The impact of processing on the phenolic acids, free betaine and choline in Triticum spp. L. whole grains and milling by-products

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Abstract: Wheat (Triticum spp. L.) is an important source of nutrients and bioactive compounds with recognized beneficial effects. Wheat undergoes several processes with the final aim of separating the endosperm from the outer layers, usually discarded. In this study, free and bound phenolic acids profile, betaine and choline contents were quantified in six wheat species, in the corresponding milling by-products as well as in flour/semolina. Bound PAs accounted for about 80% of total PAs, being ferulic acid the most represented (67-73 % of total PAs). Bread wheat grain totalized the highest content of total PAs (1209.31±7.3 µg g-1 d.w.). Betaine and choline are abundantly present in wheat species. In general, the highest content of bioactive compounds was found in bran (3 times higher than whole grains), emphasizing the good nutritional profile of these by-products. The milling process leads to a severe reduction of phenolic acids and methyl-donors in the end-products.

Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

Parma, 17.10.2019

Dear Editor,
On behalf of my coauthors, I'm submitting the revised version of our manuscript. The text has been revised according to the suggestion of reviewers. We are grateful for the efforts in increasing the quality of our work. Along the text small changes have been done to improve and clarify the discussion (all tracked). The comments moved directly in the text by reviewer#1 have been taken into consideration and all addressed. Those moved by reviewer#3, are discussed below.
Best Regards
Chiara Dall'Asta

Reviewer #3: Dear Editor and Author (s),
My apologies for slow response. Please see the presented comments into the MS and also:

1. In all of the MS, the discussion should be improved.

2. All figures and Tables need to be modified for uniformity and clarity.

This MS is based on a huge amount of different data, and we are aware that formatting them for clarity and consistency can be an issue. Therefore, we've put particular attention in Table/Figure reporting and, at best of our knowledge, the current layout is the best we can achieve. We will be more than willing to follow any reviewer's suggestion to improve clarity.

3. In Introduction section, the author (s) should explain about the classification of phenolic compounds in the plants.

Honestly, we don't think this should be included in the manuscript, or extensively treated, for meeting the expectation of the average Food Chemistry reader. The MS is already quite long, and adding info on phenolics in plants would steel lines useful for other less well-known points.

4. It will be better to provide important mean values in the Abstract.

We have improved our abstract accordingly

5. The term 'secondary metabolites' is often replaced by 'specialised metabolites' these days. What do you think?

We amended the text accordingly, introducing the concept for those readers that are less familiar with the term.

Highlights

- The milling process leads to a severe reduction of phenolic acids and methyl-donors in the end-products.
- Data confirmed that the content of bioactive compounds in bran is significantly higher than other by-products
- Among by-products, emmer bran was found as the highest in PAs

The impact of processing on the phenolic acids, free betaine and choline in *Triticum* spp. L. whole grains and milling by-products.

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Abstract

Wheat (*Triticum* spp. L.) is an important source of nutrients and bioactive compounds with recognized beneficial effects. Wheat undergoes several processes with the final aim of separating the endosperm from the outer layers, usually discarded. In this study, free and bound phenolic acids (PAs) profile, betaine and choline contents were quantified in six different wheat species (durum and bread wheat, *turanicum* wheat, einkorn, emmer and spelt), the corresponding milling by-products (bran, middlings, aleurone and I, II and III steps of debranning) and flour/semolina, using UHPLC-MS/MS methods. The bound form of phenolics was the component present in higher concentration (80% of the total, in average) and ferulic acid was the most abundant compounds, representing between 67-73 % of total PAs. Among the species, bread wheat grain totalized the highest content of total PAs (1209.31±7.3 μg g^{-1} d.w.). Betaine and choline are abundantly present in wheat species. In general, the highest content of bioactive compounds was found in bran (3 times higher than whole grains), emphasizing the good nutritional profile of these by-products. The milling process leads to a severe reduction of phenolic acids and methyl-donors in the end-products.

Key words: wheat milling, by-products, whole grains, *Triticum* spp., phenolic acids, betaine, choline.

Abbreviation Used

<LOQ, below the limit of quantification; 4-HB, 4-hydroxybenzoic acid; Caff, caffeic acid; c-Fer, *cis*-Ferulic acid; Dif, diferulates; d.w., dry weight; ESI, electrospray ionization; HILIC, hydrophilic interaction liquid chromatography; UHPLC, ultra-high performance liquid chromatography; MS, mass spectrometry; MRM, multiple reaction monitoring; PAs, phenolic acids; p-C, *para*-Coumaric acid; SD, standard deviation.; Sin, sinapic acid; t-Fer, *trans*-Ferulic acid.

1 Introduction

Wheat is one of the most important crops in the world. Wheat cereals belong to the *Triticum* spp. genus which comprehend several species, *Triticum turgidum* subsp. *durum* Desf. (known as durum wheat) and *Triticum aestivum* L. (known as bread wheat) are the most widely known, mainly because of their end use to produce many commodities such as pasta, bread and baked products (Shewry et al., 2013). In addition to these species, today there is a growing interest on the so-called "ancient" wheat, which are apparently more tolerant to abiotic and biotic stresses, like drought, pests, cold, and heat (Arzani & Ashraf, 2017). Moreover, greater health related properties than the classic bread and durum wheat have been reported for these wheat species (Arzani & Ashraf, 2017; Longin et al., 2016). The so-called "ancient" wheat include *T. turgidum* subsp. *turanicum, T. monococcum* (einkorn), *T. turgidum* subsp. *dicoccum* (emmer) and *T. aestivum* subsp. *spelta* (spelt). Genetically, these species embrace all levels of ploidy. In fact, durum wheat, *turanicum* and emmer are tetraploids (2n=4x=28; genomes AABB), einkorn is diploid (2n=2x=14; genome AA) and bread wheat and spelt are hexaploids (2n=6x=42; genomes AABBDD) (Arzani, 2011). In terms of morphology, wheat grain is mainly structured in three parts, the endosperm (81-84%), that plays a role as storage of energy (starch granules), the bran (14-16%), which is composed by the outer layers protecting the grain, and the germ (2-3%), in which the genetic material is enclosed. However, for their suitability as ingredient in food preparation, the whole grain undergoes several processes generally defined as milling. During this procedure a large amount of by products are generated, mainly composed by the most outer layer of the seeds. It has been determined that the by-products stream account for about 23-27% of the milling output (Serna-Saldivar, 2012). Nowadays these by-products are mainly directed to the feed industry, nevertheless they preserve their nutritional quality. In fact, several recent studies have stressed the role of cereal grain consumption against cardiovascular diseases (Katcher et al., 2008), colorectal cancer and other health issues (Schatzkin et al., 2007). The beneficial properties are mainly associated with the occurrence of bioactive compounds and fibres. These molecules represent the product of the plant specialized metabolism, since it is a form of adaptation to specific ecological situation (Pichersky & Lewinsohn, 2011). Among the bioactive compounds are the phenolic acids (PAs), from either hydroxybenzoic (i.e.: p-hydroxybenzoic acid, vanillic acid and syringic acid) and hydroxycinnamic (i.e.: p-coumaric acid and ferulic acid) acid classes, recognized for their antioxidant activity and protective effects towards the oxidation processes. In plants, mainly two fractions of these compounds are present, a bound (most abundant) strictly bonded to polysaccharides that compose the cell wall, and a free component (Liyana-Pathirana & Shahidi, 2007) mainly present in the endosperm. Furthermore, wheat cereal products are also important sources of other non-essential nutrients such as betaine and its precursor, choline. They are chemically very similar, and they have been reported to exert a wide range of beneficial effects in humans (Craig, 2004). Betaine plays an important role in the conversion and detoxification of homocysteine to methionine in human liver and kidneys, acting as methyl group donor (De Zwart et al., 2003; Ross, Zangger, & Guiraud, 2014). Likewise, choline is well metabolized in humans and converted in acetylcholine and phosphatidylcholine, which are important for the normal functions of cells (Zeisel, 2006). These compounds are mainly concentrated in the most external layers of the seed, such as the bran, germ and mostly in the aleurone. This means that highly refined products are more likely to be deficient in bioactive compounds (Andersson et al., 2013). However, numerous studies reported a high variance in terms of bioactive compounds concentration, either in whole grain and by-products (Abdel-Aal et al., 2001; Li, Shewry, & Ward, 2008). This is mainly due to the different wheat genotype, growing location, environment, and the interaction between these factors (Laddomada et al., 2017; Yu, Haley, Perret, & Harris, 2004). In fact, as reported by Fernandez-Orozco, Li, Harflett, Shewry, & Ward, 2010, certain genotypes were more resistant to harsh environmental conditions affecting mostly the free (soluble) phenolic component. Therefore, in order to increase the knowledge about the occurrence of bioactive compounds, the main objectives of this paper were (1) to determine the concentration of principal phenolic acids, free betaine and choline in six different *Triticum* species and (2) to monitor their distribution within the major fractions of the caryopsis produced after an industrial-scale milling process, using a UHPLC-MS/MS analytical method.

