



Metabolomic insights into *Triticum aestivum* L.: comparative profiling of growth stages and parts using hydrophilic extracts

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Abstract

Despite wheat being a vital global food source, its metabolomic profile across different growth stages and anatomical parts (e.g., germ and bran) remains underexplored, particularly in relation to its known antioxidant potential. In this study, an untargeted, comparative metabolomic analysis of hydrophilic extracts from wheat was performed across ten distinct growth stages and the metabolomes were further related with in vitro antioxidant activity. Using GC-MS, principal component analysis (PCA) revealed distinct grouping of samples from early root stages (stages 1 and 2) and the aerial part of stage 3. Variable importance in projection (VIP) scores identified five key discriminant metabolites, including sugars, phenolic acids, and amino acids. In contrast, LC-MS/MS-based PLS-DA clearly separated seed parts from other tissues, with VIP scores highlighting flavonoids and phenolic acids as primary contributors to variation. Among the examined stages and tissues, the aerial part of stage 3 showed the highest total phenolic content and strongest antioxidant activity, indicating its potential nutritional value despite being typically disregarded. A total of 29 and 43 compounds were annotated via GC-MS and LC-MS/MS, respectively, including several known for antioxidant activity such as l-proline, sucrose, myo-inositol, caffeic acid derivatives, coumaric acid isomers, catechin, and vitexin. This study is the first to use an untargeted metabolomic approach to explore the differences in phytoconstituents across wheat growth stages and parts, highlighting the antioxidant potential and health benefits of these segments and expanding the interest in the nutritional value of wheat beyond the most commonly ingested seeds and germ.

Keywords Antioxidant · Food · Growth stages · Metabolomics · Wheat

Abbreviations

ABTS	2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
AlCl ₃	Aluminum chloride
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemical Industry
BHA	Butylated hydroxyanisole

DPPH	2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay
EC ₅₀	Effective concentration 50
FRAP	Ferric reducing antioxidant power
GC	Gas chromatography
HPTLC	High-performance thin-layer chromatography
IMS	Ion mobility spectrometry

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IS	Internal standard.
MS	Mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
PLS-DA	Partial least squares discriminant analysis
QC	Quality control
RES	Resveratrol
RI	Refractive index
ROS	Reactive oxygen species
SEM	Standard error mean
TFC	Total flavonoid content
TOSC	Modified total oxyradical scavenging capacity
TPC	Total phenolic content
UPLC	Ultra-high-performance liquid chromatography
VIP	Variable importance for projection

Introduction

Wheat (*Triticum aestivum*, L.) belongs to one of the most diverse and substantial families, Poaceae [1]. It is the most common strategic cereal crop and staple food for humans [2] with almost two billion people (36% of the world population) consuming it as their primary source of nutrition [3]. Morphologically, a central stem emerges from the mature wheat plant, from which leaves arise on opposite sides. Tillers, transverse branches that protrude from the main stem of the wheat plant, can grow leaves on opposing sides of their central stem in the same way as the main stem produces leaves. Alternating leaves with the blade and sheath are found on the stem. As for the seed, bran coat and endosperm make up the wheat plant's caryopsis (kernel or grain or seed) that surround the embryo (germ). The endosperm, the middle layer of kernel, makes up 83% of the wheat grain and contains mostly carbohydrates and protein necessary for the plant's development. Finally, the germ (embryo) which is the inner part of the grain, makes up a minor portion of the grain [4]. Wheat encompasses a myriad of metabolite classes including vitamins, minerals, amino acids, alkaloids, carbohydrates, dietary fiber components and many other secondary metabolites [5]. Different wheat stages were reported to contain several polyphenols such as phenolic acids, flavonoids, lignans, stilbenes, non-phenolic metabolites and other polyphenols [6]. This wide array of compounds resulted in medicinal importance and many associated with pharmacological effects such as anti-inflammatory, anti-diabetic, anti-hypercholesterolemia, energy booster, antioxidant and immunomodulatory [3]. Phenolic compounds are known for their health-promoting ability protecting against various degenerative disorders, cancer and diabetes due to their anti-inflammatory [7, 8] and

their strong antioxidant properties. Those are assessed using established assays namely FRAP (ferric reducing antioxidant power), DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) and modified total oxyradical scavenging capacity (TOSC) assay [9, 10]. In the last 20 years, omics has asserted itself as a vital tool in understanding the internal systems of several plant functions. Metabolomics refers to the fundamentally thorough, unbiased, high-throughput analysis of complex metabolite mixtures typical of plant extracts [2].

Despite literature reports various biological activities of wheat extract, yet it does not relate the metabolome of different parts and growth stages with antioxidant activities from a comparative perspective. This research aims to conduct an untargeted metabolomic profiling of different stages and parts of hydrophilic wheat extracts (aerial parts, bran, groats and germ, roots and seeds) using GC-MS (gas chromatography) and UPLC-MS/MS (Ultra-high performance liquid chromatography-MS/MS) followed by estimation of total phenolic and flavonoid contents in order to relate the in vitro antioxidant potential to the metabolites' profiles of each of the stages and parts of wheat. This comprehensive data collection provides the opportunity to investigate the progression of growth across different plants that are reflected in the metabolomic profile and further application of these analyses methods on other plants to further understand their metabolomes, thereby relating them with their biological effects. With further future biological validation and application, the outcome of this research can also be a prelude to production of a new food supplement.

Experimental

Chemicals

Butylated hydroxyanisole (BHA), potassium persulfate and quercetin dihydrate were purchased from SD Fine Chem Limited, India. 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) was purchased from Alfa Aesar, United States and aluminum chloride hexahydrate from Fisher Scientific, United Kingdom. Acetic acid, ammonium acetate, copper chloride, ethanol 96%, ferrous sulfate, petroleum ether HPLC grade, potassium acetate, sodium acetate and sodium carbonate anhydrous were purchased from ADWIC El Nasr Pharmaceutical Company, Egypt, while tripyridyltriazine (TPZT) and trolox were from Acros Organic, Belgium. Ferric chloride hexahydrate was purchased from Alpha Chemika, India, and 2,2-Diphenyl-1-picrylhydrazyl (DPPH), acetonitrile, alkane standard C8-C20, folin, formic acid, formol, gallic acid, methanol HPLC grade, neocuproine

hydrate and N-methyl-N-(trimethylsilyl) trifluoroacetamide 1% trimethylchlorosilyl from Sigma Aldrich, Germany. The internal standard resveratrol was purchased from Science & Technology Co., Ltd, China.

Plant material

Different wheat (*Triticum aestivum* L.) stages of the winter cultivar (cultivated using raised bed method in fluvisols soil) were collected (Giza, Egypt, 29.945986019124348, 31.24183203766764) over a period of 4 months (January to April). Stage 1, collected in January, represented early vegetative growth (wheatgrass) and corresponded to BBCH stage 13–14. Stage 2, collected in March, reflected an intermediate phase of active stem elongation at 50 cm height corresponding to BBCH stage 32–33. Stage 3, collected in April, represented the mature vegetative phase just before flowering, at approximately 60 cm height, corresponding to BBCH stage 47–49. (Fig. 1). Assigned BBCH stages were approximate and based on the observed morphological differences. The chosen stages represent developmental transitions from early growth to pre-reproductive maturity, selected to observe the metabolic changes in the wheat plant. The total samples were 10 representing different growth phases in addition to seeds, germ, groats and bran. They were identified by Prof. Mosaad Abdel Aleem, Professor at Agricultural Research Center (ARC), Egypt, with a voucher number (M152). For each stage, the whole plant was extracted from the soil and the roots (which this study is the first to investigate) were separated manually from the rest of the parts (aerial parts) to obtain the roots and aerial parts of the 3 stages and were washed with water to remove soil parts, air-dried in the shade, powdered and stored at 25 °C.

Plant extraction procedure

The plants' materials (250 g) were defatted using petroleum ether. A hydroalcoholic fraction was obtained using maceration and extraction of plant material by 90% methanol-water at room temperature until full exhaustion. Subsequently, the extraction solutions were concentrated using a rotavapor (Vacuum controller (V-850), Vacuum pump (V-700), Rotavapor (R-210) and heating bath (B-491), BUCHI, Switzerland) at 58 °C under reduced pressure to yield a crude extract that was stored at -20 °C. A total of 10 fractions were obtained in triplicates and used for further analysis; whole seeds, bran, germ, groats, stage 1 aerial part, stage 1 roots, stage 2 aerial part, stage 2 roots, stage 3 aerial part and stage 3 roots, with average % yield weight/dry weight of 1.93%, 4.3%, 18.27%, 1.88%, 22.53%, 14.4%, 13.84%, 9.62%, 16.95% and 6.65%, respectively. To facilitate identification across figures and analyses, sample labels were abbreviated using consistent codes, B1–B3 represent triplicate samples of bran extract; GR1–GR3 for groat extract; GE1–GE3 for germ extract; S1–S3 for seed extract; R1-1 to R1-3 for root stage 1 extract; R2-1 to R2-3 for root stage 2 extract; R3-1 to R3-3 for root stage 3 extract; L1-1 to L1-3 for aerial parts stage 1 extract; L2-1 to L2-3 for aerial parts stage 2 extract; and L3-1 to L3-3 for aerial parts stage 3 extract. These codes are used consistently throughout the figures to indicate sample type and biological replicates.

