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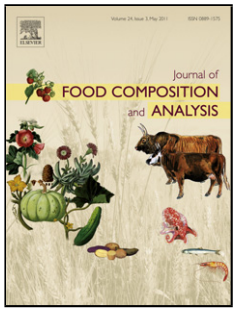

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What's new on total phenols and γ-oryzanol derivatives in wheat? A comparison between modern and ancient varieties

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Highlights

- High rate plants/m^2 promoted higher phenolic content in some ancient wheat varieties
- Campestanyl and sitostanyl ferulates were the main steryl ferulates in wheat
- Steryl ferulates were proposed as markers for cereals differentiation
- Ancient whole-wheat pasta showed 50% higher steryl ferulates than modern one

Abstract

A big challenge for our century concerns discovering new foods, including re-discovering ancient wheat. Phenolic and steryl ferulates composition of Tuscan and Campania wheat samples was evaluated by HPLC-DAD-MS/MS. Cinnamic acids and flavonoids were expressed as ferulic acid and schaftoside equivalents, respectively. Agronomic conditions with a higher rate of plants/m^2 promoted higher amounts of phenols in most of the Tuscan varieties. The ancient varieties Gentil Rosso (1137 µg/mg dry weight) and Carosella 2 (854 µg/mg dry weight) showed the highest phenols amount, determined by HPLC-DAD. Steryl ferulates were expressed as ferulic acid equivalents. Campestanyl and sitostanyl ferulates were found as main steryl ferulates compounds in wheat, differently from rice, millet and sorghum; 10 steryl ferulates and 2 caffeoyl phytosterols

were tentatively identified. Total steryl ferulates in Tuscan and Campania wheat samples ranged from 37.6 to 62.3 µg/g dry weight and from 14.4 to 56.6 µg/g dry weight, respectively. Ancient whole wheat pasta showed 50% higher steryl ferulates with respect to modern whole wheat pasta. The study compared for the first time the content of γ-oryzanol derivatives in ancient and modern wheat varieties.

Keywords: acid hydrolysis; steryl ferulates; HPLC-DAD-MS/MS; ferulic acid; ancient wheat; polyphenols.

1. Introduction

Food products derived from cereal grains constitute a major part of the daily diet and wheat is considered the most important cereal crop worldwide (Shewry & Hey, 2015). Wheat is a leading grain for consumption due to its nutrient profile, relatively easy harvesting, storage, transportation and processing, compared to other grains (Poutanen, 2012).

From a botanical point of view, wheat includes several species belonging to the genus *Triticum*. The best known species are *Triticum aestivum* L. and *Triticum durum* L. The former, also known as "common wheat", "bread" or "soft wheat", is a hexaploid wheat that constitutes 95% of the total wheat production, and its flour is suitable for bread and bakery products. The remaining 5% includes *Triticum durum* L., a tetraploid wheat also known as "durum wheat", which is traditionally used for the production of pasta (Dhanavath & Prasada Rao, 2017; Dinu, Whittaker, Pagliai, Benedettelli & Sofi, 2018). A further classification recognizes wheat as "ancient", which has remained genetically unchanged over the last hundred years, or "modern" grains, which has been extensively modified and subject to cross-breeding during the “Green Revolution” (Dinu et al., 2018). Indeed, in the last century, wheat breeding efforts concentrated on yield increases, typically using high-energy inputs in terms of fertilizers, herbicides, insecticides and fungicides, to produce modern varieties characterized by genetic uniformity and adaptation to conventional agriculture (Arzani & Ashraf, 2017; Dinu et al., 2018; Zamaratskaia, Gerhardt & Wendin, 2021). This agronomic trend is commonly referred to as the Green Revolution, when Mendel’s laws were systematically applied to increase production and technological quality of wheat grains. Today, most wheat species are hybrids created from ancient wheat over the last 100 to 150 years. Compared to ancient varieties, the modern ones are characterized by several advantages such as higher yields, tolerance to environmental stresses, lower susceptibility to pathologies and insects, higher glutenin content leading to technological improvement in the quality of bread and pasta (Mefleh et al., 2019). Although these modern wheat varieties have a positive response in terms of

production compared with the ancient wheat, several differences have been highlighted regarding the nutritional value and the technological performances between ancient and modern wheat varieties (Dinu et al., 2018; Ruisi et al., 2021). It is worth to be mentioned that the literature studies are mainly focused on ancient wheat varieties as emmer (*Triticum dicoccum* L.), einkorn (*Triticum monococcum* L.) and spelt (*Triticum spelta* L.).

Considering the starch content, lower amounts have been found in ancient wheat samples, with respect to modern ones (Arzani et al., 2017). However, the content of the slowly digested amylose in ancient varieties as Khorasan (*Triticum turgidum* L.) was significantly higher than amylopectin, decreasing both glucose and insulin post prandial levels (Sofi et al., 2013). When analyzing the lipid fraction, it was also observed that einkorn (*Triticum monococcum* L.) contains approximately 50% more lipids than modern grains, with higher amount of monounsaturated fatty acids and approximately 21% less saturated fatty acids (Hidalgo, Brandolini & Ratti, 2009). These values are associated with a reduction in total cholesterol, LDL cholesterol and triglycerides with a marked improvement in the lipid profile of diabetic patients or those with a high risk of cardiovascular diseases (Dinu et al., 2018). With regard to the mineral content, modern grain samples have lower amounts, especially in iron and zinc, than ancient ones (Erba, Hidalgo, Bresciani & Brandolini, 2011; Zhao et al., 2009). The lack of these elements in populations of developing countries reinforces the need to support local and diversified cereal crops rich in such micronutrients. Considering the carotenoids fraction, ancient grain samples showed 8-10 times higher levels of lutein, the main carotenoid found in cereals, than modern grain, contributing to enhance the antioxidant properties of the former (Ziegler et al., 2015).

Although a number of studies have been published focusing on the content of bioactive molecules of emmer (*Triticum dicoccum* L.), einkorn (*Triticum monococcum* L.) and spelt (*Triticum spelta* L.), less information is available on heritage cultivars of *Triticum aestivum* L. and *Triticum durum* L. Furthermore, definitive comparisons of these species grown together in randomised field plots are currently rare (Shewry, 2018). The reason behind this is that ancient wheat is usually grown in organic or traditional low-input farming systems, while modern wheat is usually bred for high-input intensive systems (Dinu et al., 2018; Hidalgo et al., 2009).