2 Material and Methods

2.1 Raw materials

All samples, whole grains and the corresponding milling by-products, were produced in industrial-scale durum and bread wheat mills. All durum wheat mills are equipped with the three-step debranning technology (Delfino&Giancaspro, Italy) that, in one case, is also combined to air-classification by a SeparMicroSystems (Italy) turbo-separator. For each cereal species different types of industrial-scale by-products were produced:

- *Triticum turgidum* subsp. *durum* Desf.*:* whole grain (WG), semolina (S), bran (B), fine bran (FB), middlings (M), aleurone (A), I, II and III steps of debranning (I°, II° and III° respectively);
- *T. aestivum* L.*:* whole grain (SWG), refined flour (F), bran (SB) and middlings (SM);
- *T. turgidum* subsp. *turanicum:* whole grain (TWG), semolina (TF), bran (TB) and middlings (TM);
- *T. monococcum* L.*:* whole grain (MWG), wholemeal flour (WMF) and middlings (MM);
- *T. turgidum* subsp. *dicoccum:* whole grain (DWG), flour (DF), bran (DB) and middlings (DM);
- *T. aestivum* subsp. *spelta:* whole grain (SpWG), refined flour (SpF), bran (SpB) and middlings (SpM).

On arrival, the whole grain samples were ground with an IKA a11 basic lab mill (IKA Mills) to particle size <200 mm, vacuum-packed in polyethylene bags and kept at -20°C prior to analysis. The proximate composition is reported as Supplementary Information (**Table 1S**).

<u>Production of by-products</u>: The wheat lots came from the 2015-2016 crop and were a commercial blend of different varieties. Durum wheat commercial lots were from either Italy (Marche, Emilia Romagna and Apulia regions) or the US (Northern Plains, North Dakota, ND) and collected from both Italian and American industrial milling plants. Commercial lots of all the other *Triticum* species were from Italy. Sampling: For each fraction, five sub-samples of the same lot were collected at different times and

combined into one. A brief description of the milling by-products and the flow charts for the different wheat species are provided in **Table 2S** and **Figure 1AS, BS** and **CS**, respectively.

2.2 Proximate composition

The analyses of moisture content (ISTISAN 1996/34, met. B), total nitrogen (ISTISAN 1996/34, conversion factor: 5.70), crude fat content (ISTISAN 1996/34, met. A), ash content (ISTISAN 1996/34) and total dietary fiber (AOAC 985.29:1997) were carried out by an external accredited laboratory of food analysis, which follows the official analytical methods used in food chemical control (Onori, Orefice & Stacchini, 1996). Then, the carbohydrate content was calculated by difference.

2.3 Chemicals

HPLC-grade acetonitrile (>99.9%), ethyl acetate (>99.8%), formic acid (>95.0%), acetic acid, hydrochloric acid (HCl, 37.0%), methanol (>99.9%), sodium hydroxide (NaOH, >98.0%), phenolic acid standards (caffeic acid >98%, p-hydroxybenzoic acid >99%, p-coumaric acid >98%, sinapic acid >98% and trans-ferulic acid >99%) betaine solution (0.1 M) and choline chloride (>99%) were purchased from Sigma-Aldrich (St. Louis, Missouri, US). The *cis*-ferulic acid was obtained by complete conversion of a trans-ferulic acid solution under UV light.

2.4 Extraction and analysis of free and bound phenolic acids

Phenolic acids were extracted and analyzed according to Verma, Hucl, & Chibbar, (2009). Briefly, for the extraction of free phenolic compounds, 1 g of sample was extracted with 4 mL of a mixture of methanol/water (7/3 v/v) on a shaker at 200 strokes/min for 10 min at room temperature, followed by centrifugation at 4000 rpm for 10 min. After that, 1 mL of the supernatant was collected and brought to dryness under a gentle Nitrogen flow. The residue was dissolved with 200 µL of acidified water (0.2% of formic acid) before the analyses. For the extraction of the bound phenolic fraction, the sample residue remained after free phenolic compound extraction was further hydrolyzed with 20 mL of 2N sodium hydroxide at room temperature for 1 h. After alkaline hydrolysis, the pH of the mixture was adjusted to 3 by adding an opportune volume of 6N chloridric acid. The bound phenolic fraction was then recovered with 10 mL of ethyl acetate, shaking samples for 10 minutes at 200 strokes/min. After a centrifugation step at 4000 rpm for 10 min, the supernatant was recovered and evaporated to dryness under vacuum, then the residue was dissolved with 500 µL of acidified water (0.2% of formic acid). For the separation of the analytes, a RP-C18 SunShell column (2.6µm, 100x2.10 mm; ChromaNik Technologies, Osaka, Japan) was used applying as eluents bi-distilled water (phase A) and methanol (phase B), both acidified with formic acid (0.1%). The elution gradient started from 2% of B and, after an initial isocratic step of 1 min, increased at 30% in 13 min; then at 20 min the percentage of B was further increased at 80% and, after a flashing step of 2 minutes, the initial conditions were restored. The total run time was 30 minutes, the column temperature was set at 35°C while the flow was maintained at 0.3 mL min^{-1}. For each sample, 2 µL were injected. Hydroxycinnamic acids were monitored in negative ionization mode (spray voltage = 3500 V), with the capillary temperature at 270 °C, the vaporizer temperature was kept at 200 °C, the sheath gas flow was set at 50 units and the auxiliary gas flow at 5 units; the other parameters such as S-Lens RF amplitude and Collision Energy (CE) values were obtained and set by tuning methanolic solutions of each considered molecule (1 mg L^{-1}) employing an automatic function of Xcalibur software (Thermo Fisher Scientific Inc., San Jose, CA, USA). Detection was carried out using SRM modality, using the following transitions: 4-hydroxybenzoic acid (4-HB) *m/z* 137 → 93 (CE = 34 eV), *m/z* 137 → 65 (CE = 17 eV); p-coumaric acid (p-C) *m/z* 163 → 119 (CE = 14 eV), *m/z* 163 → 93 (CE = 34 eV); caffeic acid (Caff) *m/z* 179 → 135 (CE = 19 eV), *m/z* 179 → 107 (CE = 24 eV); ferulic acid (t-Fer) *m/z* 193 → 178 (CE = 18 eV), *m/z* 193 → 134 (CE = 14 eV), *m/z* 193 → 134 (CE = 19 eV); sinapic acid (Sin) *m/z* 223 → 208 (CE = 15 eV), *m/z* 223 → 164 (CE = 18 eV), *m/z* 223 → 120 (CE = 34 eV). Dimeric ferulic (Dif) acids with respective $[M-H]^-$ value of *m/z* 385 were analyzed in full scan MS^2 mode and quantified as ferulic acid equivalents. For quantification, two different calibration sets were prepared using acidified water as solvent (0.2% of formic acid): one with a calibration range of 0.05 - 5 µg gr $^{-1}$ and one in the range of 5 - 100 µg gr $^{-1}$ for free and bound phenolic compounds respectively, obtaining a good linearity ($R^2 > 0.99$) for both calibration ranges.

2.5 Extraction and analysis of free betaine and choline

The extraction of betaine and choline from cereal samples was performed following the procedure proposed by Bruce, Guy, Rezzi, & Ross, (2010) with some modifications. Briefly, 1 gram of fine grounded sample was extracted with 15 mL of a 50% methanol/water solution. As betaine and choline may not be fully released from the sample matrix, samples were extracted sequentially six times, and extracts were pooled. Samples were then vortexed for 2 min in a vortex mixer and then centrifuged (10 min, 4°C, 4000 rpm). The supernatant was then transferred to LC-MS vials and opportunely diluted for analysis. For the separation of the analytes, a HILIC XBridge BEH column (2.5µm, 150x3 mm; Waters, Massachusetts, USA) was used applying as eluents acetonitrile (phase A), bi-distilled water (phase B) both acidified with formic acid (0.2%) and 20 mM ammonium formate 1% formic acid (phase C). The elution gradient started from 1% of B, 10% of C and 89% of A and, after an initial isocratic step of 1 min, B increased at 10% in 3 min; then at 5 min the percentage of B was further increased at 63% and, after a flashing step of 2 minutes, the initial conditions were restored. The total run time was 13 minutes, the column temperature was set at 35°C while the flow was maintained at 0.4 mL min^{-1}. For each sample, 3 µL were injected. Betaine and choline were monitored in positive ionization mode (spray voltage = 4000V), with the capillary and vaporizer temperature at 325°C, the sheath gas flow was set at 50 units and the auxiliary gas flow at 5 units; the other parameters such as S-Lens RF amplitude and Collision Energy (CE) values were obtained and set by tuning methanolic solutions of each considered molecule (1 µg mL^{-1}) as described for the phenolic acids content. Detection was carried out using SRM modality, using the following transitions: betaine m/z 118 → 42 (CE = 32 eV), m/z 118 →58 (CE = 24 eV), m/z 118 →59 (CE = 19 eV); Choline m/z 104 → 58 (CE = 33 eV), m/z 104 → 60 (CE = 25 eV). For quantification, a calibration set was prepared for betaine and choline (10-1000 µg g^{-1}) using commercial standards.

2.6 Statistical analyses

Sample extraction and analysis was performed in triplicate. Differences in the content of bioactive compounds between cereal species and among milling by-products were determined using one-way analysis of variance ANOVA with the *Tukey-b's* post-hoc test. Correlation between total, free and bound phenolic acids was performed following the Pearson correlation test. Results were considered significant at $p < 0.05$ (reject the null hypothesis). All statistical analyses were performed using IBM SPSS statistics 21 software.