In vitro assays for determination of total phenolic content and antioxidant activity

Determination of total flavonoid and total phenolic contents

Total phenolic content (TPC) was determined colorimetrically in triplicates ($n = 3$) using Folin-Ciocalteu method according to [11, 12]. TPC was expressed in terms of μg of gallic acid equivalent per milligram of plant extract (μg

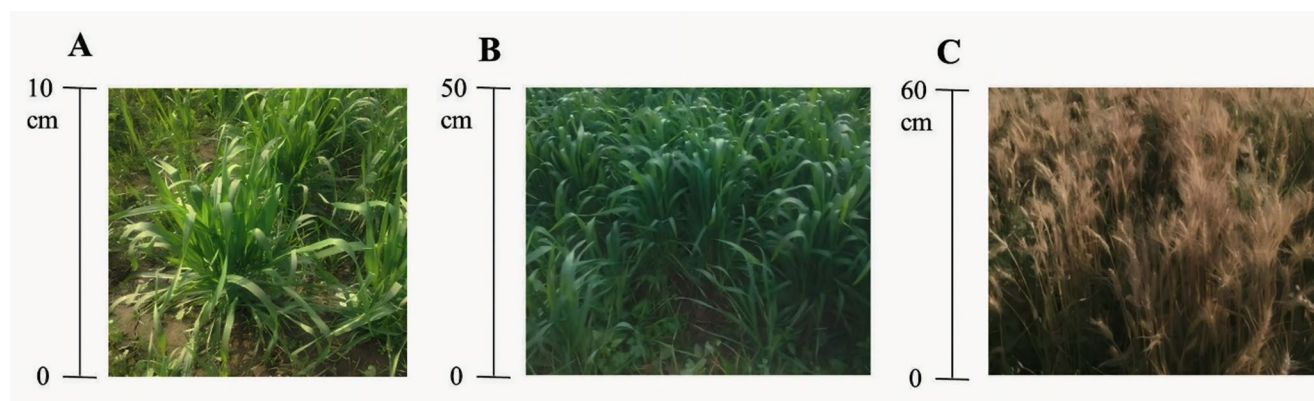


Fig. 1 Developmental stages of wheat: (A) stage (1) 10 cm in height, (B) stage (2) 50 cm in height, and (C) stage (3) full maturity 60 cm

GAE/mg extract) as a quantification factor. Total flavonoid content (TFC) was estimated using AlCl_3 colorimetric method [13]. TFC was expressed in terms of μg of quercetin equivalent per milligram of dry weight of plant extract (μg quercetin/mg extract). Gallic acid and quercetin standard calibration curves with the serial dilutions ranging from 12.5 to 200 $\mu\text{g/mL}$ and from 6.25 to 100 $\mu\text{g/mL}$, respectively (Supplementary material 1 – Fig. S1).

Evaluation of antioxidant activity of extracts

The antioxidant activities of different extracts were conducted in triplicates ($n = 3$) and evaluated using four in vitro assays; CUPRAC, FRAP, ABTS and DPPH. The CUPRAC method is based on the reduction of a cupric neocuproine complex (Cu(II)-Nc) by antioxidants to the cuprous form (Cu(I)-Nc) [14], BHA was used as reference standard. The extracts' antioxidant activities were described in terms of μg of quercetin equivalent per milligram of dry weight of the plant extract (μg quercetin/mg extract). The FRAP assay uses the reduction of ferric ions (Fe^{3+}) to ferrous (Fe^{2+}) as the indicator for the reaction and is linked to a color change where an electron donating antioxidant in the reaction mixture reduces the ferric ion [15]. The total electron donating capacity of the reductive antioxidants in the sample added in the reaction is the limiting factor. Ferrous sulfate (FeSO_4) was used for the calibration curve and the extracts' antioxidant activities were described in terms of μg of FeSO_4 equivalent per mg dry weight of the plant extract (μg FeSO_4 /mg extract). ABTS [2,2'-azinobis-(3-ethylbenothiazoline-6-sulphonate)] assay is based on the evaluation of antioxidants' efficiency to scavenge the $\text{ABTS}^{\cdot+}$ radical generated in an aqueous or organic phase [16]. Quercetin was used for the standard calibration curve and the extracts' antioxidant activities were described in terms of μg of quercetin equivalent per mg dry weight of the plant extract (μg quercetin/mg extract). DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay is based on the radical scavenging activity of the tested substance [17]. The % scavenging activity of extracts was determined with trolox as reference and the EC_{50} of the extracts with the highest percentage were obtained and compared to that of trolox. BHA, trolox, quercetin and ferrous sulphate standard calibration curves are shown in Supplementary material 1 – Fig. S2.

Sample derivatization and GC-MS analysis

The hydroalcoholic fraction of wheat parts were derivatized and analyzed following a reported protocol [18]. Samples were dissolved in 75 μL 90% MeOH followed by addition of 17 μL of the selected internal standard resveratrol (RES) (1 mg/mL) in a glass extraction tube. After solvent evaporation,

the dry residue was methoximated by 70 μL methoxamine HCl in pyridine (20 $\mu\text{g/mL}$) and incubated for 90 min at 37 °C in a heat block (Thermo Scientific Digital Dry bath). The second derivatization reaction involved silylation by addition of 120 μL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% trimethylchlorosilyl (TMCS) to the mixture and incubation for 30 min at 37 °C. MSTFA was chosen as the derivatizing reagent as it is a universal derivatizing agent suitable for screening of unknown metabolites in the tested extracts [19].

Derivatized samples (1 μL) were injected onto the GC column DB-5 MS capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness) using an autosampler (Agilent 7693 A, Agilent Technologies, USA) in a specific predetermined order (Supplementary material 1, Table S1) where the injection runs started by alkane standard (for retention index calculation), solvent run, the internal standard (RES), then alternating 2 quality control (QC) samples, 5 extracts samples, and ended with a solvent run.

The gas chromatograph (Agilent 8890, Agilent Technologies, USA) was coupled to a triple quadrupole MS (Agilent 7010B, Agilent Technologies, USA). The injection port, transfer line and ion source temperatures were set to 280 °C, while the quadrupole was set to 150 °C. Helium was used as carrier gas at a constant flow rate of 1 mL/min. The temperature gradient involved a flat step at 70 °C for 1 min followed by linear increment at 5 °C/min up to 300 °C held for 2 min. The spectrometer was operated in electron-impact (EI) mode at 70 eV ionization voltage. The mass spectra were obtained at 2.21 scan/s and the scan range was 15 to 1000 amu. The solvent delay for pyridine sample solvent was 4.5 min.

Sample Preparation and UPLC/PDA/ESI-MS/MS analysis

Triplicates of the 10 residues of hydroalcoholic extracts were dissolved in 90% MeOH at a concentration of 1 mg/mL. Quality control (QC) samples were prepared by pooling 1 mL of each of these solutions together.

To prepare the vials for injection, 990 μL of each extract or QC sample was added to 10 μL of RES stock to obtain a final volume of 1 mL (final concentration of IS was 3 $\mu\text{g/mL}$), followed by filtration through PTFE micropore filter (0.22 μm pore size, Sigma Aldrich, Germany). The injection runs were in a specific order that started with no injection, followed by solvent, internal standard (resveratrol), then alternating 2 QC and 5 extracts samples, and ended with a solvent injection run (Supplementary material 1, Table S1).

Waters ACQUITY Xevo TQD system consisted of ACQUITY UPLC H-class system, ACQUITY photodiode array (PDA) detector and XEVO TMTQD triple-quadrupole

tandem mass spectrometer with electrospray ionization (ESI) (Waters Corp., Milford, MA, USA). Agilent Eclipse Plus C18 column (250 mm x 4.6 mm column, 5 μ m) was used to separate the analytes (Agilent, USA). System operation and data acquisition were controlled using MassLynx 4.1 software (Waters).

The liquid chromatographic method followed a reported protocol [20]. The solvent system consisted of 0.01% formic acid (FA) in water (mobile phase A) and 0.01% FA in acetonitrile (mobile phase B) at flow rate was 0.5 mL/min. The linear gradient was 5%- 95% B in 40 min, flushed back to 5%B and conditioned for 3 min. After injection (10 μ L), the compounds were detected at 100–900 m/z. Full mass spectral scan was carried out in negative mode (DIA mode) at capillary voltage 3.06 kV, cone voltages (20–40 V), source temperature 150 $^{\circ}$ C, and desolvation gas temperature 500 $^{\circ}$ C. Nitrogen was used as desolvation and cone gas at flow rate of 1000 and 1 L/h, respectively. For the PDA detector, the wavelength range spanned 100–900 nm at a resolution of 1.2 nm. A fresh stock solution of RES 300 μ g/mL in 90% methanol was used as the internal standard. The metabolites were tentatively identified by literature comparison.

Multivariate statistical analysis of GC-MS and LC-MS/MS data

Triplicates of the 10 extracts (total $n=30$) were analyzed to ensure adequate statistical power and cost effectiveness while limiting the solvent use.

