Determination of Benzoxazinoids in Spring and Winter Varieties of Wheat Using Ultra-Performance Liquid Chromatography Coupled with Mass Spectrometry

I. Kowalska* and M. Kowalczyk

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

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The composition and concentration of natural products largely depend on a plant part, development stage, cultivar, and growing conditions. This study evaluated the influence of cultivars and production systems on the composition of natural products (benzoxazinoids) in wheat aerial parts. The determination of benzoxazinoids was performed by combining pressurized liquid extraction, ultra-performance liquid chromatography, and tandem mass spectrometry. Six benzoxazinoids were identified and quantitated in wheat varieties. Significant differences were observed among the examined varieties. The average concentrations of total researched compounds were definitely higher in the organically produced spring wheat cultivars than in the winter ones. The content of these compounds in the same varieties grown under organic and conventional systems showed their higher content under the organic one. The main benzoxazinoids detected in wheat varieties were 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (DIMBOA-Glc) and 6-methoxy-2-benzoxazolinone (MBOA). The richest sources of benzoxazinoids were Brawura, Łagwa, and Kandela (52.46, 34.67, and 30.14 µg/g dry weight [DW], respectively).

Keywords: Benzoxazinoids, UPLC–MS/MS, wheat varieties, crop production system

Introduction

Mature grains of rye, wheat, and maize have been recently discovered to contain natural products (secondary metabolites) of the benzoxazinoid type. They are divided into groups according to their structures: benzoxazolinones such as 6-methoxy-2-benzoxazolinone (MBOA); hydroxamic acids including 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), and their corresponding 2-β-D glucosides (DIBOA-Glc and DIMBOA-Glc); and lactams such as 2-hydroxy-1,4-benzoxazin-3-one (HBOA) [1]. Hydroxamic acids in the plants are found in the β-glucosides form. In cereal crops, benzoxazinoids are mainly found in the vegetative parts — roots and leaves [2]. They are also present in cereal foods, such as bread [3, 4].

Benzoxazinoids have been reported to have antiallergenic, anti-inflammatory, anticancer, and appetite-suppressing effects in human [5, 6]. They represent group of compounds with remarkable pharmacological properties. These compounds can be responsible for favorable effects of whole grain products on health problems such as obesity, allergy, and inflammatory diseases. On the other hand, benzoxazinoid metabolites have been typically analyzed due to their importance in plant biochemistry and physiology. Being highly bioactive molecules, they are used by plants as allelochemicals, for a defence against predators and infections. They play a major part in the interaction and communication among plants, microbes, and insects, as well as in the formation of resistance mechanisms and quality traits of plants [7]. Benzoxazinoid composition and concentration largely depend on a plant part, development stage, cultivar, and growing conditions [2, 8, 9]. Growing wheat varieties with high levels of particular hydroxamic acids could reduce the problems with diseases, pests, and weeds and thus limit the use of pesticides [1, 10]. A possible use of allelochemicals as substitutes for pesticides in crop protection has created a need for their qualitative and quantitative analyses.

Analyses of benzoxazinoid metabolites have been performed due to their importance in plant biochemistry and physiology, as highly bioactive molecules used by plants as allelochemicals for the defence against predators and infections [1]. Due to their specific bioactivity against microbial pests and weeds, benzoxazinoids have been studied in terms of their potential agronomic use as natural herbicides in weed control, for example, by incorporating the green plant parts into the soil [11]. The analysis of these compounds relies mostly on gas chromatography coupled with mass spectrometry (MS) or liquid chromatography (LC) coupled with ultraviolet detection. To enhance the sensitivity and selectivity of these analyses, new methodologies (e.g., LC coupled with MS and tandem MS) have been developed [12, 13]. In the present study, an ultra-performance liquid chromatography (UPLC)–tandem MS (MS/MS) was applied to study the chemical composition of benzoxazinoids.

