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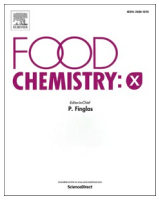

Determination of free and bound antioxidants in Kamut® wheat by HPLC with triple detector (DAD-CAD-MS)

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ABSTRACT

Kamut® wheat (*Triticum turgidum* ssp. *turanicum*), an ancient, underutilized cereal, offers potential health benefits due to its phenolic compounds. This study aimed to investigate the antioxidant potential of Kamut® wheat's free and bound phenolic extracts using an HPLC system equipped with three detectors. The bound extracts, released after alkaline hydrolysis, exhibited higher total phenolic and flavonoid content compared to the free extracts ($p < 0.05$). The total antioxidant capacity of bound extracts was six-fold greater than in free extracts ($p < 0.05$). The main antioxidants in free extracts were tyrosine, phenylalanine, tryptophan, and apigenin. In bound extracts, ferulic acid, its dimers and trimer were present. Kamut® wheat exhibited a source of dietary antioxidants and should be considered a potential ingredient for the development of functional foods. Also, the HPLC-triple detector system is effective for in-depth profiling of antioxidant compounds, paving the way for future research on similar grains.

1. Introduction

The ancient grain Kamut® wheat (*Triticum turgidum* ssp. *turanicum*), popularly referred to as Khorasan wheat, stands out as a rich source of phenolic compounds. These compounds are potent antioxidants with established health benefits (Shewry & Hey, 2015). While ancient cereals like Kamut® were once staple foods, their consumption has declined in favor of domesticated wheat varieties, which provide higher yields and superior technological quality (Tran et al., 2020). However, a renewed interest has emerged in those ancient wheat cultivars over modern ones, driven by their distinctive antioxidant profiles, especially the phenolic acids. Such profiles are believed to offer health advantages over modern wheat varieties (Dinu et al., 2018).

The health advantages of phenolic compounds involve their capacity to neutralize oxidative stress within the human body. This is accomplished either by donating a proton or an electron to radicals and other reactive oxygen species (ROS) (Kruk et al., 2022). Phenolic compounds are broadly categorized into free and bound forms based on their extraction method. While free phenolic compounds reside in the plant cell's cytoplasm and dissolve in hydroalcoholic solvents (Alara et al., 2021), bound phenolic compounds are mainly esterified to cell wall polysaccharides, and require a hydrolysis process for their extraction (Ding et al., 2020). A hydrolysis process typically involves acid or alkaline treatment, effectively releasing bound phenolic compounds into the aqueous phase (Kim et al., 2006).

The main phenolic component in cereal grains is ferulic acid (Bourne & Rice-Evans, 1998), contributing up to 90 % of the total phenolic content (Boz, 2015; Gorinstein et al., 2008), although, its concentration can vary significantly across varieties (Rao et al., 2018; Xia et al., 2022). In addition to ferulic acid, cereals are also a source of other types of antioxidants, including flavonoids, lignans, stilbenes (Dinelli et al., 2009), hydroxycinnamic acid, like p-coumaric, and dihydroxybenzoic acid, like vanillic (Adom et al., 2003). Furthermore, the germ and endosperm of cereals abound in lipophilic antioxidants, like tocopherols and tocotrienols (Falk et al., 2004; Hidalgo et al., 2006; Kamal-Eldin & Appelqvist, 1996).

Despite the richness of polyphenols in Kamut® wheat, identifying its main antioxidant compounds remains limited. While some studies have investigated its total polyphenol content (Suchowilska et al., 2020), others have focused on its environmental adaptability (Di Loreto et al., 2017), or its nutritional value in comparison with conventional wheat (Dinu et al., 2018; Thorup et al., 2014). Instead, this study aims to comprehensively profile both the free and bound phenolic fractions from Kamut® wheat using an advanced HPLC system with three detectors; diode-array (DAD), CoulArray (CAD), and high-resolution mass spectrometry (HRMS) detectors. This will allow the screening,

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quantification, identification, and characterization of their antioxidant potential (Ding et al., 2022). This research seeks to elevate Kamut® wheat's status in the food industry as a source of bioactive compounds, potentially leading to the development of new natural food preservatives and functional ingredients by exploiting the health benefits of this ancient grain.

2. Materials and methods

2.1. Chemicals and reagents

Folin–Ciocalteau reagent, 2,2- diphenyl-1-picrylhydrazyl (DPPH, purity higher than 98 %), 2,2′- azobis (2-amidinopropane) dihydrochloride (AAPH), sodium phosphate dibasic, potassium phosphate monobasic, sodium carbonate, sodium acetate, aluminum chloride, fluorescein, 2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97 %), acetic acid, hydrochloric acid, sodium hydroxide, methanol, vanillic acid, syringic acid, quercetin, gallic acid, *p*-coumaric acid, and ferulic acid were obtained from Sigma-Aldrich (Merck, Italy). Mass spectrometry grade methanol was purchased from Merck (Germany). Milli-Q water was purified by Sartorius arium® mini (Germany).

2.2. Samples

Khorasan wheat (*Triticum turanicum*) samples were obtained from Molino Merano (Lana, Italy). The kernels were oven-dried (IKA 125, Milan, Italy) for 15 h at 40 °C and then milled with a millstone mill (Mill Hawos Mühle 2, Hawos Kornmühlen GmbH, Italy) and sieved (Retsch, AS 200, Germen) to obtain a uniform size (100–250 μm). The flour (5 g) was then defatted with 150 mL hexane using a Soxhlet apparatus for 3 h and dried in a hood at room temperature overnight. Defatted flour was stored in the dark at ambient temperature.

