

Article

Not peer-reviewed version

Phenolic Profile Dynamics during Grain Development in Hull-Less Food Barley Genotypes with Varying Grain Colors

Iván Friero , Alba Macià , María Paz Romero , [Ignacio Romagosa](#) , [Mariona Martínez-Subirà](#) , [Marian Moralejo](#)

*

Posted Date: 23 May 2024

doi: 10.20944/preprints202405.1487.v1

Keywords: food barley; phenolic compounds profile; antioxidant capacity; grain development; immature grains; UPLC-MS/MS



Preprints.org is a free multidiscipline platform providing preprint service that is dedicated to making early versions of research outputs permanently available and citable. Preprints posted at Preprints.org appear in Web of Science, Crossref, Google Scholar, Scilit, Europe PMC.

Copyright: This is an open access article distributed under the Creative Commons Attribution License which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Article

Phenolic Profile Dynamics during Grain Development in Hull-Less Food Barley Genotypes with Varying Grain Colors

Iván Friero, Alba Macià, María Paz Romero, Ignacio Romagosa, Mariona Martínez-Subirà* and Marian Moralejo*.

Universitat de Lleida – AGROTECNIO-CERCA Center, Av. Alcalde Rovira Roure, 191, 25198 Lleida, Spain

* Correspondence: marian.moralejo@udl.cat (Marian Moralejo), mariona.martinez@udl.cat (Mariona Martínez-Subirà).
Equally contributed

Abstract: Barley is rich in phenolic compounds, providing health benefits and making it a valuable addition to a balanced diet. However, most studies focus on these compounds at barley's final maturity, neglecting their synthesis during grain development and its impact on barley quality for food applications. This study investigates phenolic profiles during grain development in four hull-less barley genotypes with different grain colors, specifically bred for food applications. The objectives were to determine the phenolic profile and identify the optimal maturity stage for maximum phenolic content and antioxidant capacity. Using UPLC-MS/MS and *in-vitro* antioxidant capacity assays, results show that total phenolic compounds decrease as grain matures due to increased synthesis of reserve components. Flavan-3-ols, phenolic acids, and flavone glycosides peaked at immature stages, while anthocyanins peaked at physiological maturity. The harvest stage had the lowest phenolic content, with a gradient from black to yellow, purple, and blue genotypes. Antioxidant capacity fluctuated during maturation, correlating positively with phenolic compounds, especially bound phenolic acids and anthocyanins. These findings suggest that early harvesting of immature grain can help retain bioactive compounds, promoting the use of immature barley grains in foods. To support this market, incentives should offset costs associated with decreased grain weight.

Keywords: food barley; phenolic compounds profile; antioxidant capacity; grain development; immature grains; UPLC-MS/MS

1. Introduction

Barley (*Hordeum vulgare* L.) is a versatile cereal crop primarily used for animal feed and malting, with growing interest as a food source for its health benefits. Barley is rich in bioactive compounds such as dietary fiber, β -glucans, arabinoxylans, and phenolic compounds (PCs) [1]. For human consumption, barleys with high β -glucan content are preferred due to their associated health benefits, such as reducing the risk of cardiovascular disease by lowering blood cholesterol levels [2,3]. Additionally, PCs are valued for their antioxidant properties and potential health benefits including anti-inflammatory, antitumor, and antimicrobial properties [4,5].

PCs are secondary plant metabolites, crucial for defense against stresses and environmental adaptation [6,7], and they provide structural support when cross-linked with cell wall polysaccharides [8]. In barley grains, these compounds may include phenolic acids, anthocyanins, flavonoids, and flavones, categorized as free, sugar-conjugated, or insoluble bound [9]. Insoluble bound phenolics, which predominate, significantly contribute to overall antioxidant activity [10]. The color of barley grains is closely linked to their phenolic composition, with flavonoids contributing to yellow, purple, and blue colors, and melanins, derived from polymerized PCs, leading to black grains [11,12]. Purple and blue grains high in anthocyanins, as well as black grains rich in phytomelanins, have been identified as promising ingredients for the development of cereal-based functional foods due to their natural antioxidant properties [11,13].

During maturation, physical and chemical composition of cereal grains undergo substantial modifications affecting nutritional value. Barley grain maturation involves three distinct phases: the initial lag phase with fertilization and rapid cell division, the grain-filling phase with the accumulation of reserve components, and the maturation-drying phase marked by the loss of water content until reaching physiological maturity [14].

While cereals are commonly consumed as fully mature grains, immature cereal grains are attracting interest for their nutritional value and digestibility. However, extensive research on the chemical compositions, bioactive compounds, characteristics, and potential applications of immature cereal grains is lacking [15]. Some studies report that immature wheat grains contain more phenols than mature grains [16,17], and whole immature rice grains have higher levels of vitamin B, and tocopherols [18]. Previous researchers found that immature corn seeds possess high concentrations of reducing sugars, high protein contents with biological activity, and higher antioxidant activity than mature grains [19]. For barley, previous studies showed that total phenolic content gradually decreases throughout grain development, accompanied by a decline in antioxidant capacity [20]. However, comprehensive investigations into the dynamic changes of specific PCs such as phenolic acids, flavan-3-ols, flavones, and anthocyanins, and their corresponding antioxidant activities during the barley grain development are lacking.

In the present study, four hull-less genotypes differing in grain color and designed for food purposes were selected to investigate the variation in their phenolic profile during grain development. The aim was to identify the grain growth stage with the highest phenolic content and associated antioxidant capacity, to evaluate its potential use as active ingredients with health-promoting properties. To our knowledge, this is the first comparative study of major health-promoting PCs in barley genotypes specifically bred for human food. These findings will contribute to understanding the phenolic dynamics of barley grains, providing valuable insights into the optimal harvesting times to maximize their nutritional value for functional food application.

2. Materials and Methods

2.1 Experimental Conditions

The field experiment was carried out in collaboration with Semillas Batlle S.A. in Bell-lloc d'Urgell, Lleida, Spain, under irrigation and well-fertilized conditions. The sowing date was 15 December 2022. The genotypes were grown in four main plots size of 4 x 1.5 m with a seedling rate of 350 seeds/m². Biotic interferences were mitigated by implementing standard practices for weed, insect, and disease control.

2.2. Plant Material

Four barley genotypes were used in this study, differing in number of rows, type of starch and grain color:

- DHL-190849: Hull-less, two-rowed, normal endosperm, and yellow grain double-haploid advanced breeding line.
- Rajapani®: Hull-less, six-rowed, normal endosperm, and blue grain registered Spanish variety.
- DHL-151340: Hull-less, two-rowed, normal endosperm, and purple grain double-haploid advanced breeding line.
- DHL-191250: Hull-less, two-rowed, normal endosperm, and black grain double-haploid advanced breeding line.

One hundred individual spikes of each genotype were marked at the heading to monitor grain growth. At seven-day intervals during grain development (heading until harvest from mid-April to late June), ten spikes were collected from each genotype. Four stages of grain development were selected according to the moisture content of the grains: milky (58-62 %), softy (49-51 %), physiological maturity (35 %), and harvest (12-14 %) as described by Ferreira et al. [21]. Spikes were frozen, freeze-dried (Lyobeta 15 TELSTAR Lyophilizer, Terrassa, Spain), and maintained at -20 °C

until analysis. Prior to analysis, thousand grain weight (TGW) was determined with a Marvin system (GTA Sensorik GmbH, Neubrandenburg, Germany) according to the standard MSZ 6367/4-86 (1986) method (Table S1). Then, the grains were milled to a particle size smaller than 0.5 mm (Foss Cyclotec 1093TM mill, Foss Iberia, Barcelona, Spain).

2.3. Phenolic Compounds and Anthocyanin analysis by Ultra-Performance Liquid Chromatography (UPLC-MS/MS)

Free phenols including anthocyanins and bound PCs were extracted from barley according to Martínez et al. [22]. Samples were analyzed using an AcQuity Ultra-Performance TM liquid chromatography coupled to a tandem mass spectrometer (UPLC-MS/MS) from Waters (Milford, MA, USA) as described in Martínez et al. [22]. Quantification was based on 0.01–173 mg/L calibration curve of standard compounds with results expressed as µg/g dry samples. Limits of detection (LOD) ranged from 0.003 to 0.058 mg/L and limits of quantification (LOQ), from 0.010 to 0.173 mg/L. Table S2 shows the selected reaction monitoring (SRM) conditions, the cone voltage and collision energy used for the quantification as well as fragmentation products (MS2) used for identification of the PCs. This table also indicates the specific phenolic standard compound in which each phenolic has been quantified. PCs were also expressed as µg/grain by multiplying absolute flour measurements by the thousand grain weight (TGW) and dividing the results by 1000.

2.4. In-Vitro Antioxidant Capacity

Antioxidant capacity (AC) was measured with the Oxygen Radical Absorbance Capacity (ORAC) according to Huang et al. [23]. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the control. Antioxidant capacity was expressed as µmols Trolox equivalent/g. The antioxidant capacity was determined using a FLUOstar OPTIMA fluorescence reader (BMG Labtech) controlled by the OPTIMA 2.10R2 software.

2.5. Statistical Analysis

All statistical analyses were conducted using JMP® Pro 16 (SAS Institute Inc., Cary, NC, USA). Samples were analyzed in triplicate and the results presented as the mean value. A general linear model was performed to determine significant differences with genotypes and grain development stages considering fixed and sampling dates random factors. Mean comparison tests were assessed by the Tukey–Kramer’s honestly-significant-difference tests (HSD) and p-values of less than 0.05 were considered statistically significant. The standard error of the difference (SED) was also included for each variable.

3. Results and Discussion

3.1. Deposition Pattern of Total Phenolic Compounds

In this study, total phenolic compound (PCs) content in both free and bound fractions was analyzed across distinct stages of grain development in four barley genotypes differing in grain color. Total PCs were quantified as the combined sum of both free and bound extracts and expressed as µg/g of flour (Table 1).