Aerial parts have been chosen as a starting material for the extraction, since they are a feeding source of many insects, and therefore are responsible for many interactions between plants and the environment. Changes in the benzoxazinoids composition, even if of minor importance to humans, can severely influence the ecosystem either by destabilizing balance between insects and hosts or by influencing plant resistance to biotic and abiotic stresses. Due to the fact that the presence of natural compounds depends on many factors, it was important to identify and quantify benzoxazinoids in wheat aerial parts (more than booting stage), in different varieties (winter or spring) and farming systems (organic or conventional).

Experimental

Chemical Reagents. Ultra-gradient grade acetonitrile, mass spectrometry grade formic acid, and deionized water (resistivity 18.2 MΩ cm at 25 °C) were used to prepare UPLC solvents. Chloroform, methanol, and acetic acid were purchased from J.T. Baker (Deventer, Netherlands). Other chemical reagents were

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

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purchased from POCH S.A. (Gliwice, Poland). The benzoxazinoid standards (DIBOA, DIMBOA, HBOA, MBOA, DIMBOA-Glc) were obtained from the FATEALLCHEM project (Fate and Toxicity of Allelochemicals in Relation to Environment and Consumer).

Plant Material. Winter wheat: Arkadia, Bamberka, Banderola, Jantarka, Julius, Sailor, KWS Ozon, Muszelka, Ostroga, Rokosz, Skagen, and Smuga and spring wheat: Bombona, Brawura, Trappe, Hewilla, Kandela, Katoda, Łagwa, Monsun, Ostka Smolicka, Parabola, Tybalt, Werbena, and Żura were grown at the experimental field of the Institute of Soil Science and Plant Cultivation — State Research Institute 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 the conventional system, too. All of the tested varieties are commercially important, and twenty of them are present on the Polish National List of Varieties. In the ecological system of farming (potato – spring wheat – red clover with grass grown two years – winter wheat + catch crop), seed dressing, mineral fertilization, herbicides, fungicides, and the growth regulator were not used. Ecologic fertilization includes only manure application (30 t/ha) before potato. The conventional system of farming (winter rape – winter wheat – spring wheat) is conducted as an intensive crop production technology [14]. These two production systems were located on the same type of soil. Wheats were cultivated on 1 ha experimental fields, one field for each production system. The terms of sowing and harvesting were the same for all the tested cultivars. Each cultivar section contained 6 plots (30 m²) from which crops were harvested. The plant materials were collected from each variety at flag leaf sheath opening — BBCH 47 (Biologische Bundesanstalt, Bundessortenamt and Chemical Industry) scale. The samples of aerial parts from all 6 harvest plots were taken and mixed together. The obtained mixtures were used for the preparation of aerial parts extracts to create a representative pool for each cultivar.

The aerial parts of winter and spring wheats, voucher number TRIT 25/14, were harvested at the principal growth stage in June, 2014. The voucher samples have been deposited at the Department of Biochemistry and Crop Quality of Institute (Pulawy). 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).

Extraction of Wheat Samples. The powdered wheat aerial parts (100 mg) were blended with diatomaceous earth and extracted with 70% methanol in cells of an accelerated solvent extraction system (ASE 200, Dionex, Sunnyvale, CA). The extractions were carried out at 10 MPa operating pressure at 40 °C. After evaporation to dryness using a vacuum evaporator, the extracts were reconstituted in 1 mL of methanol containing 0.1% (v/v) acetic acid and stored at –20 °C.

UPLC–MS/MS Analysis of Benzoxazinoids. A Waters ACQUITY UPLC system (Waters, Milford, MA) with a triple quadrupole mass spectrometer (Waters TQD) was used for quantitative analyses. Plant material extracts were centrifuged