2.3. Extraction of free phenolic (FP) compounds

Defatted flour fractions (1 g) were mixed with 10 mL of 80 % methanol for 45 min, at 250 rpm with a VWR advanced digital shaker (VWR, Milan, Italy) at room temperature (Fig. 1). The suspension was

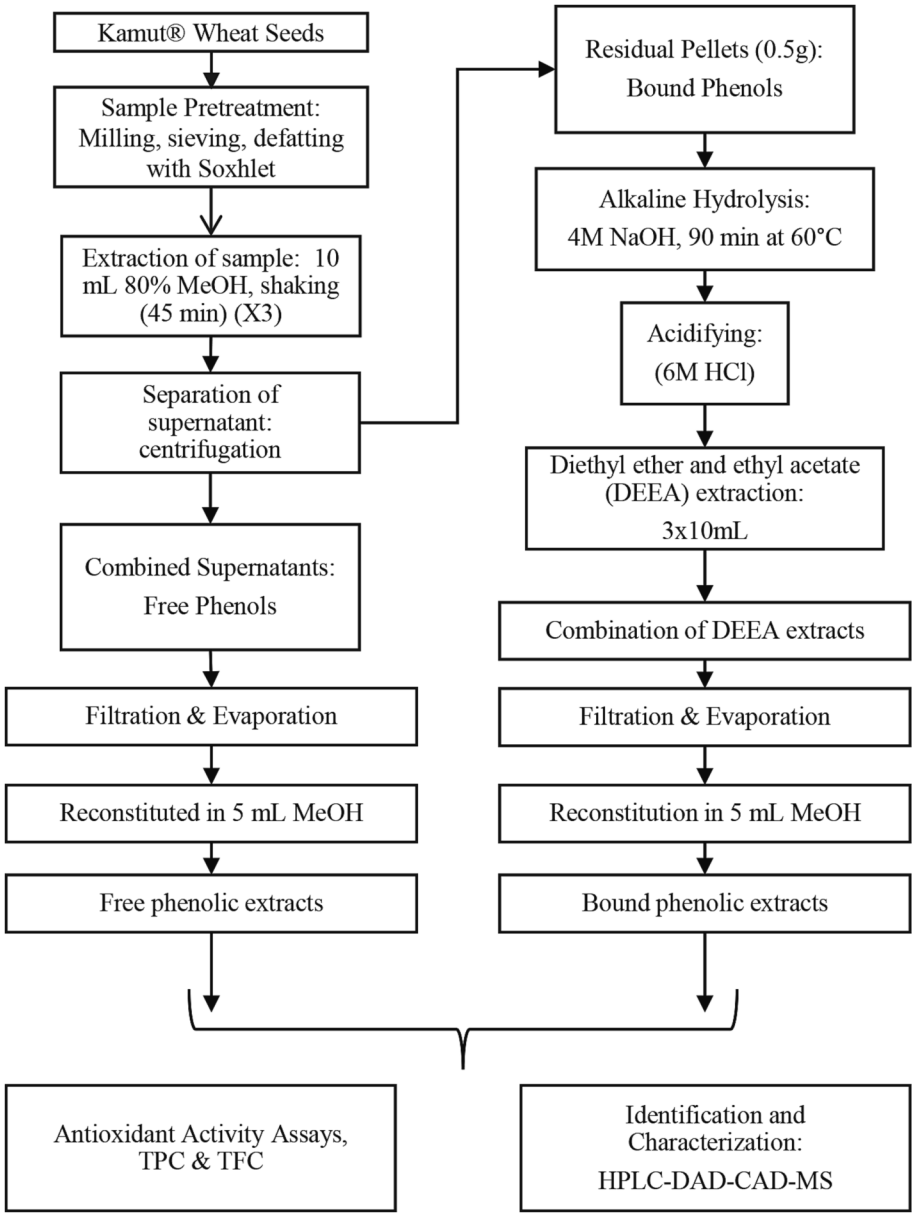

Fig. 1. Flow chart of extraction of free and bound antioxidant extracts from Kamut® wheat.

centrifuged at 10,000 rpm and 4 °C (SL 16R Centrifuge, Thermo Scientific, USA) for 10 min, and the supernatant (free phenolic extracts) was collected. The remaining pellets were re-extracted twice with 10 mL of 80 % methanol, and all supernatants were combined. Free phenolic extracts were then filtered through a Whatman No. 1 filter paper and evaporated under nitrogen until dryness at 25 °C using a MultiVap (LabTech Srl., Sorisole, Italy). The concentrated extract was reconstituted with methanol to a final volume of 5 mL. The free phenolic compounds were then stored at − 80 °C until further use.

2.4. Extraction of bound phenolic (BP) compounds

The solid residues obtained after the free phenolic extraction were dried in a fume hood at 25 °C for 12 h. Following drying, the dried pellets were subjected to alkaline hydrolysis to extract the bound phenolic compounds. A 4.0 M NaOH solution was prepared and added to 0.5 g of the dried pellets at a 1:20 w/v ratio. This mixture was placed in a water bath set at 60 °C and allowed to hydrolyze for 90 min. After completion, the mixture was acidified to pH ~ 2.0 using 6.0 M HCl. The acidified sample (5 mL) was then extracted with 10 mL of diethyl ether and ethyl acetate mixture (1:1 v/v). The extraction was repeated two times. The aliquots from each extraction were combined and evaporated to dryness under nitrogen at 25 °C using the MultiVap. The resultant dried extract was then reconstituted in 5 mL of HPLC-grade methanol. The methanolic sample extracts were then stored at −80 °C in cryogenic glass vials until further analysis. Each extraction procedure was performed in triplicates.