GC–MS raw data files were converted via reifycs file converter (<https://www.reifycs.com/AbfConverter/>) to analysis base file (ABF) files and processed using the open-source MS-DIAL ver. 4.8 software (<https://systemsomicslab.github.io/compms/msdial/main.html>) for peaks deconvolution, filtering, alignment, and normalization using locally estimated scatterplot smoothing (LOESS) and internal standard method. The dataset was exported to Metaboanalyst ver 6.0 online platform (<https://www.metaboanalyst.ca/>) to perform multivariate analysis via (Principal component analysis

(PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) in correlation with the antioxidant activity of the extracts [2]. The Variable Importance for Projection (VIP) scores were used to determine the compounds that contributed to the observed PLS-DA pattern. For ease of interpretation, all annotated compounds were assigned short codes, which are used consistently across the VIP plots. These codes and compound details are summarized in Table 1.

The LC-MS/MS raw chromatographic and mass spectral data were processed using Mzmine 4.80 open-source software (<https://mzmine.github.io/download.html>) where they were filtered, deconvoluted, de-isotoped and aligned (Supplementary material 1). Afterwards, the exported dataset was normalized using the QC samples and used for multivariate analysis via Metaboanalyst version 6.0 online platform (Supplementary material 1).

Statistical analysis for the in vitro assays

Data generated from in vitro assays are expressed as mean $\pm$ standard error of the mean (SEM) and multiple comparisons between the extracts was done using Tukey multiple comparisons test. A p -value < 0.05 was considered statistically significant.

Results and discussion

In vitro assays for evaluation of total phenolic, total flavonoid contents, and antioxidant potential

The total phenolic and flavonoid content for the 10 hydroalcoholic extracts was evaluated along with the antioxidant potential using four different assays; CUPRAC, FRAP (reducing activity evaluating assays), DPPH and ABTS (radical scavenging evaluating assays).

Extract of wheat aerial part of stage 3 showed the highest total phenolic content of 89.935 ± 9.597 μ g gallic acid/

Table 1 Annotation of 5 out of the top 15 VIP compounds with assigned short codes detected by PLS-DA using GC-MS data set. The annotation was done by comparison with the NIST20 library (match > 700)

Serial	Code	Annotated VIP compound	RT	Experimental RI	Library RI	References
1	M590	D-(+)-Turanose, octakis(trimethylsilyl) ether	39.451	2616.09 (2354.49-2877.69)	2693.1	***
2	M591	Sucrose, 8TMS derivative	39.551	2619.57 (2357.67-2881.47)	2712	[72]
3	M449	Gallic acid, 4TMS derivative	27.945	1878.94 (1691.14-2066.74)	1981	[73]
4	M416	D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether (isomer 1)	24.973	1804.97 (1624.5-1985.3)	1792.1	[74]
5	M422	D-(-)-Tagatofuranose, pentakis(trimethylsilyl) ether (isomer 1)	24.991	1806.99 (1626.39-1987.59)	1800.6	***

*** not previously reported in wheat

mg extract (Table 2) significantly different from the other extracts (Table S2), while methanolic extract of whole seeds showed the lowest total phenolic content. Furthermore, it was concluded that the polyphenol content in aerial parts of the 3 stages was higher than their respective roots parts in the 3 stages. This is due to the fact that polyphenols usually accumulate in the central vacuoles of guard cells and epidermal cells and subepidermal cells of leaves and shoots [21]. Moreover, the most mature aerial parts of wheat-aerial parts stage 3- contains the highest levels of phenolics and antioxidants compared to the other stages due to increased exposure to environmental and physiological stress, at which the ROS accumulate and trigger the plant's defense mechanism via the phenolics, which decrease the oxidative damage and protect from pathogens. In contrast, stages like grains, groats, germ, and less mature aerial parts are more protected, leading to lower demand for defense-related metabolites [22].

On the other hand, the aerial part of stage 2 showed the highest total flavonoid content of 19.462 ± 0.478 μg quercetin/mg extract and a significant difference from the other extracts except for aerial part of stage 3 (supplementary material 1, Table S2). However, roots part of stage 3 showed the lowest total flavonoid content. It can be concluded that flavonoid content in aerial parts of the 3 stages was higher than all other plant parts due to the fact that flavonoids are also a type of polyphenols, so they accumulate in the leaves and shoots of plants.

Stage 3 aerial part extract showed the highest antioxidant activity with concentrations of 51.494 ± 13.965 μg BHA/ mg extract using CUPRAC assay compared to the other extracts and a significant difference from the other extracts (supplementary material 1, Table S3). This reducing power can be attributed to the total phenolic contents in these extracts,

where stage 3 aerial part also showed the highest total phenolic content expressed in gallic acid equivalent concentration. FRAP assay also depends on the reducing power of the studied extract. Stage 3 aerial part methanolic extract showed the highest reducing power with 705.386 ± 39.960 μg FeSO_4 / mg extract, and a significant difference from the other extracts (supplementary material 1, Table S2). As in CUPRAC assay, the reducing power is attributed to the total phenolic contents in these extracts, where stage 3 aerial part showed the highest total phenolic content expressed in gallic acid equivalent concentration. DPPH assay depends on the radical scavenging power of the tested extracts [23]. A screening step was done for the ten extracts to test for their antioxidant potential using DPPH assay, where 65% DPPH % scavenging was considered as the cutoff for the extract to be considered with antioxidant potential [24], with the aerial part of stage 2 showing the highest % scavenging at $84.07\% \pm 4.557\%$. The results showed that four methanolic extracts had antioxidant potential; wheat germ, bran, stage 1 aerial part and stage 2 aerial part. When the EC_{50} was obtained for the extracts with the highest % scavenging, it was observed that the bran extract showed the highest EC_{50} of 859.75 $\mu\text{g}/\text{mL}$ followed by wheat germ with 776.224 $\mu\text{g}/\text{mL}$, aerial part of stage 1 with 451.943 $\mu\text{g}/\text{mL}$ and the aerial part of stage 2 showed the lowest EC_{50} of 286.301 $\mu\text{g}/\text{mL}$, compared to EC_{50} of trolox, which was 9.849 $\mu\text{g}/\text{mL}$. The results showed that stage 2 aerial part had the lowest EC_{50} and therefore showed the highest radical scavenging activity and highest antioxidant potential using DPPH assay. This could be attributed to its high content of total flavonoid content as expressed in quercetin equivalent concentration. ABTS assay also depends on the radical scavenging activity of the studied extracts. In this assay, stage 3 aerial part extract showed the highest radical scavenging activity of

Table 2 Total phenolic content (TPC) using FCR assay in Gallic acid equivalent concentration and total flavonoid content (TFC) using AlCl_3 assay in Quercetin equivalent concentration and antioxidant effects using CUPRAC assay in BHA equivalent concentration, FRAP assay in FeSO_4 equivalent concentration, DPPH assay expressed as % scavenging and ABTS assay in Quercetin equivalent concentration

Plant sample	Total phenolic content (μg gallic acid/ mg extract)	Total flavonoid content (μg quercetin/mg extract)	CUPRAC (μg BHA/ mg extract)	FRAP (μg FeSO_4 / mg extract)	ABTS (μg quercetin/ mg extract)	DPPH (% scavenging)
Seeds	Tends to 0*	$3.296 \pm 0.121^{\#}$	$2.559 \pm 0.337^*$	$11.772 \pm 2.516^*$	15.417 ± 1.134	$26.00\% \pm 1.813\%$
Groats	$9.145 \pm 0.336^*$	$4.603 \pm 0.314^{\#}$	$5.512 \pm 3.693^*$	$23.737 \pm 3.701^*$	12.167 ± 3.899	$31.26\% \pm 2.723\%$
Germ	$2.263 \pm 1.047^*$	$5.693 \pm 0.173^{\#}$	$13.616 \pm 6.627^*$	$30.912 \pm 1.046^*$	12.389 ± 1.375	$67.81\% \pm 6.600\%$
Bran	$8.446 \pm 0.845^*$	$5.105 \pm 0.264^{\#}$	$28.490 \pm 2.271^*$	$68.579 \pm 7.277^*$	23.306 ± 1.139	$68.60\% \pm 2.618\%$
Aerial part stage 1	$22.371 \pm 2.074^*$	$9.810 \pm 0.925^{\#}$	$19.777 \pm 5.547^*$	$88.772 \pm 6.670^*$	6.917 ± 1.128	$80.35\% \pm 4.223\%$
Roots part stage 1	$3.016 \pm 1.406^*$	$6.869 \pm 0.335^{\#}$	$5.835 \pm 3.722^*$	$30.579 \pm 3.583^*$	$2.306 \pm 0.147^*$	$55.90\% \pm 1.217\%$
Aerial part stage 2	$21.887 \pm 1.699^*$	19.462 ± 0.478	$12.577 \pm 3.791^*$	$88.386 \pm 5.874^*$	8.681 ± 2.510	$84.07\% \pm 4.557\%$
Roots part stage 2	$5.704 \pm 3.597^*$	$3.514 \pm 0.095^{\#}$	$5.312 \pm 4.453^*$	$125.737 \pm 2.006^*$	$1.722 \pm 1.970^*$	$43.57\% \pm 0.987\%$
Aerial part stage 3	89.935 ± 9.597	17.401 ± 0.696	51.494 ± 8.063	705.386 ± 23.071	24.361 ± 3.096	$64.97\% \pm 1.784\%$
Roots part stage 3	$59.363 \pm 3.073^*$	$2.492 \pm 0.106^{\#}$	$21.726 \pm 3.630^*$	$82.491 \pm 2.406^*$	9.472 ± 3.476	$42.53\% \pm 1.632\%$

BHA=Butylated hydroxyanisole equivalent, FeSO_4 =ferrous sulfate equivalent. Values are presented as Mean $\pm$ SEM, *significantly different from the methanol extract of aerial part stage 3 (p -value < 0.05), $^{\#}$ significantly different from the methanol extract of aerial part stage 2 (p -value < 0.05)

$24.36 \pm 5.362 \mu\text{g}$ quercetin/ mg extract, with a significant difference only from the roots of stages 1 and 2 extracts (Supplementary material 1, Table S2). It was also concluded that the roots parts of the 3 stages showed the lowest antioxidant activity due to their low polyphenols and flavonoid contents.

