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A comparative analysis of primary and specialized metabolites in durum wheat grains reveals striking differences across durum emmer wheat, landraces and modern cultivars

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ABSTRACT

A comprehensive profiling of primary and specialized metabolites was carried out on fifty durum wheat accessions of different geographical origin, including ancient emmer, traditional landraces and modern cultivars, grown in a single cropping season, at the same site, and under uniform agronomic management, to reduce within-trial environmental heterogeneity and avoid confounding between genotype and environment. Proteins, gluten, and starch were analysed as primary metabolites, while free and bound phenolic acids, carotenoids, and anthocyanins were identified and quantified as specialized metabolites.

The results revealed that domestication and modern breeding practices have resulted in a reduction in phenolic acid levels, particularly the bound form of ferulic, vanillic and *p*-coumaric acids, coupled with an increase in carotenoid content, especially β-carotene and lutein. However, no notable variations were observed in macronutrient composition across the germplasm categories. Although an increase in carotenoid content may offer nutritional benefits, the concurrent reduction in the overall content of phenolic bioactive compounds observed over time raises concerns about genetic erosion. The higher levels of bound phenolic acids found in ancient spelt varieties, particularly *p*-coumaric and ferulic acids, support their use as valuable sources of genetic variation for biofortification strategies. The ancient and traditional wheat varieties included in this study could be incorporated into breeding programmes to improve the nutritional quality of modern durum wheat cultivars, particularly for use in Mediterranean diets.

1. Introduction

Durum wheat (*Triticum turgidum* ssp. *durum* (Desf.) Husn.), is the most important cultivated tetraploid wheat species and a staple food in the Mediterranean Basin, where it serves as a major source of calories and proteins. Its relevance is deeply rooted in the traditional Mediterranean diet, particularly through products such as pasta and couscous.

Among the kernel edible parts, the endosperm accounts for approximately 90% of the grain's dry weight and primarily stores starch. Starch digestibility, governed by its composition and structure (i.e., amylose/amylopectin ratio and crystallinity), plays a key role in human glycaemic response. The Digestible and Resistant Starch Assay (DSTRS) procedure has been developed to partition starch into three fractions according to its digestibility: rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) (McCleary et al., 2020). These differ in their physiological impact, with RDS quickly causing a spike in blood glucose, SDS leads to a more gradual release, and RS showing health-promoting properties such as improved glycaemic control by enhancing the fibre content thus promoting satiety; it attracts growing interest for the nutraceutical potential and for its functional benefits in food processing (Kahraman et al., 2019).

Wheat proteins are highly concentrated in the aleurone layer, which often separates during milling, but are also found in the endosperm.

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Abbreviations:

Amylase Resistant Starch RS
Digestible and Resistant Starch Assay DSTRS
Domesticated Emmer Wheat DEW
Durum Wheat Cultivars DWC
Durum Wheat Landraces DWL
Free F
Insoluble-Bound IB
Interquartile Range IQR
Not detected n.d.
Oxygen Radical Absorbance Capacity ORAC
Principal Component Analysis PCA
Rapidly Digestible Starch RDS
Slowly Digestible Starch SDS
Total Anthocyanin Content TAC
Total Carotenoid Content TCC
Total Carotenoid Index TCI
Total Digestible Starch TDS
Total Phenolic Acid Index TPAI
Total Starch TS
Trolox Equivalents TE

Based on their solubility, these categorised into four fractions: albumins, globulins, gliadins, and glutenins, the latter two forming gluten. The total protein content and composition are crucial for dough elasticity, rheological and technological properties of wheat-based doughs and vary according to genotype and environment (Arzani and Ashraf, 2017; Poggi et al., 2022).

Beyond macronutrients, whole wheat grain is also a valuable source of bioactive compounds like phenolic acids, carotenoids, and flavonoids, many of which are concentrated in the bran fractions - especially the pericarp and aleurone layer. The latter is rich in minerals, phytates, B vitamins (such as niacin and folates), and lipidic compounds (including plant sterols).

These bioactive compounds have been reported to contribute to several health benefits, such as reduced risks of cardiovascular diseases and type II diabetes (Boukid et al., 2018). Phenolic compounds, the most abundant class of specialized metabolites in wheat, are primarily found in the pericarp and aleurone layer. They occur in three forms: free, conjugated to low molecular weight molecules (e.g., sugars, sterols), and bound to cell wall polysaccharides, such as arabinoxylans, the latter accounting for up to 80% of total content (Li et al., 2008). This group of phenolic compounds has recently received great attention from a nutraceutical point of view, due to its antioxidant, anti-inflammatory and anti-carcinogenic properties (Hernandez-Espinosa et al., 2020). Carotenoids contribute to the characteristic yellow hue of pasta, which is an important feature that impacts consumers' choices, and they also function as provitamins and antioxidants (Ficco et al., 2014). Different types of flavonoids, although present in lower concentrations compared to the above-mentioned compounds, include anthocyanins found both in the pericarp and in the aleurone, where they confer the purple pigmentation to some Ethiopian wheat accessions (Gamel et al., 2023).

By simplifying the evolutionary process that has occurred over the centuries in tetraploid wheat, as a result of the anthropic pressure, two main domestication steps can be identified. The first one marked the beginning of the cultivation of tetraploid wheat, with the birth of the first domesticated emmer wheat (DEW) species from the wild ones, followed by the transition from emmer to durum wheat landraces (DWL) (Mandrioli et al., 2024). Then, in the 20th century, modern breeding programmes carried out during the Green Revolution led to a new severe reduction in the biodiversity of cultivated tetraploid wheat, as DWL were progressively replaced by semi-dwarf, high-yielding durum wheat cultivars (DWC), selected for agronomic traits such as lodging resistance and harvest index. Yield-related traits were thus given priority, strongly contributing to the so-called domestication syndrome (Poggi et al., 2023).