Table 1. Free, bound, and total phenolic content (µg/g flour) in barley flour of four barley genotypes at different stages of grain development.

| | DHL-190849 | Rajapani® | DHL-151340 | DHL-191250 |
|-------------------------|------------|-----------|------------|------------|
| | Yellow | Blue | Purple | Black |
| Free phenolic compounds | | | | |
| Milky | 1431 A a | 1039 A c | 922 B c | 1284 A b |
| Softy | 1071 B b | 757 B c | 1036 A b | 1223 A a |

| | | | | |
|--------------------------|-----------|-----------|-----------|-----------|
| PM | 549 C c | 430 C d | 689 C b | 927 B a |
| Harvest | 568 C b | 308 D c | 342 D c | 972 B a |
| Bound phenolic compounds | | | | |
| Milky | 2106 A b | 1282 AB c | 2371 A ab | 2509 A a |
| Softy | 2079 A b | 1289 AB c | 2168 A ab | 2287 B a |
| PM | 1457 B b | 1456 A b | 1643 B a | 1606 C ab |
| Harvest | 1341 C b | 1201 B c | 1462 B a | 1362 D ab |
| Total phenolic compounds | | | | |
| Milky | 3537 A ab | 2321 A c | 3193 A b | 3793 A a |
| Softy | 3150 B b | 2047 B c | 3204 A b | 3493 B a |
| PM | 2006 C b | 1886 B b | 2332 B a | 2534 C a |
| Harvest | 1909 C b | 1509 C d | 1788 C c | 2325 C a |

Mean values followed by different letters are significantly different according to Tukey–Kramer’s HSD for $\alpha = 0.05$. Uppercase letters show the results of tests for differences among stages of maturation within each genotype. Lowercase letters compared mean phenolic compound content across genotypes for individual stages of maturation. PM: Physiological Maturity.

Free, bound, and total PCs exhibited significantly higher levels during the milky and softy immature stages across three of the four studied genotypes. These results align with previous findings in other cereals such as maize, wheat, and rice [17,24,25]. At the harvest stage, significant differences in total PCs were observed among genotypes with black genotype DHL-191250 exhibiting the highest content, followed by yellow, purple, and blue genotypes in decreasing order. These contents surpassed those reported by Ge et al. [26] and Kim et al. [27] for colored barleys, where black genotypes showed the lowest total PCs levels compared to blue and purple ones.

When comparing the total PCs content between the immature milky stage and the final harvest stage, a decrease ranging from 35 to 46 % across genotypes was observed. Among these, the black genotype DHL-191250 exhibited the smallest. Both total free and bound phenols, exhibited different decreasing trends across genotypes during grain maturation, affecting total PCs. In the case of the blue genotype Rajapani®, the bound-to-free ratio peaked during the two final maturity stages, exceeding 70 %. This fact suggests that during grain development, free PCs decrease drastically compared to bound phenolics, increasing the bound-to-free phenols ratio, as has been reported in wheat [17]. Conversely, the black genotype DHL-191250 showed the opposite trend, with the bound-to-free phenols ratio decreasing as maturation progressed from 49 % in the milky stage to 29 % at harvest, demonstrating that in this genotype, free phenols did not decline as in the other genotypes. In contrast, the purple genotype DHL-151340 experienced a different trend due to the accumulation of anthocyanins in the central stages of grain development, as will be detailed in subsequent Section 3.2.4. For this genotype, the bound-to-free ratio initially favored bound PCs at the milky stage, starting at 61 %. However, once grains reached the softy stage and anthocyanins accumulated, this ratio decreased, reaching a maximum bound-to-free ratio of 52 %, then increased again to 76 % at harvest.

To explore the decline in PCs during maturation it was hypothesized that this could be due to a dilution effect caused by the rise in grain weight during development, as observed in colored wheat genotypes by Ma et al. [28]. Therefore, the total PCs levels in flours were assessed relative to their grain content. The results of the total phenolic content per grain showed a deposition pattern different from that observed in flour, with maximum peaks occurring in post-milky stages such as softy or physiological maturity (Figures 1A, and B).

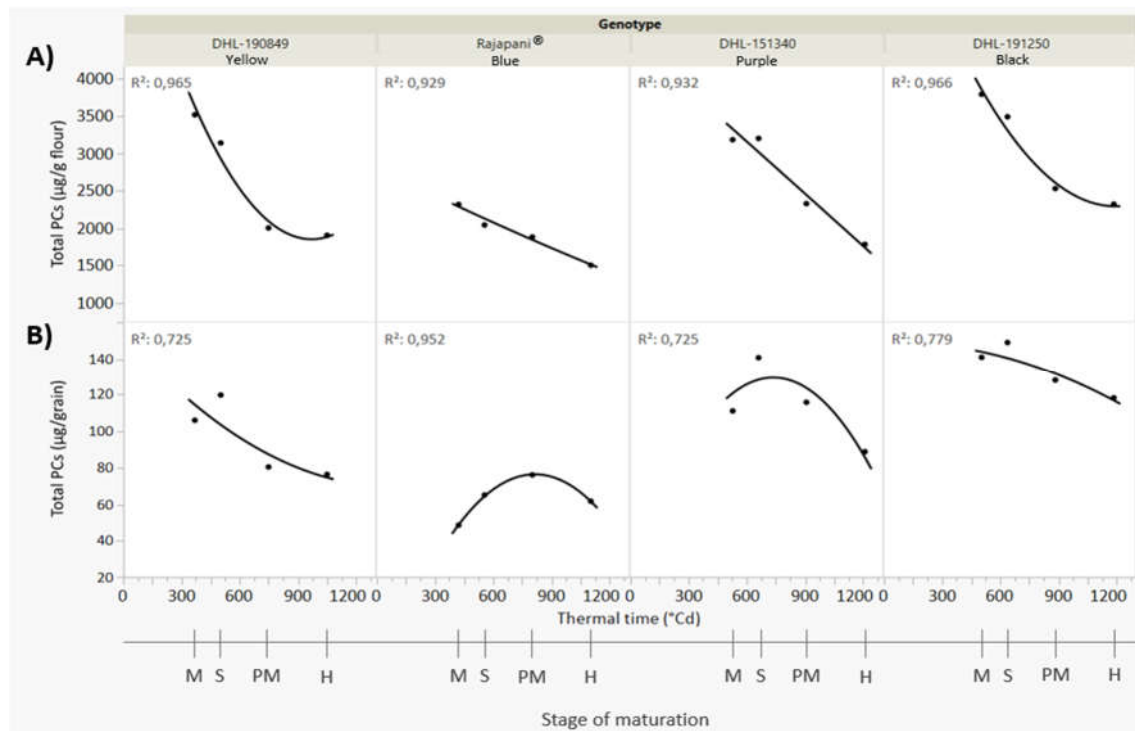


Figure 1. Total phenolic compounds (PCs) of four barley genotypes at different stages of the grain development. A) µg of PCs/g flour. B) µg of PCs/grain. Solid lines represent the best fit (second-degree polynomial curve). M: Milky, S: Softy, PM: Physiological Maturity, H: Harvest. Thermal time (°Cd) was determined using maximum and minimum daily temperatures with a base temperature of 0 °C, using growing degree-days after heading.

These findings suggest a continuous synthesis of some PCs during grain development that can extend until physiological maturity as seen in Rajapani® and the purple genotype DHL-151340. Thus, the significant reduction in PCs from immature to mature stages observed in flour could be attributed to a dilution effect, as starch accumulates in the growing endosperm. Once the grain reaches physiological maturity and grain filling is complete, the decrease in PCs is not significant compared to the harvest stage. However, exceptions are observed in genotypes containing anthocyanins such as Rajapani® and DHL-151340. In these cases, there was a substantial reduction in these compounds from physiological maturation to harvest stage, contributing to the significant decrease in total PCs.

Some researchers suggest that the decrease in phenolic content during grain development may result from physiological factors, such as reduced photosynthesis or oxidative metabolism during the grain dehydration process [29]. Several authors have speculated that carbohydrates, such as sucrose, might enhance the activity of phenylalanine ammonia lyase, an important enzyme during PCs biosynthesis, and stimulate phenolic compound production during grain maturation [30,31]. However, as grain filling progresses into the later stages, the availability of carbohydrates could be compromised, particularly during starch synthesis, which could potentially affect phenolic compound formation [28,32]. Identifying the optimal developmental stage of cereal grains with the highest contents of PCs is essential from both nutraceutical and pharmaceutical perspectives. PCs, renowned for their antioxidant properties and health benefits, play a vital role in protecting against chronic diseases such as cardiovascular ailments and certain types of cancer [4]. Understanding the peak content of these compounds would allow researchers and the industry to enhance crop management to preserve these compounds in their most beneficial state. This could enhance the nutritional value of food products and facilitate the development of potentially healthy components derived from barley.

3.2. Dynamics of Specific Phenolic Compounds across Grain Development Stages

3.2.1. Flavan-3-ols

Flavan-3-ols stand out as a significant category of flavonoids within barley grains [33]. The analysis of the four barley genotypes revealed the presence of 10 different flavan-3-ols, all detected in their free extract. These compounds constituted between 81 % and 99 % of the total free PCs, as detailed in Table 2.

Among the different compounds identified, catechin, procyanidin B3, prodelfphinidin B4, and catechin glucoside were the most representative, while other compounds such as procyanidin B3, prodelfphinidin B3, procyanidin-diglucoside, procyanidin C2, and some dimers and trimers of these components were detected in minor quantities. A significant decrease in total flavan-3-ols was observed throughout grain development across genotypes as illustrated in Figure 2.

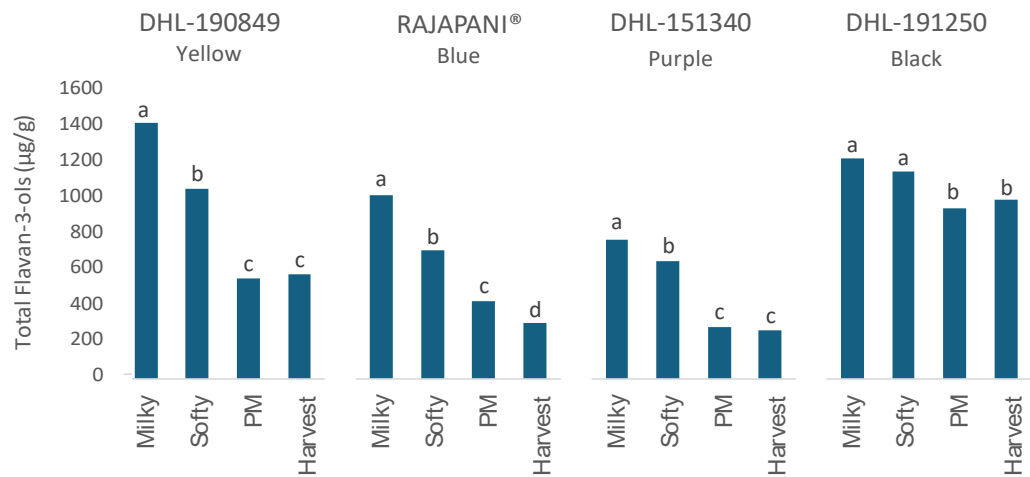


Figure 2. Total Flavan-3-ols in flours of four barley genotypes at different stages of the grain development. Statistic was performed in total flavan-3-ols. Different letters at the same genotype mean significant differences between stages (Tukey–Kramer’s HSD for $\alpha = 0.05$). PM: Physiological Maturity.