for 20 min at 23,000 × g in 4 °C and separated on a Waters BEH C18 column (1.7 μm, 50.0 × 2.1 mm) with a 7-min-long linear gradient from 3% to 10% of acetonitrile containing 0.1% (v/v) formic acid (solvent B) in deionized water containing 0.1% formic acid (solvent A). All separations were carried out at 50 °C, at the flow rate of 700 μL/min. Injection volume was 2.5 μL; each analysis was repeated two times. Effluent from column was introduced into the ion source of the mass spectrometer, which operated in the negative ion electrospray mode with the following parameters: desolvation temp., 400 °C; desolvation gas flow, 1000 L/h; cone gas flow, 100 L/h; capillary voltage, –2.8 kV; source temp., 130 °C; and Radio frequency (RF) lens, 100 mV. Nitrogen was used as the nebulizing gas, and argon, at the flow of 0.1 mL/min, was used as the collision gas. Concentrations of analyses were estimated using multiple reaction monitoring. Cone voltage and collision energy were optimized for each compound to attain the maximal response. Quantitation method was calibrated from the standard solutions of DIBOA, DIMBOA, HBOA, DIMBOA-Glc, and MBOA. DIMBOA-Glc was used as a reference standard for DIBOA-Glc quantitation. Identity of DIBOA-Glc and its retention time as well as fragmentation pattern were established using authentic DIBOA-Glc standard prepared in small quantity from rye seedlings according to the protocol of Macias et al. [15]. Calibration was performed between 2.5 and 30.0 ng/μL, and it was found to be linear only within this range. The samples with concentrations of analytes higher than 30 ng/μL were appropriately diluted (typically between 2 and 10 times) using 0.1% formic acid and reanalyzed. Details of calibration equations as well as limits of detection and quantitation are shown in Table 1. Waters MassLynx 4.1 SCN 849 software was used for data acquisition and processing.

Statistical Analysis. Each calibration curve was constructed by running standards of different concentrations, in triplicate. All wheat samples were prepared, and UPLC analyses were performed in triplicate. Results were subjected to one-way analysis of variance (ANOVA) analysis (Statgraphics Stratus), and means were compared using a multicomparison Tukey's test. Statistical significance was declared at $P \leq 0.05$.

Results and Discussion

Analysis of benzoxazinoids was possible after the development of a new method of UPLC–MS/MS. The composition and content of these compounds in aerial parts (13 varieties of spring wheat, 12 varieties of winter wheat grown in ecological system, including four of them grown also in a conventional system) were determined (Figure 1). Six benzoxazinone derivatives, including lactam (HBOA), hydroxamic acids and their glycosides (DIBOA, DIBOA-Glc, DIMBOA, DIMBOA-Glc), and benzoxazinolinone (MBOA), were analyzed (Tables 2 and 3). DIMBOA-Glc and MBOA were the major metabolites detected in wheat varieties. The total benzoxazinoid content differed widely among the evaluated varieties (in organic system), and the minimum and maximum values were 0.13 and 52.46 μg/g dry weight (DW) for Banderola and Brawura varieties, respectively. Significant differences between spring and winter wheat varieties, especially in

Table 1. Parameters for quantitation of benzoxazinoids in wheat samples

Compound	RT (min)	Parent (M–H) [–] ion <i>m/z</i>	Fragment ion <i>m/z</i>	CE (eV)	Cone voltage (V)	Calibration equation	<i>r</i> ²	LOD (ng/μL)	LOQ (ng/μL)
HBOA	2.10	164	108	15	30	$y = 73.688 - 35.865$	0.9980	0.91	2.73
DIBOA	2.30	180	134	6	20	$y = 229.86 + 145.90$	0.9950	1.88	5.65
DIBOA-Glc	2.80	342	162	15	25				
DIMBOA	3.70	210	149	6	15	$y = 107.37 + 22.018$	0.9978	1.28	3.84
DIMBOA-Glc	4.60	372	149	15	35	$y = 107.26 + 20.187$	0.9911	0.97	2.91
MBOA	5.20	164	149	15	20	$y = 113.20 - 61.705$	0.9968	1.45	4.35