3 Results and Discussion

3.1 Whole grains and flours/semolina phenolic acids content

The phenolic acids profile of whole grain *Triticum* spp. differs significantly between species, as reported in **Table 1** and **2**. Regarding the soluble free component, the predominant PAs were t-Fer and Sin, as already reported by (Moore et al., 2005). Free t-Fer acid was the most abundant compound in all species (**Table 2**), except for *T. turanicum*, for which sinapic acid was found in higher concentration. 4-HB, p-C and caffeic acids were found as the lowest PAs in whole grains. Although p-C was quantified in durum and bread wheat, in the other whole grains it was below the limit of quantification (0.05 µg g $^{-1}$). In addition, in einkorn, emmer and spelt the free caffeic acid content was significantly higher than in durum, bread and *turanicum* wheats. Moreover, when the phenolic acids content of semolina and flours was compared to the respective whole grain, a not negligible difference was noticed. In fact, in durum, bread and *turanicum* wheat flours only the free form of caffeic acid was quantified. While in einkorn, emmer and spelt flours also t-Fer and Sin acids were found. The bioactive compound loss is mainly attributable to the milling process resulting in a separation of the outer layers from the starchy endosperm. Besides, the contribution of the soluble PAs component differs for each compound and for each wheat species. For example, the free form of 4-HB accounts between 4.5-11.7 %, 0.7-3.6 % for p-C, 18.8-73.7 % for Caff, 0.3-0.5 % and 1.8-21.5 % for t-Fer and Sin, respectively. These results are in accordance with other studies (Mpofu, Sapirstein, & Beta, 2006; Okarter, Liu, Sorrells, & Liu, 2010). The presence of a high free phenolic content is important from the nutritional point of view. In fact, in this form they can be easily absorbed in the human intestine exerting their beneficial functions.

The bound and conjugated phenolic acids are the components present in higher concentration in cereals. These compounds are strictly linked to the fibrous elements of the seed, like lignins, cellulose, arabinoxylans and sometimes to sugar moieties. As for the free component, the most abundant PAs found in whole grains were t-Fer and Sin, while the least concentrated ones were p-C, 4-HB and caffeic acids. Interestingly, among the secondary phenolic acids, p-C was the most abundant in whole grains, in particular in emmer, indicating that this PA in cereals is mainly found in the bound form. Likewise, the t-Fer is mostly present in its matrix-linked form, accounting between 99.5-99.7 % of the total. These results were also reported in the study of Nicoletti et al., (2013). On the contrary, the bound form of caffeic acid in einkorn accounts only for the 26.3 %, indicative of a high free content of this compound in einkorn. Furthermore, the isomer of t-Fer, *cis*-Ferulic acids (c-Fer) could be found in cereals. Arguably, the c-Fer is the isomer found in the lowest concentration, nevertheless it was highly present in almost all the whole grain species except for the *spelta* wheat, in which it was found as <LOQ, as reported in **Table 2**. The highest content of this compound was found in the bread wheat whole grain. In addition, the dimers (Dif) of t-Fer, composed by two moieties of ferulic acid with *m/z* 385 (**Figure 2**), can be also found in cereals. Here it was highly concentrated in durum, bread and *turanicum* whole grains, and significantly less abundant in einkorn, emmer and spelt whole grains. Moreover, a remarkable content of diferulates was also found in flours. These compounds are strictly linked to the cell wall polysaccharides and the 5-5- and 5-8-dehydrodiferulate esters are the mostly reported (Li et al., 2008). Shibuya, (1984) reported the correlation between the occurrence of this compounds in the starchy endosperm and the presence of high carbohydrates content.

Moreover, the differences in phenolic content found in the end-products, flours and semolina, were more pronounced, stressing the effect of the processing. In fact, very low quantity of PAs was found in flours and semolina, due to the high refining degree. Instead, wholemeal flour produced from einkorn showed no differences in terms of PAs content with respect to the whole grains. The latter remarks the fact that the most of bioactive components were found in the outermost layers of the kernel (Siebenhandl et al., 2007). It is worth to mention that of from one side the milling process improves the technological and sensorial quality of cereals, from the other side beneficial compounds are lost.

The total phenolic acid content (TPA) is represented by the sum of the free and bound form of these compounds. This last component gave the highest contribution to this parameter. In fact, a positive correlation was found between the TPA and both the bound (r=0.57) and free (r=0.56) components of PAs. However, significant differences in TPA between whole grains were found (**Figure 3**). The highest TPA was obtained by the bread wheat (1209.31±7.3 μg g $^{-1}$ d.w.), followed by the durum wheat (811.4±137.7 μg g $^{-1}$ d.w). Nevertheless, no significant difference was found between *turanicum* (608.1±13.5 μg g $^{-1}$ d.w), einkorn (596.4.0±64.6 μg g $^{-1}$ d.w), emmer (508.3±24.4 μg g $^{-1}$ d.w) and spelt (568.6±60.4 μg g $^{-1}$ d.w). These findings are consistent with the studies conducted by Brandolini, Castoldi, Plizzari, & Hidalgo, (2013), in which they performed a two-year evaluation of phenolic acids composition, total polyphenols content and antioxidant activity on einkorn, emmer, durum and bread wheat. Since ferulic acid is the most common phenolic acid found in cereals (Okarter et al., 2010), its total content in whole grains was similar and ranged between 89.5 % (emmer) to 93 % (bread wheat), as reported by Brandolini et al., (2013). Besides, the effect of the milling process on the total amount of the PAs were calculated. The sequential removing of the bran fraction caused an important decrease of these bioactive compounds in the flour and semolina samples. For example, in durum wheat semolina and bread wheat flour a 94 % loss of total PAs was detected. Likewise, the same trend was seen in *turanicum* semolina (62%) and spelt flour (82%); however, the milling effect was less marked for emmer flour (11%). This high variance could be due to many variables, such as the seed morphology and shape, but also the technology used to separate the external tissues from the endosperm (Beta, Nam, Dexter, & Sapirstein, 2005). However, when part of these outer layers, richer in PAs, are preserved in the flour, this decrease is negligible. For instance, the PAs loss in einkorn wholemeal flour was the 2 %.

3.2 Milling by-products phenolic acids content

Cereal milling by-product streams can differ in function of the seed shape and morphology and in relation to the milling technology used. In general, bran and middlings are produced during the kernel fractionation. The most abundant PAs were the t-Fer and Sin whereas 4-HB, Caff and p-C were the least abundant. Nevertheless, differences among wheat species were found as reported in **Table 1** and **2**. Free t-Fer and Sin were found in higher amount in bran than the middlings by-product. In particular, they were more abundant in bread wheat bran and, in order, in *turanicum*, durum, spelt, emmer and einkorn bran. 4-HB was more concentrated in bran fractions and p-C was less variable between by-products. Interestingly this p-C was quantified below the limit of quantification (calculated as the slope of calibration curve divided by the standard deviation of response, multiplied by ten, as suggested by the ICH – International Conference on

Harmonisation) in bran and middlings of bread wheat and in spelt bran. In addition, the highest amount of caffeic acid was found in spelt bran. Regarding the durum wheat fractions, since different milling process is commonly applied, also different by-product side streams are produced. In our case, aleurone tissue, I, II and III steps of debranning were obtained from the milling processing of whole durum wheat grain. Overall, the I and II steps of debranning were the fractions in which free phenolic acids were more abundant, followed by the aleurone layer. However, the percentage in terms of weight of these fractions in respect to the whole is very low (around 10 % as sum of the three steps), thus also the contribution to the total phenolic acids content is negligible. Beside this, the *c*-Fer and diferulates were most abundant in the aleurone fraction. This cereal grain component is nowadays largely used for the production of wheat bread since it could confer a better sensorial quality (Bagdi et al., 2016) and an increased bioavailability (Bresciani et al., 2016)of bioactive compounds in respect to the wheat bran addition.

Bran, middlings and other cereal milling by-products are mainly composed by several layers of fibrous material as reported in **Table S1.** Therefore, a higher contribution of the bound phenolic component was expected. The general composition was similar to the whole grain, represented by the major PAs as t-Fer, its dimers, c-Fer and Sin, and minorities as p-C, 4-HB and caffeic acids. As for whole grains, the p-C acid was the most abundant among the secondary PAs, found in highest concentration in emmer bran. Since the ferulic acid is the most abundant PA found in cereals, the sum of *cis* and *trans* isomers accounts between 67-73 % of total PAs, in accordance to Gallardo, Jiménez, & García-Conesa,(2006). However, the bound form of this PA was preponderant, ranging between 97-99 %. In fact, the bound and insoluble phenolic compounds are mainly found linked by means of ester or ether bonds to the five carbon atoms sugars of arabinoxylans, as described by Smith & Hartley, (1983). The highest content of t-Fer was found in emmer bran, followed by spelt bran and *turanicum* bran. The dimeric form of ferulic acid was also found in milling fractions, in particular the highest value was measured in *turanicum* bran where it was present entirely in the bound form. Among by-products, the highest concentration of bound PAs was found in the bran fraction. Regarding durum wheat by-products, aleurone showed the highest content of all bound phenolic acids, indicating that these compounds are strictly connected to the polysaccharides of the cereal grain cell wall. Overall, I, II and III steps of debranning bound PAs content did not significantly differ from the aleurone layer. In fact, since the debranning process starts from the outer to the inner part of the kernels, possibly a small portion of aleurone would be present which can influence the latter composition.