Table 2. Free phenolic compounds contents (µg/g) in four barley genotypes at different stages of maturation.

| | Flavan-3-ols | | | | | | | | | Phenolic acids | | | | | | | | Flavone glycosides | | Total free phenols* | |
|---------------------|--------------|---------|---------|---------|----------|---------|--------|---------|---------|----------------|--------|--------|-------|-------|------------|-------|--------|--------------------|--------|---------------------|----------|
| | Cat | Cat-g | Pc B3 | Pc B2 | Pd B4 | Pd B3 | Pc-d | Pc C2 | C-C-GCs | Ferulic | isoF | TriF | p-Cm | Caff | Sinap-hexo | p-OHB | Vanill | Syring | Lut-g | | Isosc-g |
| DHL-190849 (Yellow) | | | | | | | | | | | | | | | | | | | | | |
| Milky | 469.6 a | 87.0 a | 346.3 a | 14.2 a | 476.2 a | 21.3 a | nd | 10.0 a | nq | 15.7 b | 7.6 a | 1.4 a | 1.2 b | nd | 31.5 a | 1.3 a | 3.1 a | nd | nq | 2.1 ab | 1430.8 a |
| Softy | 216.6 b | 75.0 b | 303.9 b | 6.3 b | 403.8 b | 20.6 a | nd | 7.8 a | nq | 19.2 a | 6.1 b | 1.7 a | 1.6 a | nd | 13.3 b | 1.2 a | 3.0 a | nd | nq | 2.7 a | 1070.5 b |
| PM | 81.8 c | 41.0 c | 181.2 c | 4.0 c | 252.1 c | 10.6 b | 4.7b | 4.2 b | nd | 2.4 c | nd | nd | nd | nd | nd | nd | 1.7 b | nd | nq | 1.9 b | 549.3 c |
| Harvest | 94.7 c | 46.2 c | 149.4 d | 5.7 bc | 234.3 c | 11.4 b | 7.3 a | 3.9 b | nd | 1.5 c | nd | nd | nd | nd | nd | nd | 1.3 b | nd | nq | 1.5 b | 567.8 c |
| SED | 11.89 | 2.80 | 9.28 | 0.42 | 12.90 | 0.96 | 0.29 | 0.65 | | 0.35 | 0.43 | 0.21 | 0.05 | | 2.09 | 0.18 | 0.30 | | | 0.23 | 28.06 |
| Rajapani® (Blue) | | | | | | | | | | | | | | | | | | | | | |
| Milky | 441.5 a | 43.3 ab | 330.8 a | 13.0 a | 138.8 a | 6.4 ab | nd | 9.0 ab | nd | 36.4 a | 14.7 a | 11.6 a | 2.1 a | nd | nd | 3.5 a | 7.3 a | 1.2 a | nd | 1.7 a | 1038.9 a |
| Softy | 308.8 b | 32.9 c | 211.0 b | 8.9 b | 112.7 b | 7.9 a | nd | 7.5 b | nd | 30.4 b | 16.6 a | 7.3 b | 2.5 a | nd | nd | 2.2 b | 4.7 b | 1.0 a | nd | 2.1 a | 754.7 b |
| PM | 41.7 c | 45.3 a | 199.6 b | 7.0 bc | 105.9 b | 4.3 b | 8.3 a | 9.8 a | nd | 3.2 c | nd | nd | nd | nd | nd | nd | nd | nd | nd | 1.0 b | 423.3 c |
| Harvest | 33.3 c | 42.0 b | 138.0 c | 5.7 c | 71.4 c | 1.5 b | 7.2 a | 7.5 b | nd | 0.9 c | nd | nd | nd | nd | nd | nd | nd | nd | nd | 1.2 b | 306.9 d |
| SED | 12.92 | 0.99 | 10.12 | 0.73 | 5.72 | 0.54 | 0.86 | 0.55 | | 0.79 | 1.32 | 0.76 | 0.30 | | | 0.31 | 0.51 | 0.12 | | 0.13 | 29.34 |
| DHL-151340 (Purple) | | | | | | | | | | | | | | | | | | | | | |
| Milky | 195.0 a | 56.4 a | 233.8 a | 11.0 a | 242.5 a | 1.9 a | nd | 9.7 a | nd | 31.8 a | 4.9 a | 2.2 a | 3.5 a | 0.7 b | nd | 2.0 b | 4.7 ab | nd | 10.5 c | 2.5 a | 815.0 a |
| Softy | 108.5 b | 58.5 a | 258.9 a | 8.7 b | 188.7 b | 9.7 b | 3.9 a | 7.4 b | nd | 25.9 b | 3.8 b | 1.2 b | 2.7 a | 2.1 a | nd | 3.1 a | 6.7 a | nd | 26.7 b | 3.1 a | 711.6 b |
| PM | 38.4 c | 35.8 b | 134.2 b | 4.1 c | 55.8 d | 5.5 c | 3.3 a | 6.0 bc | nd | 10.6 c | 1.4 c | nd | 1.5 b | 0.6 b | nd | 1.8 b | 4.2 b | nd | 42.3 a | 2.4 ab | 344.2 c |
| Harvest | 24.8 c | 43.7 b | 129.9 b | 4.6 c | 81.1 c | 1.9 d | 6.2 a | 4.7 c | nd | 6.3 d | nd | nd | nd | nd | nd | 0.9 c | 3.2 b | nd | 13.5 c | 1.9 b | 311.0 d |
| SED | 4.87 | 3.00 | 7.53 | 0.47 | 3.04 | 0.65 | 0.73 | 0.49 | | 0.99 | 0.24 | 0.08 | 0.31 | 0.17 | | 0.14 | 0.55 | | 1.44 | 0.18 | 7.98 |
| DHL-191250 (Black) | | | | | | | | | | | | | | | | | | | | | |
| Milky | 170.5 a | 115.6 a | 434.3 a | 13.7 ab | 414.9 a | 24.3 a | nd | 13.6 b | 9.7 a | 28.0 a | 14.7 a | 3.4 a | 2.9 a | nd | 47.9 a | 1.9 a | 4.9 a | nd | nd | 5.6 a | 1283.9 a |
| Softy | 117.5 b | 114.2 a | 420.4 a | 15.4 a | 395.0 ab | 23.4 ab | nd | 16.3 a | 7.5 b | 30.6 a | 15.5 a | 1.7 b | 2.7 a | nd | 41.6 b | 0.9 b | 5.4 a | nd | nd | 4.9 a | 1222.6 a |
| PM | 82.2 c | 97.8 a | 311.2 b | 12.0 ab | 396.0 ab | 19.7 c | 17.8 a | 12.0 b | 10.3 a | 1.0 b | 1.9 b | nd | nd | nd | nd | nd | nd | nd | nd | 2.2 b | 927.3 b |
| Harvest | 89.8 c | 114.6 a | 324.2 b | 11.1 b | 375.3 b | 20.7 bc | 19.0 a | 13.8 ab | 9.1 ab | 1.5 b | 1.3 b | nd | nd | nd | nd | nd | nd | nd | nd | 1.7 b | 972.0 b |
| SED | 4.87 | 5.10 | 13.19 | 1.03 | 6.99 | 0.74 | 1.15 | 0.72 | 0.50 | 1.07 | 0.80 | 0.34 | 0.46 | | 1.22 | 0.15 | 0.41 | | | 0.23 | 31.32 |

Results are presented as the mean. Means within a column followed by different letters indicate significant differences according to Tukey–Kramer’s HSD for $\alpha = 0.05$. SED: standard error of the difference between means. **Flavan-3-ols**: (Cat: Catechin, Cat-g: Catechin-glucoside, Pc B3: Procyanidin, Pc B2: Procyanidin B2, Pd B4: Prodelphinidin B4, Pd B3: Prodelphinidin B3, Pc-d: Procyanidin-diglucoside, Pc C2: Procyanidin C2, C-C-GCs: sum of C-C-GC/GC-C-C and C-GC-C); **Phenolic acids** (Ferulic: Ferulic acid, isoF: Iso-Ferulic acid, TriF: Tri-Ferulic acid, p-Cm: p-Coumaric acid, Caff: Caffeic acid, Sinap-hexo: Sinapoyl-hexose, p-OHB: p-hydroxybenzoic acid, Vanill: Vanillic acid, Syring: Syringic acid); **Flavone glycosides**: (Lut-g: Luteolin-O-glucoside, Isosc-g: Isoscorparin-7-glucoside). nd: not detected. nq: not quantified. PM: Physiological Maturity. *Anthocyanins not included.

The milky stage of the early development showed the highest content of total flavan-3-ols across genotypes, with yellow genotype DHL-190849 reaching the maximum value followed by the black, blue, and purple genotypes in decreasing order. The greatest reduction in flavan-3-ols content occurred during the immature stages leading up to physiological maturity, reaching a maximum decrease at harvest stage of 59 %, 69 %, 65 %, and 19 % for yellow, blue, purple, and black genotypes respectively.

At harvest, the black genotype DHL-191250 exhibited the highest total flavan-3-ols level reaching 967.6 µg/g of flour, while the purple genotype displayed the lowest with a final level of 264.5 µg/g of flour. These results are consistent with those found by Lin et al. [34], where barley genotypes with black grain showed higher flavonoid content than genotypes with other colors.

Regarding specific flavan-3-ols, all quantified components exhibited a significant decrease with grain development across genotypes, except for procyanidin-diglucoside. (Table 2). This compound was not detected in the two immature stages across all genotypes but showed a significant increase with maturation, reaching its peak value at the harvest stage. In contrast, procyanidin C2, remained statistically constant during grain development in both Rajapani® and DHL-191250. Dimers and trimers procyanidins were solely quantified in the black genotype, showing no statistical variances between the milky and harvest stages. At harvest, the levels of procyanidin B3 and prodelphinidin B4 in DHL-191250 were notably higher compared to other genotypes, with contents of 324.2 and 375.3 µg/g of flour, respectively. This represents a reduction of 25 % and 10 %, respectively, from the milky to the harvest stages. In contrast, reductions in other genotypes ranged from 45 % to 60 % for procyanidin B3, and from 45 % to 55 % for prodelphinidin B4. This trend may explain why the black genotype DHL-191250 maintains high levels of total flavan-3-ols during maturation compared to other genotypes.