In this context, establishing the amount of phenolic compounds in ancient and modern wheat samples grown together in the same field can be valuable in order to select specific cereal grains suitable for the production of health-promoting staple food (Gotti et al., 2018). Phenolic compounds as phenolic acids and flavonoids are mainly found in the outer layer of the caryopsis. Most of them are bound by ester and ether linkages with cell wall polysaccharides (Memon et al., 2020; Wang et al., 2020). Among wheat's bioactive compounds, steryl ferulates, which are esters of phytosterols

with ferulic acid, can be considered a class of phenolic compounds, due to their phenolic moiety (Hakala et al., 2002; Ziegler, Schweiggert, Würschum, Longin & Carle, 2016). Wheat bran fractions are high in steryl ferulates, which contribute up to 17% of total phytosterols (Nyström, Paasonen, Lampi, & Piironen, 2007). Research into steryl esters has mainly focused on rice and its γ-oryzanol content (Cho, Lim, Rehman, Farooq & Lee, 2019; Waraksa et al., 2019) because of its demonstrated beneficial effects on human health (Andersson, Dimberg, Åman, & Landberg, 2014; Burlando, B., & Cornara, L. 2014;). γ-Oryzanol, initially reported as single component, is now known to be a mixture of 10 molecules, with cycloartenyl ferulate, 24–methylene cycloartanyl ferulate and campesteryl ferulate as major components (Sawada et al., 2021). The health benefits associated with γ-oryzanol include antinflammatory, antioxidant and chemopreventive properties (Burlando, & Cornara, 2014; Kim, Kang, Nam, & Friedman, 2012; Mattei et al., 2021). It has also been proposed in the treatment of diabetes mellitus and prostate cancer (Kozuka et al., 2015; Son, Rico, Nam, & Kang 2011). Furthermore, free or esterified plant sterols/stanols have been recognized by the European Food Safety Authority for decreasing of plasma cholesterol and for maintenance of normal blood cholesterol levels (Andersson et al., 2014; EFSA, 2010, 2011; Commission Regulation No. 432/2012). Despite the beneficial properties of these compounds, quantitative studies on cereals other than rice are currently rare. In particular, little information is available on the steryl ferulates in ancient and modern grains.

The aim of this research was to investigate on the different classes of phenolic compounds, including phytosterols bound to ferulic acid, of several ancient wheat species grown in Tuscany and Campania regions. The comparison between ancient and modern wheat samples was carried out evaluating plants grown together in the same field. In particular, the Tuscan varieties were cultivated under two different agronomic conditions: with a low and with a high rate of plants per m^2. Two acidic hydrolyses were compared in terms of recovered phenols, to define the best extractive procedure. The extractive method which showed the highest recovery of phenols was applied to Tuscan and Campania wheat samples. The steryl ferulates composition of the ancient and modern wheat samples was compared with those obtained for other common cereals as rice, millet, sorghum and spelt. Finally, to estimate the actual potential intake of steryl ferulates in the diet, four different types of pasta made by ancient and modern wheat were analysed. All the analyses were performed by HPLC-DAD-MS/MS also applying High Resolution Mass Spectrometry (HRMS) (i.e., HPLC-HRMS/MS).

2. Materials and methods

2.1 Chemicals

Ultrapure water was obtained by the Milli-Q-system (Millipore SA, Molsheim, France). All solvents with analytical HPLC grade, sulfuric acid (95.0-98.0 %), sodium hydroxide (≥ 98 %), and ferulic acid standard were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Standard of schaftoside was purchased from Extrasynthese (Genay, France).

2.2 Samples

A total of 21 wheat samples were collected. Eight ancient and two modern wheat varieties were purchased in 2018 from Cesa (Arezzo, Tuscany, Italy), and were cultivated under two densities: d1= 250 plants/m^2 (density usually applied for the ancient varieties) d2= 350 plants/m^2 (density usually applied for the modern varieties). The samples were grown in a medium-textured soil tending to loamy, in a low-input system. The climate of the growing area is Mediterranean, characterized by a daily average temperature of 14°C with amount of precipitation of approx. 990 mm/year. Eight ancient and three modern wheat varieties were cultivated in 2019 in Caselle in Pittari (Salerno, Campania, Italy). The ancient and modern varieties included in the study are listed in Table 1 and were as follows. Ancient varieties from Tuscany: Gentil Rosso (GRd1 and GRd2); Verna (VRd1; VRd2); Frassineto (FRd1; Frd2); Bianconostrale (BNd1; BNd2); Inalettabile (ILd1; ILd2); Andriolo (ADd1; ADd2); Sieve (SVd1; SVd2) and Gentil Bianco (GBd1; GBd2). Modern varieties from Tuscany: Control (COd1; COd2) and Bologna (BOd1; BOd2). Ancient varieties from Campania: Carosella 1 (CAR1), Carosella 2 (CAR2), Russulidda (RUS), Ianculidda 1 (IA1), Ianculidda 2 (IA2), Annibale (AN), Saragolla Rossa (SR), Cappelli (CA). The samples Carosella 2 and Ianculidda 2 were a mix of Carosella 1 and Ianculidda 1, respectively, with other caryopses of ancient wheat. Modern varieties from Campania: Ambrogio (AM), Aureo (AU) and Bologna (BOR). The samples were grown in a low input system in a calcareous soil (flysch). Generally, the amount of precipitation is between 500 and 1200 mm/year.

All wheat samples were *Triticum aestivum* L. with the only exceptions of SR, CAR, and AU from Campania. Concerning the other cereals (Table 1), two Nigerian millet samples of different varieties (i.e., finger millet (FM) and pearl millet (PM)), one Nigerian sorghum sample (S), one commercial spelt sample (SP) from Garfagnana (Italy) and one commercial whole rice sample (R) from Pavia (Italy), were collected. Finally, four different samples of pasta were collected: one based on a modern whole wheat (MWWP), one produced with a modern wheat (MWP) both purchased from local Italian market; one sample based on ancient whole wheat (AWWP) purchased from the

Italian farm Terre e Tradizioni (Sicily, Italy); and the last sample produced with an ancient wheat (AWP) purchased from the Italian farm Floriddia (Pisa, Italy).

2.3 Extraction of total phenolic compounds

Total phenolic compounds. All the Tuscan wheat samples cultivated under two different densities were treated with an acidic hydrolysis (Method A). Briefly, 1 g of defatted flour was suspended in 25 mL of acidic MeOH (1.05 M H_2SO_4), and the solution was sonicated for 135 min at 40 °C.