2.5. Determination of total phenolic content

Total phenolic content was determined based on Margraf et al. with small modifications (Margraf et al., 2015). Approximately, 130 μL of the methanolic sample extracts or gallic acid standard was mixed with 130 μL of Folin–Ciocalteu reagent (2 M) and 1 mL of distilled water in 5 mL glass tubes. A blank sample was prepared identically, having 130 μL of MilliQ water instead of the methanolic sample extracts. The mixture was vortexed for 10 s and then incubated for 5 min before adding 130 μL of saturated sodium carbonate solution (20 % w/v). The mixtures were vortexed again and incubated in the dark for 2 h at 25 °C. Then 200 μL were transferred to a 96-well microplate and the absorbance of each well was measured at $\lambda = 765$ nm (Tecan, Infinite M Nano+, Switzerland). From the absorbance measurements, the mean readings of two wells were used and the total phenolic content was expressed as milligrams of gallic acid equivalents per 100 g dry weight of Khorasan wheat flour (mg GAE/100 g DW) based on a calibration curve made with a series of gallic acid standards (in the range of 0–0.136 mg/mL). Analysis was conducted in triplicates and results were expressed as mg GAE/100 g DW (their mean value ± standard deviation) for each of the free and bound phenolic extracts.

2.6. Determination of total flavonoid content

The total flavonoid content (TFC) was determined based on Sharanagat et al. with some slight modifications (Sharanagat et al., 2019). Exactly 100 μL of methanolic sample extract was mixed with 400 μL of 99.5 % methanol, 100 μL of $AlCl_3$ (10 %), and 100 μL of sodium acetate (0.1 M). A blank sample was prepared identically, having 100 μL of MilliQ water instead of the sample extract. The mixture was vortexed and then incubated at 25 °C in the dark for 45 min. After incubation, 250 μL was transferred to a 96-well microplate and the absorbance of each well was measured at 415 nm (Tecan, Infinite M Nano+, Switzerland). The total flavonoid content was expressed as mg of quercetin equivalent (QE) per 100 g DW, based on a calibration curve of quercetin ranging from 0 to 0.15 mg/mL. Analysis was conducted in triplicates (their mean value ± standard deviation) for each of the free and bound phenolic extracts.

2.7. Oxygen radical absorbing capacity

The oxygen radical absorbance capacity (ORAC) assay was conducted according to Prior et al.'s method using the Tecan Infinite M Nano + spectrophotometer (Switzerland) (Prior et al., 2005). Trolox standard solutions (concentration range from 0 to 200 μM), or sample extract (diluted 1:20) were first mixed with fluorescein solution (3.2 μM). The reaction mixture (50 μL sample and 50 μL fluorescein) was incubated at 37 ℃ in a dark 96-well microplate for 15 min before adding 100 μL of freshly prepared AAPH solution (200 mM) in phosphate buffer (75 mM, 7.0 pH). For a total of 90 min, the microplate was shaken for 10 s and the fluoresceine intensity was measured every 30 s. The emission wavelength was set at 520 nm, while the excitation wavelength was set at 485 nm. The total antioxidant capacity was calculated from the difference between the area under the curve of the experiment with the samples and that of the blank solution. Analysis was conducted in triplicates and results were expressed as μM of Trolox equivalents per 100 g of dry weight sample (μM TE/100 g DW) (their mean value ± standard deviation) for each of the free and bound phenolic extracts.

2.8. DPPH radical scavenging activity

The free radical scavenging activities of the free and bound phenolic extracts were determined with 2,2-diphenyl-1-picrylhydrazyl stable radical (DPPH·), according to the method of Sharma and Bhat with minor modifications (Sharma & Bhat, 2009). Briefly, a DPPH stock solution was freshly prepared at a concentration of 2.5 mM in methanol. This stock solution was then diluted with methanol to obtain a 200 μM working solution. Then, 100 μL of the DPPH working solution was added with 100 μL of each free or bound phenolic extract. A blank sample was prepared identically, but with 100 μL of MilliQ water replacing the sample extract. The resulting mixture was incubated for 60 min in the dark at 25 °C. The absorbance was measured at 515 nm (Tecan, Infinite M Nano+, Switzerland). A standard curve was prepared using Trolox standards with concentrations ranging from 0 to 100 μM. Analyses were performed in triplicates and the results for both free and bound phenolic extracts were expressed as mean value ± standard deviation in μM Trolox equivalents per 100 g of dry weight sample (μM TE/100 g DW).

2.9. HPLC-DAD-CAD analysis

All the extracts were analyzed using an Agilent 1260 Infinity HPLC equipped with a binary pump and an autosampler that had a temperature control system set at a constant temperature of 4 °C. The HPLC system was equipped with a DAD (Agilent Technology, USA), a Kinetex Biphenyl column (100 × 2.1 mm, 2.6 μm particle size, equipped with a pre-column, Phenomenex, USA). The separation was conducted at 30 °C. The gradient method consisted of mobile phase A: Milli-Q water with 0.5 % acetic acid (v/v), and mobile phase B: methanol with 0.5 % acetic acid (v/v). The elution gradient had a constant flow rate of 0.3 mL/min and was programmed as follows: 0–1 min, 10 % B; 1–5 min, 10–30 % B; 5–10 min, 30–40 % B; 10–13 min, 40–45 % B; 13–18 min, 45–50 % B;18–27 min, 50–70 % B; 27–40 min, 70–50 % B; 40–48 min, 95–10 % B; 48–51 min, 10 % B. Before injection, the Khorasan wheat extracts were diluted 5 times with 90 % of phase A and 10 % of phase B. Sample injection volume was 20 μL and the spectra for wavelengths between 190 and 400 nm were recorded by the DAD. The eluting analytes were determined at 280 nm, 330 nm, and 365 nm.