3.2.2. Phenolic Acids and Aldehydes

Phenolic acids, comprising hydroxybenzoic and hydroxycinnamic acids, emerge as the predominant PCs in barley, as noted by Šimić et al. [35]. These acids were detected in both free and bound phenolic fractions across all genotypes, regardless of the maturation stage (Tables 2, and 3). During grain development, a significant decline in total, free, and bound phenolic acids and aldehydes was evident across genotypes, as illustrated in Figure 3.

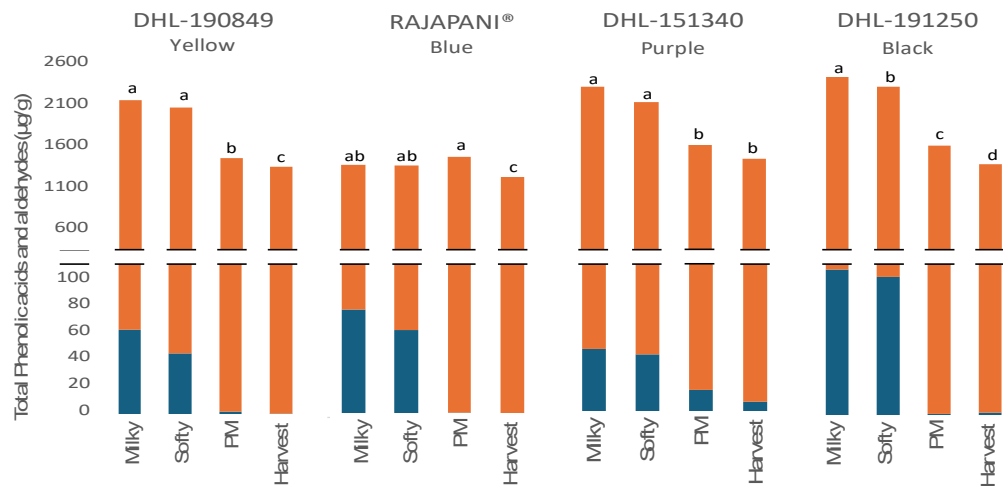


Figure 3. Total Phenolic acids and aldehydes in flours of four barley genotypes at different stages of the grain development. Orange bars: Bound extract. Blue bars: Free extract. Statistic was performed in total phenolic acids. Different letters at the same genotype mean significant differences between stages (Tukey–Kramer’s HSD for $\alpha = 0.05$). PM: Physiological Maturity.

At the milky stage, the black genotype DHL-191259 exhibited the highest level of total phenolic acids, followed by purple, yellow, and blue genotypes in descending order. Similar to flavan-3-ols,

the most significant decrease in total phenolic acids and aldehydes occurred during the two immature stages leading up to physiological maturity. However, Rajapani® maintained consistent levels of total phenolic acids and aldehydes throughout maturation, experiencing only a slight but significant decrease of 12 % from the milky to the harvest stage. At harvest, the purple genotype DHL-151340 showed the highest total phenolic acids and aldehydes, 1470.7 µg/g of flour, while Rajapani® exhibited the lowest, 1201.3 µg/g of flour. These values are consistent with those reported by Holtekjølen et al. [36] for various barley genotypes, which ranged from 604 to 1346 µg/g of flour.

Although there was a significant decrease in total free phenolic acids during physiological maturity and harvest, the predominant presence of total bound phenolic acids ensured that the overall total remained relatively stable, especially during grain development. More than 95 % of the total phenolic acids and aldehydes were quantified in the bound extract through the stages of maturity. The ratio of bound-to-free of total phenolic acids increased with maturity, rising from 94 % in the milky stage to nearly 100 % at the harvest stage across all genotypes. This increase can be attributed to the significant decrease in total free PCs during the last two maturity stages across all genotypes, resulting in contents that were almost negligible compared to the total bound phenolic acids.

Out of the 16 phenolic acids and aldehydes identified, nine were detected in the free extracts (Table 2). Among these, six were hydroxycinnamic acids, primarily including ferulic acid and its isoform, along with a trimer of it, *p*-coumaric acid, caffeic acid, and synapoyl-hexose. Additionally, three hydroxybenzoic acids were identified, including *p*-hydroxybenzoic, vanillic, and syringic acids. Total free phenolic acids constituted less than 8 % of the total free PCs. Other researchers have also noted that free phenolic acids represent a small portion of the total phenolic acids content in barley [37]. Ferulic acid emerged as the predominant constituent, with contents ranging from 0.9 to 36.4 µg/g of flour in the harvest and milky stages, respectively. As reported by Ou & Kwok [38] ferulic acid is rarely found in the free extract as it is predominantly bound to other compounds in cell walls, such as oligosaccharides and polysaccharides. Synapoyl-hexose exhibited an even higher content during the milky stage compared to ferulic acid, reaching a maximum content of 47.9 µg/g of flour in the black genotype DHL-191250. Caffeic acid was solely detected in the purple genotype DHL-151340, reaching its peak content at the softy stage and becoming undetectable at the final stage of maturation.

Concerning hydroxybenzoic acids, syringic acid was only measured in Rajapani® at very low content during the two immature stages of development. Among the phenolic acids identified in the free extract, both hydroxycinnamic and hydroxybenzoic exhibited a significant decreasing trend with grain development, with the majority decreasing in physiological maturation and harvest. It is noteworthy that the presence of *p*-hydroxybenzoic acid in the free fraction was previously linked to colored barley grains as reported Deng et al. [9]. However, in this study, we detected this compound in its free form across all different genotypes.

Regarding total bound phenolic acids and aldehydes, 15 were detected in the bound extract (Table 3). Across genotypes and maturity stages, total bound PCs were predominantly represented by phenolic acids, consistent with previous findings in barley [22,39,40]. During grain development, ferulic acid emerged as the predominant component, constituting over 60 % of the total phenolic acids and aldehydes at the milky stage across genotypes. This percentage increased to 65 % at the harvest stage in Rajapani® and the purple genotype DHL-151340. Comparable findings were previously documented in highland barleys at harvest stages [40,41]. Ferulic acid decreased significantly across genotypes from the milky stage to harvest, except in Rajapani®. Total bound phenolic acids remained relatively constant during maturation for this genotype, primarily due to the behavior of ferulic acid as the major constituent. Ferulic acid exhibited its highest content peak at physiological maturity in this genotype, reaching 944.4 µg/g of flour. This contrasts with the other genotypes where the milky stage showed the highest content. Iso-ferulic acid, the second major constituent, followed a similar trend. However, distinct patterns were noted for the dimers and trimers of ferulic acids. Di-ferulic, tri-ferulic, and decarboxylated di-ferulic acids exhibited a notable increase from the milky stage through maturation, peaking during the softy and physiological maturity stages before declining towards the final stage of maturity. In Rajapani®, decarboxylated di-ferulic acid peaked at the final maturity stage, showing a significant increase from 21 µg/g of flour in the milky stage to 35.2 µg/g of flour in the harvest stage. Similarly, both *p*-coumaric and *m*-

coumaric acids displayed a comparable trend, significantly increasing from the milky stage during the intermediate stages of development. Despite the observed trend in these phenolic acids, the dominant presence of ferulic acid and its significant decrease during maturation across genotypes made it challenging to discern the increasing trend of the remaining phenolic acids during the development of total phenolic acids. Among hydroxycinnamic acids, additional compounds with negligible contents were detected, such as sinapic, caffeic, cinnamic, and feruloyl pentose acids. Sinapic acid was exclusively detected in the purple and black genotypes, with contents of 4.2 and 2.7 $\mu\text{g/g}$ of flour, respectively. In contrast, cinnamic acid was found in all genotypes except Rajapani®, reaching its highest content in the purple genotype DHL-151240 at 3.5 $\mu\text{g/g}$ of flour.

Concerning hydroxybenzoic acids, the same three components identified in the free extract were found in the bound extract, including *p*-hydroxybenzoic, vanillic, and syringic acids. Significant differences were observed in hydroxybenzoic acids during maturation, with all constituents experiencing a significant increase across genotypes. Butsat et al. [42] reported a significant increase in bound *p*-hydroxybenzoic, vanillic, and syringic acids during grain development in rice husks. In our study, this increase was particularly pronounced in vanillic acid, which exhibited a content three times higher at harvest compared to the milky stage across genotypes, reaching a maximum value of 9.8 $\mu\text{g/g}$ of flour in the purple genotype DHL-151340. A similar trend was observed in wheat-colored genotypes, where bound vanillic acid reached its peak content at the end of maturation due to upregulated expression of the TaC3H1 gene associated with its accumulation [28]. Similarly, *p*-hydroxybenzoic acid also showed a significant increase, rising nearly six-fold in the purple genotype DHL-151340 from the milky stage to harvest. Despite the significant increase experienced by hydroxybenzoic acids, their final contribution to total bound phenolic acids was less than 1% across maturity stages.

Only one aldehyde, syngaraldehyde, was identified in the bound extract. This compound showed a notable increase until the harvest stage across genotypes, except in the black genotype DHL-191250, where it peaked at a maximum value of 2.5 $\mu\text{g/g}$ of flour. Consistent with prior research, its overall contribution to total bound phenolic acids was marginal.

