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

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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, Lagwa, 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

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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, Jantaraka, Julius, Sailor, KWS Ozoń, Muszelka, Ostroga, Rokosz, Skagen, and Smuga and spring wheat: Bombona, Brawura, Trappe, Hewilla, Kandelka, Katoda, Lagwa, 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 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 + potato) was used. Ecologic fertilization includes only mineral fertilization, herbicides, fungicides, and the growth regulator were not used. Ecologic fertilization includes only mineral fertilization, herbicides, fungicides, and the growth regulator were not used. Ecologic fertilization includes only mineral fertilization, herbicides, fungicides, and the growth regulator were not used.

**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 benzoxazolinone (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

| Compound | RT (min) | Parent (M+H) ion m/z | Fragment ion m/z | CE (eV) | Cone voltage (V) | Calibration equation | r² | 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             | y = 107.37 + 22.018 | 0.9978 | 1.28         | 3.84        |
| DIMBOA    | 3.70     | 210                 | 149            | 6       | 15             | y = 107.26 + 20.187 | 0.9911 | 0.97         | 2.91        |
| DIMBOA-Glc| 3.72     | 372                 | 149            | 6       | 35             | y = 113.20 + 61.705 | 0.9668 | 1.45         | 4.35        |
| MBOA      | 5.20     | 164                 | 149            | 15      | 20             | y = 113.20 + 61.705 | 0.9668 | 1.45         | 4.35        |
concentration of DIMBOA-Glc, were observed. The content of DIMBOA-Glc ranged from 0.00 to 46.22 μg/g DW for spring wheat (Table 2) and from 0.13 to 15.03 μg/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 μg/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 μg/g DW. According

Table 2. Content of benzoxazinoids (μg/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 ± 1.78 | <LOD     | 6.23 ± 0.09 | <LOD     | 52.46 ± 1.87 |
| Łagwa           | <LOD     | <LOD     | 26.38 ± 0.87 | <LOD     | 8.30 ± 0.49 | <LOD     | 34.67 ± 1.36 |
| Kandela         | <LOD     | <LOD     | 13.63 ± 0.56 | <LOD     | 16.51 ± 0.45 | <LOD     | 30.14 ± 1.01 |
| Tybalt          | <LOD     | <LOD     | <LOD       | <LOD     | 24.44 ± 0.91 | <LOD     | 24.44 ± 0.91 |
| Katoda          | <LOD     | <LOD     | 14.74 ± 0.34 | <LOD     | 3.84 ± 0.02 | <LOD     | 18.57 ± 0.36 |
| Hewilla         | <LOD     | <LOD     | 11.00 ± 0.21 | <LOD     | 4.61 ± 0.08 | <LOD     | 15.61 ± 0.29 |
| Monsun          | <LOD     | <LOD     | <LOD       | <LOD     | 15.46 ± 0.67 | <LOD     | 15.46 ± 0.67 |
| Oska Sm.        | <LOD     | <LOD     | 11.98 ± 0.23 | <LOD     | 3.27 ± 0.01 | <LOD     | 15.25 ± 0.24 |
| Trappe          | <LOD     | <LOD     | 8.04 ± 0.13  | <LOD     | 5.50 ± 0.10 | <LOD     | 13.54 ± 0.23 |
| Zara            | <LOD     | <LOD     | 2.82 ± 0.01  | <LOD     | 10.13 ± 0.44 | <LOD     | 12.94 ± 0.45 |
| Bombona         | <LOD     | <LOD     | 5.65 ± 0.09  | <LOD     | 6.27 ± 0.09 | <LOD     | 11.92 ± 0.18 |
| Werbena         | <LOD     | <LOD     | 0.26 ± 0.00  | <LOD     | 6.59 ± 0.02 | <LOD     | 6.86 ± 0.02  |
| Parabola        | <LOD     | <LOD     | <LOD       | <LOD     | 4.93 ± 0.03 | <LOD     | 4.93 ± 0.03  |

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

Table 3. Levels of benzoxazinoids (μg/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 ± 0.14 | <LOQ     | 6.87 ± 0.01 | 0.89 ± 0.05 | 21.41 ± 0.20 |
| Rokosz          | <LOD     | <LOD     | 15.03 ± 1.05 | <LOD     | 6.12 ± 0.11 | <LOD     | 21.14 ± 1.16 |
| Smuga           | <LOD     | <LOD     | 6.78 ± 0.28  | <LOD     | <LOD     | <LOD     | 6.78 ± 0.28  |
| Arkadia         | <LOD     | <LOD     | 0.32 ± 0.07  | <LOD     | 6.09 ± 0.35 | <LOD     | 6.41 ± 0.42  |
| Muszelka        | <LOD     | <LOD     | 4.48 ± 0.08  | <LOD     | <LOD     | <LOD     | 4.48 ± 0.08  |
| KWS Ozon        | <LOD     | <LOD     | 2.98 ± 0.04  | <LOD     | <LOD     | <LOD     | 2.98 ± 0.04  |
| Jantarka        | <LOD     | <LOD     | 2.77 ± 0.03  | <LOD     | <LOD     | <LOD     | 2.77 ± 0.03  |
| Julius          | <LOD     | <LOD     | 2.21 ± 0.05  | <LOD     | <LOD     | <LOD     | 2.21 ± 0.05  |
| Skagen          | <LOD     | <LOD     | 1.98 ± 0.07  | <LOD     | <LOD     | <LOD     | 1.98 ± 0.07  |
| Ostroga         | <LOD     | <LOD     | 1.15 ± 0.09  | <LOD     | <LOD     | <LOD     | 1.15 ± 0.09  |
| Bamborka        | <LOD     | <LOD     | 1.08 ± 0.09  | <LOD     | <LOD     | <LOD     | 1.08 ± 0.09  |
| Banderola       | <LOD     | <LOD     | 0.13 ± 0.09  | <LOD     | <LOD     | <LOD     | 0.13 ± 0.09  |

Values expressed as mean ±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 ≤ 0.05).
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, Lagwa, and Kandel variety 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 benzoxazinones 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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