For the electrochemical detection of antioxidant compounds in the samples, the analysis was conducted with a CAD detector (Thermo Fisher Scientific, USA), that was coupled with the HPLC-DAD. The CAD consisted of 16 porous graphite working electrodes with an associated palladium pseudo-reference electrode. The palladium reference electrode exhibited a half-wave potential shift of −360 mV, when compared with an Ag/AgCl electrode, as tested with ferrocene methanol standard (Ding et al., 2022). The channels of the detector were set from −50 to +

700 mV with 50 mV increments at a constant temperature of 25 °C. The quantification of the eluted compounds was done based on Faraday's law. Data processing was done using the CoulArray® data station 3.10. and R software (4.2.2). The package (MALDIquant) was used for the determination of the peak height, and area (Gibb & Strimmer, 2012).

2.10. HPLC-DAD-MS^2 analysis

The antioxidants were identified using an Ultimate 3000 HPLC coupled to a Q-Exactive Orbitrap (Thermo Fisher Scientific, USA) equipped with an ESI source in positive and negative mode, for accurate mass measurements. The column and gradient method was identical to the HPLC-DAD-CAD. The operation parameters for the full MS were as follows: capillary voltage 4 kV; sheath gas (nitrogen) was kept at 10 arbitrary units, and the capillary temperature was maintained at 320 °C. With a resolution of 70,000, and an automatic gain control (AGC) target set at $5{\cdot}10^5$, the MS range was from 75 to 1000 *m/z*, and a maximum injection time of 50 ms. An inclusion list of target species was created based on the full MS scan and a data-dependent (dd) MS/MS (MS^2) analysis was carried out to obtain fragmentation patterns of targeted species in both negative and positive ionization mode. The dd-MS^2 parameters were as follows: an AGC target 10^5, a maximum injection time of 50 ms, resolution at 17500, and an isolation window at 4.0 *m/z*. The acquired MS data and results were collected and analysed using Xcalibur 4.3.73 and Compound Discoverer 3.3.1 software (Thermo Fisher Scientific, USA).

2.11. Statistical analysis

The results obtained were reported as mean ± standard deviation (SD) for triplicate analyses. The significance of differences between free and bound phenolic extracts was compared using one-way ANOVA with Tukey's procedure at $p < 0.05$, using OriginPro 2021, version 9.8.0.200, OriginLab Corporation, USA.

3. Results and discussion

3.1. Antioxidant assays

The antioxidant properties of Kamut® wheat were evaluated. Table 1 reports the results of total phenol content (TPC), total flavonoid content (TFC), and total antioxidant capacity (TAC) for the free and bound phenolic extracts. The bound TPC (174.1 ± 9.3 mg GAE/ 100 g DW) was six times higher than the free phenolics (28.8 ± 2.2 mg QE/ 100 g DW), in agreement with other studies (Dinelli et al., 2009). The TPC value was higher than that reported by Žilić et al. (Žilić et al., 2012) and Dinelli et al. (Dinelli et al., 2009), but lower than those reported by Yu et al. (Yu et al., 2013). Also, the amount of TFC was six times higher in the bound fraction (45 ± 3.8 mg GAE/ 100 g DW) than in the free fraction (7.2 ± 0.9 mg QE/ 100 g DW), in agreement with previous works (Yang et al., 2018).

Table 1
Total phenol content, total flavonoid content, DPPH, and ORAC results of free and bound phenolic extracts of Kamut® wheat.

Extract	TPC mg GAE / 100 g DW	TFC mg QE / 100 g DW	DPPH μM TE / 100 g DW	ORAC μM TE / 100 g DW
Free Phenols	28.8 ± 2.2^{B}	7.2 ± 0.9^{B}	46.7 ± 1.8^{B}	767.5 ± 88^{B}
Bound Phenols	174.1 ± 9.3^{A}	45 ± 3.8^{A}	268.4 ± 4.2^{A}	3740 ± 270^{A}

TPC: Total phenolic content; DPPH: DPPH antioxidant assay; TFC: Total flavonoid content; ORAC: Oxygen radical absorbance capacity; GAE: Gallic acid equivalent: TE; Trolox equivalent; QE: Quercetin equivalent; DW: Dry weight. In a column mean ± SD (n = 3) that do not share a letter in the superscript are significantly different ($p < 0.05$, Tukey's test).

The DPPH assay, which measures free radical scavenging capacity, indicated that bound phenolics (268.4 ± 4.2 μM TE/ 100 g DW) had a significantly higher antioxidant capacity than free phenolics (46.7 ± 1.8 μM TE / 100 g DW). The ORAC assay results further corroborated this trend, showing a total antioxidant capacity for the bound phenolic extracts (3740 ± 270 μM TE/ 100 g DW) higher than that of free phenolic extracts (767.5 ± 88 μM TE/ 100 g DW). In detail, the bound phenolic content was significantly higher ($p < 0.05$) than the free phenolic based on TPC, TFC, DPPH, and ORAC. These findings highlight the greater antioxidant potential of Kamut® wheat, which can be attributed to the high content of bound phenolics, compared to free phenolics.

3.2. Analysis of free and bound phenolic compounds by HPLC-DAD-CAD and HPLC-DAD-MS^2

Both free and bound phenolic extracts underwent analysis using HPLC with three detectors: DAD, CAD, and MS, as previously described (Ding et al., 2022). The absorbance signal of the free and bound extracts of Kamut® wheat are shown in Fig. 2A and Fig. 4A respectively, obtained via HPLC coupled with a DAD detector at 280 nm UV wavelength. These chromatograms also show the corresponding HPLC-CAD signals in red, representing, respectively, the free and bound phenolic extracts of Kamut® wheat. These signals were generated using 16 coulometric detectors in series, each poised at an increasing applied potential from −50 mV to + 700 mV with 50 mV increments (relative to a Pd reference electrode).