The total phenolic acids content in wheat milling by-products progressively increased from the inner (endosperm) to the outer layers (bran and germ) of the seed, as depicted in **Figure 3**. The TPA content differs significantly between cereal species and the highest amount was found in emmer (2955,07±25,9 µg g $^{-1}$ d.w, bran) and *turanicum* wheat (2550,15±66,47 µg g $^{-1}$ d.w, middlings). The by-products commonly called "bran" is composed by several layers including the aleurone, testa and pericarps, as shown in **Figure 1**. They could differ between species in terms of thickness and composition and could represent a good source of bioactive compounds (Hidalgo & Brandolini, 2008; Serna-Saldivar, 2012). Furthermore, a very high concentration of phenolic compounds was seen in bran and middling samples. These compounds could play an important role in terms of plant physiology. In fact, the accumulation of ~~secondary~~specialized metabolites is a recognized strategy for plant protection against pathogens or/and biotic/abiotic stimulus (Agrios, 2005). The latter is probably the reason why phenolic acids are more concentrated in the outer layers of the seed, which constitute the first barrier between the core and the environment. In addition, each by-products account between 12-14 % of the total weight of the grain, nevertheless they are used in large amount as ingredients or supplements. For example, the concentration factor, in percentage, ranged between 206-607 % in bran samples, and 177-452 % in middling samples. Nevertheless, the seed size plays an important role during the milling process. In fact, in function of the dimension and the shape of the kernel, the portion removed during the processing could be different. In this way, grains that are bigger have a higher portion of endosperm, on the contrary in smaller grains like einkorn, the bran fraction will account for more weight (Brandolini & Hidalgo, 2011). In this way, a higher concentration factor should not be intended as a higher phenolic content, which has to be related to the % (w/w) of tissue removed from the kernel.

3.3 Free betaine and choline content

3.3.1 Whole grain and flour/semolina

Methyl donor compounds, like betaine and choline, play important role in human diet and wheat is likely to be the main dietary source (Likes, Madl, Zeisel, & Craig, 2007). **Figure 4** shows the free betaine (A) and choline (B) content of whole grains and corresponding milling by-products. The highest content of betaine was found in durum wheat whole grain (757.13±15.96 µg g $^{-1}$ d.w), while the highest choline content in emmer whole grain (26.47±1.93µg g $^{-1}$ d.w). Flour and semolina samples had the lowest betaine and choline content, meaning a detrimental effect caused by the sequential removing of the seed outer layers. For example, the betaine and choline concentration in spelt flour (360,61±53.78 and 9.94±0.73 µg g $^{-1}$ d.w, respectively), corresponds to a loss of 27 and 45 %, respectively, as compared to the whole grain. Overall, the pauperization effect caused by the milling determined a decreased betaine and choline content, which ranged between 23-94 % and 28-67 %, respectively. These results are in agreement with those reported by Likes, Madl, Zeisel, & Craig, (2007). However, the variability in betaine and choline content can differ in respect to cereal genotypes and in function of the growing conditions (Corol et al., 2012). In addition, as discussed by Burg, Ferraris, & Dmitrieva, (2007) and Ross et al., (2014), betaine acts as an osmolyte in plants, hence its occurrence is related to the osmotic stresses and proper cell volume regulations. In this study the betaine content of bread wheat was 367.56±17.88 µg g $^{-1}$ d.w. Similar values for bread wheat whole grain were found by Kojić et al., (2017), who studied the betaine levels in cereals pseudocereals and their products. However, the betaine content in spelt grain (495.82±84.03 µg g $^{-1}$ d.w) was lower than the level found in that study. Furthermore, *turanicum*, einkorn and emmer free betaine content was 378.61±32.69, 415.17±0.86 and 427.91±6.34 µg g $^{-1}$ d.w, respectively. Choline content was lower and ranged between 17.37±1.16 (*turanicum*) – 22.55±0.89 µg g $^{-1}$ d.w (durum wheat). These findings are slightly lower than the results reported by Ross et al., (2014). Nevertheless, the high results variability could be due to the different extraction and analysis methods. However, in this study, the importance of the sequential extraction of the matrix is underlined as explained by Bruce et al., (2010) and Hefni, McEntyre, Lever, & Slow, (2016).

3.3.2 Milling by-products

The determination of specialized metabolites of different cereal fractions obtained by technological process can be useful for the evaluation of nutrients distribution and availability and also can provide information about the degree of the milling step. Between the two methyl-donor compounds, betaine occurs in higher concentration than choline. The content of the free betaine in cereal milling by-products was higher in bran, as already reported in the study conducted by Likes et al., (2007), Graham, Hollis, Migaud, & Browne, (2009), Ross et al., (2014) and Kojić et al.,(2017).

The highest content of free betaine was found in bread wheat bran (1720,05±160.94 µg g $^{-1}$ d.w) while choline was the highest in durum wheat bran (81.71±3.70 µg g $^{-1}$ d.w) and emmer middlings (78.2±0.20 µg g $^{-1}$ d.w). In addition, also spelt bran appears to be a good source of betaine (1464.12±115.78 µg g $^{-1}$ d.w). These findings are slightly lower than those reported by Bruce et al., (2010), however they fall in the range proposed by Patterson et al., (2008). Nevertheless, they were very different to the results found by Zeisel, (2006) and Filipčev, Kojić, Krulj, Bodroža-Solarov, & Ilić, (2018). The latter could be related to the fact that different methods were employed, although more studies must clarify this high variability. In addition, it is possible that an important portion of these compounds is present in bound or linked form in cereals. Interestingly, the choline content in middlings of ancient wheat species appears to be richer than in the bran fraction, while this trend was opposite in durum and bread wheats. In relation to the durum wheat milling process, the II step of debranning was found to be significantly richer in betaine and choline content, confirming that methyl-donors are mostly present in the outer layer of cereal grain.

4 Conclusion

In conclusion, a wide variability in terms of bioactive compounds was found among the *Triticum* L. species and by-products. Regarding phenolic acids, the soluble component was highly lower than the bound and insoluble component, representing in average ~ 80 % of the total phenolic acids. Among them, ferulic acid was the predominant, especially in the outer layers (bran and middlings) where it was found strictly bound to the matrix (~ 80 %). In addition, also ferulic acid *cis* isomer and its dimeric form were found in high amount. Bread wheat whole grain had the highest total phenolic acids content, nevertheless when by-products were compared, emmer bran was the highest one; this fact underlines the importance of the shape, dimension and processing variables. In relation to the free betaine and choline content, in this study we confirmed the relevance of cereal grains and by-products as good sources of these compounds. The distribution trend reflects the one of phenolic compounds, decreasing toward the inner part of the seed. Overall, the milling process extremely reduced the content of bioactive compounds, meaning that the end-

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products were poor in these substances. However, the re-integration of the outer layers to produce a wholemeal flour allowed to overcome the problem, as seen for einkorn wholemeal flour. Finally, information regarding the occurrence of these type of compounds in Triticum flours and by-products could be useful to the determination of accurate levels of intake.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting Information description

Appendix A

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Figure captions

Figure 1. Simplified anatomy of wheat seed.

Figure 2. Chromatogram and mass spectrum of the full scan analysis of the ion *m/z* 385 (diferulate). The EIC (extracted ion chromatogram) of ion *m/z* 193 (ferulic acid) is represented in red. The ion fragments surrounded by red circles represent the characteristic fragmentation pattern of the ferulic acid.

Figure 3. Total phenolic acids (TPA) content of *Triticum* whole grains and milling by-products. Different letters on top of bars indicate a significant difference ($p < 0.05$) using Tukey-b's post-hoc test. The contribution of the different phenolic acids on the total content is also showed.

Figure 4. The free betaine (A) and choline (B) content in whole grain and milling fractions of wheat species. The results are expressed on a d.w. basis. Different letters on the top of the bars indicate a significant difference ($p<0.05$) using the *Tukey-b's* post hoc test.

Tables

Table 1. Free and bound phenolic acids content (µg g $^{-1}$ d.w.) of different whole grain *Triticum* L. species and corresponding milling by-products.