Table 3. Bound phenolic compounds contents (µg/g) in four barley genotypes at different stages of maturation.

| | Phenolic acids | | | | | | | | | | | | | | | Flavone glycosides | Total bound phenols |
|---------------------|----------------|---------|----------|---------|---------|--------------|--------------|---------|-------|--------|-----------|---------------|----------|--------|----------|--------------------|---------------------|
| | Ferulic | isoF | DiF | TriF | DC DiF | <i>p</i> -Cm | <i>m</i> -Cm | Sinapic | Caff | Cinna | Ferul-pen | <i>p</i> -OHB | Vanillic | Syring | Syngaral | Isosc-g | |
| DHL-190849 (Yellow) | | | | | | | | | | | | | | | | | |
| Milky | 1364.8 a | 377.9 a | 249.5 ab | 21.4 c | 28.6 b | 9.9 b | 0.8 b | nd | 0.3 a | nd | nd | 1.5 b | 1.6 d | 0.5 c | 0.6 c | nd | 2106.3 a |
| Softy | 1418.2 a | 423.6 a | 255.1 a | 37.0 b | 36.2 a | 9.4 b | 0.9 b | nd | 0.1 a | nd | 0.3 ab | 1.6 b | 3.0 c | 0.7 bc | 1.1 b | 0.2 a | 2078.6 a |
| PM | 866.6 b | 258.6 b | 211.8 bc | 51.8 a | 36.5 a | 16.5 a | 3.8 a | nd | 0.2 a | 0.4 a | 0.4 a | 2.5 a | 7.0 a | 0.9 ab | 1.3 a | 0.3 a | 1457.1 b |
| Harvest | 833.3 b | 243.0 b | 180.3 c | 33.2 b | 33.1 ab | 7.2 c | 0.8 b | nd | 0.2 a | 0.3 a | 0.2 b | 1.8 b | 5.3 b | 1.1 a | 1.2 a | 0.2 a | 1341.1 c |
| SED | 70.59 | 24.57 | 11.16 | 1.75 | 1.94 | 0.64 | 0.06 | | 0.04 | 0.04 | 0.04 | 0.10 | 0.31 | 0.10 | 0.05 | 0.03 | 28.01 |
| Rajapani® (Blue) | | | | | | | | | | | | | | | | | |
| Milky | 884.8 ab | 229.3 a | 122.0 b | 10.4 c | 21.0 b | 12.7 b | 1.5 a | nd | 0.2 a | nd | nd | 1.4 c | 1.5 b | 0.6 b | 1.3 b | 0.1 a | 1282.4 ab |
| Softy | 823.5 ab | 228.3 a | 133.7 b | 14.5 bc | 23.2 b | 12.9 b | 1.9 a | nd | 0.2 a | nd | nd | 1.8 b | 2.4 b | 0.8 ab | 0.5 c | 0.1 a | 1289.3 ab |
| PM | 944.4 a | 227.0 a | 188.3 a | 49.2 a | 24.6 b | 15.4 a | 1.5 a | nd | 0.2 a | nd | 0.2 a | 2.2 a | 5.2 a | 1.1 a | 2.4 a | 0.2 a | 1456.1 a |
| Harvest | 799.2 b | 192.2 a | 133.5 b | 25.0 b | 35.2 a | 13.7 ab | 1.5 a | nd | 0.2 a | nd | 0.2 a | 2.1 ab | 4.9 a | 1.2 a | 2.3 a | 0.1 a | 1200.6 b |
| SED | 38.87 | 13.34 | 10.23 | 2.63 | 2.66 | 0.66 | 0.15 | | 0.02 | | 0.03 | 0.10 | 0.28 | 0.14 | 0.12 | 0.03 | 62.58 |
| DHL-151340 (Purple) | | | | | | | | | | | | | | | | | |
| Milky | 1461.6 a | 414.1 a | 345.8 b | 56.8 b | 50.0 b | 27.0 bc | 2.0 c | 3.8 a | 0.2 a | 3.5 a | nd | 1.3 d | 2.7 d | 0.8 a | 1.3 b | 0.04 b | 2371.1 a |
| Softy | 1240.0 b | 328.1 b | 398 .9 a | 82.3 a | 63.1 a | 32.3 a | 3.1 b | 4.1 a | 0.2 a | 3.3 a | 1.6 a | 2.8 c | 5.2 c | 0.9 a | 1.8 ab | 0.07 ab | 2167.8 a |
| PM | 1006.0 c | 165.4 c | 299.4 c | 62.0 b | 51.6 b | 31.7 ab | 4.0 a | 3.6 a | 0.1 a | 2.2 b | 0.7 b | 5.9 b | 8.4 b | 1.0 a | 2.1 a | 0.08 a | 1642.8 b |
| Harvest | 954.8 c | 106.4 d | 242.3 d | 58.7 b | 48.5 b | 22.5 c | 1.5 c | 4.2 a | 0.2 a | 2.1 b | 0.0 b | 7.6 a | 9.8 a | 1.1 a | 1.9 a | nd | 1461.5 b |
| SED | 39.67 | 8.97 | 7.91 | 4.65 | 2.51 | 1.34 | 0.19 | 0.04 | 0.03 | 0.22 | 0.18 | 0.28 | 0.35 | 0.10 | 0.12 | 0.01 | 61.57 |
| DHL-191250 (Black) | | | | | | | | | | | | | | | | | |
| Milky | 1526.0 a | 663.6 a | 231.2 b | 21.0 b | 46.3 b | 13.7 b | 2.1 b | nd | 0.2 a | nd | nd | 1.4 a | 2.3 d | 0.9 a | 0.8 c | 0.2 a | 2509.3 a |
| Softy | 1320.0 b | 438.4 b | 396.4 a | 36.5 a | 58.4 a | 20.3 a | 3.2 a | 4.1 a | 0.4 a | 0.3 a | 0.6 a | 1.6 a | 3.6 c | 1.0 a | 1.8 b | 0.3 a | 2286.6 b |
| PM | 960.7 c | 347.5 c | 220.0 bc | 24.8 b | 36.3 c | 8.5 c | 1.2 c | 3.8 a | 0.2 a | 0.2 b | 0.3 b | 1.4 a | 6.4 a | 1.0 a | 2.5 a | 0.3 a | 1606.3 c |
| Harvest | 851.4 d | 247.2 d | 186.2 c | 21.2 b | 31.2 c | 10.5 bc | 1.3 c | 2.7 b | 0.2 a | 0.2 ab | nd | 1.4 a | 5.3 b | 1.0 a | 1.8 b | nd | 1361.6 d |
| SED | 28.59 | 17.86 | 10.75 | 2.37 | 2.52 | 0.87 | 0.17 | 0.27 | 0.07 | 0.01 | 0.04 | 0.12 | 0.21 | 0.13 | 0.07 | 0.05 | 49.85 |

Results are presented as the mean. Means within a column followed by different letters indicate significant differences according to Tukey–Kramer’s HSD for $\alpha = 0.05$. SED: standard error of the difference between means. **Phenolic acids:** (Ferulic: Ferulic acid, isoF: Iso-Ferulic acid, DiF: Di-Ferulic acid, TriF: Tri-Ferulic acid, DC DiF: Decarboxylated Di-ferulic acid, *p*-Cm: *p*-Coumaric acid, *m*-Cm: *m*-Coumaric acid, Sinapic: Sinapic acid, Caff: Caffeic acid, Cinna: Cinnamic acid, Ferul-pen: Feruloyl-pentose, *p*-OHB: *p*-hydroxybenzoic acid, Vanillic: Vanillic acid, Syring: Syringic acid, Syngaral: Syngaraldehyde); **Flavone glycosides:** (Isosc-g: Isoscoparin-7-glucoside). nd: not detected. nq: not quantified. PM: Physiological Maturity.

3.2.3. Flavone Glycosides

Figure 4 illustrates the total flavone glycosides values in barley flours across maturation, calculated as the sum of free and bound extracts.

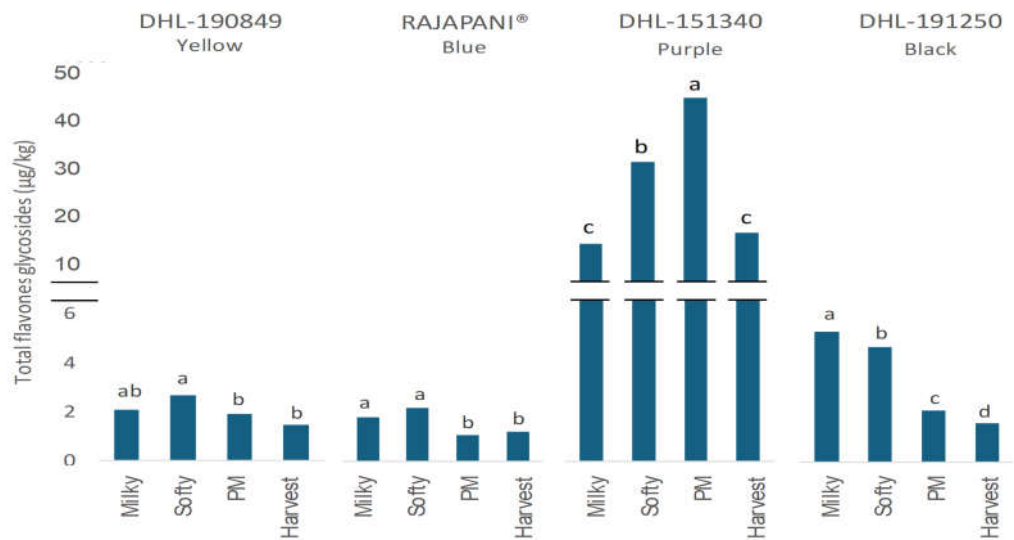


Figure 4. Total flavone glycosides in flours of four barley genotypes at different stages of the grain development. Statistic was performed in total flavone glycosides. Different letters at the same genotype mean significant differences between stages (Tukey–Kramer’s HSD for $\alpha = 0.05$). PM: Physiological Maturity.

Total flavone glycosides constituted less than 2 % of total PCs across genotypes and maturity stages, with the purple barley DHL-151340 showing the highest values, reaching a peak of 45.7 µg/g of flour during physiological maturation. Interestingly, this genotype displayed a distinct trend compared to others, as total flavone glycosides decreased significantly throughout the maturity stages until the harvest stage. This suggests an ongoing synthesis of these compounds in the purple genotype DHL-151340 until physiological maturation

In the free extract, only two different constituents of total flavone glycosides were detected (Table 2). Among them, luteolin-*O*-glucoside was exclusively detected in the purple genotypes DHL-151340 reaching a maximum content of 42.3 µg/g of flour at the physiological maturity stage. At this point, this compound accounts for over 12 % of total free PCs, surpassing the relevance of all free phenolic acids to total PCs. This compound has been exclusively detected in the bound extract of colored barleys and minor proportions in the free extract [9]. Isoscoparin-7-*O*-glucoside was the additional constituent identified in this group. Unlike luteolin-*O*-glucoside, this compound was detected across genotypes, peaking at 5.6 µg/g of flour in the black genotype DHL-191250 at the milky stage. In comparison to luteolin-*O*-glucoside, isoscoparin-7-*O*-glucoside demonstrated a significant decrease across genotypes during maturation. Regarding the bound extract, only isoscoparin-7-*O*-glucoside was detected among flavone glycosides (Table 3).

3.2.4. Anthocyanins

Anthocyanins are water-soluble pigments classified as flavonoids in plants, responsible for the orange, pink, red, violet, and blue colors [43]. Six distinct families of anthocyanins were identified in the blue variety Rajapani® and the purple genotype DHL-151340, as shown in Table 4.