Figure 1. Multiple-reaction monitoring chromatograms of benzoxazinoid derivatives present in aerial parts extracts of Sailor variety (upper right corner of each panel shows m/z parent and daughter ion as well as the maximal signal level)

concentration of DIMBOA-Glc, were observed. The content of DIMBOA-Glc ranged from 0.00 to 46.22 $\mu\text{g/g}$ DW for spring wheat (Table 2) and from 0.13 to 15.03 $\mu\text{g/g}$ DW for winter wheat (Table 3). MBOA was detected in all the tested spring

wheat varieties, its content changing from 3.27 to 24.44 $\mu\text{g/g}$ DW, whereas this compound was not detected in most of winter wheat varieties. Only one of winter wheat cultivars (Sailor) contained DIBOA-Glc, in the amount of 0.89 $\mu\text{g/g}$ DW. According

Table 2. Content of benzoxazinoids ($\mu\text{g/g}$ DW) in spring wheat varieties growing in organic farming system

Spring varieties	HBOA	DIBOA	DIMBOA-Glc	DIMBOA	MBOA	DIBOA-Glc	Total
Brawura	<LOD	<LOD	46.22 $\pm$ 1.78	<LOD	6.23 $\pm$ 0.09	<LOD	52.46 $\pm$ 1.87
Łagwa	<LOD	<LOD	26.38 $\pm$ 0.87	<LOD	8.30 $\pm$ 0.49	<LOQ	34.67 $\pm$ 1.36
Kandela	<LOD	<LOD	13.63 $\pm$ 0.56	<LOD	16.51 $\pm$ 0.45	<LOQ	30.14 $\pm$ 1.01
Tybałt	<LOD	<LOD	<LOD	<LOD	24.44 $\pm$ 0.91	<LOD	24.44 $\pm$ 0.91
Katoda	<LOD	<LOD	14.74 $\pm$ 0.34	<LOD	3.84 $\pm$ 0.02	<LOD	18.57 $\pm$ 0.36
Hewilla	<LOD	<LOD	11.00 $\pm$ 0.21	<LOD	4.61 $\pm$ 0.08	<LOQ	15.61 $\pm$ 0.29
Monsun	<LOD	<LOD	<LOD	<LOD	15.46 $\pm$ 0.67	<LOQ	15.46 $\pm$ 0.67
Ostka Sm.	<LOD	<LOD	11.98 $\pm$ 0.23	<LOD	3.27 $\pm$ 0.01	<LOQ	15.25 $\pm$ 0.24
Trappe	<LOD	<LOD	8.04 $\pm$ 0.13	<LOD	5.50 $\pm$ 0.10	<LOQ	13.54 $\pm$ 0.23
Żura	<LOD	<LOD	2.82 $\pm$ 0.01	<LOD	10.13 $\pm$ 0.44	<LOQ	12.94 $\pm$ 0.45
Bombona	<LOD	<LOD	5.65 $\pm$ 0.09	<LOD	6.27 $\pm$ 0.09	<LOQ	11.92 $\pm$ 0.18
Werbena	<LOD	<LOD	0.26 $\pm$ 0.00	<LOD	6.59 $\pm$ 0.02	<LOD	6.86 $\pm$ 0.02
Parabola	<LOD	<LOD	<LOD	<LOD	4.93 $\pm$ 0.03	<LOD	4.93 $\pm$ 0.03

Values expressed as mean $\pm$ SD ($n = 3$); <LOQ, below limit of quantification; <LOD, below limit of detection.

Table 3. Levels of benzoxazinoids ($\mu\text{g/g}$ DW) in winter wheat varieties growing in organic farming system