Successively, the Tuscan wheat samples GRd2, VRd2, FRd2, BNd2, ILd1, ADd2, SVd1, GBd2, COd1 and BOd2, and all the wheat varieties from Campania were treated according to Balli et al. (2020) with an acidic hydrolysis previously optimized on millet (Method B). Briefly, 1 g of defatted sample was extracted with 25 mL of acidic MeOH (1.20 M H_2SO_4) and sonicated for 180 minutes at 55 °C. All samples were centrifuged (5000 rpm for 10 min) to recover the supernatant to be used for HPLC analyses.

2.4 Determination of steryl ferulates

Steryl ferulates determination was performed on a part of Tuscan samples (GRd2, VRd2, FRd2, BNd2, ILd1, ADd2, SVd1, GBd2, COd1 and BOd2), on all the Campania wheat samples, and on finger millet (FM), pearl millet (PM), whole sorghum (S) and whole spelt (SP) samples. A whole rice (R) sample was used to produce an extract to be used as reference internal sample. Furthermore, steryl ferulates were determined in all the four pasta samples.

Extraction was carried out according to Cho et al. (2019). Briefly, 5 g of powdered milled sample were extracted thrice (20 min each) at 40°C with the aid of ultrasonic bath, using 50 mL, 30 mL and 20 mL of dichloromethane-methanol (2:1 v/v), respectively. The extracts of each cycle of extraction were combined and dried with a rotary evaporator under vacuum at 40°C; the sample was re-dissolved in 1 mL of methanol, centrifuged (13148×*g* for 10 min) and analyzed by HPLC-DAD, HPLC-DAD-MS/MS and HPLC-HRMS/MS.

2.5 HPLC-DAD and HPLC-DAD-MS analyses

All the extracts were analyzed using a HP 1200L liquid chromatograph equipped with a DAD detector (Agilent Technologies, Palo Alto, CA, USA) with a Raptor ARC-18 column (150 × 3 mm, 5 µm, Restek, USA). The gradient method for the phenolic extracts was the same proposed by Balli et al. (2020): solvent A (CH_3CN), solvent B (0.1% $HCOOH/H_2O$), with A from 0.10 to 10% in 5 min, from 10 to 15% in 5 min, from 15 to 30% in 10 min, from 30 to 35% in 5 min, from 35 to

40% in 3 min, from 40 to 45% in 3 min, from 45 to 100% in 11 min and 5 min of final plateau. Total time 47 min, equilibration time 5 min, flow rate 0.4 mL/min and injection volume 10 µL.

The method for steryl ferulates was an isocratic elution with solvent A (CH_3OH), solvent B (CH_3CN), 55:45 v/v. Total time of analysis 15 min, flow rate 0.6 mL/min and injection volume 20 µL. The chromatograms were recorded at 330 nm and 350 nm.

The HPLC-DAD-MS analyses of phenolic and steryl ferulates extracts were performed using the same column and chromatographic conditions previously described. HPLC-DAD-MS system was from Waters and composed by 2695 HPLC, 2996 PAD and 4 micro MS equipped with Zspray ESI source. The ESI interface parameters were capillary 3.50 kV, cone 76 V, source temperature 120 °C, desolvation gas temperature 350 °C, cone gas flow 25 (L/h), desolvation gas flow 370 (L/h). Data were acquired in negative ion mode from 150 m/z to 800 m/z, at 0.5 sec/scan rate. The chromatograms were recorded at 330 nm.

2.6 HPLC-HRMS/MS

Steryl ferulates extractes were analysed by HPLC-ESI-HRMS and MS/HRMS on a LTQ-Orbitrap coupled to a Dionex Ultimate 3000 (Thermo Scientific, Bremen, Germany). The chromatographic parameters were the same described in paragraph 2.5. Sheath, auxiliary and sweep gas flow rates were 38, 23 and 2 (arbitrary units), respectively. Negative ion ESI source parameters were: source voltage 4.9 kV, capillary voltage -41 V, tube lens -93 V, capillary temperature 285°C.. Data were acquired in data dependent acquisition mode; after a survey scan from 250 to 800 m/z recorded at 100000 resolution (at 400 m/z), the three more intense ions in the scan, in the range from 540 to 650 m/z, were sequentially isolated and fragmented, with a 2.4 mass isolation width, a normalised collision energy of 30%, Q value was 0.2 and activation time 25 msec. The CID mass spectra were recorded in the orbitrap analyser operating at 15000 resolution (at 400 m/z). After two consecutive MS/MS experiments, the precursor ions were inserted in the exclusion list for 15 sec, allowing MS/MS fragmentation of less intense precursor ions (minimum signal intensity 1000 counts for triggering MS/MS experiments on singly charged ions). The mass spectrometer was calibrated with the standard mixture indicated by the producer immediately before the acquisition of the samples.

2.7 Quantitation of phenolic acids, flavonoids and steryl ferulates by HPLC-DAD

Phenolic acids were quantified using a five-point calibration curve with ferulic acid as external standard (purity ≥ 99%) at 330 nm, linearity range 0.00-0.41 µg (R^2=0.9999). The content

in steryl ferulates was determined using the same calibration curve: a multiplicative factor was applied for correcting the results based on the molecular weight of the identified molecules.

The content of flavonoids was determined using a six-point calibration curve of schaftoside standard (purity ≥ 95%) at 350 nm, linearity range 0.00-0.39 µg, (R^2=0.9999).

The Supplementary file contains the calibration curves of ferulic acid and schaftoside (Figure S1) and the parameters of method validation for the two standards in Table S1. The table reports the range of linear calibration, linearity (in terms of R^2), sensitivity (in terms of slope of the calibration curve), Limit of Quantification (LOQ, estimated according to the Eurachem Guide (Magnusson, & Ornemark, 2014)), Limit of Detection (LOD, estimated as one third of the LOQ), and accuracy (in terms of trueness and precision). Trueness and precision were calculated by performing sextuplicate analyses of samples spiked at two levels. They were prepared using flours previously confirmed as free from the two standards and from any interfering peak. The low concentration level was 1.15 × LOQ, while the high concentration level was the penultimate point of the calibration line.

2.8 Statistical methods

Each experiment was performed in triplicate and results were expressed as mean ± SD using EXCEL software (version 2013) in-house routines. One-way ANOVA and *F*-test ($p < 0.05$) by Microsoft Excel statistical software and Fisher's LSD (DSAASTAT software v. 1.1, Onofri, Pisa, 2007) were used to identify significant differences between quantitative data.

3. Results and Discussion

The principal goal of the study was to give an insight into the phenolic compounds present in modern and ancient wheat samples, by comparing two groups of modern and ancient wheat varieties grown in the same field. The results of this research are related to the specific agricultural/environmental practices applied for comparing different wheat varieties grown together in the same agronomic conditions. Surely, the processing differences, age of product and storage conditions, are additional factors that could influence the total phenolic amount (Shewry et al., 2010). The samples were grown in two Italian regions in which wheat is cultivated (i.e., Tuscany and Campania). After the choice of the samples, the second step was to investigate on the extractive method in order to select the best one between two different procedures, both involving an acidic hydrolysis step.