Black peaks on the chromatogram represent all compounds in the free and bound phenolic extracts of Kamut® wheat, inclusive of those highlighted by the CAD. In contrast, the HPLC-CAD exclusively displays peaks possessing some redox activity (Razem et al., 2022). Thus, the CAD detector is more selective than the DAD towards antioxidants.

Accordingly, the HPLC-DAD was initially used to screen the overall bioactive profile within both extracts. This was followed by selecting the redox active species using the HPLC-CAD, which demonstrates electron transfer capacity and, thus, can act as antioxidants. The peaks observed via the HPLC-CAD were quantified based on Faraday's law with eq. (1).

$$Q = NFn \tag{1}$$

Where, Q is the area under the peak as total charge, n signifies the number of moles of electrons involved in a redox event, N is the quantity of analyte determined by the injection volume and concentration (in moles), and F is Faraday's constant, which is 96,485 Coulomb per mole (Hicks et al., 2017). For the quantification of the antioxidant compounds detected, the total charge was accumulated from the area under the curve from the 16 channels of the CAD. The n was assumed as 2 electrons based on a previous study done by Hicks et al. (Hicks et al., 2017). Accordingly, the concentration of the antioxidant compounds was determined. Then, the HPLC-DAD-MS^2 was subsequently used to identify the electroactive compounds observed in the HPLC-CAD, by comparing the retention times, and the fragmentation mass patterns with the relevant literature and available standards.

Table 2 summarizes the results of the antioxidant compounds detected in both free and bound phenolic extracts of Kamut® wheat. To facilitate easy referencing, compounds in the chromatograms of both free and bound extracts have been alphabetically and numerically labeled and organized by retention time. Notably, the bound phenolic extract was richer in antioxidant compounds. This finding can be explained, considering that most of the phenolic acids in Kamut® wheat are mainly bonded to polysaccharides and lignin, and become available only after alkaline hydrolysis (Pérez-Jiménez et al., 2014). Lignin is present in the aleurone and outer pericarp layer of the wheat grain and not in the endosperm and germ (Lebert et al., 2022). Our study focuses on fractions between 100 and 250 μm in size, which is endosperm-

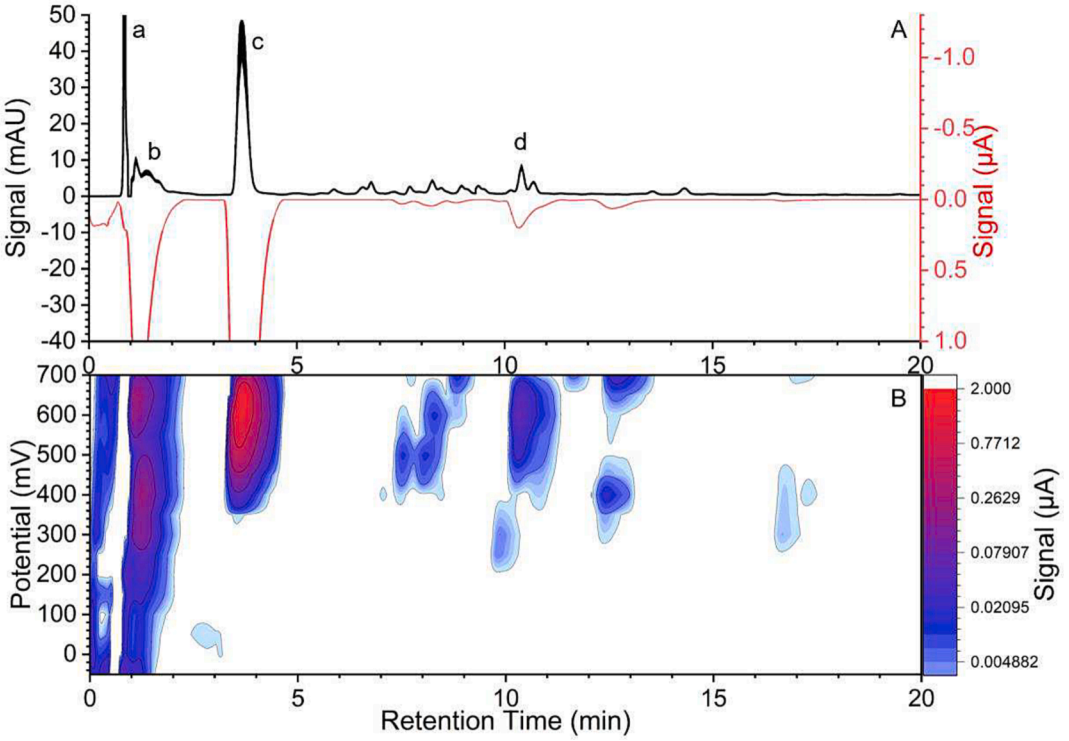

Fig. 2. (A) HPLC-DAD chromatogram of free extracts of Kamut® wheat measured at 280 nm (black), and the accumulated sum of current obtained from the 16 CAD channels (red). a tyrosine, b phenylalanine, c tryptophan, d apigenin 6-C-arabinoside 8-C-glucoside; (B) Contour Plot of peaks detected by CAD.

derived flour.