Since the 1960s, when semi-dwarf cultivars were introduced precisely as a result of a very stringent selection for yield parameters (e.g., thousand kernel weight), the bioactive compound concentration in wheat grain started to decrease significantly, suggesting a "dilution effect", i.e., a lower density of bioactive compounds (e.g., phenolic acids, carotenoids, flavonoids) in favour of the starch in the grain. Thus, an enrichment in durum wheat nutritional value, referred to as 'biofortification', gained great interest in the last decades (Hernandez-Espinosa et al., 2020). In this scenario, DEW and DWL, having a higher genetic variability than DWC, represent a promising tool for wheat grain biofortification (Adhikari et al., 2022).

Breeding cereal crops with increased bioactive compounds concentration requires knowledge of the variability among different germplasm resources. This highlights the importance of studies analysing variability from a genetic point of view (Maccaferri et al., 2019) and the content of bioactive compounds across different wheat varieties (Boukid et al., 2019; Hidalgo et al., 2006).

In this study, we performed a comprehensive profiling of primary metabolites (starch and proteins) and bioactive compounds (phenolic acids, carotenoids, and anthocyanins) in fifty durum wheat accessions, including emmers, traditional landraces and modern cultivars. Besides falling into different categories (DEW, DWL, and DWC), the selected fifty accessions had different geographical origins. All accessions were grown in a single site and cropping season under uniform agronomic management. This experimental design was intentionally adopted to ensure that all seed samples compared in the study developed under the same environmental and management conditions. By fixing environment and management, any confounding effects arising from differences in climate, soil, agronomic practices, or seasonal variability were avoided, allowing direct and meaningful comparisons among genotypes within a well-defined agroecological context.

Under these shared conditions, observed differences in metabolite accumulation cannot be attributed to environmental or management variability, but rather reflect genuine genotypic differences in the capacity to produce and accumulate primary and secondary metabolites. This provides clear evidence for the existence of genetic variability among the analysed accessions, which represents exploitable information for breeding strategies aimed at nutritional improvement and phenolic acid biofortification.

2. Materials and methods

2.1. Plant material

The plant material included fifty tetraploid wheat accessions from the UNIBO-Durum Panel association mapping population, selected to represent a broad spectrum of the genetic diversity within the tetraploid wheat gene pools. The panel included sixteen DWC (*T. turgidum* ssp. *durum*), thirty landraces (DWL), comprising accessions of *T. turgidum* ssp. *durum*, *Triticum turgidum* ssp. *turanicum* (n = 2), *Triticum turgidum* ssp. *carthlicum* (n = 3), and *Triticum aethiopicum* Jakubz. (n = 1), and four accessions of DEW (*Triticum dicoccon* Schrank).

Data regarding category, species and geographical origin of each sample analysed are reported in Table 1. Seed grains were produced in a field trial conducted during the 2019/2020 agronomic season at the Cadriano experimental station (44° 330 '0300 '' N, 11° 240 '3600 '' E), University of Bologna (Italy). The site has a sub-humid climate, with an average annual air temperature of 12.8 °C and a mean annual precipitation amount of 924 mm. Experimental layout was a randomized complete block design with three replicates. Each plot measured 1.5 m × 0.5 m, with a density of 22 plants/m^2. Fertilization was applied at sowing with 112 kg N ha^{-1} and 56 kg P_2O_5 ha^{-1}. Weeds, diseases and

Table 1
List of the accessions analysed. For each accession, the code number, category, and country of origin are provided. DWC = durum wheat cultivar; DWL = durum wheat landrace; DEW = Durum Emmer Wheat. N/A = Not Available.

Sample	Accession name	Category	Country	Sample	Accession name	Category	Country
1	EP 004	DWL	Ethiopia	26	CRESO	DWC	Italy
2	EP 041	DWL	Ethiopia	27	HAURANI	DWL	Syria
3	EP 051	DWL	Ethiopia	28	KRONOS	DWC	Desert
4	EP 054	DWL	Ethiopia	29	KYPEROUNDA	DWL	Cyprus
5	EP 067	DWL	Ethiopia	30	MONASTIR	DWC	France
6	EP 075	DWL	Ethiopia	31	IRIDE	DWC	Italy
7	EP 084	DWL	Ethiopia	32	MARCO AURELIO	DWC	Italy
8	EP 090	DWL	Ethiopia	33	ODISSEO	DWC	Italy
9	EP 098	DWL	Ethiopia	34	NEODUR	DWC	Italy
10	EP 111	DWC	Ethiopia	35	RUSSELLO SG7	DWL	Italy
11	EP 115	DWC	Ethiopia	36	SARAGOLLA	DWC	Italy
12	EP 124	DWC	Ethiopia	37	SIMETO	DWC	Italy
13	EP 140	DWL	Ethiopia	38	STRONGFIELD	DWC	Canada
14	EP 156	DWL	Ethiopia	39	LD 186	DWL	Morocco
15	EP 163	DWL	Ethiopia	40	LD 207	DWL	Turkey
16	EP 193	DWL	Ethiopia	41	TETRA IPK 251	DWL-aethopicum	Ethiopia
17	KUBANKA (LD127)	DWL	Kazakhstan	42	SSD 502	DWL	N/A
18	AG189	DWL- carthlicum	Georgia	43	TC 4	DWL-carthlicum	Russian Federation
19	AUREO	DWC	Italy	44	TC 8	DWL-carthlicum	Turkey
20	CAPPELLI	DWL	Italy	45	TDS 234	DEW	Fertile Crescent
21	DAURUR	DWC	France	46	AUS 26460	DWL	Egypt
22	SVEVO	DWC	Italy	47	AUS 26628	DWL	Russian Federation
23	TETRA IPK 814	DWL- turanicum	Iraq	48	DIC UNIBO 8	DEW	Italy
24	TETRA IPK 815	DWL- turanicum	Russian Federation	49	DIC UNIBO 100	DEW	Serbia
25	TRINAKRIA	DWL	ICARDA	50	DIC UNIBO 126B	DEW	Iran

insect's pest pressure were negligible throughout the crop cycle, making it unnecessary to carry out weeding or phytosanitary interventions. The experimental site was characterized by high uniformity, thereby minimizing environmental variability experienced by different genotypes. Moreover, each trait was evaluated in triplicate, to ensure data robustness.