Among the detected phenols, the group of steryl ferulates is one of the least investigated so far in wheat samples, and no data are available in the literature for a comparative evaluation in

ancient and modern varieties. Consequently, this study tried to answer some questions: i) whether the chromatographic pattern of steryl ferulates in different cereals, such as rice, millet, and sorghum is typical of each grain and different from that of wheat; ii) whether the total amount of steryl ferulates in modern and ancient varieties varies consistently. Finally, to preliminary evaluate the actual intake of steryl ferulates in the diet deriving from wheat, four different types of pasta obtained from ancient and modern grain were included in the study and analyzed with the same analytical methodologies applied to flour.

The description of the samples included in this study is reported in Table 1. All extracts of the samples listed in Table 1 were analysed by HPLC-DAD-MS, while the HPLC-HRMS/MS was used to identify the steryl ferulates. Concerning quantitation, the commercial standard of schaftoside was used to quantitate the flavonoids, specifically schaftoside itself and isoschaftoside, while ferulic acid was selected to quantitate cinnamic derivatives and steryl ferulates. This choice was done because the ferulic moiety is present in the structure of almost all the detected molecules, and the absorption of the steroyl moiety at the selected wavelength of 330 nm is negligible.

3.1 Total phenolic compounds

The chromatographic profiles of modern and ancient wheat varieties from Tuscany and Campania presented the same ten compounds (Supplementary file, Figure S2): two flavonoids (compounds **1** and **2**), 7 cinnamic derivatives (compounds **3**, **5-10**) and one resorcinol derivative (**4**). All compounds were tentatively identified by their retention time, UV-Vis and mass spectra and the previous published data on wheat samples (Balli et al., 2019; Bueno-Herrera & Perez-Magarino, 2020). The identified phenolic compounds are listed in Table 2a.

One of the main limitations that disincentives the cultivation of ancient grains is related to their reduced yield per unit area, with respect to modern species (Migliorini et al., 2016; Mefleh et al., 2019). In this context, samples from an intensive (density 2), and a traditional crop (density 1) applied to the Tuscan wheat samples, were compared to verify if this condition could influence the plants' phenols production. All the samples were treated with acidic hydrolysis (Method A) for the recovery of the total phenols in a single extractive step. The quantitative evaluations, for both the densities, are reported in Figure S3. The total phenolic amounts ranged from 665 µg/g to 1019 µg/g dry weight (dw), with higher values for the ancient variety VR and the modern variety Control from density 1. As concern the two agronomic treatments, VR, IL, SV, GB and BO showed a similar phenolic content in both density 1 (250 plants/m^2) and density 2 (350 plants/m^2). On the contrary, the results highlighted greater amounts of total phenols in GR, FR, BN, and AD grown with a higher rate of plants per m^2 (density 2) with respect to the same varieties grown under normal

treatment (density 1). These latter result confirmed a certain capability of plants to increase phenolic compounds expression in stressful conditions and the possibility of use high rates of plants/m^2 in the cultivation of ancient wheat (Laddomada et al., 2021).

As a second step, we decided to verify whether the hydrolytic method (Method B), previously optimized by Response Surface Methodology for millet (Balli et al., 2020), was also suitable for wheat. To this aim, a pool of samples from Tuscany (GRd2, VRd2, FRd2, BNd2, ILd1, ADd2, SVd1, GBd2, COd1 and BOd2) were also analysed with method B. The total phenolic amounts recovered with the two acidic hydrolysis approaches (methods A and B) were compared (Figure S4). The total phenolic compounds recovered with the Method B were significantly higher in GRd2, BNd2, ILd1 and SVd1, while they were not significantly different for all the other varieties; the only exception was COd1, which showed a higher amount with method A.

In light of these results, Method B was chosen for the analysis of the Campania wheat samples. The total phenolic compounds ranged from 675 µg/g in FRd2 to 1137 µg/g in GRd2 in Tuscan wheat samples and from 560 µg/g in SR to 854 µg/g in CAR2 in Campania wheat (Table 3a-3b). Among the Tuscan samples, it was possible to distinguish three different groups of wheat samples on the basis of their total phenolic content: i) VRd2 (901 µg/g), BNd2 (1049 µg/g) and GRd1 (1137 µg/g) with higher values, ii) COd1 (851 µg/g), ILd1 (852 µg/g) and GBd2 (854 µg/g) with medium to high values, and iii) BOd2 (783 µg/g), ADd2 (811 µg/g) and SVd1 (813 µg/g) with lower phenolic content, and FRd2 sample with the lowest phenolic content (675 µg/g). Also the Campania wheat samples, with lower values with respect to the Tuscan ones, could be divided in three different groups: i) AN (851 µg/g) and CAR2 (854 µg/g) with the highest phenolic content, ii) AM (801 µg/g), CA (808 µg/g), BOR (812 µg/g) and IA1 (816 µg/g) with medium to high values, and iii) IA2 (750 µg/g), AU (779 µg/g), RUS (778 µg/g) and CAR1 (792 µg/g) with lower phenolic content. The SR had a much lower phenolic content (560 µg/g) with respect to the other varieties. The total phenolic content were expressed on dry weight.

These results highlighted that the total phenolic content is strictly dependent on wheat variety and on the specific agronomic/environmental practice applied. Noteworthy, in both Tuscany and Campania, ancient wheat varieties belonged to the group with the highest phenolic content. Our values were in the same range of those obtained by Gotti et al. (2018) and higher than those collected in the EU Healthgrain project (Li, Shewry, & Ward 2008).

3.2 Steryl ferulates

In order to evaluate the steryl ferulates profile, the wheat samples from Tuscany and Campania were treated according to Cho et al. (2019). A whole rice (R) sample was used as laboratory internal

reference standard. The identification of each compound was confirmed by the comparison of the retention time of the related peak with those present in γ-oryzanol from rice, and by HPLC-DAD-MS and HPLC-HRMS/MS analyses (Table 2b). The preliminary HPLC-DAD-MS analyses allowed identifying 10 compounds (Table 2b, matrix R), which presented a characteristic loss of 15 Da attributable to the ferulic acid's methyl radical group (Figures S5-S8), in accordance with the literature (Ziegler, Schweiggert & Carle 2015; Tsuzuki et al., 2018).