Metabolomic profiling of wheat hydroalcoholic extracts

Various analytical methods are used to generate data sets for metabolomic profiling of wheat such as NMR, HPTLC, MS, GC-MS and LC-MS highlighting that no single analytical platform can comprehensively capture the entire metabolome [25, 26]. In our study, we used both GC-MS and LC-MS/MS for untargeted data acquisition, and unsupervised (e.g. PCA) and supervised (e.g. PLS-DA) analyses in order to explore similarities and differences in the metabolite's profiles of different stages and parts of wheat and relate these profiles to the antioxidant activity.

Quality control (QC) samples were essential in the multivariate statistical analysis to evaluate the stability, reproducibility, and reliability of the analytical system throughout data acquisition. Apart from their importance in peak alignment, normalization, and drift correction, the high clustering of QC samples in the multivariate score plots indicated analytical stability and reproducibility, confirming that the observed variations among samples were primarily biological rather than analytical. Moreover, QC-derived features were only used for quality assurance and normalization and were excluded from downstream biological analyses and interpretation [27].

GC-MS based metabolomic profiling

To impart volatility and thermal stability to the polar metabolites including phenolic compounds, a two-step derivatization was performed first via methoximation of carbonyl functional groups using methoxyamine in pyridine followed by silylation of alcoholic and phenolic functional groups under non-aqueous conditions using N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA).

The GC-MS method was optimized to separate compounds according to their boiling points and vapor pressure, using an oven temperature program ranging from 70 °C to 300 °C at a rate of 5 °C/min. This strategy enabled comprehensive profiling of the diverse chemical classes of compounds present in wheat extracts. Low start temperatures (70 °C) in the chromatograph allowed detection of highly volatile compounds such as derivatized small amino acids and organic acids. As the temperature increased, elution of less volatile and larger compounds including sugars, sugar

alcohols, sugar acids, polyols, fatty acids, phenolic acids, phosphate esters, and disaccharides (Table 3).

Throughout the 10 growth stages, a total of 29 TMS-derivatives of sugars and their derivatives, amino acids, fatty acids, and, small molecular weight organic acids were annotated relying on the comparison of their mass spectra to NIST20 library using a minimum match cutoff value of 700 (as per NIST guidelines), which indicated a fair level of confidence in the metabolite annotation [28] (Supplementary material 2 – Fig. S1). Moreover, the retention indices (RI) (Table 3) confirmed the annotation by normalizing instrument variables, allowing for the comparison of retention data across and facilitating the comparison of retention characteristics [29]. The RI of annotated compounds was calculated using Van der Dool and Kratz formula used in temperature gradients [30] referring to alkane standard mixture that was injected at the beginning of the sequence runs. An additional advantage of our approach is that the reference RI values from the library were obtained using the same column composition as in our GC-MS analysis, further enhancing the reliability of our compound annotation. Tolerance value of $\pm 10\%$ was allowed for the calculated RI relative to the reported RI as guided in the Human Metabolome Database (https://hmdb.ca/spectra/c_ms/search).

Kahkimov et al. [31] reported GC-MS metabolomic profiling of phenolics and organic acids in grains of wheat, barley, oat, and rye via comparison of RI with NIST05 and Golm Metabolite Databases leading to annotation of 89 metabolites at level 2, including citric acid, and various sugars such as D-fructose, α -D-glucopyranose, phenolic acids and esters for caffeic acid, ferulic acid, sinapic acid, amino acids such as L-proline and L-serine, sugars and short chain fatty acids including 1-monopalmitin, all of which have been annotated in our current study (Table 3). Differences in annotated secondary metabolites generally arise from variation in cultivation conditions, derivatization efficiency and reference library. In our study, we relied on the most recent version of NIST library and we took in consideration the deviation of experimental retention indices from the reference ones leading to ignoring more than 50 chromatographic peaks that did not meet our set quality limits for annotation. The literature reports other compounds that we failed to find in our extracts such as benzeneacetaldehyde, (E)-2-heptenal, methyl hexadecanoate, β -cyclocitral, and benzaldehyde that were all detected by GC-IMS (Gas chromatography-ion mobility spectrometry) that is highly sensitive for detection of trace volatile flavor substances [32]. Since the aim in the current study is to profile secondary metabolites, GC-MS was preferred over GC-IMS to allow detection of the less volatile derivatized sugars and alkaloids [33, 34].

Table 3 List of annotated metabolites from methanolic extracts of different wheat growth stages and parts by GC-MS. Metabolite annotation was based on retention time, retention index and EI-MS NIST20 library (match score > 700)

#	Metabolite	RT	Peak area %		Growth stages			Parts			Roots stage 2	Aerial part stage 2	Roots stage 3	Aerial part stage 3	Roots stage 3	Experimental RI ± 10%	Library RI	References
			Seeds	Bran	Germ	Groats	Aerial part stage 1	Roots stage 1	Aerial part stage 2	Roots stage 2								
1	L-Alanine, TMS derivative	5.927											Low sensitivity			1009.357 (908.4-1110.2)	931.8	[75]
2	Lactic Acid, 2TMS derivative	7.173	2.06							1.06						1035.288 (931.7-1138.7)	1051	[76]
3	L-Proline, TMS derivative	9.615											Low sensitivity			1086.046 (977.4-1194.6)	1178.3	[77]
4	2-Amino-2-cyclopropylacetic acid, TMS	9.746											Low sensitivity			1088.771 (979.9-1197.5)	1143.6	[78]
5	L-Valine, 2TMS derivative	10.52					1.34			2.14			Low sensitivity			1207.161 (1087.6-1329.2)	1208.4	[77]
6	L-Proline, 2TMS derivative	12.81								2.72						1248.3 (1123.5-1373.1)	1293.8	[77]
7	Serine, 3TMS derivative	14.46								1.99						1280.05 (1152.05-1408.05)	1369.5	[77]
8	Malic acid, 3TMS derivative	17.73	0.3							1.71		1.19	0.91			1446.008 (1301.4-1590.6)	1479	[79]
9	L-5-Oxoproline, 2TMS	18.44											2.01			1460.65 (1314.59-1606.71)	1524.2	***
10	Phenylalanine, 2TMS derivative	20.88					Low sensitivity						Low sensitivity			1611.908 (1450.8-1773.008)	1636	[80]
11	Asparagine, 3TMS derivative	21.95					Low sensitivity			1.72			3.83			1636.15 (1499.5-1772.7)	1666	[77]
12	Adonitol, 5TMS	23.01								0.31		1.89				1660.086 (1494.08-1826.08)	1698.9	[74]
13	Phosphoric acid, bis(trimethylsilyl) 2,3-bis[(trimethylsilyl)oxy]propyl ester	23.95	Low sensitivity		0.90											1681.29 (1513.19-1849.39)	1743.1	***
14	L-Glutamine, 3TMS derivative	24.25							0.94							1688.068 (1519.2-1856.8)	1768.3	[77]
15	D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether (isomer 2)	24.78	0.48	Low sensitivity		0.55	3.08			13.73		2.31				1800.2 (1620.2-1980.2)	1800.3	[74]
16	D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether (isomer 1)	24.98	0.95		0.57					11.01						1805.17 (1624.6-1985.6)	1792.1	[74]
17	D-(-)-Tagatofuranose, pentakis(trimethylsilyl) ether (isomer 1)	25.02					1.42			3.52		9.29				1806.02 (1625.4-1986.6)	1800.6	-

Table 3 (continued)