2.2. Chemicals

All pure analytical standards (≥99.5% purity) for phenolic acids, carotenoids, and anthocyanins were purchased from Extrasynthese (Lyon, France). Methanol, ethanol, acetic acid, sodium hydroxide beads (≥98%), hydrochloric acid (37%, w/w), *o*-phosphoric acid (75% w/w), diethyl ether, ethyl acetate, acetone, calcium chloride, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium hydroxide, maleic acid, 3′,6′-dihydroxyspiro[2-benzofuran-3,9′-xanthene]-1-one (fluorescein), 6-hydroxy-2,5,7,8-tetramethyl-3,4-dihydrochromene-2-carboxylic acid (Trolox), 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), HPLC-grade acetonitrile, acetone and water were purchased by Merck Italia (Milan, Italy).

2.3. Protein and gluten determination

Total protein and wet gluten content were assessed non-destructively via Near-Infrared spectroscopy (NIR) in three replicates. Nir Control Plus (Isoelectric Inc.) was used. Protein content has been expressed as a percentage of grain dry weight, while wet gluten content was reported on a 14% moisture basis, according to the approved AACC methods 38-12 (AACC, 2000).

2.4. Starch quantification

Starch fractions, including Total Starch (TS), Total Digestible Starch (TDS), Rapidly Digestible Starch (RDS), Slowly Digestible Starch (SDS) and amylase Resistant Starch (RS) were determined according to the AOAC Method 2017.16 (McCleary, 2019), employing the 'Digestible and Resistant Starch Assay Kit' (Megazyme).

2.5. Preparations of extracts enriched in phenolic acids, carotenoids, and anthocyanins

The extraction of phenolic acids was performed according to Antognoni et al. (2019). Two fractions were prepared, one enriched in free (F) phenolic acids, and the other in insoluble-bound (IB) ones, released after an alkaline hydrolysis.

Carotenoids were extracted according to Atienza et al. (2007), with some modifications. The procedure was conducted in semi-darkness to preserve carotenoids from degradation. One g of ground material was extracted with 4 mL of acetone containing 0.1% of ascorbic acid, to avoid carotenoid oxidation. The extract was vortexed for 2 min, sonicated for 1 min, centrifuged at 3000×*g* for 5 min at 4 °C, and the supernatant was dried under N_2. The residue was dissolved in 0.5 mL of acetone and stored at −30 °C until spectrophotometric and HPLC-DAD analysis.

Anthocyanins were extracted according to the method of Abdel-Aal and Hucl. (1999). Three grams of sample were extracted with 24 mL acidified methanol (methanol and 1 N HCl, 85:15, v/v), vortexed for 1 min, and stirred for 30 min at 4000 rpm on an orbital shaker (Duomax 1030, Heidolph, Germany). Then the extract was centrifuged at 2700×*g* for 20 min at 5 °C, and the supernatant kept at the same temperature for 48 h to promote protein precipitation. A second centrifugation was carried out, and the supernatant evaporated. The dried extract was solubilized in 1 mL of acidified methanol (methanol: 1 N HCl, 85:15, v/v) and filtered (0.45 μm) for the subsequent analysis.

2.6. HPLC-DAD analysis of phenolic acids and carotenoids

Phenolic acids and carotenoids were analysed by HPLC-DAD according to Antognoni et al. (2019) and Ceccarini et al. (2019). For phenolic acids, calibration curves of standards were built injecting 80-0.625 ppm of each compound. Quantification was based on peak area measured at 254 nm for *p*-hydroxybenzoic acid, at 280 nm for gallic, vanillic and *p*-coumaric acid, and at 329 nm for neochlorogenic, chlorogenic, caffeic, ferulic and sinapic acid. The sum of all individual phenolic acid concentrations was calculated and used to express the total phenolics acid index (TPAI) for each extract. For carotenoids, the quantification was performed using calibration curves prepared with different concentrations of zeaxanthin, lutein, β-cryptoxanthin and β-carotene (Mellado-Ortega and Hornero-Méndez, 2012). Calibration curves for zeaxanthin and β-cryptoxanthin were prepared in the range 25 – 0.1 ppm, while for lutein and β-carotene the range was 50 – 0.5 ppm. The sum of all individual carotenoid concentrations was calculated and used to express the total carotenoid index (TCI) for each extract.

2.7. Spectrophotometric determination of total carotenoid content and total anthocyanin content

Total carotenoid content (TCC) was quantified at 450 nm using a Jasco V-630 spectrophotometer (Tokyo, Japan). Lutein was used as reference compound building a calibration curve between 30 and 0.1 ppm. Samples were diluted 1:20 and results expressed as mg of lutein equivalents/g DW. Total Anthocyanin Content (TAC) was determined using cyanidin-3-*O*-glucoside (kuromanin) as reference compound. A calibration curve was built with different concentrations of kuromanin, from 10 to 0.625 ppm, and reading the absorbance at 535 nm. The results are expressed as milligrams of kuromanin equivalents per gram of dry weight (mg of kuromanin equivalents/g DW).

2.8. Oxygen radical absorbance capacity (ORAC) assay

ORAC assay was performed using a Viktor X3 microplate reader (PerkinElmer, Turku, Finland) based on the method of Ou et al. (2001). Data were expressed as μmol Trolox equivalents (TE) per gram of dry weight (μmol TE/g DW).

2.9. Statistical analysis

Principal Component Analysis (PCA) was developed using SIMCA® software v. 17.0.2 (Umetrics, Sweden). The model was built based on twenty-one variables and fifty observations. The variables were the following: (F *p*-OH-benzoic acid, F *p*-coumaric acid, IB vanillic acid, IB *p*-coumaric acid, IB ferulic acid, IB sinapic acid, antioxidant activity (ORAC), β-carotene, zeaxanthin, lutein, TCC, TAC, TPAI, TCI, proteins, wet gluten, RDS, SDS, TDS, RS, and total starch. F neochlorogenic acid, IB *p*-hydroxybenzoic acid and β-cryptoxanthin were not included, since these variables had several missing values, thus invalidating the model. Data were log-transformed and subject to UV (Unit Variance) scaling.