The tentative identification of the steryl ferulates was performed by HPLC-HRMS/MS analyses (Figure 1). The MS/MS product spectra of compounds **1s**, **2s**, **5s**-**10s** showed a loss of 15 Da and a fragment at 193 m/z attributable to the ferulic acid moiety and a less intense fragment at 175 m/z (Figures 1a). The MS/MS spectra of compounds **3s** and **4s** showed the loss of 15 Da and a fragment at 175 m/z (Figures 1b).

The chromatographic profiles (Figure 2) of ancient and modern wheat samples showed two main compounds tentatively identified as campestanyl ferulate (**9s**), partially co-eluting with traces of sitosteryl ferulate (**8s**), and sitostanyl ferulate (**10s**) (Table 2b). Several differences in the relative abundances of steryl ferulates were observed with respect to rice, in which cycloartenyl ferulate (**3s**) and 24-methylencycloartanyl ferulate (**4s**) were found as the most abundant molecules. These results suggested the possibility of using steryl ferulates as useful markers for the recognizing of different types of cereals. To confirm this hypothesis, further cereals other than rice and wheat were analysed: two millet, one sorghum and one spelt samples.

Spelt (SP), together with wheat, belongs to *Triticum* genus and presented the same compounds of ancient and modern wheat varieties. Finger Millet (FM), Pearl Millet (PM) and Sorghum (S) showed unique chromatographic profiles, with compounds not found in γ-oryzanol from rice, confirming the possibility of using the steryl ferulates as markers to quickly differentiate cereals. Compound **1*s** with a molecular weight of 562 Da was only found in sorghum. The MS/MS spectrum showed the deprotonated ion at 561 m/z with empirical formula $C_{37}H_{53}O_4$ and the fragment ion at 179 m/z with empirical formula $C_9H_7O_4$, attributable to a caffeic acid moiety (Table 2b). The same behavior was observed for compound **3*s** (Table 2b), found in sorghum and millet, with a deprotonated ion at 575 m/z with empirical formula $C_{38}H_{55}O_4$ and the caffeic acid moiety at 179 m/z (Figure 1c). These molecules were tentatively identified as sterol esterified with caffeic acid. Compound **5*s** was only found in millet and its identification is still ongoing (Table 2b).

The total content of steryl ferulates ranged from 37.6 µg/g to 62.3 µg/g and from 14.4 µg/g to 56.6 µg/g in Tuscan and Campania wheat samples, respectively (Table 4a-d). The total steryl ferulates content was expressed on dry weight. The quantities are in the same range of those previously reported by the literature (Hakala et al., 2002) and slightly lower than those found by

Nurmi et al. (2012). Higher values were found for five ancient wheats (VRd2 ILd1-ADd2-SVd1-GBd2) with respect to the modern ones (COd1-BOd2) from Tuscany. A different behavior was highlighted for the Campania wheat grain samples: i) similar values between four ancient (CAR1; CAR2; IA1; AN) and 2 modern (AM; AU) grains; ii) lower amounts for the old varieties IA2, SR and CA and iii) higher amounts for the old variety RUS and the modern variety BOR. These results highlighted that the total steryl ferulates amount strictly depend on wheat variety.

The total steryl ferulates content in wheat samples resulted higher than in sorghum (6.8 µg/g) and spelt (4.5 µg/g) but lower in comparison to rice (89.1 µg/g) (Table 4c). Noteworthy, finger millet from Nigeria was found to be rich in total steryl ferulates, with values similar to those obtained from rice (Table 4c).

To understand if the steryl ferulates were hydrolyzed in form of ferulic acid and extracted together with phenolic compounds during the acidic hydrolysis (paragraph 3.1), the extractive procedure proposed by Cho et al. (2019) was also applied on the residue of total phenols extraction, starting from the defatted flour. The results highlighted the absence of steryl ferulates in the residue of total phenols extraction, meaning that steryl ferulates could be removed by the defatted process or could be extracted by acidic hydrolysis. To exclude one of the two hypotheses, the extraction of steryl ferulates was performed starting from the defatted flours. The results highlighted the presence of steryl ferulates (data not shown) in the same quantities reported in Table 4, confirming that the defatting process was not able to remove these compounds from the flours. It was possible to assess that the total phenolic content obtained after acidic hydrolysis includes the ferulic acid moiety of steryl ferulates.

3.3 Steryl ferulates composition in old and modern wheat pasta

Steryl ferulates's beneficial properties on human health, in particular the antioxidant, anti-inflammatory and the capability of maintain normal cholesterol blood levels, have been extensively reviewed (Burlando, & Cornara, 2014). Determining the concentration of steryl ferulates in pasta samples, as daily consumed cereal staple food, is useful to estimate the amount actually taken of these beneficial molecules. Therefore, this part of the work was focused on a preliminary measurement of the steryl ferulates profile in commercial pasta samples, being aware that the variability determined by several factors such as processing differences, age of product and storage, was not evaluated in this work.

MWWP and AWWP, together with MWP and AWP were analysed and compared in terms of their steryl ferulates composition (Table 4d). Both the not whole meal pasta were poor in steryl ferulates: in particular, steryl ferulates were completely absent in MWP. This latter result was

expected, considering the absence of the bran fractions where steryl ferulates are mainly concentrated (Nyström et al., 2007). As concern the MWWP and AWWP, 50% higher amount of steryl ferulates were found in AWWP (57 µg/g), with respect to MWWP (29 µg/g) (Table 4d). It was possible to hypothesize that the different technological approaches in the production of modern pasta with respect to ancient one, could lead to a loss of steryl ferulates. Temperature could represent a crucial parameter that contributes to steryl ferulates degradation, as previously reported in the literature (Soto-Jover, Boluda-Aguilar & Lopez-Gomez, 2016). One of the main differences in the two production processes is the drying temperature and time: high temperature (120° C) and short time for modern wheat, low temperature (40°C) and long time for ancient wheat (Piwińska, Wyrwisz, Kurek & Wierzbicka 2016). Considering pasta as a leading staple food, the daily consumption of ancient whole wheat pasta could represent a good source of steryl ferulates. These results are only preliminary and future studies require the inclusion of a larger number of samples to further investigate the mean steryl ferulates content of different type of pasta.

4. Conclusions

Composition and amount of phenolic and steryl ferulates in ancient and modern wheat grains could represent one of the possible criteria for selecting one variety rather than another. Indeed, when analyzed through extraction under suitable acidic conditions combined with HPLC-MS analysis, these two groups of molecules were demonstrated to be closely variety dependent in cereals. Overall, a little but significant increase in total phenolic content was found in several ancient varieties with respect to modern ones. Noteworthy, these latter results are strictly dependent on wheat variety and on the specific agronomic/environmental practice applied. Therefore, future perspectives will be to enlarge the number of analysed wheat varieties also considering several different agronomic/environmental factors.