Table 4. Anthocyanins contents (µg/g) in two barley genotypes at different stages of maturation.

| Anthocyanins | | | | | | | |
|---------------------|-----------|---------------|-----------|--------------|------------|-----------|--------------------|
| | Cyanidins | Pelargonidins | Peonidins | Delphinidins | Petunidins | Malvidins | Total anthocyanins |
| Rajapani® (Blue) | | | | | | | |
| Milky | 0.29 d | 0.01 d | 0.01 d | 0.06 d | 0.01 c | 0.001 c | 0.37 d |
| Softy | 1.95 b | 0.10 b | 0.09 b | 0.32 b | 0.05 a | 0.004 b | 2.51 b |
| PM | 5.36 a | 0.25 a | 0.13 a | 0.44 a | 0.06 a | 0.010 a | 6.34 a |
| Harvest | 0.85 c | 0.04 c | 0.02 c | 0.23 d | 0.02 b | 0.001 c | 1.16 c |
| SED | 0.054 | 0.002 | 0.030 | 0.005 | 0.001 | 0.000 | 0.054 |
| DHL-151340 (Purple) | | | | | | | |
| Milky | 103.88 c | 1.93 b | 1.19 c | 0.96 c | 0.19 b | 0.09 c | 108.24 c |
| Softy | 301.87 b | 14.57 a | 2.58 b | 3.66 a | 0.62 a | 0.67 a | 323.97 b |
| PM | 323.60 a | 13.73 a | 3.06 a | 3.41 b | 0.58 a | 0.51 b | 344.89 a |
| Harvest | 27.35 d | 1.60 b | 0.94 d | 1.09 c | 0.14 c | 0.10 c | 31.21 d |
| SED | 2.588 | 0.213 | 0.038 | 0.054 | 0.011 | 0.016 | 2.769 |

Results are presented as the mean. Means within a column followed by different letters indicate significant differences according to Tukey–Kramer’s HSD for $\alpha = 0.05$. SED: standard error of the difference between means. PM: Physiological Maturity.

The purple barley DHL-151340 exhibited significantly higher levels of total anthocyanins in comparison to the blue variety Rajapani®, with a peak in physiological maturity of 344.9 $\mu\text{g/g}$ of flour. Both genotypes demonstrated a significant increase in anthocyanin content during maturation, reaching a maximum at physiological maturity. However, this content significantly decreased at the harvest stage, dropping by 81 % in Rajapani® and 91 % in DHL-151340. Contrary to the proposal by Knievel et al. [44], the possibility of a dilution effect during grain maturation can be discarded, as the reduction in anthocyanin content occurred after the grain filling period concluded. This observation suggests a significant degradation of anthocyanins in the final stages of maturation. Kohyama et al. [45] associated this reduction, occurring just before harvest, in purple hull-less varieties with processes such as degradation, diacylation, or insolubilization of anthocyanins. Bustos et al. [46] reported a similar pattern in total anthocyanins in purple wheat, while Martínez-Subirá et al. [20] observed the same trend barley genotypes. In both cases, the peak content of anthocyanins was reached at physiological maturity, followed by a significant decrease until harvest. Additionally, according to Li et al. [47], anthocyanin biosynthesis and accumulation take place during the middle and late filling stages of the grains, followed by degradation until maturity. Among the two anthocyanin-containing genotypes studied, the cyanidin family was predominant. However, in the case of the blue variety Rajapani®, the second most represented family was delphinidins, while in the purple genotype DHL-151340, it was pelargonidins. Cyanidin-dimalonylglucoside emerged as the main constituent, followed by cyanidin-malonylglucoside, representing over 70 % and 13 % of total anthocyanins at the peak content, respectively (Table S3). Similar results were reported by Kohyama et al. [45] in hull-less waxy varieties. Conversely, other authors have reported cyanidin rutinoside as the predominant anthocyanin in other cereals such as wheat, responsible for the purple color [48].

3.3. Dynamics of Antioxidant Capacity during Barley Grain Development

The total antioxidant capacity (AC) of barley flours during maturation is depicted in Figure 5. At the milky stage, the yellow genotype DHL-190849 demonstrated the highest total antioxidant capacity (AC) among the examined genotypes, measuring 230.6 μmol s Trolox/g. However, it also exhibited the lowest AC across genotypes at the harvest stage, with a value of 86.5 μmol s Trolox/g. Consequently, it experienced the most substantial reduction in AC during maturation, decreasing by 62 %. A similar trend was observed for Rajapani®, which displayed a continuous reduction in AC throughout maturation, reaching the lowest total AC at harvest. In contrast to these two genotypes, purple DHL-151340 and black DHL-191250 genotypes showed an increase in total AC during maturation, reaching peaks of content in the softy stage. The significant rise in AC observed in the purple genotype can be attributed to the increase in anthocyanin content during maturation, as previously reported in barley [49] and maize [50]. In the black genotype, elevated levels of total phenolic acids during immature stages led to a consistent AC during this period. Additionally, the

synthesis of phytomelanins in black barley, as suggested by Nowak et al. [11], may enhance AC due to their antioxidant properties. Nevertheless, further research is required to elucidate the role of these components in AC.

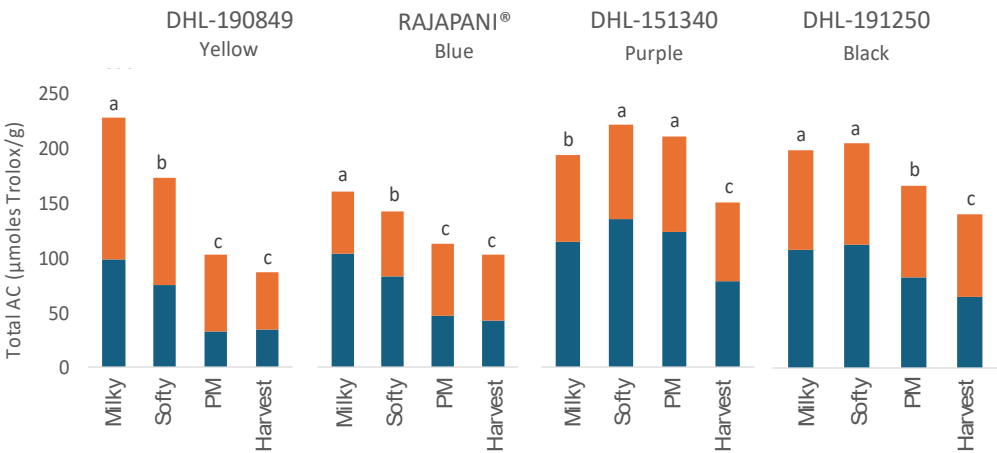


Figure 5. Total antioxidant capacity in barley flours of the four genotypes at different stages of the grain development. Orange bars: Bound extract. Blue bars: Free extract. Statistic was performed in Total AC. Different letters at the same genotype mean significant differences between stages (Tukey–Kramer’s HSD for $\alpha = 0.05$). PM: Physiological Maturity.

In terms of the total AC, the bound and free fractions were not equally represented across the studied genotypes. Despite DHL-190849 and Rajapani® showing a similar decreasing trend in total AC, they exhibited different patterns in the distribution of bound and free fractions during maturation. The yellow genotype DHL-190849 consistently exhibited a higher representation of bound fractions across all maturity stages, with a bound-to-free ratio ranging between 56 % and 69 % throughout maturation. In contrast, Rajapani® showed a higher proportion of free AC during the immature stages. However, at physiological maturity and harvest, bound AC surpassed free AC in representation. A similar trend to Rajapani® was observed in the bound- to- free AC representation in the black genotype DHL-191250. In the purple genotype DHL-151340, the proportion of free AC predominated over bound AC during all maturity stages, attributed to the predominant content of anthocyanins among all studied PCs during maturation.

At the harvest stage, notable variations in total antioxidant capacity were observed across genotypes. Specifically, the purple DHL-151340 and the black genotype DHL-191250 exhibited the highest antioxidant content, surpassing that of the blue and yellow genotypes. These findings align with those of Dang et al. [51], who reported that purple highland barley had the highest overall antioxidant capacity, followed by black highland barley, with blue and yellow highland barley showing lower levels. The results suggest that the antioxidant capacity of barley is significantly influenced by the grain color, attributed to differences in the composition and content of PCs.

3.4. Correlations between Phenolic Compound Families and Antioxidant Capacity across Grain Development

Figure 6 shows a heatmap correlation between different PCs families and the total AC of the studied genotypes during grain development. Previous studies have highlighted the significant antioxidant activity of PCs in barley [40]. These observations find support in the present study, where AC positively correlated with all detected families of PCs in the barley genotypes. However, the significance level of the positive correlation between AC and phenolic compound families was different. The strongest positive correlation was found between AC and the content of anthocyanins ($r=0.853$) and bound phenolic acids ($r=0.775$).

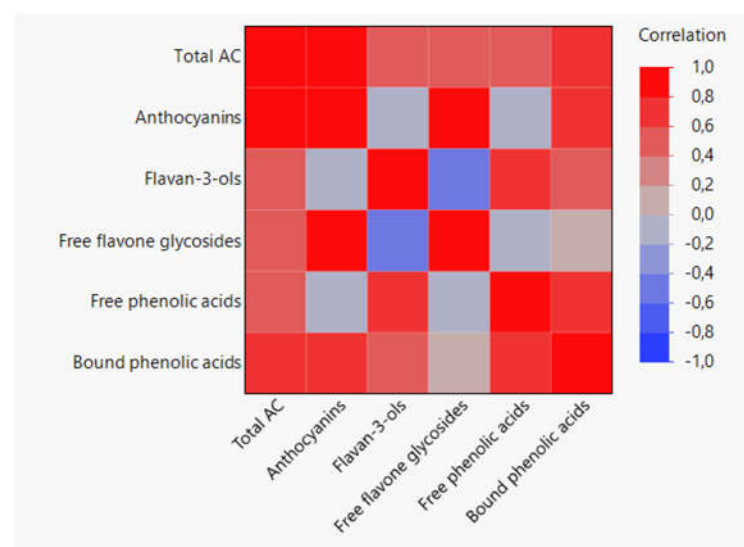


Figure 6. Pairwise correlation heatmap relating the different families of phenols and total antioxidant capacity (AC) analyzed in four barley genotypes during grain development.