The statistical significancy of differences between the accessions categories (DWC, DWL, DEW) for the analysed primary and secondary metabolites was checked using one-way ANOVA test. Before this was performed, the necessary assumption of normality and homoscedasticity were verified using the Shapiro-Wilk and Levene tests, respectively. If assumptions were violated, the Kruskal–Wallis or Welch's test was used instead of ANOVA. When significant differences emerged among categories, proper post-hoc test was used to separate the means (Tukey test in case of ANOVA, Dunn's post hoc rank sum comparison in case of Kruskal-Wallis, Duncan-Waller Test in case of Welch one-way test).

3. Results and discussion

3.1. PCA model for primary and secondary metabolites in wheat seeds

To provide a comprehensive overview of the biochemical diversity among the fifty wheat accessions, a PCA was conducted based on both primary storage components and bioactive secondary metabolites. The PCA model was developed using as X variables the concentration of the targeted phenolic acids (either in their F or IB form) and β-carotene, zeaxanthin, lutein total anthocyanin and total carotenoid content, antioxidant activity, TPAI, TCI, proteins, gluten and the different starch types (SDS, RDS, TDS) recorded for each germplasm. The results from ORAC assay were included as an overall indicator of antioxidant capacity, rather than as a nutrient-specific metric. As such, ORAC values should be interpreted as an integrative functional response that reflects the combined contribution of multiple antioxidant classes, rather than as a direct proxy for individual bioactive compounds.

The Score plots in Fig. 1a and c, coloured according to accession type and geographic origins, respectively, showed distinct clustering patterns reflective of compositional similarities. The sample distribution along the score plots was determined by the variables, whose effects are represented in the loading plot (Fig. 1b). The latter provides information on the significance of each variable's contribution to the principal components.

As a general trend, the PCA showed that the main drivers of compositional variance among the studied accessions were secondary metabolites rather than primary ones. The unsupervised model highlighted a marked difference between DEW and both DWC and DWL accessions. PC1, explaining the 28.4 % of variation, was primarily driven by TPAI (calculated as the sum of the detected phenolic acids contents), IB *p*-coumaric acid, IB vanillic acid, IB ferulic acid and β-carotene. Moving from negative to positive values along PC1, an inverse trend was clearly observed: β-carotene content decreased, while the total phenolic acids index and the concentration of the above-mentioned bound phenolic acids increased. PC2, explaining the 19.2% of the total variance, was associated with variation in lutein, zeaxanthin, TCI, protein and gluten content. Accessions on the positive side of PC2 resulted enriched in lutein, zeaxanthin and TCI (calculated as the sum of the detected targeted carotenoids content), while those on the negative side had higher protein and wet gluten levels.

Fig. 1a shows that DEW accessions, characterized by high levels of IB ferulic (885-1142 μg/g DW), IB vanillic (10.3-18.14 μg/g DW), and IB *p*-coumaric acids (747-1102 μg/g DW) and elevated TPAI (1724-2316 μg/g DW), alongside with low β-carotene concentrations (not detected in sample 45 and 48, while 0.02 and 0.2 μg/g DW in sample 49 and 50, respectively) clearly clustered in the two right quadrants. In particular, accessions 48 and 49 appeared as outliers beyond the Hotelling's ellipse, which represents the 95% of the confidence region, due to the exceptionally high concentrations of these phenolic acids. Conversely, DWCs were clustered in the two left quadrants, displaying elevated β-carotene (1.46 μg/g DW on average) and the lowest levels of the targeted phenolic acids. Conversely, a few cultivars (e.g., Aureo, Cappelli, Saragolla, Simeto) grouped in the fourth quadrant since they had a low concentration of lutein (<1.78 μg/g DW) and zeaxanthin (<0.018 μg/g DW). Notably, the Italian Aureo (accession 19), and the Turkish landrace 40, resulted outliers due to their low content of all targeted carotenoids, including β-carotene. DWLs were distributed in all four quadrants, confirming that local varieties represent an intermediate step between ancient emmer wheat and modern cultivars.

ORAC was included in the multivariate analysis model as a complementary, integrative indicator of overall antioxidant capacity, providing a functional response of the wheat matrix rather than a compound-specific analytical target. However, the differences among the analysed samples concerning the antioxidant activity and TAC were not outstanding. Analysis of the PCA model correlation matrix (Fig. S1), based on Pearson's test, showed only weak to moderate associations between ORAC and lutein ($r = 0.40$) and TCI ($r = 0.39$). This indicates