A more in-depth investigation has been carried out on the profile of steryl ferulates in wheat. The findings on wheat were compared to the profile of rice and other cereals such as millet and sorghum. The results allowed pointing out different compositions among different cereals, and suggested the possibility of using these compounds as markers for cereals differentiation.

Authors contributions

Diletta Balli: data curation, writing, formal analyses, conceptualization. **Lorenzo Cecchi**: data curation, writing. **Giuseppe Pieraccini:** methodology, formal analysis. **Marzia Innocenti:** conceptualization, supervision. Stefano Benedettelli: sampling, formal analyses. **Nadia Mulinacci**: conceptualization, reviewing and editing.

Declaration of interest

Authors declare no conflict of interest.

Consent for publication

All authors have reviewed and approved the final version of the manuscript for publication.

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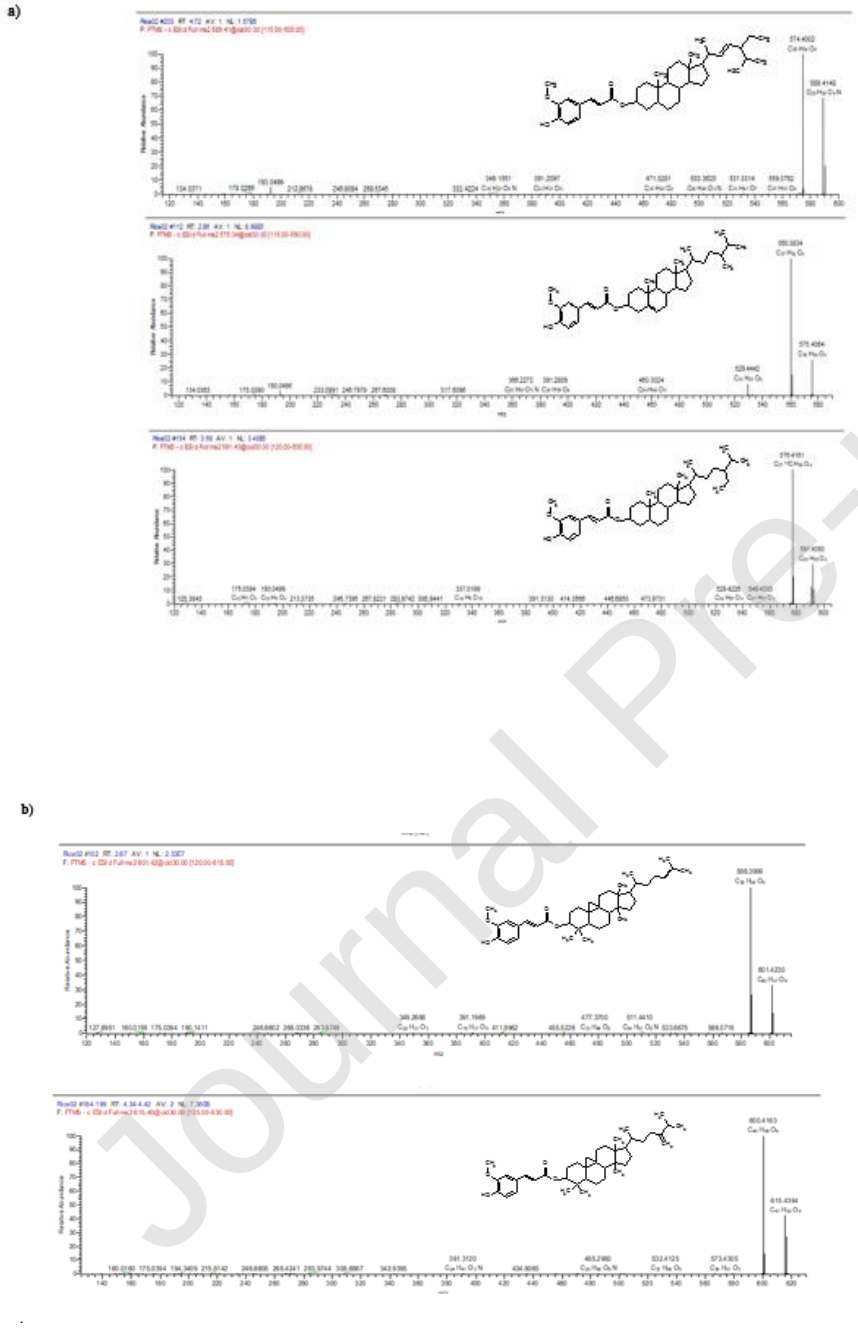
Figures Caption

Figure 1. MS^2 spectra of steryl ferulates. All compounds have been tentatively identified. A) Compounds **1s**, Δ7-stigmastenyl ferulate, **6s**, campesteryl ferulate and **10s,** sitostanyl ferulate. B) Compounds **3s**, cycloartenyl ferulate, **4s,** 24-methylencycloartanyl ferulate. C) Compounds **1*s** and **3*s** identified as caffeoyl phytosterols.



c)

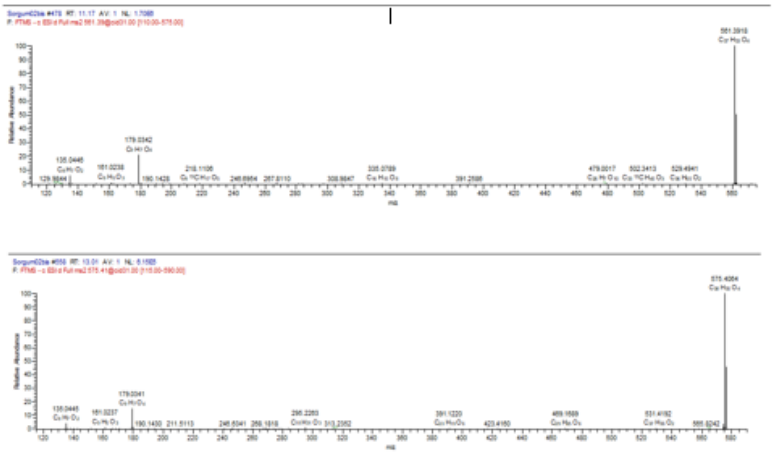


Figure 2. Steryl ferulates HPLC profiles at 330 nm of ancient wheat (AW), modern wheat (MW), spelt (SP), sorghum (S), finger millet (FM) and rice (R). All compounds have been tentatively identified. **1s**: Δ7-stigmastenyl ferulate; **1*s**: caffeoyl phytosterol (t.i.); **2s**: stigmasteryl ferulate; **3s**: cycloartenyl ferulate; **3*s**: caffeoyl phytosterol; **4s**: 24-methylencycloartanyl ferulate; **5s**: Δ7-campestenyl ferulate; **5*s**: unknown; **6s**: campesteryl ferulate; **7s**: sitostenyl ferulate; **8s**: sitosteryl ferulate; **9s**: campestanyl ferulate; **10s**: sitostanyl ferulate