#	Metabolite	RT	Peak area %		Germ	Groats	Aerial part stage 1	Roots stage 1	Aerial part stage 2	Roots stage 2	Aerial part stage 3	Roots stage 3	Experimental RI $\pm 10\%$	Library RI	References
			Seeds	Bran											
18	D-Fructose, 5TMS derivative	26.58						0.29			1.57		1845.07 (1660.5-2029.5)	1805.1	[81]
19	D-(+)-Galactopyranose, 5TMS derivative (isomer 2)	26.62						4.56					1845.91 (1661.4-2030.4)	1889.1	***
20	β -D-(+)-Mannopyranose, 5TMS derivative	26.62					Low sensitivity						1845.91 (1661.4-2030.4)	1890.3	***
21	D-Glucitol, 6TMS (isomer)	27.42			0.47								1865.85 (1679.3-2052.3)	1981	[82]
22	Gallic acid, 4TMS derivative	27.95	3.02	Low sensitivity	1.44	2.55	Low sensitivity	1.17	1.60	Low sensitivity	1.23	Low sensitivity	1879.16 (1691.2-2067.06)	1978.6	[73]
23	β -D-(+)-Talopyranose, 5TMS derivative (isomer)	28.39								0.64			1890.01 (1701.01-2079.01)	1895.9	***
24	β -D- Glucopyranose, 5TMS derivative	28.39					Low sensitivity	9.28			8.81		1890.01 (1701.01-2079.01)	1971.3	***
25	D-Galactonic acid, 6TMS	28.66			0.72								1896.61 (1707.01-2086.21)	1991.6	[83]
26	Myo-Inositol, 6TMS	30.33			1.16								2041.75 (1837.6-2245.8)	2130	[84]
27	9,12-Octadecadienoic acid (Z, Z)-, TMS derivative	32.68	0.64		0.71	0.71							2206.12 (1985.5-2426.7)	2208.5	[85]
28	Sucrose, 8TMS derivative	38.15								Low sensitivity	0.13		2473.31 (2226.01-2720.61)	2712	[86]
29	1-Monopalmitin, 2TMS derivative	38.84					Low sensitivity				0.39		2495.37 (2245.87-2744.87)	2574.1	[87]
30	D-(+)-Turanose, octakis(trimethylsilyl) ether	39.433	2.26	2.22	12.52	14.25	1.76	8.30	6.47	5.10	7.0	4.85	2615.67 (2354.11-2877.23)	2693.1	***

*** not previously reported in wheat

To relate growth stages to their chemical profiling and their antioxidant potential, the GC-MS based multivariate statistical analysis was employed. Unsupervised PCA (Fig. 2A) was conducted where PC1 and PC2 explained 34% and 26.5% of the variance, respectively. Along PC1, roots' stages 1, 2 and aerial part of stage 3 were discriminated from all other extracts. This variance was explained by the generated loadings plot (Fig. 2B), where D-(-)-fructofuranose, pentakis(trimethylsilyl) ether (isomer 1) (M416) and D-(+)-turanose, octakis(trimethylsilyl) ether were observed in those 3 extracts. As for PC2, seeds, roots' stages 2, 3 were discriminated from the remaining extracts (Fig. 2A). Notably, it was found that D-(-)-fructofuranose, pentakis(trimethylsilyl) ether (isomer 1) (M416) was the cause of this variance. The loadings plot metabolites codes are shown in Supplementary material-1 Table S3. In order to reveal a possible relation between the ten wheat growth stages' metabolites with their antioxidant activity, partial least square discriminant analysis (PLS-DA) supervised method was used. When the antioxidant potential of extracts was experimentally assessed, ABTS and DPPH did not reveal significant differences between various extracts. Hence, the current study used CUPRAC and FRAP to reflect the antioxidant activity. Referring to the results of these two assays, aerial parts at stage 3 exhibited

the highest antioxidant potential. Hence, PLS-DA (Fig. 3A) was constructed by the data matrix where X represents the peak area of metabolites and Y, the antioxidant potential as response categorical variable. In this model, the aerial parts stage 3 sample was denoted as "Higher", representing the highest antioxidant activity, while the remaining samples were denoted as "Lower", representing lower antioxidant activity compared to aerial parts stage 3. It is clear that aerial parts stage 3, roots stages 1–2 were separated from other stages along component 1, while roots stage 1 and germ were distinguished from others along component 2. Discriminative metabolites contributing to the observed separation were identified through VIP scores, filtered by a statistical significance threshold of $p < 0.05$, highlighting the most discriminatory attributes (Fig. 3B). Among the top detected 15 VIPs, five metabolites were annotated. One of them had a VIP score ≥ 1.0 , namely D-(+)-Turanose, octakis(trimethylsilyl) ether (code: M590), while four compounds Sucrose 8TMS derivative (code: M591), and gallic acid 4TMS derivative (code: M449), D-(-)-fructofuranose pentakis(trimethylsilyl) ether (code: M416), and D-(-)-tagatofuranose pentakis(trimethylsilyl) ether (code: M422) had scores slightly below this threshold (Table 1). Despite this, they were retained due to their statistical significance, supporting their contribution to group discrimination. The

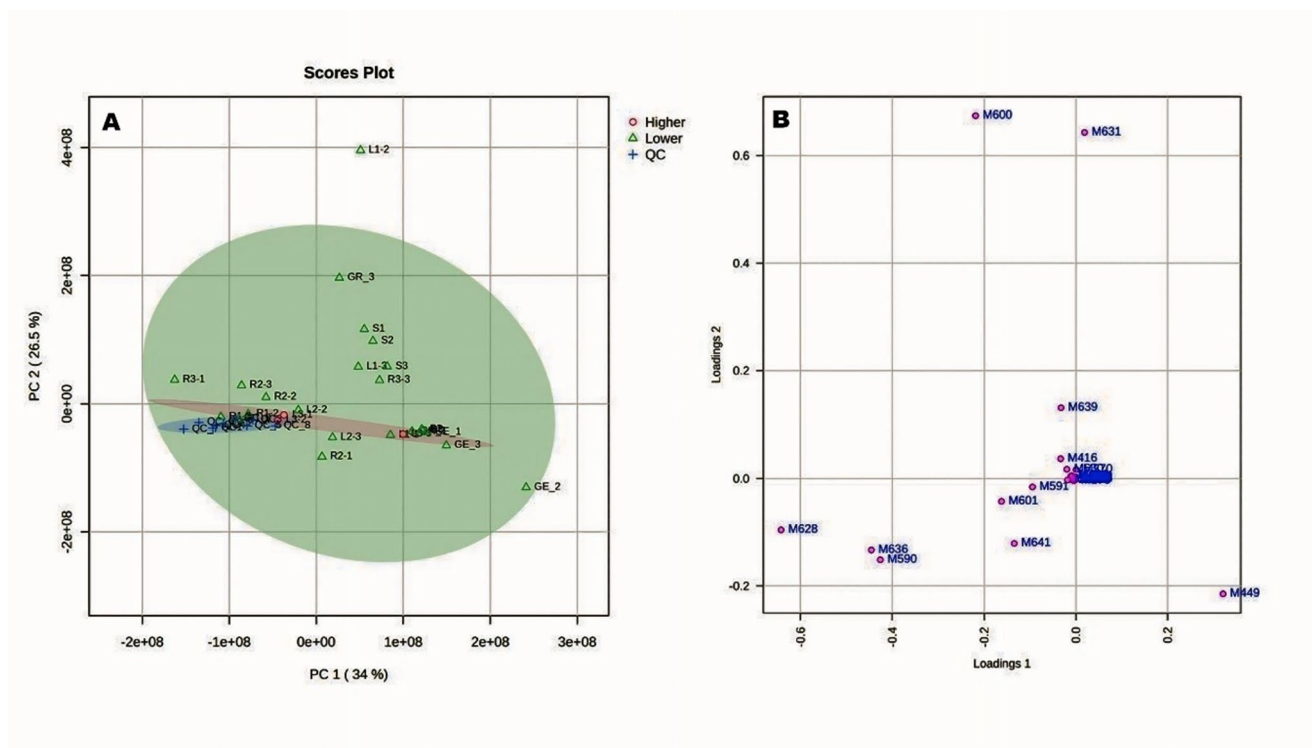


Fig. 2 (A) 2D score plot of the three component PCA model based on data from GC-MS data. The metabolome groups are located at the distinct positions in two-dimensional space described by two vectors of principal component 1 (PC1)=34% and principal component 2 (PC2)=26.5%, sample codes: B=bran, GR=groats, GE=germ,

S=seeds, R1/R2/R3=root extracts at stages 1, 2, and 3, respectively; L1/L2/L3=aerial parts at stages 1, 2, and 3. Numbers indicate biological replicates, (B) 2D PCA loadings plot between the selected PCs based on data from GC-MS data, Metabolite codes correspond to those listed in Supplementary Material 1, Table S3