Winter varieties	HBOA	DIBOA	DIMBOA-Glc	DIMBOA	MBOA	DIBOA-Glc	Total
Sailor	<LOD	<LOQ	13.65 $\pm$ 0.14 ^d	<LOQ	6.87 $\pm$ 0.01 ^b	0.89 $\pm$ 0.05	21.41 $\pm$ 0.20 ^d
Rokosz	<LOD	<LOD	15.03 $\pm$ 1.05 ^d	<LOD	6.12 $\pm$ 0.11 ^a	<LOD	21.14 $\pm$ 1.16 ^d
Smuga	<LOD	<LOD	6.78 $\pm$ 0.28 ^c	<LOD	<LOD	<LOQ	6.78 $\pm$ 0.28 ^c
Arkadia	<LOD	<LOD	0.32 $\pm$ 0.07 ^a	<LOD	6.09 $\pm$ 0.35 ^a	<LOQ	6.41 $\pm$ 0.42 ^c
Muszelka	<LOD	<LOD	4.48 $\pm$ 0.08 ^{bc}	<LOD	<LOD	<LOQ	4.48 $\pm$ 0.08 ^{bc}
KWS Ozon	<LOD	<LOD	2.98 $\pm$ 0.04 ^{ab}	<LOD	<LOD	<LOQ	2.98 $\pm$ 0.04 ^{ab}
Jantarka	<LOD	<LOQ	2.77 $\pm$ 0.03 ^{ab}	<LOD	<LOD	<LOQ	2.77 $\pm$ 0.03 ^{ab}
Julius	<LOD	<LOD	2.21 $\pm$ 0.05 ^{ab}	<LOD	<LOD	<LOQ	2.21 $\pm$ 0.05 ^{ab}
Skagen	<LOD	<LOD	1.98 $\pm$ 0.07 ^{ab}	<LOD	<LOD	<LOQ	1.98 $\pm$ 0.07 ^{ab}
Ostroga	<LOD	<LOD	1.15 $\pm$ 0.09 ^a	<LOD	<LOD	<LOD	1.15 $\pm$ 0.09 ^a
Bamberka	<LOD	<LOD	1.08 $\pm$ 0.09 ^a	<LOD	<LOD	<LOQ	1.08 $\pm$ 0.09 ^a
Banderola	<LOD	<LOD	0.13 $\pm$ 0.09 ^a	<LOD	<LOD	<LOQ	0.13 $\pm$ 0.09 ^a

Values expressed as mean $\pm$ SD ($n = 3$); <LOQ, below limit of quantification; <LOD, below limit of detection. Means in a column without a common superscript letter differ significantly ($P \leq 0.05$).

Figure 2. Comparison of the content of main benzoxazinoids in the same cultivars grown under organic and conventional system

to scientific literature [2, 16, 17], the concentration of benzoxazinone in aerial parts of the plant increases suddenly a few days after germination and then decreases progressively with plant age. In the research presented by Villagrasa et al. [2], the major metabolite detected in wheat foliage was MBOA. In the studies of Mogensen et al. [10], in organically grown wheat, DIMBOA-Glc (the precursor of DIMBOA) was detected in all varieties at growth stage 21 and in one variety at growth stage 31. In our research (stage 47), DIMBOA-Glc was detected in all varieties. HBOA (DIMBOA metabolites) was detected in low concentrations at growth stages BBCH 9-10 (10–12 mg/kg DW) and 12 (4–6 mg/kg DW) and was not found at later growth stages. In our research, HBOA was not detected. In the current study, in organic system, the total content of benzoxazinones was definitely lower in the winter wheat varieties than in the spring ones. The comparison of the content of these compounds in the same varieties grown under organic and conventional systems showed their higher content under the organic one (Figure 2). This may be related to the increase in the content of secondary metabolites under stressful conditions.

Conclusion

In this study, content of benzoxazinoids of Polish spring and winter wheat was reported. The average concentrations of total researched compounds were definitely higher in spring wheat cultivars than in winter ones. It can be concluded that the Brawura, Łagwa, and Kandela varieties had the highest content of benzoxazinoids (52.46, 34.67, and 30.14 µg/g DW, respectively). UPLC combined with MS/MS could be applied for a complete characterization of benzoxazinoids in alcoholic extracts from wheat varieties. The high concentration benzoxazinoids in wheat aerial parts can play a crucial role in forming plant resistance against pests and protecting them against diseases. In the future, they can also affect the quality of the grains.

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