Wheat species	Milling product	4-HB		p-C		Caff		Sin	
		Free	Bound	Free	Bound	Free	Bound	Free	Bound
Durum wheat	WG	0.14±0.07d	1.45±0.25d	0.14±0.02cde	6.51±+0.60c	0.12±0.00e	0.46±0.08cd	0.66±0.48c	24.91±2.93de
	S	<LOQ	<LOQ	0.06±0.01e	0.18±0.26cd	0.13±0.00e	<LOQ	<LOQ	4.12±0.57e
	B	0.61±0.05b	5.81±0.47ab	0.22±0.04cd	13.50±0.09ab	0.25±0.00cd	1.45±0.02ab	7.06±0.14b	75.86±4.28b
	FB	0.25±0.03d	4.22±0.42abc	0.11±0.03de	9.85±0.34abc	0.20±0.06de	1.13±0.11abc	2.37±1.34de	62.71±2.87bc
	M	0.40±0.05bc	3.22±0.94c	0.18±0.02cde	7.90±4.33bc	0.28±0.01c	1.00±0.37bc	3.74±0.17cd	58.55±9.71bc
	A	0.46±0.23bc	5.91±0.96a	0.36±0.07ab	14.52±2.44a	0.18±0.01e	1.82±0.41a	4.91±1.29bcd	120.29±25.60a
	I	1.43±0.05a	3.98±0.43abc	0.47±0.01a	9.54±0.87abc	0.43±0.01a	0.95±0.06bc	11.19±0.29a	35.60±1.99cde
	II	1.43±0.10a	4.95±0.27abc	0.27±0.01bc	10.53±0.07abc	0.36±0.01b	1.46±0.10ab	6.42±0.85bc	63.42±3.55bc
	III	0.46±0.01bc	3.86±0.23bc	0.20±0.03cd	10.44±0.88abc	0.28±0.00c	1.30±0.16ab	3.56±0.13cd	53.88±1.66bcd
Bread wheat	BWG	0.11±0.05b	2.30±0.07b	0.07±0.01	5.02±0.44b	0.31±0.06b	1.22±0.03b	1.19±0.08c	40.29±3.24ba
	F	<LOQ	0.30±0.04b	<LOQ	<LOQ	0.14±0.01b	<LOQ	<LOQ	2.38±0.25b
	BB	0.48±0.19a	11.73±3.69a	0.08±0.02	16.91±5.34a	0.78±0.14a	4.92±1.4a	7.93±1.42a	123.77±49.82a
	BM	0.19±0.04b	5.02±0.12b	0.09±0.01	9.12±0.35b	0.39±0.00b	0.87±0.10b	3.35±0.37b	84.96±1.94ba
Turanicum	TWG	0.10±0.02b	1.48±0.29c	0.08±0.01	4.44±0.06c	0.12±0.00	0.53±0.05b	3.71±0.92b	13.42±1.16b
	TF	<LOQ	0.63±0.00c	<LOQ	1.15±0.27d	0.12±0.00	0.16±0.01c	<LOQ	4.78±0.86b
	TB	0.63±0.25a	10.87±0.39b	0.19±0.09	20.78±0.34b	0.14±0.01	3.16±0.05a	6.08±1.23a	59.70±1.57a
	TM	0.41±0.10b	12.70±0.46a	0.16±0.05	25.71±1.39a	0.14±0.00	3.07±0.13a	2.99±0.41b	68.36±6.65a
Einkorn	MWG	0.18±0.07	1.52±0.56b	0.06±0.01	4.65±2.03b	1.07±0.12	0.38±0.13b	0.54±0.23b	29.01±9.89b
	WMF	0.29±0.12	1.41±0.07b	0.09±0.01	5.42±0.27b	1.22±0.09	0.58±0.00b	1.12±0.16b	29.77±0.30b
	MM	0.51±0.17	5.84±0.32a	0.16±0.09	19.47±0.06a	1.70±0.73	5.33±0.07a	1.56±0.85a	116.11±0.45a
Emmer	DWG	0.11±0.03	2.28±0.06c	0.07±0.00	11.12±0.64c	0.35±0.01	1.10±0.13c	1.14±0.02	29.49±7.91c

	DF	<LOQ	1.26±0.09d	<LOQ	3.23±0.14d	0.26±0.02	0.70±0.05c	0.36±0.03	21.92±0.67c
	DB	0.27±0.06	14.84±0.10a	0.09±0.00	43.05±1.63a	0.45±0.01	4.27±0.30a	2.05±0.56	169.28±5.95a
	DM	0.25±0.17	7.63±0.29b	0.08±0.03	17.26±0.9b6	0.51±0.22	2.90±0.09b	1.45±0.79	140.41±1.61b
Spelt	SpWG	0.09±0.03c	1.55±0.01c	0.05±0.00	5.15±0.47c	0.44±0.00c	0.82±0.24c	0.84±0.01c	22.80±1.67c
	SpF	<LOQ	<LOQ	<LOQ	<LOQ	0.16±0.02d	<LOQ	<LOQ	3.82±0.12d
	SpB	0.73±0.21a	9.14±0.28a	0.07±0.02	21.04±0.51a	1.02±0.06a	7.54±0.53a	3.19±0.36a	115.42±3.08a
	SpM	0.49±0.15b	4.09±0.11b	0.10±0.00	10.55±0.14b	0.93±0.02b	5.02±0.22b	2.38±0.03b	75.00±2.17b

4-HB: 4-hydroxybenzoic acid; p-C: p-Coumaric acid; Caff: caffeic acid; Sin: sinapic acid. Results are expressed as μg gr $^{-1}$ d.w. Different letters in the same column indicate a significant difference ($p<0.05$) using Tukey-b's post-hoc test. <LOQ: 0.05 μg g $^{-1}$ d.w.

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Table 2. Content (µg g $^{-1}$ d.w.) of the different ferulic acid forms in *Triticum* L spp. whole grains and corresponding milling by-products.

Wheat species	Milling product	t-Fer		c-Fer	Dif
		Free	Bound		
Durum wheat	WG	1.72±0.01d	369.70±67.3bc	91.56±1.84cd	314.07±65.26c
	S	<LOQ	45.37±2.33c	<LOQ	<LOQ
	B	6.74±0.41b	1093.59±63.15a	235.83±8.44bc	564.46±19.66ab
	FB	2.19±1.28d	759.44±3.85ab	214.18±0.76bc	394.43±26.64bcd
	M	3.38±0.45cd	722.59±312.56ab	225.46±94.68bc	441.26±1.82bcd
	A	2.92±0.38d	1094.20±249.70a	430.18±94.91a	665.89±138.49a
	I	12.45±0.38a	623.29±33.98ab	201.25±16.58bc	342.02±27.61cd
	II	4.94±0.06c	965.59±5.62a	289.83±15.54b	554.38±32.05ab
	III	2.42±0.29d	821.00±3.01ab	268.85±14.26b	522.86±29.44abc
Bread wheat	BWG	1.94±0.65b	564.15±0.88c	253.89±11.93b	338.80±0.06a
	F	<LOQ	19.00±1.53d	16.73±1.79c	34.04±1.99b
	BB	9.52±1.56a	1330.79±7.93a	445.95±1.87a	533.25±172.83a
	BM	3.07±0.74b	1148.41±63.76b	273.97±40.49b	616.47±34.32a
Turanicum	TWG	1.02±0.29c	208.33±15.05c	46.44±0.47c	345.28±0.62c
	TF	<LOQ	66.40±3.03d	29.62±0.62d	104.82±14.72d
	TB	6.80±1.82a	1571.14±59.63a	352.18±28.27b	822.67±56.58a
	TM	3.84±0.60b	1331.04±74.22b	442.59±10.05a	659.14±9.77b
Einkorn	MWG	1.08±0.35	336.37±1.2b	88.07±1.21c	133.43±52.28b
	WMF	0.80±0.13	219.67±3.36c	117.49±3.92b	119.03±46.97b
	MM	1.34±0.32	1239.70±10.94a	421.75±1.61a	325.64±14.32a
Emmer	DWG	1.19±0.08b	310.66±3.28c	64.25±10.52c	113.44±18.92c
	DF	0.69±0.00b	176.90±8.67d	37.32±0.01d	161.37±2.6b
	DB	2.90±0.24a	1894.73±0.26a	338.00±34.87a	485.13±2.69a

	DM	1.90±0.73ab	1340.13±4.94b	188.03±7.21b	500.91±0.10a
Spelt	SpWG	1.27±0.28c	300.64±36.32c	21.26±1.92c	234.94±22.34b
	SpF	0.60±0.01d	32.39±3.69d	<LOQ	43.83±1.63c
	SpB	4.73±0.13a	1696.15±33.59a	230.24±16.79a	580.57±64.49a
	SpM	3.31±0.01b	1033.24±28.57b	50.48±14.69b	517.23±11.02a

t-Fer: *trans*-ferulic acid; *c*-Fer: *cis*-ferulic acid; Dif: diferulic acid. Results are expressed a µg/gr d.w. Different letters in the same column indicate a significant difference ($p<0.05$) using *Tukey-b's* post-hoc test. <LOQ: 0.05 µg g $^{-1}$ d.w.