Previous research suggests that the ability of PCs to scavenge free radicals is influenced by their structural composition, such as the presence of methoxy and hydroxyl groups in phenolic acids [52], or the substituents of the heterocyclic and B rings in flavonoids [53]. As it was previously commented in Section 3.2.2, bound phenolic acids represent the major constituents among the PCs, contributing significantly to greater AC. Skroza et al. [54] reported that hydroxycinnamic acids exhibit greater antioxidant efficacy compared to hydroxybenzoic acids, with ferulic and sinapic acids displaying better AC than vanillic or syringic acids. Among the bound phenolic acids identified in this study, hydroxycinnamic acids, particularly ferulic acids and their derivatives, were predominant during grain development. Consequently, the robust positive correlation observed between phenolic acids and AC could specifically be attributed to hydroxycinnamic acids. These compounds were predominant in the purple DHL-151340 genotype, followed by the black DHL-191250 genotype, while the yellow DHL-190849 exhibited lower AC despite having similar hydroxycinnamic acid levels to the black genotype (Table 3, and Figure 5). The differences in AC observed between the black DHL-191250 and yellow DHL-190849 genotypes could be attributed to phytomelanins, which are responsible for the black color of the grain. Nowak et al. [11] have reported a strong correlation between antioxidant activity and phytomelanins in black barley genotypes, indicating the strong antioxidant properties of these compounds. Among flavonoids, anthocyanins have been associated to high AC [49], especially when a catechol structure is present in the B ring, as is the case of cyanidin [53]. Our findings support this association, as cyanidins were the predominant anthocyanins identified in barley grains, particularly in the DHL-151340 genotype, which displayed significantly elevated AC.

Interestingly, flavan-3-ols were negatively correlated with flavone glycosides (-0.454) and a similar trend was observed with anthocyanins that, despite not being significant, showed a low negative value (pairwise correlation equal to -0.101). It was noted that the two genotypes containing anthocyanins, Rajapani® and DHL-151340, exhibited lower levels of flavan-3-ols, particularly in the case of DHL-151340. This genotype also showed the highest content of flavone glycosides. In our previous study, we also observed a pigmented barley genotype containing anthocyanins (Hindukusch), which displayed lower levels of flavan-3-ols and higher levels of flavone glycoside compared to non-pigmented varieties [20]. This pattern is also evident in other crops. Bars-Cortina et al. [55] showed that white-fleshed apples have higher levels of flavan-3-ols than red-fleshed apples. The biosynthesis pathways for flavone glycosides, flavan-3-ols, and anthocyanins share some common steps, particularly involving the precursor compounds in the phenylpropanoid pathway [26]. Although each pathway employs some common substrates and enzymes, there may be competition for these resources within the plant. Deluc et al. [56] suggested that proanthocyanidins (oligomers or polymers of flavan-3-ols) and anthocyanins may accumulate simultaneously if there is enough substrate available, but they may compete when the substrate is lacking. Additionally,

regulatory mechanisms within the plant may prioritize the synthesis of certain compounds over others. Zhang et al. [57] indicated that proanthocyanidins and anthocyanins exhibit a competitive relationship in apple peel under light stress, and the inhibition of proanthocyanidins accumulation aids the rapid synthesis of anthocyanins. The negative correlation observed in this study suggests a potential competitive relationship among flavone glycosides, flavan-3-ols, and anthocyanins for shared precursor molecules within the phenylpropanoid pathway, particularly in colored barley. Further research is needed to deepen our understanding of how these flavonoids interact within barley during grain development.

4. Conclusions

Four hull-less barley genotypes differing in grain color exhibited distinct phenolic compound profiles among themselves and across different stages of grain development. Notably, immature barley grains exhibited higher contents of PCs in both bound and free extracts compared to mature grains. Moreover, a dilution effect was observed when expressing contents as $\mu\text{g}/\text{grain}$, suggesting ongoing synthesis of PCs during grain development, extending even until physiological maturity, particularly in the Rajapani® variety.

At the harvest stage, the total PCs content followed a gradient from black to yellow, purple, and blue genotypes. However, the maturation process did not uniformly affect all PCs, as evidenced by the divergent trends observed in individual compounds. Particularly, ferulic acid in the bound extract, and catechin, procyanidin B3, and prodelphinidin B4 in free extract, emerged as influential contributors shaping the decreasing behavior of total PCs during grain development. This fact potentially masks the increasing contents of other minor constituents during grain maturation. Anthocyanins reached their maximum peak of content at physiological maturity in blue and purple genotypes, with the latter exhibiting the highest content. Additionally, a significant correlation between PCs and AC was observed, particularly bound phenolic acids and anthocyanins, with the highest values occurring at immature stages, except for the purple genotype, attributed to the high anthocyanin synthesis during the central maturity stages.

These findings provide valuable insights into the dynamics of phenolic compound accumulation and decline during barley grain development, which is essential for maximizing its nutritional benefits. Additionally, they highlight the potential use of immature barley grains as a functional ingredient in the development of barley-based foods. Notably, the highest levels of PCs in barley grains were reached before the harvest stage, particularly in immature grains. Among the studied genotypes, purple DHL-151340 and black DHL-191250 emerge as the most promising sources of PCs and high antioxidant capacity in their immature stages. Therefore, these genotypes offer significant potential for enhancing the nutritional value and health benefits of barley-based products, making them ideal candidates for further research and development in functional food applications. Consequently, if a market for barley food ingredients develop, early harvesting of non-mature grain should be considered to maximize AC. Possible incentives should be implemented to offset the costs associated with decreased grain weight. Further research is needed to explore these possibilities.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1. Thousand Grain Weight (TGW) of four barley genotypes at different maturation stages.; Table S2. SRM conditions and MS2 fragments used for the quantification and identification of phenolic compounds in barley samples.; Table S3. Anthocyanins contents ($\mu\text{g}/\text{g}$) in four barley genotypes at different stages of maturation.

Author Contributions: Conceptualization, I.F.; methodology, I.F., A.M., and M.M-S.; validation, M.P.R., and M.M.; formal analysis, I.F., A.M., and M.M-S.; investigation, I.F., and M.M-S.; resources, M.P.R., and M.M.; data curation, I.R., and M.M-S.; writing—original draft preparation, I.F.; writing—review and editing, M.P.R., I.R, M.M-S., and M.M.; supervision, M.M.; project administration, M.P.R, and M.M.; funding acquisition, M.P.R, and M.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by project PID2020-113009RB-I00 from the Spanish Ministry of Economy and Competitiveness. Iván Friero Moreno was supported by a pre-doctoral fellowship (PRE2021-100591). Mariona Martínez-Subirà received postdoctoral support (Margarita Salas) from European Union-Next Generation EU, Ministerio de Universidades y Plan de Recuperación, Transformación y Resiliencia.

Data Availability Statement: The data are available from the corresponding authors.

Acknowledgments: Our thanks to Semillas Batlle, SA (Bell-lloc d'Urgell, Lleida, Spain) for providing the food barley genotypes used in this work. The authors would like to thank Dr. Roxana Savin for her guidance in the design of the experiment and support.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Baik BK, Ullrich SE. Barley for food: Characteristics, improvement, and renewed interest. *Journal of Cereal Science*. 2008 Sep 1;48(2):233–42.
2. Food and Drug Administration, HHS. Food labeling: health claims; soluble fiber from certain foods and risk of coronary heart disease. Interim final rule. *Fed Regist*. 2008 Feb 25;73(37):9938–47.
3. EFSA. Scientific Opinion on the substantiation of health claims related to beta-glucans from oats and barley and maintenance of normal blood LDL-cholesterol concentrations (ID 1236, 1299), increase in satiety leading to a reduction in energy intake (ID 851, 852), reduction of post-prandial glycaemic responses (ID 821, 824), and “digestive function” (ID 850) pursuant to Article 13(1) of Regulation (EC) No 1924/2006 EFSA [Internet]. 2011 [cited 2024 February 20]. Available from: <https://www.efsa.europa.eu/en/efsajournal/pub/2207>
4. Neelam N, Khatkar A, Sharma KK. Phenylpropanoids and its derivatives: biological activities and its role in food, pharmaceutical and cosmetic industries. *Critical Reviews in Food Science and Nutrition*. 2020 Sep 7;60(16):2655–75.
5. Raj R, Shams R, Pandey VK, Dash KK, Singh P, Bashir O. Barley phytochemicals and health promoting benefits: A comprehensive review. *Journal of Agriculture and Food Research*. 2023 Dec 1;14:100677.
6. Kumar K, Debnath P, Singh S, Kumar N. An Overview of Plant Phenolics and Their Involvement in Abiotic Stress Tolerance. *Stresses*. 2023 Sep;3(3):570–85.
7. Labudda M, Muszyńska E, Gietler M, Różańska E, Rybarczyk-Płowska A, Fidler J, et al. Efficient antioxidant defence systems of spring barley in response to stress induced jointly by the cyst nematode parasitism and cadmium exposure. *Plant Soil*. 2020 Nov 1;456(1):189–206.
8. Houston K, Learmonth A, Hassan AS, Lahnstein J, Looseley M, Little A, et al. Natural variation in HvAT10 underlies grain cell wall-esterified phenolic acid content in cultivated barley. *Front Plant Sci*. 2023 May 10; 14:1095862.
9. Deng J, Xiang Z, Lin C, Zhu Y, Yang K, Liu T, et al. Identification and quantification of free, esterified, and insoluble-bound phenolics in grains of hullless barley varieties and their antioxidant activities. *LWT*. 2021 Nov 1;151:112001.
10. Suriano S, Iannucci A, Codianni P, Fares C, Russo M, Pecchioni N, et al. Phenolic acids profile, nutritional and phytochemical compounds, antioxidant properties in colored barley grown in southern Italy. *Food Research International*. 2018 Nov 1;113:221–33.
11. Nowak R, Szczepanek M, Kobus-Cisowska J, Stuper-Szablewska K, Dziedziński M, Błaszczuk K. Profile of phenolic compounds and antioxidant activity of organically and conventionally grown black-grain barley genotypes treated with biostimulant. *PLOS ONE*. 2023 Jul 12;18(7):e0288428.
12. Shoeva OY, Mursalimov SR, Gracheva NV, Glagoleva AY, Börner A, Khlestkina EK. Melanin formation in barley grain occurs within plastids of pericarp and husk cells. *Sci Rep*. 2020 Jan 13;10(1):179.
13. Martínez-Subirà M, Romero MP, Puig E, Macià A, Romagosa I, Moralejo M. Purple, high β -glucan, hullless barley as valuable ingredient for functional food. *LWT*. 2020 Sep 1;131:109582.
14. Celestina C, Hunt J, Brown H, Huth N, Andreucci M, Hochman Z, et al. Scales of development for wheat and barley specific to either single culms or a population of culms. *European Journal of Agronomy*. 2023 Jul 1;147:126824.
15. Ranathunga RAA, Suwannaporn P. Young cereal grains as a new source of healthy and hypoallergenic foods: a review. *J Food Sci Technol*. 2022 Sep 1;59(9):3336–48.
16. Paradiso A, Cecchini C, De Gara L, D'Egidio MG. Functional, antioxidant and rheological properties of meal from immature durum wheat. *Journal of Cereal Science*. 2006 Mar 1;43(2):216–22.
17. Barros Santos MC, Ribeiro da Silva Lima L, Ramos Nascimento F, Pimenta do Nascimento T, Cameron LC, Simões Larráz Ferreira M. Metabolomic approach for characterization of phenolic compounds in different wheat genotypes during grain development. *Food Research International*. 2019 Oct 1;124:118–28.
18. Ji CM, Shin JA, Cho JW, Lee KT. Nutritional evaluation of immature grains in two Korean rice cultivars during maturation. *Food Sci Biotechnol*. 2013 Aug 1;22(4):903–8.
19. Xu J guo, Hu Q ping, Wang X dong, Luo J yang, Liu Y, Tian C rui. Changes in the Main Nutrients, Phytochemicals, and Antioxidant Activity in Yellow Corn Grain during Maturation. *J Agric Food Chem*. 2010 May 12;58(9):5751–6.