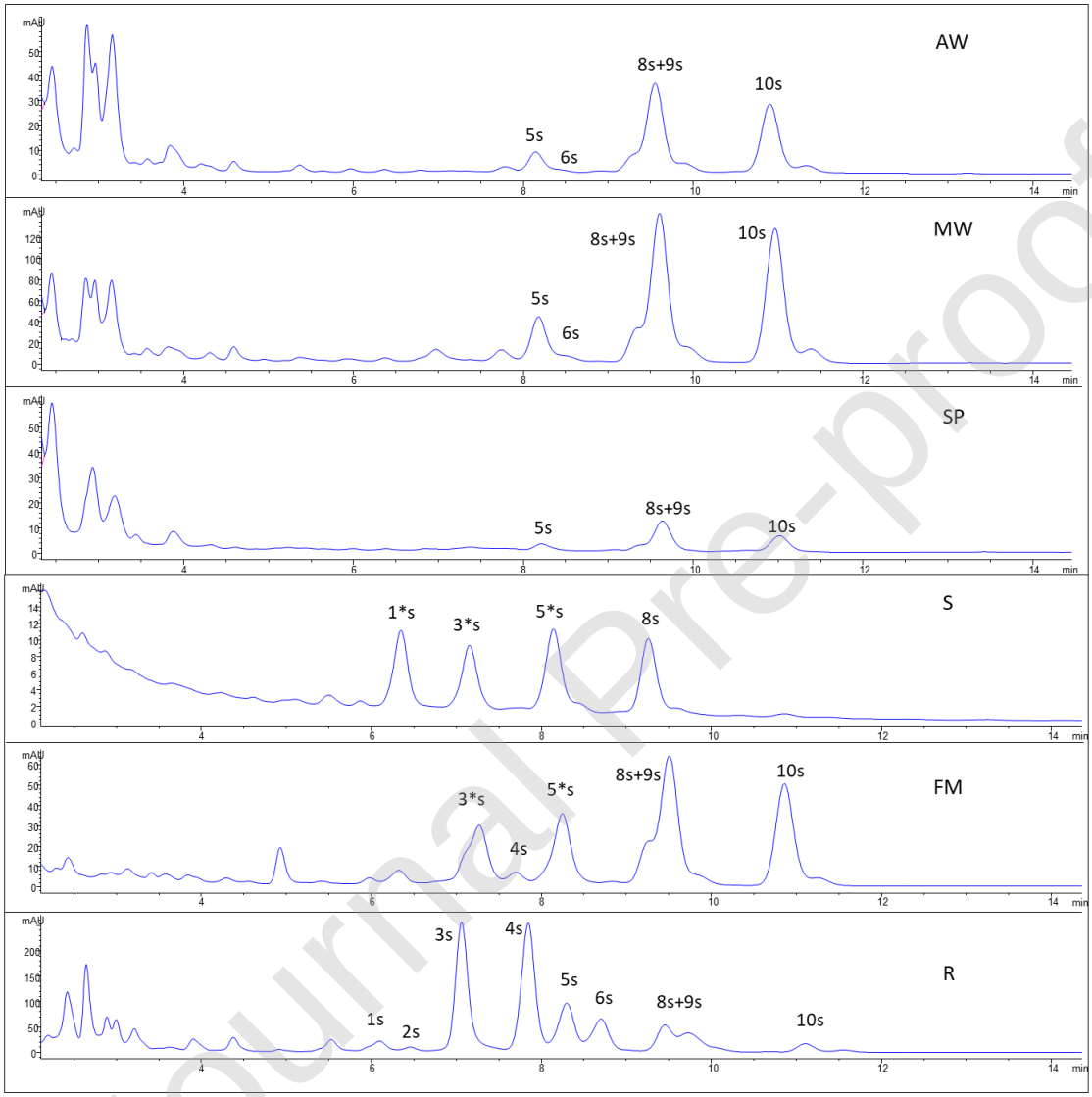


Table 1. Analysed samples, acronyms and their geographical origin.

**two agronomic treatments: density 1 = 250 plants/m^2; density 2 = 350 plants/m^2*

Samples	Acronyms	Geographical origin
Ancient wheat		
Gentil Rosso*	GR	Cesa, Arezzo, Italy
Verna*	VR	Cesa, Arezzo, Italy
Frassineto*	FR	Cesa, Arezzo, Italy
Bianconostrale*	BN	Cesa, Arezzo, Italy
Inallettabile*	IL	Cesa, Arezzo, Italy
Andriolo*	AD	Cesa, Arezzo, Italy
Sieve*	SV	Cesa, Arezzo, Italy
Gentil Bianco*	GB	Cesa, Arezzo, Italy
Carosella 1	CAR1	Caselle in Pittari, Salerno, Italy
Carosella 2	CAR2	Caselle in Pittari, Salerno, Italy
Russulidda	RUS	Caselle in Pittari, Salerno, Italy
Ianculidda1	IA1	Caselle in Pittari, Salerno, Italy
Ianculidda 2	IA2	Caselle in Pittari, Salerno, Italy
Annibale	AN	Caselle in Pittari, Salerno, Italy
Saragolla Rossa	SR	Caselle in Pittari, Salerno, Italy
Cappelli	CA	Caselle in Pittari, Salerno, Italy
Modern wheat		
Control*	CO	Cesa, Arezzo, Italy
Bologna*	BO	Cesa, Arezzo, Italy
Ambrogio	AM	Caselle in Pittari, Salerno, Italy
Aureo	AU	Caselle in Pittari, Salerno, Italy
Bologna	BO	Caselle in Pittari, Salerno, Italy
Other cereals		
Rice	R	Italian Local Market
Finger millet	FM	Nigeria
Pearl millet	PM	Nigeria
Sorghum	S	Nigeria
Spelt	SP	Garfagnana, Italy
Pasta		
Modern whole wheat	MWWP	Italian Local producer
Modern wheat	MWP	Italian Local producer
Ancient whole wheat	AWWP	Italian Local producer
Ancient wheat	AWP	Italian Local producer