Figure graphics

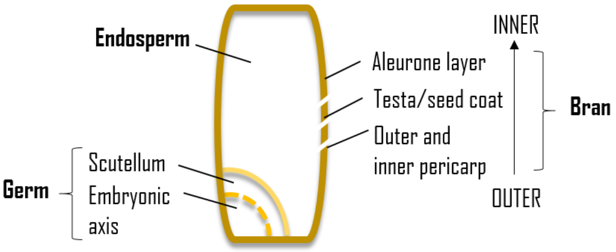


Figure 1

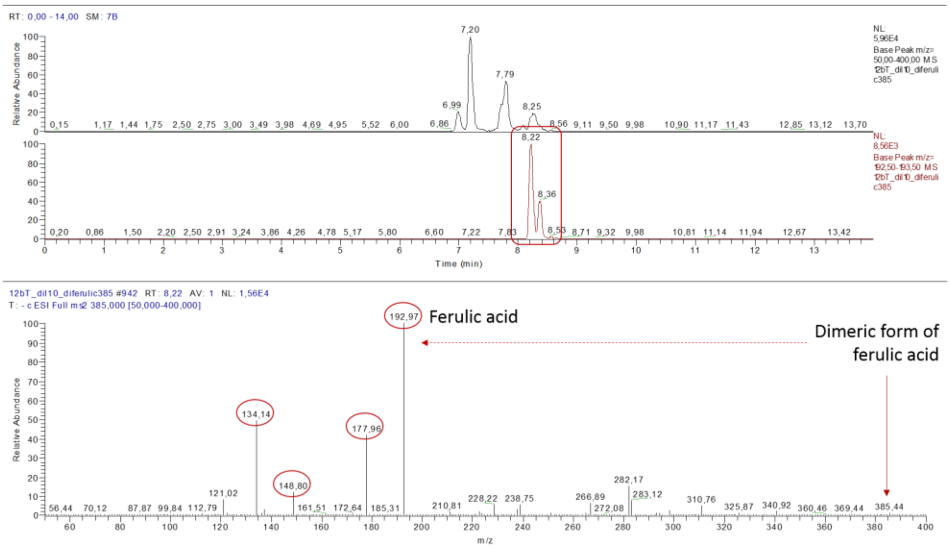
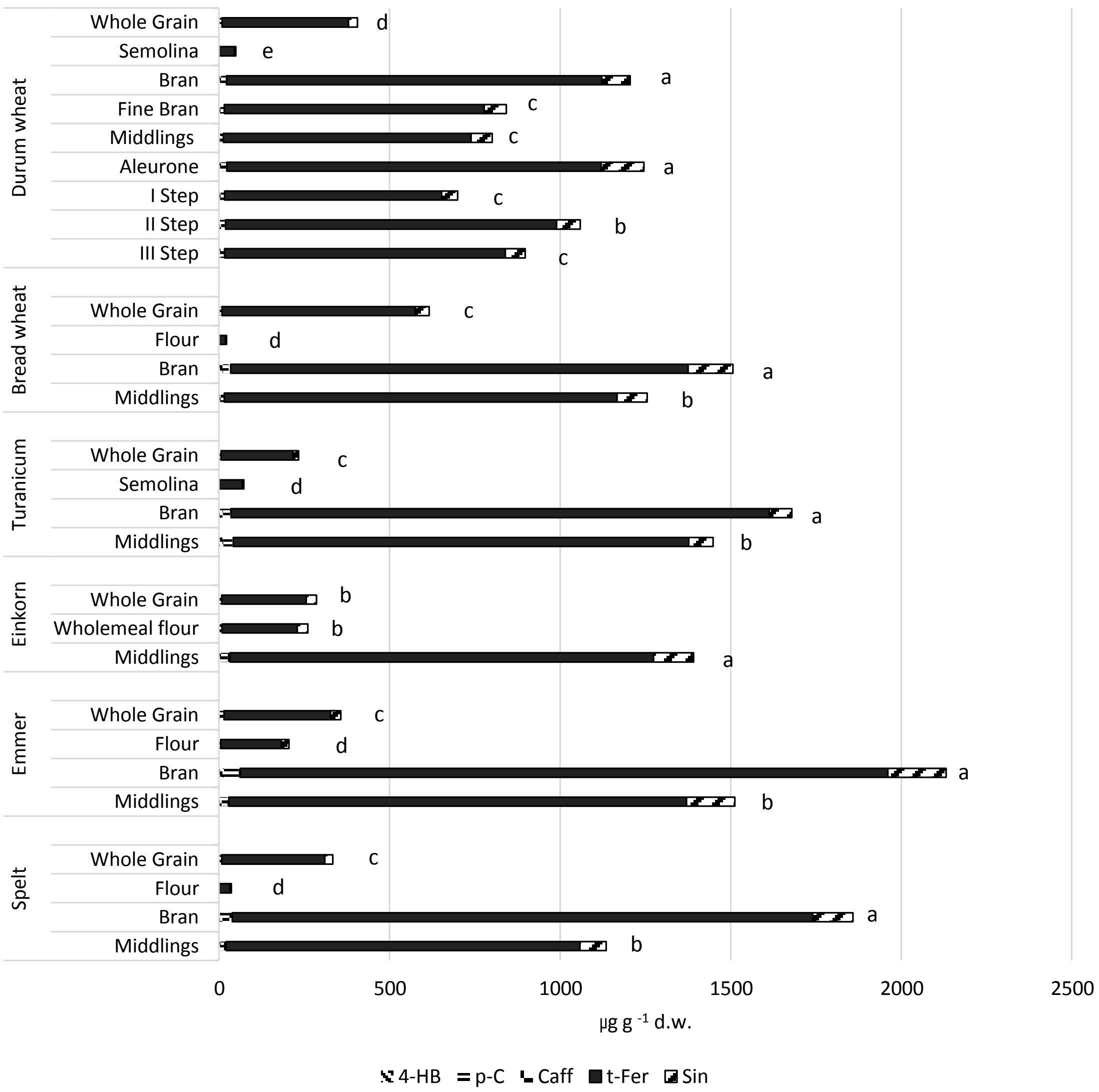


Figure 2



Durum wheat
Whole Grain
d
Semolina
e
Bran
a
Fine Bran
c
Middlings
c
Aleurone
a
I Step
c
II Step
b
III Step
c
Bread wheat
Whole Grain
c
Flour
d
Bran
a
Middlings
b
Turanicum
Whole Grain
c
Semolina
d
Bran
a
Middlings
b
Einkorn
Whole Grain
b
Wholemeal flour
b
Middlings
a
Emmer
Whole Grain
c
Flour
d
Bran
a
Middlings
b
Spelt
Whole Grain
c
Flour
d
Bran
a
Middlings
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500
1000
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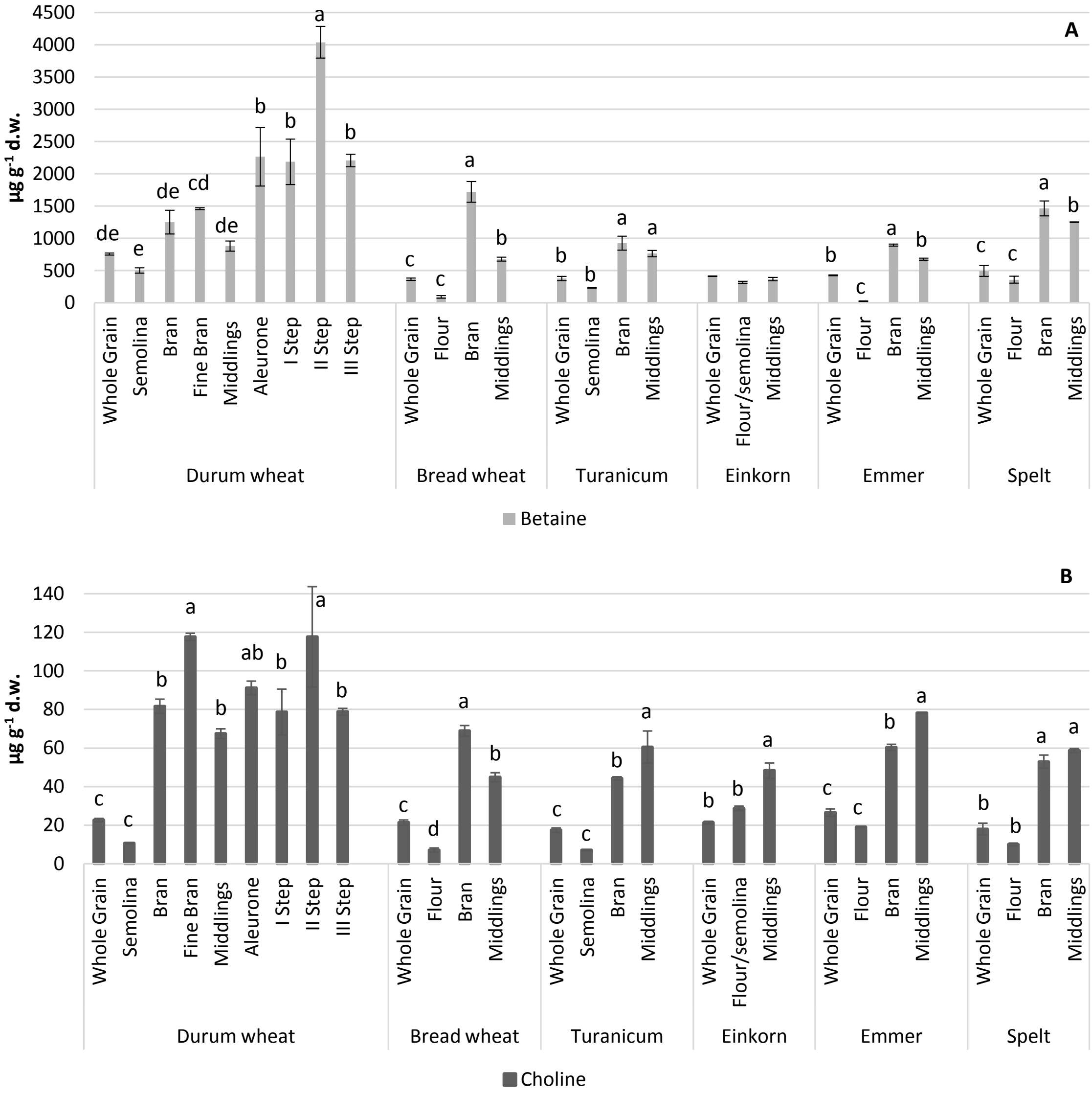
2000
2500
μg g -1 d.w.
4-HB
p-C
Caff
t-Fer
Sin