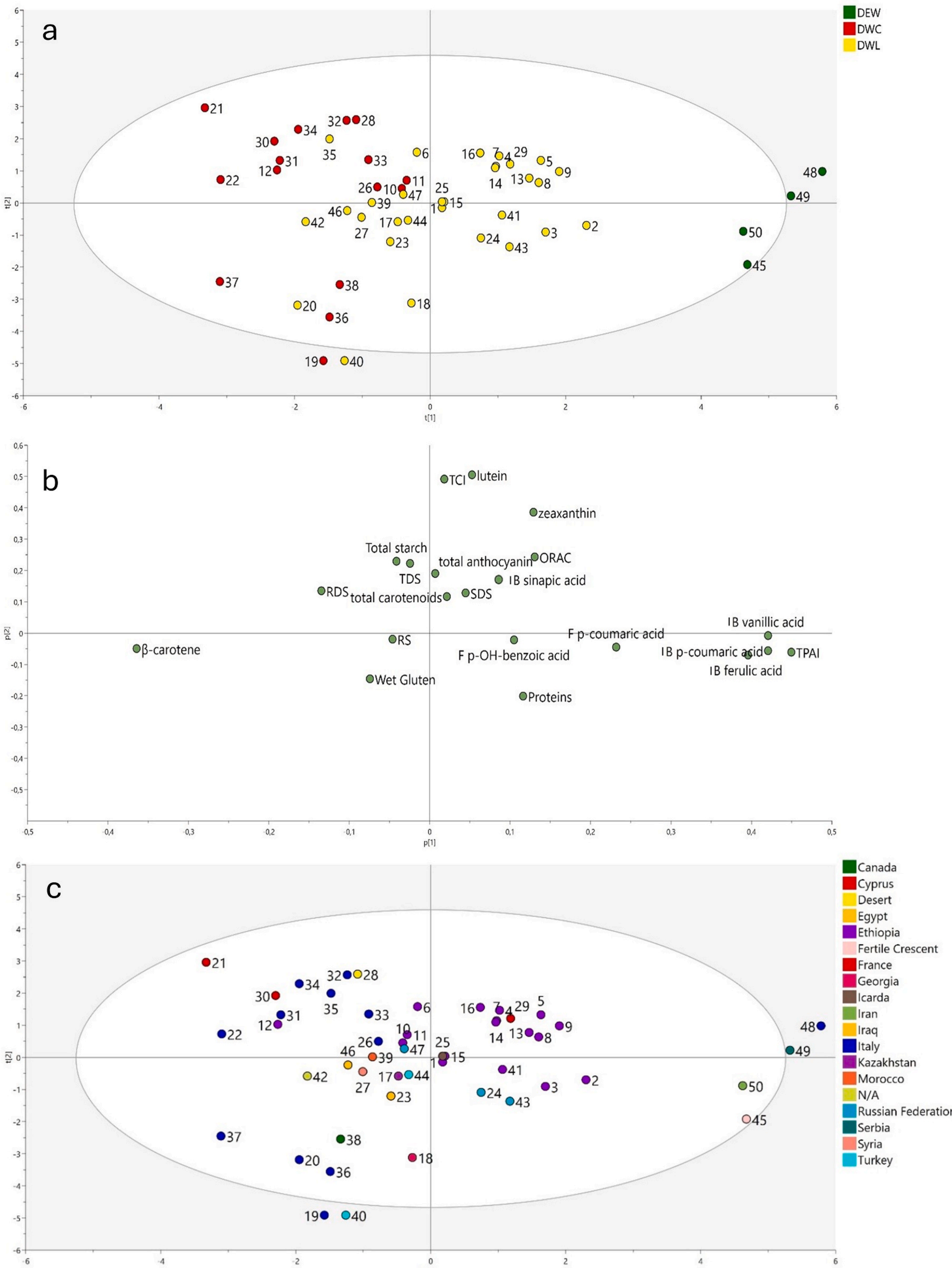

Fig. 1. a) PCA score scatter plot (PC1 *vs* PC2), based on primary and specialized metabolites present in fifty durum wheat accessions. PC1 and PC2 explained 28.4% and 19.2% of the total variance, respectively. The ellipse represents the 95% Hotelling T^2 confidence region. Samples are coloured according to the wheat category (green for DEW, yellow for DWL and red for DWC). b) PCA loading plot corresponding to the score plot shown in Fig. 1a and c summarizing the relationships among the variables in the model. The variables positioned farther from the origin contribute more strongly to sample discrimination along the respective principal component. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

that the antioxidant capacity measured by ORAC cannot be directly attributed to carotenoids alone, supporting the multifactorial nature of antioxidant activity in the wheat matrix.

Most samples came from Italy and Ethiopia (ten and sixteen out of fifty, respectively) and the geographical origin also seemed to influence clustering patterns (Fig. 1c). Germplasms from Ethiopia were distributed in three out of four quadrants and exhibited significant variability. However, the cluster in the second quadrant was composed predominantly of Ethiopian landraces (eight out of nine samples) 29. These Ethiopian landraces samples shared features such as a high level of zeaxanthin (0.16-0.21 μg/g DW), lutein (5.85-9.70 μg/g DW) and total anthocyanins (8.44-43.87 μg/g DW), underscoring their potential nutritional value. Italian accessions, including both modern cultivars and landraces (accessions 20 and 35), were placed in the left part of the graph. Most of these samples showed high content of β-carotene, i.e. sample 35 (Russello, 2.61 μg/g DW), sample 33 (Odisseo, 2.50 μg/g DW) and sample 32 (Marco Aurelio, 2.30 μg/g DW) although lutein and zeaxanthin contents were more variable. Accessions located in the first quadrant resulted particularly rich in all targeted carotenoids, in particular the samples 32 (Marco Aurelio), 34 (Neodur) and 35 (Russello). Conversely, those in the fourth quadrant, namely accessions 19 (Aureo), 20 (Cappelli), 36 (Saragolla) and 37 (Simeto) showed a scarce content of lutein (0.38-1.78 μg/g DW) and zeaxanthin (0-0.018 μg/g DW) but higher level of proteins (16.5-17.9 μg/g DW) and gluten (33.25-39.15 μg/g DW).

3.2. Main storage compounds in wheat grains

Protein content in durum wheat grain is a key determinant affecting its price, as well as that of flour and semolina. It accounts for approximately 40% of the EU Quality Index for durum wheat ("Regulation - 2237/2003 - EN - EUR-Lex," n.d.). Higher protein content significantly impacts the rheological properties of dough and the quality of pasta cooking, with increased protein leading to shorter farinogram mixing times, improved maximum consistency, and a higher tolerance index (Sissons et al., 2007). Consequently, breeders prioritise high protein content in durum wheat grain, hence assessing it is crucial. Since environmental factors have a great impact on protein levels (Rozbicki et al., 2015), this study offers useful insights by reducing environmental differences across all accessions, making it easier to identify genetic variability. In the fifty accessions analysed, average protein content was 16.53 %, ranging from 13.56% to 22.03% (Table S1). These values are consistent with the literature, which reports protein content in wheat grain ranging from 7–8% to 20–22% of dry weight (Mefleh, 2021; Shewry, 2009). Our results are also consistent with those reported by Maryami et al. (2020), who observed a mean value of 15.7% in 158 bread wheat landraces (Maryami et al., 2020).

