowalska, I.; Oleszek, W.; Stochmal, A. *Phytochem. Anal.* **2013**, *24*, 47.