588 **Figure 3**


A
µg g-1 d.w.
4500
4000
3500
3000
2500
2000
1500
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500
0
de
e
de
cd
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b
b
a
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c
c
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b
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a
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c
a
b
Whole Grain
Semolina
Bran
Fine Bran
Middlings
Aleurone
I Step
II Step
III Step
Whole Grain
Flour
Bran
Middlings
Whole Grain
Semolina
Bran
Middlings
Whole Grain
Flour/semolina
Middlings
Whole Grain
Flour
Bran
Middlings
Whole Grain
Flour
Bran
Middlings
Durum wheat
Bread wheat
Turanicum
Einkorn
Emmer
Spelt
Betaine
B
µg g-1 d.w.
140
120
100
80
60
40
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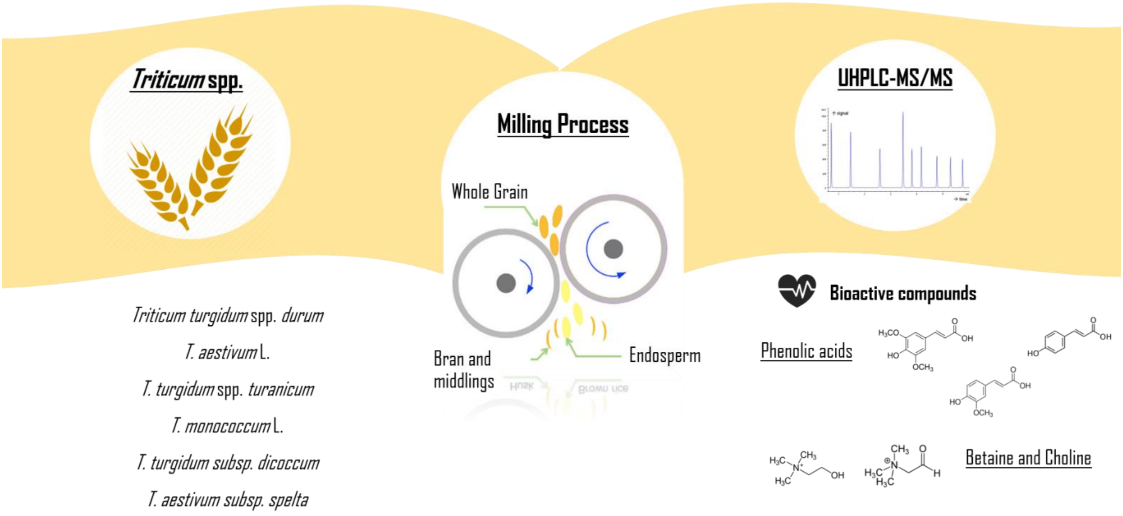
Whole Grain
Semolina
Bran
Fine Bran
Middlings
Aleurone
I Step
II Step
III Step
Whole Grain
Flour
Bran
Middlings
Whole Grain
Semolina
Bran
Middlings
Whole Grain
Flour/semolina
Middlings
Whole Grain
Flour
Bran
Middlings
Whole Grain
Flour
Bran
Middlings
Durum wheat
Bread wheat
Turanicum
Einkorn
Emmer
Spelt
Choline

Figure 4

Graphic for table of contents



The impact of processing on the phenolic acids, free betaine and choline in *Triticum* spp. L. whole grains and milling by-products.

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SUPPLEMENTARY MATERIAL

Table 1S. Proximate composition of the whole grains and milling products of wheat species.

Wheat species	Milling product	Moisture	Ash	Crude Fat	Proteins	Carbohydrate*	TDF
Durum wheat	Whole grain	14.8±0.2	1.63±0.0	1.2±0.2	10.3±1.0	72.0	16.3±1.6
	Semolina	12.7±0.2	0.8±0.0	1.6±0.1	9.6±1.1	75.3	4.7±0.5
	Bran	11.3±0.2	4.4±0.0	3.3±0.3	12.6±1.2	68.4	40.1±3.1
	Fine Bran	10.2±0.2	4.4±0.0	3.1±0.2	13.5±1.0	68.8	25.5±2.1
	Middling	9.8±0.2	3.9±0.0	3.4±0.2	12.8±1.0	70.1	31.2±1.8
	Aleurone	10.1±0.2	5.3±0.0	5.5±0.2	17.1±1.2	62.0	33.4±2.1
	I Debr	15.8±0.2	4.4±0.0	0.6±0.2	11.1±1.1	68.1	49.4±3.5
	II Debr	13.9±0.2	4.8±0.0	3.2±0.2	14.4±1.2	63.6	40.2±2.6
	III Debr	13.7±0.2	4.7±0.0	2.5±0.2	14.7±1.1	64.3	31.5±1.9
Bread wheat	Whole grain	14.4±0.2	1.63±0.0	2.5±0.2	14.2±1.1	67.3	15.5±1.6
	Flour	13.0±0.2	0.51±0.0	1.1±0.1	13.8±1.1	71.7	4.0±0.4
	Bran	12.7±0.2	6.87±0.0	3±0.2	16.3±1.2	61.2	45.2±4.5
	Middling	11.3±0.2	2.78±0.0	4.1±0.2	15.3±1.2	66.5	20.9±2.1
Turanicum	Whole grain	11.8±0.2	1.7±0.0	1.7±0.2	13.2±1.0	71.6	13.0±1.3

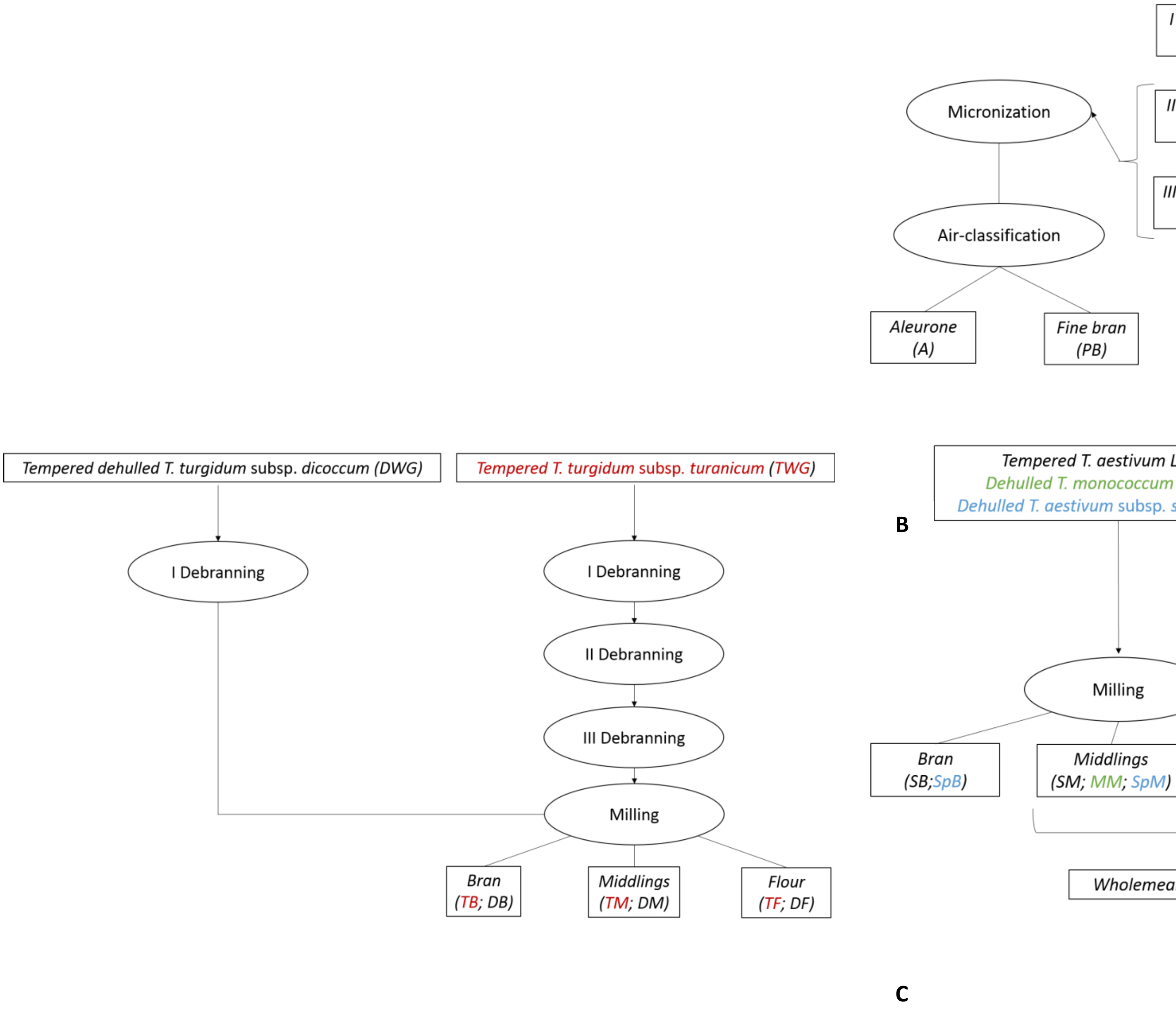
	Semolina	11.4±0.2	0.8±0.0	0.9±0.1	12.5±1.0	74.4	4.8±0.5
	Bran	9.1±0.2	4.5±0.0	3.3±0.2	14.5±1.1	68.6	40.7±1.0
	Middling	9.0±0.2	5.0±0.0	3.2±0.2	15.2±1.2	67.5	37.2±3.7
Einkorn	Whole grain	9±0.2	1.8±0.0	3.3±0.2	10.9±0.9	75.0	10.0±1.0
	WM flour	8.8±0.2	1.8±0.1	3.3±0.2	10.9±0.9	75.2	9.9±1.0
	Middling	7.5±0.2	6.2±0.0	6.0±0.3	15.5±1.2	64.8	25.7±2.5
Emmer	Whole grain	6.4±0.2	1.9±0.0	2.5±0.2	11.1±0.9	78.1	12.6±1.3
	Flour	5.9±0.2	1.4±0.0	2.00±0.2	10.8±0.9	80.0	7.1±0.7
	Bran	6.5±0.2	6.6±0.0	6.0±0.3	14.0±1.1	66.9	33.9±3.4
	Middling	5.2±0.2	5.4±0.0	6.4±0.3	14.7±1.1	68.4	23.3±2.3
Spelt	Whole grain	9.5±0.2	1.9±0.0	2.7±0.2	11.7±0.9	74.2	13.9±1.4
	Flour	8.5±0.2	0.8±0.0	1.8±0.2	10.6±0.9	78.3	4.8±0.5
	Bran	8.3±0.2	8.0±0.0	5.9±0.3	15.3±1.2	62.5	39.9±4.0
	Middling	7.5±0.2	4.9±0.0	7.0±0.3	16.4±1.3	64.1	27.4±2.7

*calculated by difference, TDF, total dietary fibre; The results are expressed as average ± MU (measurement uncertainty) (%) on a d.w. basis.