Despite the wide variation observed, statistical analysis revealed no significant differences in protein content among DWC, DWL, and DEW. This suggests that traditional germplasm and emmer wheat accessions can rival modern varieties in terms of protein content, highlighting their potential as valuable genetic resources in breeding programmes aimed at improving nutritional quality. Gluten proteins, which are primarily stored in grains, play a pivotal role in dough elasticity and rheological properties. Gluten consists of glutenin, which provides elasticity, and gliadin, which contributes to dough extensibility. The ratio of these two proteins affects dough strength, which is a key parameter for pasta quality; higher glutenin levels enhance dough performance (Sissons et al., 2007). Therefore, identifying of novel glutenin alleles and allelic combinations linked with superior quality remains an important breeding goal (Maryami et al., 2020). However, total gluten content is a critical indicator in quality assessment, given its impact on cooking quality and pasta firmness. In this study, wet gluten content ranged from 20.04% to 42.55% (Table S1). Compared to other investigation in literature (Žilić et al., 2011), this dataset showed a broader range of variation, likely due to the greater genotypic diversity in the present panel.

Among starch fractions, RS attracted mostly the interest of researchers, both for its rheological and nutritional characteristics. The RS values reported in literature vary depending on the quantification method and analysed matrix. Štěrbová et al. (2016) analysed the RS of wholemeal flour made from common wheat using the same protocol as in the present study. This allowed a comparison between the two investigations, and it was observed that the RS range between 1.8 ± 3.6 % and 16.9 ± 0.8 % based on dry matter content (Štěrbová et al., 2016). In the present study the RS concentration in grain dry matter ranged from 6.71 % to 25.49%. Interestingly, thirty-six out of fifty accessions overcame the upper threshold of the range observed by Štěrbová et al. (2016), suggesting that the germplasm selected for this study may harbour favourable alleles for RS accumulation. However, no significant differences were detected across DWC, DWL, and DEW categories ($p > 0.05$; Kruskal–Wallis test). Similarly, no statistically significant variation (checked by the Kruskal-Wallis and ANOVA) was found in the content of RDS, SDS, TDS, or TS among the three groups (additional data are given in Fig. S1).

The absence of significant differences in total starch, starch fractions, protein, and wet gluten content among the accessions investigated in this study (DWC, DWL and DEW) demonstrates that, on average, the chosen genotypes, which belong to local varieties and emmer wheat, do not possess any disadvantageous macronutrient characteristics compared to modern cultivars derived from modern breeding programmes. This finding lends support to the inclusion of landraces and emmer wheat in breeding programmes and in the pasta industry without compromising macronutrient composition. Furthermore, significant variability was observed within groups, particularly about resistant starch (RS) and protein content. This highlights the potential of specific genotypes as sources of favourable traits for developing nutritionally enriched wheat cultivars.

3.3. Bioactive compounds in wheat seeds

The concentrations of bioactive compounds in the fifty genotypes considered are reported in Tables S2 and S3. Among the phenolic acids, F and IB p-hydroxybenzoic acid, IB *p*-coumaric acid, F neochlorogenic acid, IB vanillic acid, IB ferulic acid and IB sinapic acid were quantified. In line with previous studies (Hernandez-Espinosa et al., 2020; Li et al., 2008), IB ferulic and IB *p*-coumaric acids were the most abundant phenolic acids, with concentrations ranging from 413.80 to 1142.74 μg/g, and from 5.80 to 1102.23 μg/g respectively (Table S2). IB vanillic and IB sinapic acids were detected in considerably lower quantities, ranging from 4.92 to 18.13 μg/g, and from 6.65 to 62.8 μg/g respectively. The F form of *p*-coumaric acid was detected in all samples with low variability (16.84-30.20 μg/g) compared to the IB form (5.81-1102 μg/g DW). F neo-chlorogenic acid was only detected in two samples: a *T. durum* landrace from Cyprus (accession 29) and a *T. durum* cultivar from France (accession 30). F *p*-hydroxybenzoic acid showed significant variation among germplasms, ranging from 8.77 to 167.35 μg/g (Table S2). Its IB form was detected only in two landrace samples from Ethiopia (accessions 5 and 6, at 16.95 and 18.15 μg/g, respectively). These trends are consistent with previous literature, which confirms that wheat germplasm exhibits highly variable phenolic acid contents depending on genotype, despite the general prevalence of IB over F phenolic acids (Boukid et al., 2019). Differences in the levels of selected secondary metabolites were detected among the three wheat germplasm categories (DWC, DWL and DEW), with several compounds showing statistically significant variation between groups ($p < 0.05$, Fig. 2).