3.2.1. Free phenolics

Fig. 2A shows the phenolic profile of free extracts using HPLC-DAD. In a concurrent CAD of this extract, four main peaks (a-d) were observed, each exhibiting distinctive redox activities. High-resolution mass spectrometry was next used to identify the chemical nature of those peaks. Based on the MS^2 fragmentation patterns, those four peaks were identified with their molecular ion adduct *(m/z)* as (a): tyrosine ($[M + H]^+$: 182.0811), (b): phenylalanine ($[M - H]^-$: 164.0718), (c): tryptophan ($[M - H]^-$: 203.0828), and (d) apigenin 6-C-arabinoside 8-C-glucoside, a flavonoid also known as shaftoside ($[M - H]^-$: 563.1413).

A unique feature of the CAD is to provide a hydrodynamic voltammogram (HDV) for each of the eluted compounds. This HDV plots the peak area of each eluted compound against the applied potential, serving as a tool for identifying the half-wave potential ($E_{1/2}$) of unknown redox compounds or those known structures that are not available as pure analytical standards. The $E_{1/2}$ value is a characteristic feature of any redox compound, which corresponds to the potential at which the concentration of the oxidized and reduced forms of the eluted species are equal. This specific equilibrium potential is, thus, of paramount importance as an index to differentiate those compounds based on their antioxidant potential. Table 2 compares the half-wave potential of the compounds detected in the free and bound phenolic extracts. For instance, Fig. 3A shows the HDV of tyrosine, which has an $E_{1/2}$ of + 240 mV. In comparison, phenylalanine showed an $E_{1/2}$ of + 530 mV while, tryptophan and apigenin glucoside showed slightly higher $E_{1/2}$ values of + 570 mV. Even though phenylalanine had the highest quantified result among the identified compounds in the free phenols extracts (163.97 ± 12.12 mg/kg DW), tyrosine required lower potentials to transfer electrons, and is therefore considered the strongest antioxidant among the identified compounds present in the free phenolic extracts, despite its low amount (23.79 ± 1.77 mg/kg DW).

3.2.2. Bound phenolics

The phenolic profile obtained by HPLC-DAD at 280 nm (Fig. 4A) reveals the presence of several signals. However, some of these peaks lack electrochemical activity, mostly representing organic acids (malic and jasmonic acid) and lipids. Accordingly, the corresponding CAD spectra showed only 13 peaks, five of which were unambiguously identified by matching their retention time, UV spectra, and MS^2 fragmentation of the corresponding analytical standards, with the remaining 8 compounds being putatively identified, by comparing their retention times, and fragmentation mass patterns with the relevant literature. Such peaks are shown in Table 2, along with their quantified results. Briefly, compounds 1 and 5 were identified as phenolic aldehydes, whereas compounds 2 and 3 were labelled as phenolic acids. Compounds 4, 6–8, and 10–12, were categorized as hydroxycinnamic acids (HCA) and their derivatives. Compound 9 was a flavonoid, and compound 13 was tentatively identified as a lipo-phenol, β-tocotrienol.

Compounds 2, 3, 4, 6, and 9, were positively identified as vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, and quercetin, respectively, by comparing the retention time, UV, and MS^2 fragmentations with those of authentic standards. Compound 5 exhibited a molecular ion adduct $[M - H]^-$ at 151.0400, and MS^2 fragment ions at *m/z* 136.0166 and 151.0402, which was putatively identified as vanillin (Xia et al., 2022).

Compound 6, ferulic acid, displayed the largest identified peak showing a molecular ion adduct $[M - H]^-$ at 193.0510, and MS^2 fragments at 134.0370, 178.0270, 149.0610, and 193.0488. Its electrochemical behavior revealed a half-wave potential of + 480 mV. Additionally, it contained the highest content among the other compounds, having a quantity of 319.3 ± 20.51 mg / kg DW, which is similar and greater than the amounts reported in other studies (Abdel-Aal & Rabalski, 2008; Danesi et al., 2020), but less than that mentioned by Suchowilska (Suchowilska et al., 2020).

Interestingly, compounds 7, 8, 10, and 11 exhibited identical $[M - H]^-$ at *m/z* 385.0928, corresponding to ferulic acid dehydrodimers (DFAs), exclusive to the cereals bound form (Xiang et al., 2019). By

Table 2
Antioxidant compounds identified in free and bound extracts of Kamut® wheat by high-resolution tandem mass spectrometry.