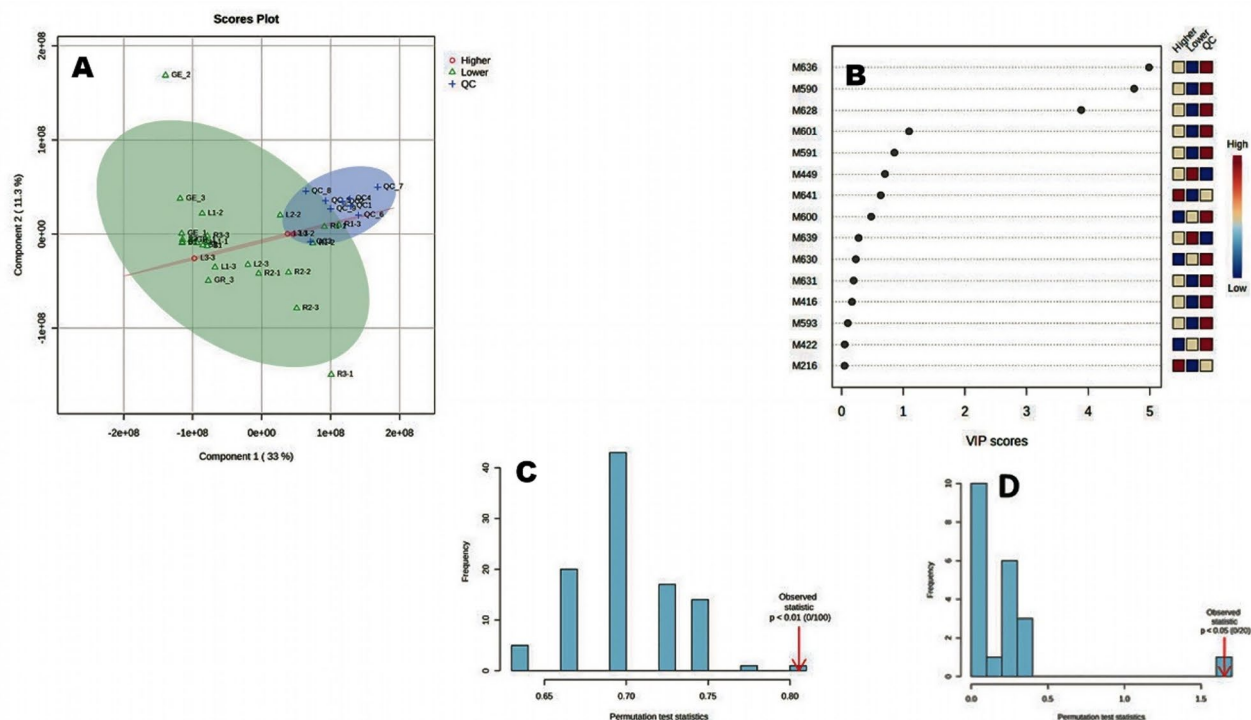


Fig. 3 (A) 2D PLS-DA scores plot of wheat growth stages with higher and lower antioxidant activity. The explained variances are shown in brackets, sample codes: B=bran, GR=groats, GE=germ, S=seeds, R1/R2/R3=root extracts at stages 1, 2, and 3, respectively; L1/L2/L3=aerial parts at stages 1, 2, and 3. Numbers indicate biological replicates. “Higher”, representing samples with the highest antioxidant activity while “Lower”, representing samples with lower antioxidant activity, (B) VIP chart shows the metabolites that separate the wheat

aerial parts stage 3 with the highest antioxidant potential from the other stages based on variable importance in projection (VIP) scores in partial least square discriminant analysis (PLS-DA), Metabolite codes correspond to those listed in Table 1, (C) PLS-DA cross validation in which R^2 and Q^2 revealed satisfactory goodness of fit and goodness of prediction, (D) PLS-DA model validation by permutation test (0/100) with $p < 0.01$, based on prediction accuracy during training

remaining 10 metabolites were not identified due to a low match score with NIST library. Model validation showed satisfactory performance with R^2 of 0.4, Q^2 of 0.38 (Fig. 3C) and a permutation test was performed to determine the importance of PLS-DA class discrimination and discard model overfitting using two different validation metrics. The first one assessed prediction accuracy during training resulting in an even stronger significance with a p -value of < 0.01 (0/100) (Fig. 3C). The second one was based on the ratio of the sum of squares within and between groups (B/W ratio), which yielded a statistically significant p -value of < 0.05 (0/20) (Fig. 3D). Class discrimination was considered statistically significant where the observed test statistic lied outside the selected confidence interval (95%) of the estimated null distribution (Fig. 3D).

UPLC/PDA/ESI-MS/MS based metabolomic profiling

UPLC/PDA/ESI-MS/MS in reversed phase mode was employed for profiling different wheat growth stages, offering the advantage of minimal sample pre-treatment compared to complex derivatization required for GC-MS. In

reverse phase mode, separation of the metabolites depended on their polarity (dipole moment), hydrophilic/hydrophobic character (logP) and acid-base properties (pKa) that all together lead to differential affinity of these compounds towards the non-polar character of the C-18 and the relatively polar character of the water-acetonitrile based mobile phase. In previous LC methods for wheat metabolite profiling, the total run time was correlated with the number of features detected. For example, Xing et al. (2020) utilized methanol for metabolite extraction and achieved a 17-min run time, annotated 1800 features in positive mode and 4303 features in negative mode [35, 36]. Zhu et al. reported 1202 metabolites in a 25-min run [26]. In contrast, our study successfully annotated 12,475 features in negative mode within a 40-min, a considerable advantage compared to previously reported methods.

The untargeted unsupervised analysis using PCA failed to show grouping of metabolomic profiles of extracts (Fig. 4A) suggesting that these groups share similar metabolite profiles. The pairwise score plots providing an overview of the various separation patterns among the most significant PCs (Supplementary material 1 – Fig. S3). While PCA

alone was insufficient to fully resolve group differences, supervised analysis such as PLS-DA and VIP score plots provided clearer discrimination and identified key metabolites contributing to group separation where supervised PLS-DA yielded a high classification performance ($R^2 = 0.95$) and revealed metabolomic patterns into four distinct groups (Fig. 4B): one encompassing wheat germ, bran, and groats; the second grouping aerial parts of all three growth stages; a third for the roots of all three stages; and the final group corresponding to the whole seed extracts. This pattern is indicative of the similarity in the metabolomic profiles of the members of the same group.

A pairwise PLS-DA score plots provided an overview of the various separation patterns among the most significant PCs (Supplementary material 1 – Fig. S4) confirmed through a dendrogram (Supplementary material 1 – Fig. S5) based on Spearman distance and Ward Distance approach clustering. Among the top 15 features with the highest VIP scores (Fig. 4C), four metabolites were annotated: caffeic acid hexoside (code 31), cyanidin hexoside (code 84),

avenanthramide isomer (code 3385), and naringin (code 1194) (Fig. 4C).

Since the LC-MS data did not exhibit a clear separation pattern in the unsupervised PCA plots, no segmentation was done for the groups based on the the antioxidant potential so as not to impose arbitrary grouping in this case to avoid overinterpretation of the data.

A permutation test ($n = 20$) was performed to determine the importance of PLS-DA class discrimination and discard model overfitting based on the ratio of the sum of squares within and between groups (B/W ratio) and an observed p -value of < 0.05 was observed. Class discrimination was considered statistically significant where the observed test statistic lied outside the selected confidence interval (95%) of the estimated null distribution (Fig. 4D). A heatmap revealed that most of VIPs were observed in the aerial part of stage 1 group (dark red in Fig. 4E). This observation conforms with the high concentrations of secondary metabolites biosynthesized during this early growth stage including caffeic acid derivatives and coumaric acid

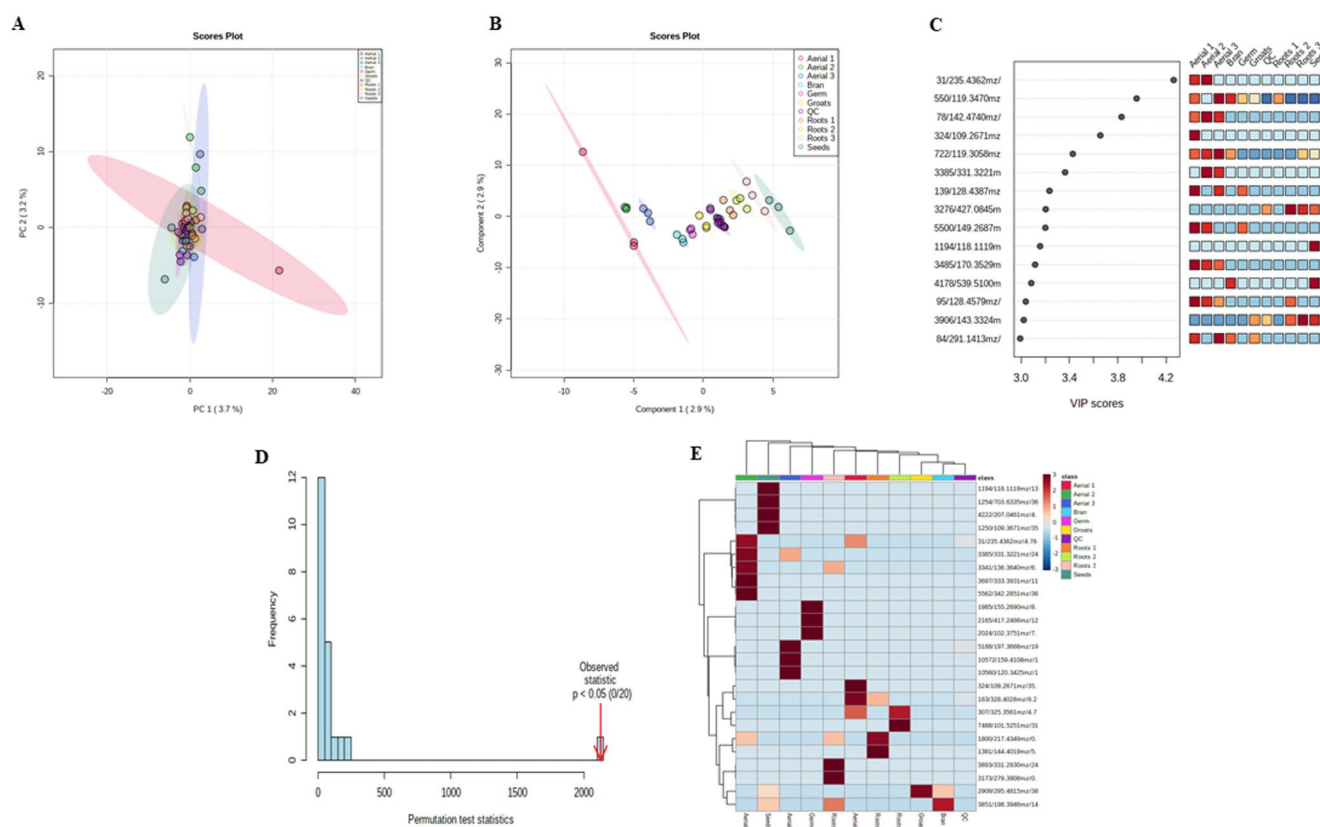


Fig. 4 Principal component analyses, VIPs, permutation test and hierarchical clustering of secondary metabolites from *Triticum aestivum* L. based on UPLC/ESI/PDA-MS/MS data, acquired in negative ion mode: **(A)** 2D PCA score plot of PC1 vs. PC2 scores, **(B)** 2D PLS-DA score plot of PC1 vs. PC2 scores. The metabolome groups are located at the distinct positions in two-dimensional space described by two vectors of principal component 1 (PC1)=3.7% and principal component 2 (PC2)=3.2% for the PCA and (PC1)=2.9% and (PC2)=2.9%