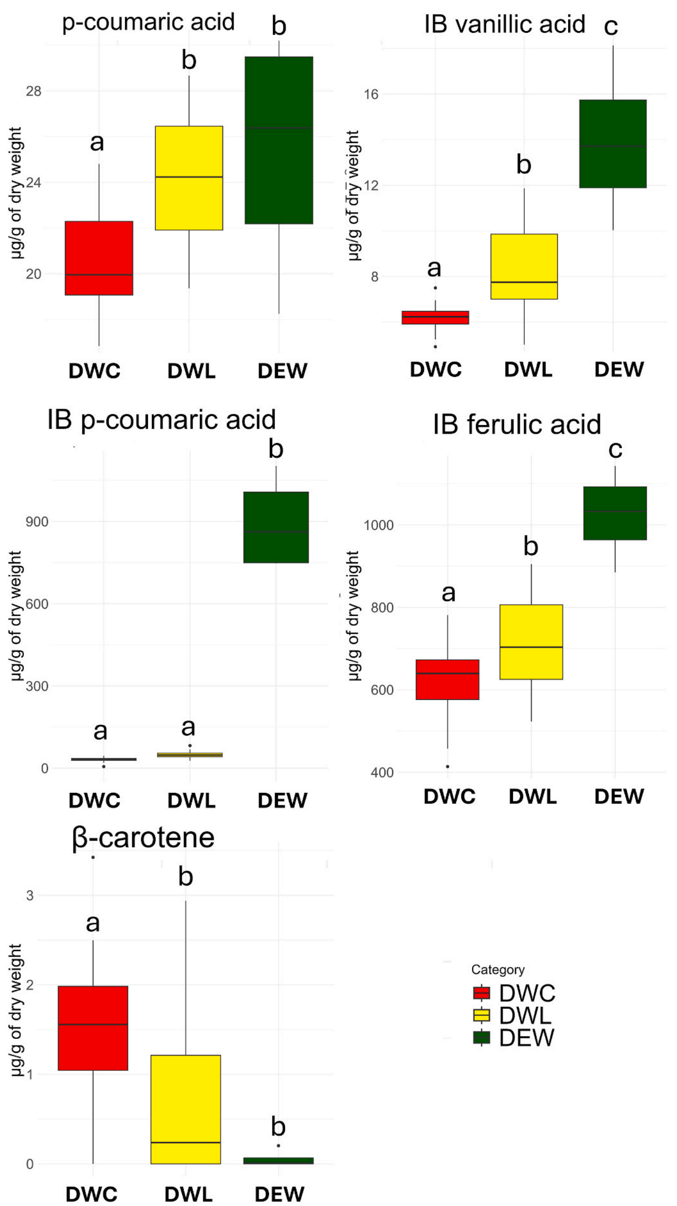

Fig. 2. Boxplot showing the distribution of selected secondary metabolites across the three wheat germplasm categories: DWC, DWL and DEW. The metabolites include individual phenolic acids (free *p*-coumaric, IB vanillic acid, IB *p*-coumaric acid, IB ferulic acid, and β-carotene. The boxes represent the interquartile range (IQR), the horizontal lines within each box indicate the medians, and the whiskers show the minimum and maximum values within 1.5 × IQR. Different lowercase letters above the boxes indicate statistically significant differences between groups according to the post-hoc test ($p < 0.05$).

The most striking differences were observed in the levels of bound ferulic and vanillic acids in DEW germplasms, which showed an average content of 1023.39 and 13.9 µg/g DW respectively, *versus* an average value of 618.83 and 6.22 µg/g DW in DWC and 716.98 and 8.33 µg/g DW in DWL. Furthermore, as already mentioned, DEWs exhibited a high concentration of IB *p*-coumaric acid, with an average content of 893.65 µg/g DW *versus* 30.68 µg/g DW for DWCs and 48.37 µg/g DW for DWLs (Table 2).

These findings are consistent with previous reports that ancient wheat species, such as domesticated emmer, tend to have higher levels of ferulic acid than modern wheat varieties (Arzani and Ashraf, 2017). Şahin and Karakas (2022), reported elevated *p*-coumaric acid levels in some ancient germplasms that outperformed ferulic acid levels (Şahin and Karakas, 2022). While genetic biofortification remains a viable strategy to enhance phenolic acid content, recent evidence emphasizes the significant role of environmental factors (Tian et al., 2022). Studies have identified strong effects of genotype (G), environment (E), agronomic practices (M), and their interactions (G × E, G × M, and G × E × M) on antioxidant levels, even in the same location, or across different years due to variations in temperature, rainfall, sunlight, pests, and disease. In the present study, all accessions were grown in a single site and cropping season under uniform agronomic conditions. This experimental design does not allow the quantification of environmental effects or G × E interactions; however, it was specifically adopted to avoid confounding between genotype and environment and to enable meaningful comparisons among genotypes under a well-defined agroecological context. Under these controlled and shared conditions, the observed variation in phenolic acid content reflects genetic differences in the expression of antioxidant traits as manifested in this specific

Table 3
Concentrations of specific carotenoids, total anthocyanins (TAC), and Oxygen Radical Absorbance Capacity (ORAC) in the germplasm categories analysed (DWC, DWL, DEW). Values are expressed as µg/g dry weight (DW) for carotenoids and TAC, and as µmol TR eq/g DW for ORAC. For each compound, data are reported as mean ± SD, with RSD (Relative Standard Deviation, %) and observed range shown in parentheses.

	DWC	DWL	DEW
β-carotene	1.95 ± 0.61 (31.20%, 1.39–3.42)	1.10 ± 0.91 (82.76%, 0.13–2.94)	0.11 ± 0.13 (114.90%, 0.02–0.20)
zeaxanthin	0.10 ± 0.05 (51.13%, 0.02–0.18)	0.15 ± 0.05 (35.20%, 0.04–0.29)	0.11 ± 0.02 (14.71%, 0.10–0.14)
lutein	9.12 ± 6.22 (68.24%, 0.36–19.91)	6.36 ± 2.40 (37.76%, 0.25–12.22)	5.98 ± 2.03 (33.91%, 3.07–7.75)
β-cryptoxanthin	n.d	n.d	0.09 ± 0.07 (80.06%, 0.04–0.20)
TAC	30.31 ± 38.96 (128.53%, 3.37–137.45)	26.17 ± 33.21 (126.92%, 2.49–154.80)	21.71 ± 11.12 (51.20%, 14.47–37.97)
ORAC	21.75 ± 7.41 (34.05%, 11.76–37.79)	22.13 ± 8.10 (36.62%, 12.86–38.38)	22.33 ± 0.90 (4.02%, 21.30–23.33)

Table 2
Concentration of phenolic acids and total phenolic acid index (TPAI), expressed as µg/g DW, in the three germplasm categories. For each compound, values represent mean ± SD, with RSD (Relative Standard Deviation, %) and range reported in parentheses.