Table 2S. Industrial milling by-products.

Fraction	**Description**	Average diameter (µm)	Median diameter (µm)	Amplitude distribution (Span)
Whole grain	Whole kernels coming from different commercial lots	-	-	-
Semolina/refined flour	Semolina is the main product of durum wheat milling while flour is the main product of wheat, einkorn, emmer, spelt and *turanicum* milling. Both semolina and flour are from the endosperm: vitreous endosperm is for durum wheat and floury endosperm is for the other species. Average particle sizes are 250 µm for semolina and 100 µm for refined flour respectively	-	-	-
Bran	Bran separated during the milling phase with particle size ranging between 0.8-1.2 mm	1050	950	1.5
Middlings	Fine bran particles and fine endosperm particles with some bran still attached	400	390	1.5
Aleurone	Fraction obtained by air-classification of the debranning fractions and containing a significant amount of aleurone layer	-	-	-
I, II and III steps of debranning	Fine bran fractions obtained through the first, second or third debranning steps having different particles sizes	680 (I), 450 (II), 310 (III)	530 (I), 370 (II), 220 (III)	2.2 (I), 2.4 (II), 3.0 (III)

Figure 1S: Flow charts of *Triticum turgidum* subsp. *durum* Desf. (A), *T. turgidum* subsp. *turanicum*, *T. turgidum* subsp. *dicoccum* (B) and *T. aestivum* L, *T. aestivum* subsp. *spelta* and *T. monococcum* L (C) milling process.



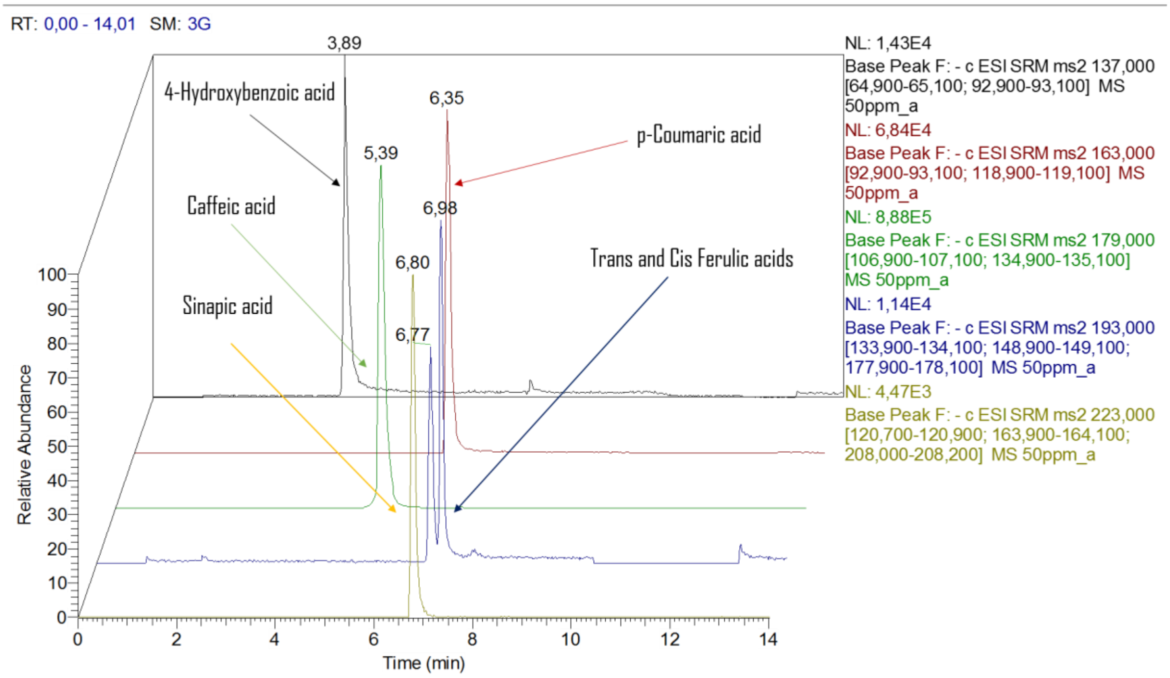


Figure 2S. Chromatograms of the monitored phenolic acids (standard reference at 50 µg g $^{-1}$).

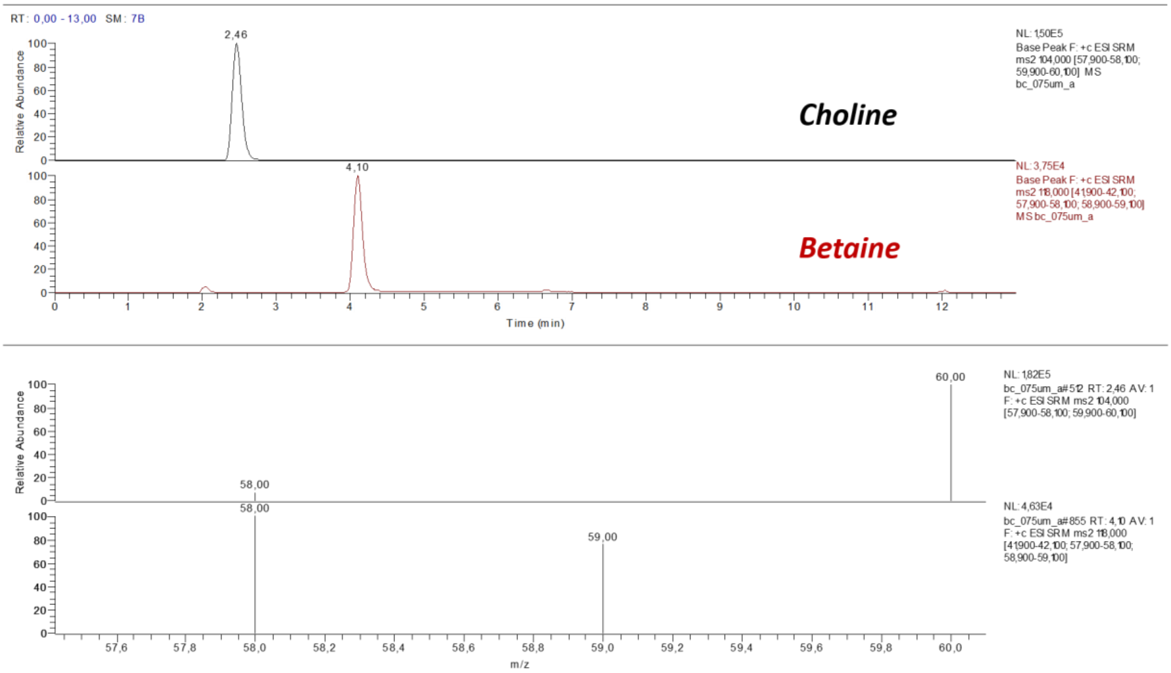


Figure 3S. Chromatogram and mass spectra of betaine and choline standard reference working solution.

***Declaration of Interest Statement**

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

RESPONSE TO REVIEWERS

Reviewer #2:

All the comments reported in the pdf file have been considered and addressed.

Reviewer #3:

In all of the MS, the discussion should be improved.

It has been done along the text

2. All figures and Tables need to be modified for uniformity and clarity.

This MS is based on a huge amount of different data, and we are aware that formatting them for clarity and consistency can be an issue. Therefore, we've put particular attention in Table/Figure reporting and, at best of our knowledge, the current layout is the best we can achieve. We will be more than willing to follow any reviewer's suggestion to improve clarity.

3. In Introduction section, the author (s) should explain about the classification of phenolic compounds in the plants.

Honestly, we don't think this should be included in the manuscript, or extensively treated, for meeting the expectation of the average Food Chemistry reader. The MS is already quite long, and adding info on phenolics in plants would steel lines useful for other less well-known points.

4. It will be better to provide important mean values in the Abstract.

We have improved our abstract accordingly

5. The term 'secondary metabolites' is often replaced by 'specialised metabolites' these days. What do you think?

We amended the text accordingly, introducing the concept for those readers that are less familiar with the term.