for the PLS-DA. The explained variances are shown in brackets, **(C)** PLS-DA-based VIPs, Code 31: caffeic acid hexoside, code 84: cyanidin hexoside, code 3385: avenanthramide 2f, and code 1194: naringin, **(D)** PLS-DA model validation by permutation tests. The p -value based on permutation is $p < 0.05$ (0/20), **(E)** Heatmap clustering of metabolites of methanolic extracts of wheat stages and parts (distance measure using Euclidean, and clustering algorithm using Ward Distance approach)

isomers that are bio-transformed into flavonoids and lignans through shikimic acid pathway that are predominant in later stages [37–39]. The total ion count chromatograms (Supplementary material 2 – Fig. S2) and PDA chromatograms (Supplementary material 2 – Fig. S3) revealed the presence of phenolic acids, flavonoids, lignans, glycosides and fatty acids (Table 4).

Metabolite profiling by LC-MS/MS

Differences in the number of annotated metabolites when compared with previous literature was associated with the use of a low-resolution instrumentation in this study and the lack of sufficient database access. Moreover, other factors could include detector sensitivity, matrix effects, data-processing parameters and software tools.

LC-MS/MS based annotation of phenolic acids derivatives in wheat

Several major peaks were annotated as phenolics with known antioxidant effect. Compound 11 $[M - H]^-$ (Table 4) appeared at m/z 341 was annotated as caffeic acid hexoside in all tested extracts except in aerial part of stage 1. Its major product ion peak at m/z 179 corresponds to deprotonated caffeic acid after loss of hexose moiety $[M - H - \text{hexose}]^-$. This hexose moiety also appears in its deprotonated form at m/z 161 [40]. Caffeic acid and its derivatives are antioxidants with proven effects on cell viability and may play a role in modulating fatty acid oxidation [41]. Compound 3 was annotated as coumaric acid isomer with $[M - H]^-$ at m/z 163 and a major product ion peak at m/z 147 corresponding to a loss of hydroxyl moiety $[M - H - \text{OH}]^-$ in addition to a fragment at m/z 119 corresponding to loss of CO_2 moiety $[M - H - 44]^-$ [42]. Coumaric acid was previously identified in wheat extracts [43] and was detected in aerial part stage 1, roots stage 1, aerial part stage 2 and 3 in this study. Among the notable activities of coumaric acid is its radical scavenging ability and enhancement of expression of antioxidant genes [44]. Caffeoylquinic acid derivative (compound 12) (Table 4) showed a molecular ion peak $[M - H]^-$ at m/z 461 and product ion peaks at m/z 191 representing the quinic acid $[M - H - 270]^-$ and 179 representing caffeoyl $[M - H - 282]^-$. These fragments inferred this compound as caffeoylquinic acid derivative that were previously identified in wheat grains [45] appearing in the aerial part of stages 2 and 3 of our study. Compound number 7 (Table 4) with a molecular ion peak $[M - H]^-$ at m/z 339 was annotated as caffeoyl-hexuronide derivative with a major ion peak at m/z 311 corresponding to loss of CO $[M - H - \text{CO}]^-$ [46]. Caffeoyl-hexuronide derivative was found

in our extracts of seeds, bran, groats, germ and aerial part stage 1 with previously proven antioxidant potential [41].

LC-MS/MS based annotation of flavonoids in wheat

Compounds numbers 14, 16 and 18 (Table 4) with anion ion peak $[M - H]^-$ at m/z 289 were annotated as catechin and its epimer. Among the characteristic fragments m/z 245 $[M - H - \text{CO}_2]^-$, m/z 229 $[M - H - \text{C}_2\text{H}_2\text{O}]^-$ and m/z 161 $[M - H - \text{CO}_2 - 2\text{C}_2\text{H}_2\text{O}]^-$ which goes in accordance with the fragmentation pathway of catechin [47, 48]. These compounds were reported previously in wheat [49] were found in aerial parts and roots of stages 1, 2 and 3. These epimers are known for their antioxidant potential through ROS scavenging, chelating metal ions, producing phase II detoxification enzymes and antioxidant enzymes, inhibiting pro-oxidant enzymes and inducing antioxidant enzymes [50].

Rutin $[M - H]^-$ (compound 21) in aerial part of stage 1 is displayed as peak 20 at m/z 609 with the major fragments at m/z 300 which corresponds to a loss of rutinose $[M - H - 308]^-$ and is representative for quercetin moiety (Table 4). In addition, further collision induced dissociation led to appearance of a fragment at m/z 178 which is a breakdown fragment of quercetin [51]. Due to its strong antioxidant effect rutin may play a preventive role in neurodegenerative disorders [52].

Compound 22 $[M - H]^-$ at m/z 563 is annotated as apigenin pentosyl hexoside (Table 4). The fragment m/z 432 corresponds to the loss of a pentosyl moiety $[M - H - \text{pentosyl}]^-$ producing a fragment at m/z 269 for apigenin aglycone [53]. In our study, this flavonoid was found in all the extracts except seeds and groats. Its glycoside and aglycone are well known for antioxidant, anti-cancer, anti-inflammatory and cardio protective properties [54].

Compound 20 with molecular ion peak $[M - H]^-$ at m/z 579 is naringin with characteristic product ion peaks at m/z 271 corresponding to loss of hexose and deoxyhexose moiety $[M - H - 308]^-$ and is representing the aglycone naringenin (Table 4). Further loss of $\text{C}_2\text{H}_2\text{O}$ moiety resulted in the appearance of a fragment at m/z 229 $[M - H - \text{gly} - \text{C}_2\text{H}_2\text{O}]^-$ [55]. The loss of a CO moiety followed by the loss of an O group and another CO moiety resulted in the appearance of a fragment at m/z 199 $[M - H - \text{gly} - \text{C}_2\text{H}_2\text{O} - \text{C}_2\text{O}_3]^-$ and the successive loss of $\text{C}_8\text{H}_8\text{O}$ moiety followed by CO_2 group resulted in a fragment at m/z 107. Also, the loss of C_3O_2 moiety from the aglycone moiety, followed by the loss of $\text{C}_2\text{H}_2\text{O}$ resulted in a fragment at m/z 161. This compound was previously reported in wheat [49] and is specifically found in this study in germ, aerial parts and roots of stages 1 and 2. This flavonoid and its glycoside (naringenin) are potent anti-inflammatory and antioxidants [56, 57].

Table 4 Peak assignment using LC-MS/MS of metabolites tentatively annotated in methanolic extracts of different wheat stages and parts (negative mode)