	DWC	DWL	DEW
F *p*-OH benzoic	27.62 ± 33.04 (119.61%, 8.77–138.69)	43.34 ± 39.91 (92.10%, 10.15–167.34)	17.31 ± 7.40 (42.76%, 9.08–25.61)
F *p*-coumaric	20.59 ± 2.23 (10.85%, 16.84–24.81)	24.16 ± 2.68 (11.08%, 19.36–28.67)	25.30 ± 5.55 (21.95%, 18.25–30.20)
IB vanillic	6.22 ± 0.64 (10.33%, 4.92–7.50)	8.33 ± 1.96 (23.49%, 5.01–11.87)	13.90 ± 3.46 (24.90%, 10.03–18.14)
IB *p*-coumaric	30.68 ± 9.24 (30.11%, 5.81–45.32)	48.37 ± 12.70 (26.26%, 27.28–82.33)	893.65 ± 175.37 (19.62%, 747.06–1102.23)
IB ferulic	618.83 ± 106.80 (17.26%, 413.80–781.29)	716.98 ± 98.56 (13.75%, 523.04–904.67)	1023.39 ± 111.33 (10.88%, 885.21–1142.74)
IB sinapic	33.79 ± 10.74 (31.77%, 15.31–48.10)	32.09 ± 15.93 (49.65%, 6.65–62.79)	28.72 ± 5.00 (17.43%, 21.82–33.76)
TPAI	763.7 ± 126.7 (16.6%, 503.15 – 921.50)	896.6 ± 116.9 (13%, 697.98 –1055.97)	2002.8 ± 258.9 (12.9%, 1724.37 – 2316.77)

environment.

Li et al. (2008) used the same strategy to assess the total phenolic acid content of 150 bread wheat genotypes grown in a single site in a single year. They observed a range of 326-1171 μg/g of dry matter, which is consistent with the amounts detected in this study (Li et al., 2008). Taken together, these findings support the interpretation that substantial genetic variability for phenolic acid accumulation can be detected under shared environmental conditions, while broader inference on environmental stability and genotype responsiveness would require multi-environment and multi-year evaluations. Results of the present study confirmed that observed variation in secondary metabolite production among selected genotypes is genetically determined, therefore representing exploitable information for biofortification breeding strategies. Further studies across multiple environments and years will be required to assess how these genetic differences are modulated by diverse agro-environmental conditions.

Regarding carotenoids, DWCs showed higher concentration of β-carotene and lutein compared to both DWL and DEW genotypes, while very similar levels of zeaxanthin were observed in all three groups (Table 3). These differences align with breeding efforts aimed at enhancing yellow pigment concentration in durum wheat to improve pasta colour. This trait has relatively high heritability and limited genotype-by-environment interaction, and there is increased market demand for bright yellow pasta products (Ficco et al., 2014).

The anthocyanin content of the fifty germplasms was also investigated. Cyanidin 3-*O*-glucoside, peonidin-3-*O*-glucoside, petunidin-3-*O*-glucoside and cyanidin 3-*O*-galactoside could not be detected by HPLC-DAD, as they were below the limit of detection. The total anthocyanin content, assayed by a spectrophotometric measurement, gave values ranging from 3.37 to 137.45 in DWCs, 2.49 to 154.80 in DWLs and 14.47 to 37.97 μg/g in DEWs (Table S3). The concentration of anthocyanins in wheat is primarily influenced by genetic factors (specific colour-forming genes) and modulated by environmental conditions (light, temperature, soil nutrients) (Tian et al., 2022). Overall, the samples analysed in this study were not particularly enriched in these compounds, especially when compared to purple or blue grain varieties, which can exceed 300 μg (Lachman et al., 2013). Finally, the antioxidant activity of the fifty germplasms was determined by an ORAC assay providing a functional perspective complementary to the targeted quantification of individual antioxidant compounds. The values ranged from 12.76 to 37.79 μmol TE/g in DWCs, from 11.76 to 38.38 μmol TE/g in DWLs and from 21.29 to 23.33 μmol TE/g in DEWs. Although significant differences were found among the samples, no marked differences were found among the three categories. These findings confirm the significant variation in antioxidant activity reported by Abdel-Aal and Rabalski (2008) between modern and primitive wheat species, independently of category.

In summary, the concentrations of bioactive compounds in wheat germplasms exhibit a broad spectrum, influenced by genetic background and modulated by environmental factors. Together, phenolic acids, carotenoids and total anthocyanin content contribute to wheat's antioxidant capacity and nutritional profile.

4. Conclusions

The analysis of fifty wheat accessions belonging to different germplasm categories (DEW, DWL and DWC) allowed the detection of genetic variability in metabolite composition in the explored agro-ecological context, providing useful information for the exploitation of this diversity in breeding programs aimed at biofortification. The results confirmed that domestication process and subsequent modern breeding, marking the transition from emmer wheat to durum wheat landraces and from durum wheat landraces to modern cultivars respectively, have led to a reduction in phenolic acids content alongside an increase in other types of metabolites, mainly carotenoids. This shift reflects the pasta industry's demand for enhanced grain yellow pigmentation. Significant differences were observed in the amounts of free *p*-coumaric, IB ferulic, vanillic and *p*-coumaric acids, while β-cryptoxanthin was detected only in the DEW and EP 156 genotypes. This highlights that the transition from ancient emmer wheat to landraces and then to modern cultivars was associated with an altered metabolite composition, reflecting a reduction in genetic variability. Indeed, these processes have led to notable genetic erosion, resulting in the loss of many alleles of interest related to grain quality traits of nutritional and nutraceutical importance.

A comparative analysis of primary metabolites among DWCs, DWLs and DEWs revealed no significant variations in protein and gluten content, nor in starch fractions. This suggests that the DWL and DEW genotypes studied could be considered suitable for pasta production without compromising quality or nutritional value. However, the DWL and DEW genotypes exhibited distinct differences in their secondary metabolite profiles, with some showing promising nutraceutical properties. These genotypes could therefore be a valuable source of beneficial allelic diversity in breeding programmes.

CRediT authorship contribution statement

Mariacaterina Lianza: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Giovanni Maria Poggi:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Simona Corneti:** Methodology, Investigation, Conceptualization. **Pietro Montalti:** Investigation. **Stefano Del Duca:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Fabiana Antognoni:** Writing – review & editing, Resources, Funding acquisition, Data curation, Conceptualization. **Iris Aloisi:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Stefano Del Duca reports financial support was provided by European Union. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcs.2026.104388.

Data availability

The data presented in this study are available on request from the corresponding author.

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