20. Martínez-Subirá M, Moralejo M, Puig E, Romero MP, Savin R, Romagosa I. Impact of Rising Temperature in the Deposition Patterns of Bioactive Compounds in Field Grown Food Barley Grains. *Plants*. 2021 Mar;10(3):598.
21. Ferreira MSL, Samson MF, Bonicel J, Morel MH. Relationship between endosperm cells redox homeostasis and glutenin polymers assembly in developing durum wheat grain. *Plant Physiology and Biochemistry*. 2012 Dec 1;61:36–45.
22. Martínez M, Motilva MJ, López de las Hazas MC, Romero MP, Vaculova K, Ludwig IA. Phytochemical composition and β -glucan content of barley genotypes from two different geographic origins for human health food production. *Food Chemistry*. 2018 Apr 15;245:61–70.
23. Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL. High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. *J Agric Food Chem*. 2002 Jul 1;50(16):4437–44.
24. Shao Y, Xu F, Sun X, Bao J, Beta T. Phenolic acids, anthocyanins, and antioxidant capacity in rice (*Oryza sativa* L.) grains at four stages of development after flowering. *Food Chemistry*. 2014 Jan 15;143:90–6.
25. Zhang S, Ji J, Zhang S, Xiao W, Guan C, Wang G, et al. Changes in the phenolic compound content and antioxidant activity in developmental maize kernels and expression profiles of phenolic biosynthesis-related genes. *Journal of Cereal Science*. 2020 Nov 1;96:103113.
26. Ge X, Jing L, Zhao K, Su C, Zhang B, Zhang Q, et al. The phenolic compounds profile, quantitative analysis and antioxidant activity of four naked barley grains with different color. *Food Chemistry*. 2021 Jan 15;335:127655.
27. Kim MJ, Hyun JN, Kim JA, Park JC, Kim MY, Kim JG, et al. Relationship between Phenolic Compounds, Anthocyanins Content and Antioxidant Activity in Colored Barley Germplasm. *J Agric Food Chem*. 2007 Jun 1;55(12):4802–9.
28. Ma D, Li Y, Ding H. Accumulation of Phenolic Compounds and Expression Profiles of Phenolic Acid Biosynthesis-Related Genes in Developing Grains of White, Purple, and Red Wheat. *Front Plant Sci*. 2016 Apr 7:528.
29. Özkaya B, Turksoy S, Özkaya H, Baumgartner B, Özkeser İ, Köksel H. Changes in the functional constituents and phytic acid contents of firiks produced from wheats at different maturation stages. *Food Chemistry*. 2018 Apr 25;246:150–5.
30. Guo R, Yuan G, Wang Q. Effect of sucrose and mannitol on the accumulation of health-promoting compounds and the activity of metabolic enzymes in broccoli sprouts. *Scientia Horticulturae*. 2011 Apr 11;128(3):159–65.
31. Solfanelli C, Poggi A, Loreti E, Alpi A, Perata P. Sucrose-Specific Induction of the Anthocyanin Biosynthetic Pathway in Arabidopsis. *Plant Physiology*. 2006 Feb 1;140(2):637–46.
32. Schulman AH, Runeberg-Roos P, Jääskeläinen M. Grain filling and starch synthesis in barley. In: Gupta AK, Kaur N, editors. *Developments in Crop Science*. Elsevier; 2000 p. 147–67. (Carbohydrate Reserves in Plants; vol. 26).
33. Idehen E, Tang Y, Sang S. Bioactive phytochemicals in barley. *Journal of Food and Drug Analysis*. 2017 Jan 1;25(1):148–61.
34. Lin S, Guo H, Gong JDB, Lu M, Lu MY, Wang L, et al. Phenolic profiles, β -glucan contents, and antioxidant capacities of colored Qingke (Tibetan hullless barley) cultivars. *Journal of Cereal Science*. 2018 May 1;81:69–75.
35. Šimić G, Horvat D, Lalić A, Koceva Komlenić D, Abičić I, Zdunić Z. Distribution of β -Glucan, Phenolic Acids, and Proteins as Functional Phytonutrients of Hull-Less Barley Grain. *Foods*. 2019 Dec;8(12):680.
36. Holtekjølen AK, Olsen HHR, Færgestad EM, Uhlen AK, Knutsen SH. Variations in water absorption capacity and baking performance of barley varieties with different polysaccharide content and composition. *LWT - Food Science and Technology*. 2008 Dec 1;41(10):2085–91.
37. Gamel T, Abdel-Aal ESM. Phenolic acids and antioxidant properties of barley wholegrain and pearling fractions. *Agricultural and Food Science*. 2012 Jun 5;21(2):118–31.
38. Ou S, Kwok KC. Ferulic acid: pharmaceutical functions, preparation and applications in foods. *Journal of the Science of Food and Agriculture*. 2004;84(11):1261–9.
39. Acosta-Estrada BA, Gutiérrez-Urbe JA, Serna-Saldivar SO. Bound phenolics in foods, a review. *Food Chemistry*. 2014 Jun 1;152:46–55.
40. Zhu Y, Li T, Fu X, Abbasi AM, Zheng B, Liu RH. Phenolics content, antioxidant and antiproliferative activities of dehulled highland barley (*Hordeum vulgare* L.). *Journal of Functional Foods*. 2015 Dec 1;19:439–50.
41. Deng XQ, Pan ZF, Li Q, Deng GB, Long H, Tashi N, et al. Nutritional components, in vitro digestibility, and textural properties of cookies made from whole hull-less barley. *Cereal Chemistry*. 2020;97(1):39–52.
42. Butsat S, Weerapreeyakul N, Siriamornpun S. Changes in Phenolic Acids and Antioxidant Activity in Thai Rice Husk at Five Growth Stages during Grain Development. *J Agric Food Chem*. 2009 Jun 10;57(11):4566–71.

43. Castañeda-Ovando A, Pacheco-Hernández Ma de L, Páez-Hernández MaE, Rodríguez JA, Galán-Vidal CA. Chemical studies of anthocyanins: A review. *Food Chemistry*. 2009 Apr 15;113(4):859–71.
44. Knievel DC, Abdel-Aal ESM, Rabalski I, Nakamura T, Hucl P. Grain color development and the inheritance of high anthocyanin blue aleurone and purple pericarp in spring wheat (*Triticum aestivum* L.). *Journal of Cereal Science*. 2009 Jul 1;50(1):113–20.
45. Kohyama N, Ono H, Yanagisawa T. Changes in Anthocyanins in the Grains of Purple Waxy Hull-less Barley during Seed Maturation and after Harvest. *J Agric Food Chem*. 2008 Jul 1;56(14):5770–4.
46. Bustos DV, Riegel R, Calderini DF. Anthocyanin content of grains in purple wheat is affected by grain position, assimilate availability and agronomic management. *Journal of Cereal Science*. 2012 May 1;55(3):257–64.
47. Li X, Qian X, Lü X, Wang X, Ji N, Zhang M, et al. Upregulated structural and regulatory genes involved in anthocyanin biosynthesis for coloration of purple grains during the middle and late grain-filling stages. *Plant Physiology and Biochemistry*. 2018 Sep 1;130:235–47.
48. Wang F, Ji G, Xu Z, Feng B, Zhou Q, Fan X, et al. Metabolomics and Transcriptomics Provide Insights into Anthocyanin Biosynthesis in the Developing Grains of Purple Wheat (*Triticum aestivum* L.). *J Agric Food Chem*. 2021 Sep 29;69(38):11171–84.
49. Bellido GG, Beta T. Anthocyanin Composition and Oxygen Radical Scavenging Capacity (ORAC) of Milled and Pearled Purple, Black, and Common Barley. *J Agric Food Chem*. 2009 Feb 11;57(3):1022–8.
50. Hu X, Liu J, Li W, Wen T, Li T, Guo XB, et al. Anthocyanin accumulation, biosynthesis and antioxidant capacity of black sweet corn (*Zea mays* L.) during kernel development over two growing seasons. *Journal of Cereal Science*. 2020 Sep 1;95:103065.
51. Dang B, Zhang WG, Zhang J, Yang XJ, Xu HD. Evaluation of Nutritional Components, Phenolic Composition, and Antioxidant Capacity of Highland Barley with Different Grain Colors on the Qinghai Tibet Plateau. *Foods*. 2022 Jan;11(14):2025.
52. Chen J, Yang J, Ma L, Li J, Shahzad N, Kim CK. Structure-antioxidant activity relationship of methoxy, phenolic hydroxyl, and carboxylic acid groups of phenolic acids. *Sci Rep*. 2020 Feb 13;10(1):2611.
53. Pietta PG. Flavonoids as Antioxidants. *J Nat Prod*. 2000 Jul 1;63(7):1035–42.
54. Skroza D, Šimat V, Vrdoljak L, Jolić N, Skelin A, Čagalj M, et al. Investigation of Antioxidant Synergisms and Antagonisms among Phenolic Acids in the Model Matrices Using FRAP and ORAC Methods. *Antioxidants*. 2022 Sep;11(9):1784.
55. Bars-Cortina D, Macià A, Iglesias I, Romero MP, Motilva MJ. Phytochemical Profiles of New Red-Fleshed Apple Varieties Compared with Traditional and New White-Fleshed Varieties. *J Agric Food Chem*. 2017 Mar 1;65(8):1684–96.
56. Deluc L, Barrieu F, Marchive C, Lauvergeat V, Decendit A, Richard T, et al. Characterization of a Grapevine R2R3-MYB Transcription Factor That Regulates the Phenylpropanoid Pathway. *Plant Physiology*. 2006 Feb 1;140(2):499–511.
57. Zhang B, Yang HJ, Qu D, Zhu ZZ, Yang YZ, Zhao ZY. The MdBBX22–miR858–MdMYB9/11/12 module regulates proanthocyanidin biosynthesis in apple peel. *Plant Biotechnology Journal*. 2022;20(9):1683–700.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.