#	RT	Metabolite	Chemical class	[M – H] [–] (m/z)	Fragment ions (m/z)	UV-Vis (λ_{max})	Peak area %			Refer-					
							Seeds	Bran	Germ	Groats	Aerial 1	Roots 1	Aerial 2	Roots 2	Roots 3
1	0.43	Melezitose	Carbohydrates	503.82	143.9	nd	-	0.3	-	-	-	-	-	-	[88]
2	0.46	Caffaric acid	Hydroxycinnamic acids	311.41	179	240, 330	-	-	-	-	4	-	-	-	[45]
3	0.52	Coumaric acid isomer	Hydroxycinnamic acid	163.17	119, 147	192	-	-	-	1.4	-	-	-	-	[89]
4	0.54	Citric acid	Tricarboxylic acid	191.07	111	nd	-	-	-	-	-	5.7	-	-	[90]
5	0.61	Dihydroferulic acid I	Phenylpropanoic acids	195.37	151, 161	nd	-	-	-	-	-	2.7	-	-	[91]
6	0.62	Sinapic acid	Hydroxycinnamic acids	223.11	179	nd	-	-	-	-	-	0.3	-	-	[92]
7	0.63	Caffeoyl hexuronide derivative	Phenolic glycoside	339.48	113, 311	240, 320	1.5	2.5	3.4	4.4	4.3	-	-	-	[51]
8	0.89	Coumaric acid hexoside	Hydroxycinnamic acid glycoside	325.31	163	nd	-	-	-	-	-	1.5	-	-	[93]
9	2.30	Apigenin di-hexoside	Flavone glycoside	594.79	269, 355, 383	nd	-	-	-	-	-	-	-	-	[94]
10	4.23	Cyanidin hexoside	Anthocyanidin glycoside	448.21	286	199, 757	-	-	-	-	7.7	13	-	13	4 7.7 [95]
11	4.79	Caffeic acid hexoside	Hydroxycinnamic acid glycoside	341.31	161, 179	190, 327	50	33	63	37	-	8.6	7.5	42.6	31.5 39.5 [40]
12	5.02	Caffeoylquinic acid derivative	Quinic acids derivative	461.29	191, 179	244, 326	-	-	-	-	-	-	1.7	-	1.6 - [51]
13	5.16	Cyanidin hexoside	Hydroxyflavonoids glycoside	48.81	286	279, 518	-	-	-	-	-	1.7	-	-	- [96]
14	5.34	(Epi)-Catechin isomer	Flavanol	289.27	161, 229, 245	278	-	-	-	-	6	6	10	2.6	- 1.5 [48, 97]
15	5.56	Dihydroferulic acid glucuronide	Phenolic glycosides	370.91	113	nd	-	-	-	-	-	-	-	-	1.4 [98]
16	6.88	(Epi)-Catechin isomer	Flavanol	289.27	115, 191, 229	202, 261, 757	-	-	-	-	3	-	-	-	2 - [48]
17	8.24	Quercetin dihexoside	Flavonol glycoside	625.29	301, 463	350	-	0.6	2.5	-	-	-	-	-	- [99]
18	10.54	(Epi)-Catechin isomer	Flavanol	289.27	137, 151, 178, 231	278	-	-	-	-	-	-	-	-	1 [48]
19	12.31	Avenanthramide isomer	Avenanthramides – Phenolic compounds	328.30	177	nd	-	1	-	-	-	-	1.1	0.9	- 1.4 [100]
20	13.18	Naringin	Flavanones	579.32	107, 161, 199, 229, 271	268, 344, 654, 661, 760	-	-	0.5	-	0.2	0.6	1.8	0.5	- - [101]
21	13.58	Quercetin rutinoside	Flavonoid-glycosides	609.05	179, 300	201, 271, 341, 661, 756	-	-	-	-	2.1	-	-	-	- [51]
22	14.01	Apigenin pentosyl hexoside	Flavone glycoside	563.52	269, 432	200, 270, 342, 652, 750	-	1	2.4	-	5	1	6	1.55	0.8 1.7 [53]
23	16.07	Luteolin glucuronide	Flavone	461.25	176, 285	201, 270, 341, 755	-	-	-	-	0.7	-	-	-	- [102]

Table 4 (continued)

#	RT	Metabolite	Chemical class	[M-H] ⁻ (m/z)	Fragment ions (m/z)	UV-Vis (λ_{max})	Peak area %	Seeds	Bran	Germ	Groats	Aerial 1	Roots 1	Aerial 2	Roots 2	Aerial 3	Roots 3	References
24	16.13	Sinapic acid hexoside	Hydroxycinnamic acids glycoside	385.28	179, 223	nd	-	-	-	-	-	-	-	1.7	-	-	-	[103]
25	16.60	Malvidin coumaryl hexoside	Anthocyanidin glycoside	637.29	329	534, 318	-	-	-	-	-	-	-	4	-	-	-	[104]
26	16.83	(Epi)-Galocatechin isomer	Flavanol	305.34	111, 179, 288	201, 654, 661	-	-	-	-	-	1.6	-	-	-	-	-	[48]
27	20.47	Procyanidin dimer digallate (B-type)	Proanthocyanidin, a biflavonoid	881.40	289, 577, 729	nd	-	-	-	-	-	-	-	-	-	-	8	[105]
28	21.91	Pinosylvin	Stilbenol	211.34	119, 167, 178, 195	211, 301, 309	-	-	-	-	-	-	-	-	-	1.2	-	[97]
29	22.72	Luteolin	Flavones	285.84	174.1	202, 347	-	-	-	-	-	1.2	-	-	-	-	-	[106]
30	23.337	Matairesinol	Lignin, phenylpropanoid.	357.04	298	230, 282	-	-	-	-	-	-	-	-	0.9	-	-	[97]
31	23.81	Arctigenin	Lignan	371.41	137	205, 231, 281	-	-	-	-	-	-	-	-	0.8	0.6	-	[107]
32	25.12	Syringaresinol	Lignan	417.01	249, 370.2	nd	-	-	1.2	-	-	-	-	-	-	-	-	[108]
33	25.23	Trihydroxy-octadec- enoic acid	Long-chain fatty acids	329.21	311	nd	-	-	-	-	-	-	-	-	-	-	3	[53]
34	34.06	Stearic acid	Long-chain fatty acids	281.24	265	nd	-	-	-	-	-	-	-	0.8	-	-	-	[53]
35	37.07	α -Tocopherol	Tocopherols	429.11	161, 219	292	-	-	-	-	-	-	1.7	-	-	-	-	[109]
36	37.25	9,10-Dihydroxy- 8-oxooctadec-12-enoic acid	Long-chain fatty acids	326.509	113	nd	-	-	-	-	-	4.5	2.4	1	1.8	-	-	[45]
37	42.46	2-Docosenoic acid	Long-chain fatty acids	337.71	113	-	-	-	-	-	-	-	-	-	-	4.7	-	[45]
38	43.00	Palmitic acid	Long-chain fatty acids	255.01	111	nd	-	-	0.6	-	-	-	-	-	-	-	-	[53]

*nd: not determined, RT: retention time

The annotated metabolites from the liquid chromatographic data sets within the 4 groups are as follows: The first group including aerial parts of the 3 stages showed similar metabolites, namely catechin and its epimer, caffeic acid derivatives, and cyanidin derivative. In the second group of germ, groats and bran, quercetin derivatives, apigenin glycosides and caffeoyl hexuronide derivative were common metabolites. In the third group of roots of all the 3 stages, coumaric acid and its isomer, naringin and avenanthramide isomer were characteristic of the group. The last group of seeds contained specifically catechin and its epimer in addition to caffeic acid derivatives.

Relation of bioactive metabolites with antioxidant activity

In accordance with the GC-MS annotated metabolites, the antioxidant potential can be attributed to the presence of L-proline in aerial parts stage 2 and roots stage 3 [58], L-valine in aerial parts stages 1,2, and roots stage 3 [59], serine in aerial parts stage 2 [60], malic acid [61] malic acid in seeds, roots stages 1 and 3, and aerial parts stages 2 and 3 [61], phenylalanine in aerial parts stage 2 and roots stage 3 [62], asparagine in aerial parts stages 1 and 2, and roots stage 3 [63], L-glutamine in aerial parts stage 2 [64], Fructose in aerial parts stage 3 [65], D-glucitol in germ [66], gallic acid in all samples [67], D-galactonic acid in germ [68], myo-Inositol in germ [69], 9,12-octadecadienoic acid (Z, Z) in seeds, germ, and groats [70], sucrose in aerial parts stages 2 and 3, and roots stage 2, 1-monopalmitin in aerial parts stages 1 and 3 [71].

Referring to LC-MS/MS annotated metabolites, the aerial part of stage 3 extract showed the highest antioxidant potential and this was contributed to its high content of caffeic acid derivatives (namely caffeic acid hexoside and caffeoyl hexuronide derivatives), which are well known for their antioxidant potential and their role in modulating fatty acid oxidation [41]. The extract of stage 2 aerial part comes in second place due to its high content of coumaric acid isomers, catechin and its epimer, and caffeic acid derivatives, with their antioxidant potentials and effect on the antioxidant enzymes [41, 44, 50].

Limitations

The main limitation in this study was the use of unit resolution LC-MS/MS and the unavailability of authentic reference standards, that was balanced by an orthogonal analytical technique, namely GC-MS. This limitation was reflected in tentative annotation of the metabolites. Moreover, in the LC-MS/MS results, a low variance was explained by PCA

which may limit the interpretability of unsupervised multivariate analyses. However, this was overcome by using the supervised multivariate analyses. Also, despite the use of permutation testing, the PLS-DA models were based on components that explain limited variance and this may have resulted in overfitting and reducing the biological relevance of the observed group separations.

Conclusion

As the demand for functional foods rises, understanding dynamic changes in plants' metabolites across different species and different growth stages using metabolomics approach becomes important in enhancing the understanding of wheat's nutritional value. The untargeted metabolomic profiling using a GC-MS and LC-MS/MS for wheat growth stages and parts led to annotation of a total of 29 secondary metabolites (GC-MS data) and 38 metabolites (LC-MS/MS data). The *in vitro* assays showed that aerial part of growth stage 3 contained the highest total phenolic content and, thereby the highest antioxidant potential. Notably, several compounds with recognized antioxidant activity were annotated, these included l-proline, malic acid, fructose, gallic acid, sucrose, 1-monopalmitin, 12-octadecadienoic acid (Z, Z), myo-inositol, D-galactonic acid, D-glucitol, L-glutamine, asparagine, phenylalanine, serine, and l-valine from GC-MS analysis. From LC-MS/MS, key antioxidant-related compounds such as caffeic acid derivatives (including caffeic acid hexoside and caffeoyl hexuronide derivatives), coumaric acid isomer, catechin, and its epimer were also annotated. These findings shed the light on the wide spectrum of nutritional value of wheat parts that are not commonly consumed.

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Data availability The generated datasets during the current study are available in the paper and supplementary material files. If another format is required, the corresponding author can submit them on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval and consent to participate Not applicable.

Consent for publication Not applicable.

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