Antioxidant Compound	Peak Label	Molecular Formula	Adduct	Theoretical *m/z*	Experimental *m/z*	Class of Antioxidant Compound	Sample	RT	Half-Wave potential (mV)	Quantification (mg/kG DW)	Δ ppm	MS/MS Fragment ions (*m/z*)
Tyrosine	*a*	C_9H_{11NO3}	[M + H]+	182.0811	182.0812	Amino acid	FP	1.27	240	23.79 ± 1.8	0.54	136.0756,165.0546,123.0439,85.0284
Phenylalanine	*b*	$C_9H_{11}N_{O2}$	[M−H]-	164.0717	164.0718	Amino acid	FP	2.185	530	18.10 ± 1.3	1.16	164.0718,147.0449,119.2470,72.0089
Tryptophan	*c*	$C_{11}H_{12}N_{2O2}$	[M−H]-	203.0826	203.0828	Amino acid	FP	3.81	570	164 ± 12	1.21	203.0823,189.2711,142.0660,116.0507
Protocatechuic aldehyde	1	$C_7H_6O_3$	[M−H]-	137.0244	137.0246	Phenolic aldehyde	BP	6.53	566	4.13 ± 0.31	1.46	137.0244,93.0349,119.7328,70.9092
Vanillic acid	2	$C_8H_8O_4$	[M−H]-	167.0349	167.035	Phenolic acid	BP	9.025	520	10.89 ± 0.81	0.86	167.0351,152.0116,123.0435,95.2313
Syringic acid	3	$C_9H_{10}O_5$	[M−H]-	197.0455	197.0459	Phenolic acid	BP	10.19	510	0.87 ± 0.07	2.03	182.0217,166.9983,136.0162,121.0297
p-Coumaric acid	4	$C_9H_8O_3$	[M−H]-	163.0405	163.025	HCA	BP	10.712	490	5.79 ± 0.44	0.76	163.0404,119.0504,126.610,86.6367
Apigenin 6-C-arabinoside 8-C-glucoside	*d*	$C_{26}H_{28}O_{14}$	[M−H]-	563.1406	563.1413	Flavone or Flavonoid glycoside	FP	11.29	570	18.4 ± 1.4	1.3	353.0669,383.0776,443.0997,563.1379
Vanillin	5	$C_8H_8O_3$	[M−H]-	151.0402	151.0401	Phenolic aldehyde	BP	11.37	490	1.18 ± 0.09	0.43	151.0402,136.0166
Ferulic acid	6	$C_{10}H_{10}O_4$	[M−H]-	193.0506	193.051	HCA	BP	12.5	480	319 ± 21	0.04	178.027,149.061,134.037,193.0488
8-8′-DFA	7	$C_{20}H_{18}O_8$	[M−H]-	385.0928	385.093	HCA	BP	14.9	385	15.53 ± 1.15	1.74	159.0455,281.082,123.0453,146.0373,257,173,119
8–5′-DFA	8	$C_{20}H_{18}O_8$	[M−H]-	385.0928	385.093	HCA	BP	15.77	340	37.03 ± 2.78	1.74	282.0901,267.0666,326.0796,297.1136,341.1027,311.0564,395.2917,364.3097
Quercetin	9	$C_{15}H_{10}O_7$	[M−H]-	301.0353	301.035	Flavonoid	BP	18.223	370	2.67 ± 0.20	2	178.9989,151.0026,121.0311,107.0133
8-O-4′-DFA benzo form	10	$C_{20}H_{18}O_8$	[M−H]-	385.0928	385.093	HCA	BP	20.42	340	15.56 ± 1.15	1.71	282.0895,383.0939,326.0796,341.1043,370.0688,267.0660
8-O-4′-DFA linear form	11	$C_{20}H_{18}O_8$	[M−H]-	385.0928	385.093	HCA	BP	21.747	370	37.56 ± 2.79	1.71	193.0507,178.0275,149.0508,134.0373
TFA	12	$C_{30}H_{26}O_{12}$	[M−H]-	577.1351	577.1362	Flavonoid glycoside	BP	26.1	340	10.08 ± 0.75	1.98	193.0506,355.0834,178.0277,134.0373,429.1339
β-tocotrienol	13	$C_{28}H_{42}O_2$	[M + H]+	411.3257	411.3258	Lipo-phenol	BP	32.19	590	60.4 ± 4.5	0.24	411.3258,277.2567,151.0896,69.0723

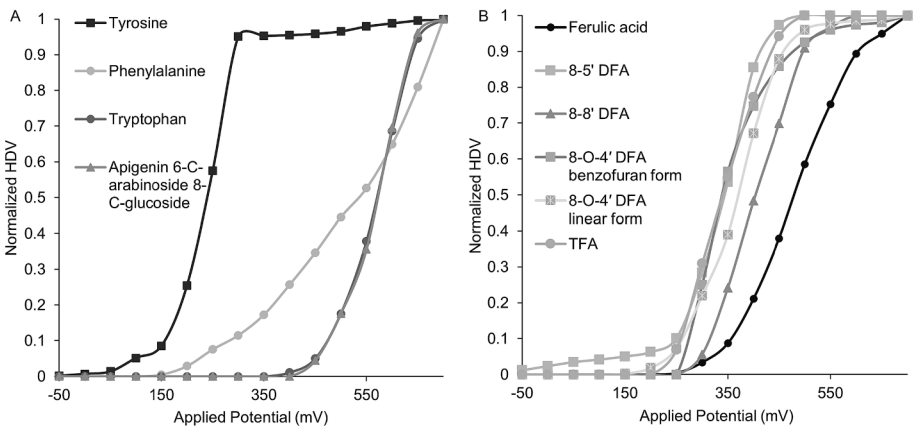

Fig. 3. (A) Hydrodynamic voltammogram of tyrosine, phenylalanine, tryptophan and apigenin 6-C-arabinoside 8-C-glucoside, obtained from free phenolic extracts of Kamut® wheat; (B) Hydrodynamic voltammogram of ferulic acid, DFAs, and TFA, obtained from the bound phenolic extracts of Kamut® wheat.

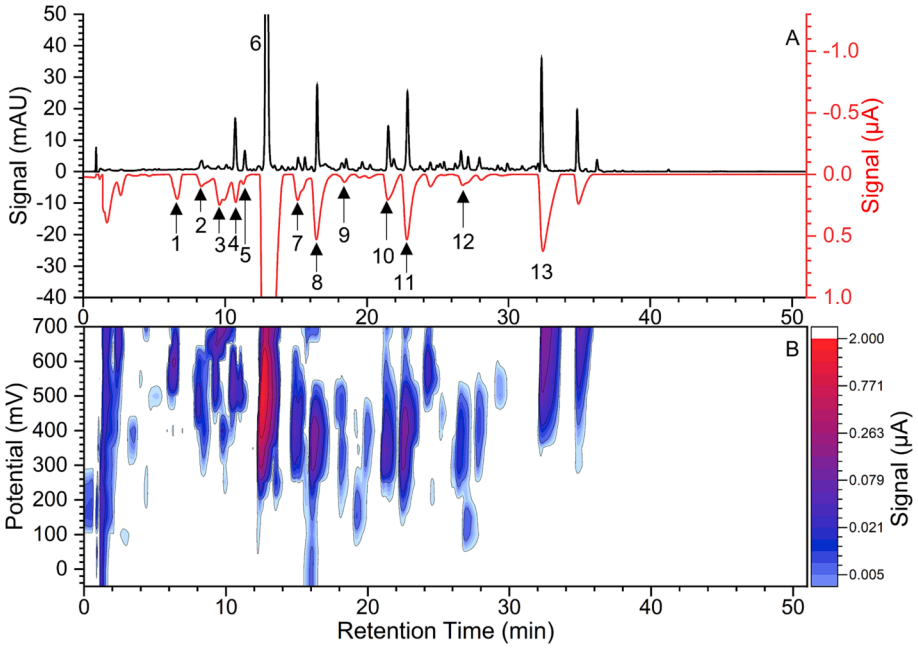

Fig. 4. (A) HPLC-DAD chromatogram of bound extracts of Kamut® wheat measured at 280 nm (black), and the accumulated sum of current obtained from the 16 CAD channels (red); 1 protocatechuic aldeheyde, 2 vanillic acid, 3 syringic acid, 4*p*-coumaric acid, 5 vanillin, 6 ferulic acid, 7 DFA 8–8, 8 8-5′-diferulic acid, 9 quercetin, 10 8-O-4′-DFA benzo form, 11 8-O-4′-DFA linear form, 12 TFA, 13 β-tocotrienol; (B) Contour Plot of peaks detected by CAD.

utilizing the UV spectra and MS fragmentation patterns, and corroborating with the literature (Callipo et al., 2010; Xia et al., 2022; Xiang et al., 2019), these DFAs were assigned as 8-8′-DFA in the linear form, 8-5′-DFA linear form, 8-*O*-4′-DFA benzofuran form, and 8-*O*-4′-DFA linear form, respectively. Notably, these dehydroferulic isomers were also detected in barley and millets (Chandrasekara & Shahidi, 2011; Hernanz et al., 2001). Previous studies have mentioned that dehydroferulic isomers showed a higher radical-scavenging efficacy than monomers (Jia et al., 2018). This increased antioxidant activity is attributed to the DFA's electron-donating methoxyl group, phenolic hydroxyl group, and stable conjugated transient structure (Jia et al., 2018). Compound 11 was tentatively identified as 8-*O*-4′-DFA in the linear form, as the fragmentation showed a distinctive product ion at *m/z* 193.0504 $[M - H-C_{10}H_8O_4]^-$, which is resulted from the cleavage of 8-O-bond between two ferulic acid structures (Dobberstein & Bunzel, 2010; Xiang et al., 2019).

The HDVs of these DFAs indicated that their half-wave potentials were consistently lower than that of ferulic acid (+480 mV). Table 2 reports the half-wave potential of all electroactive compounds from Kamut® wheat's free and bound extracts. In comparison, the bound extracts had more electroactive compounds with lower half-wave potentials than the free extracts, indicating a superior electron transfer capacity.

Compound 12 was tentatively identified as ferulic acid dehydrotrimer (TFA), as it displayed the same UV spectra as ferulic acid and had a molecular ion adduct $[M - H]^-$ of 577.1362 and MS^2 fragments at 193.0506, 355.0834, 429.1339, 134.0373 (Pedersen et al., 2015).

Interestingly, TFA exhibits a 30 mV lower half-wave potential than DFAs (~370 mV) and even lower than ferulic acid (+480 mV) (Fig. 3B), highlighting its superior electron transfer capacity. This indicates that the trimer of ferulic acid could indeed possess a better electron transfer ability than its monomeric and dimeric analogues, possibly due to the presence of multiple hydroxyl groups and electron donating methoxyl groups (Jia et al., 2018).

Compound 13 was tentatively identified as β-tocotrienol, having a *m/z* 411.3258 indicative of the $[M + H]^+$ adduct and *m/z* 433.3077

corresponding to the adduct $[M + Na]^+$ with MS^2 fragments of 411.3258, 277.2567, 151.0896, 69.0723. (Bartosińska et al., 2018; Okarter et al., 2010; Zhao et al., 2010). It displayed a high half-wave potential (+590 mV), which means it has a weak electron transfer ability, at least in the solvent system at which it has been eluted.

4. Conclusion

In this study, we investigated the free and bound phenolic compounds of Kamut® wheat using an HPLC system coupled with three detectors. Our results indicated that the bound phenolic extracts showed a significantly higher TPC, TFC, and TAC compared to free phenolic extracts. The dominant antioxidant compounds in the bound phenolic extracts were ferulic acid, 8-8′-DFA, 8-5′-DFA, 8-*O*-4′-DFA in both benzofuran and linear forms, and TFA. Instead, the main antioxidants in free extracts were tyrosine, phenylalanine, tryptophan, and apigenin 6-C-arabinoside 8-C-glucoside. Our methodology, utilizing the HPLC system with a triple detector, proved effective in screening, profiling, quantifying and identifying antioxidant compounds, even in complex matrices. This robust approach offers valuable insights despite the study's limited sample size. These findings emphasize the potential of ancient Kamut® wheat as a rich antioxidant source, suggesting its suitability for functional food development. We recommend further research into the antioxidant profiles of other wheat varieties using this triple detector approach.

CRediT authorship contribution statement

Mutasem Razem: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Ksenia Morozova:** Writing – review & editing, Visualization, Methodology, Formal analysis, Data curation. **Yubin Ding:** Investigation, Formal analysis. **Giovanna Ferrentino:** Supervision, Methodology. **Matteo Scampicchio:** Writing – review & editing, Supervision, Software, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101216.

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