Table 2. Phenolic compounds detected in the different samples: (**a**) wheat samples, and (b) steryl ferulates in different cereals. R, rice; FM, finger millet; PM, pearl millet; MW, modern wheat; AW, ancient wheat; S, shorgum; SP, spelt. Ferulic acid and schaftoside were confirmed by using commercial standards, all the other molecules have been tentatively identified.

a)

Peak n°	MW	lambda max (nm)	Parent and Fragment ions $[M-H]^-$	Identified phenolic compounds
1	564	327	563; 473; 353	isoschaftoside
2	564	270; 349	563; 473; 353	schaftoside
3	194	287sh; 323	193	ferulic acid
4	376	-	375	5-nonadecylresorcinol
5	376	287sh; 323	-	cinnamic derivative
6	194	310	193	cis ferulic acid
7	178	290sh; 310	177; 162; 145	methyl idroxycinnamate
8	208	300sh; 324	207; 192	methyl ferulate
9	578	294sh; 323	577	dehydrotriferulic acid isomer1
10	578	294sh; 326	577	dehydrotriferulic acid isomer2

b)

Steryl Ferulates	MW	$[M-H]^-$	Fragment ions (m/z)	Identified compounds	Matrices
1s	590	589	574;193;175	Δ7- Stigmastenyl ferulate	R
1*s	562	561	179	Caffeoyl phytosterol	S
2s	588	587	572;193;175	Stigmasteryl ferulate	R
3s	602	601	586;175	Cycloartenyl ferulate	R

3*s	576	575	179	Caffeoyl phytosterol	FM,PM,S
4s	616	615	600;175	24-Methylencycloartanyl ferulate	R,FM,PM,MW,AW
5s	576	575	560;193;175	Δ7- Campestenyl ferulate	R,MW,AW,SP
5[§]s	578	577		-	FM,PM
6s	576	575	560;193;175	Campesteryl ferulate	R,MW,AW,S
7s	590	589	574;193;175	Sitostenyl ferulate	R(trace)
8s	590	589	574;193;175	Sitosteryl ferulate	R,FM,PM,MW,AW,S,SP
9s	578	577	562;193;175	Campestanyl ferulate	R,FM,PM,MW,AW,SP
10s	592	591	576;193;175	Sitostanyl ferulate	R,FM,PM,MW,AW,SP

[§] not identified

Table 3. Phenolic content after acidic hydrolysis (method B) in wheat species from Tuscany (**a**) and from Campania (**b**). Data are expressed in µg/g dry weight; nd, not detected. Cinnamic derivatives and flavonoids were expressed as ferulic acid and schaftoside equivalents, respectively. All molecules have been tentatively identified; ferulic acid and schaftoside were confirmed by using commercial standards.

a) GRd2: Gentil Rosso density 2; VRd2: Verna density 2; FRd2: Frassineto density 2; BNd2: Bianconostrale density 2; ILd1: Inallettabile density 1; ADd2: Andriolo density 2; SVd1: Sieve density 1; GBd2: Gentil Bianco density 2; COd1: Control density 1; BOd2: Bologna density 2;

b) CAR1: Carosella 1; CAR2: Carosella2; RUS: Russulidda; IA1: Ianculidda1; IA2: Ianculidda2; AN: Annibale; SR: Saragolla Rossa; CA: Senatore Cappelli; AM: Ambrogio; AU: Aureo; BOR: Bologna

a)

	Ancient wheat								**Modern wheat**	
	µg/g								**µg/g**	
Phenolic compounds	**GRd2**	**VRd2**	**FRd2**	**BNd2**	**ILd1**	**ADd2**	**SVd1**	**GBd2**	**COd1**	**Bod2**
ferulic acid	7.34 ± 0.10	9.85 ± 0.08	5.67 ± 0.01	7.17 ± 0.02	19.66 ± 0.78	3.67 ± 0.02	1.99 ± 0.01	8.32 ± 0.25	16.28 ± 0.26	5.50 ± 0.01
5-nonadecylresorcinol	34.21 ± 0.04	26.53 ± 0.05	19.34 ± 1.20	33.52 ± 0.63	23.82 ± 0.32	17.86 ± 0.36	23.43 ± 1.06	30.11 ± 2.89	18.28 ± 1.24	29.68 ± 1.13
cinnamic derivative	50.89 ± 1.36	34.04 ± 2.16	29.85 ± 0.79	52.04 ± 1.14	35.32 ± 2.75	28.04 ± 2.51	26.09 ± 2.68	33.77 ± 1.52	22.10 ± 1.36	30.35 ± 2.65

cis ferulic acid	29.87 ± 0.72	19.86 ± 0.69	17.34 ± 0.96	23.35 ± 0.42	15.49 ± 1.24	14.85 ± 0.98	13.63 ± 0.45	20.29 ± 0.89		12.63 ± 0.85	17.34 ± 0.09
methyl hydroxycinnmate	42.21 ± 0.38	22.19 ± 0.42	19.34 ± 2.16	29.02 ± 3.45	24.66 ± 2.15	22.87 ± 1.13	16.95 ± 0.62	19.63 ± 0.25		24.43 ± 0.88	20.34 ± 0.29
methyl ferulate	811.87 ± 10.24	680.37 ± 2.96	469.56 ± 8.77	733.86 ± 20.31	587.19 ± 18.75	635.74 ± 22.65	627.28 ± 15.28	636.08 ± 12.44		670.47 ± 0.96	567.74 ± 15.32
dehydrotriferulic acid isomer I	41.21 ± 0.56	28.37 ± 1.12	23.01 ± 0.98	58.54 ± 2.78	27.24 ± 1.78	22.70 ± 0.32	20.11 ± 0.06	22.96 ± 0.96		25.42 ± 1.13	21.18 ± 1.45
dehydrotriferulic acid isomer II	49.22 ± 1.47	41.05 ± 0.89	38.35 ± 1.19	47.37 ± 5.13	68.97 ± 6.35	39.39 ± 4.85	35.39 ± 2.18	41.42 ± 5.12		33.90 ± 2.15	36.52 ± 0.02
other minors cinnamic derivatives	28.20 ± 1.42	8.51 ± 0.65	8.67 ± 0.74	15.01 ± 1.12	27.49 ± 1.74	9.18 ± 1.12	7.48 ± 0.65	7.98 ± 0.67		4.82 ± 0.74	17.01 ± 2.14
Total cinnamic derivatives	1066.82 ± 16.29	870.77 ± 9.02	631.13 ± 16.80	1007.06 ± 35.00	829.84 ± 35.86	794.30 ± 33.94	772.35 ± 22.99	820.56 ± 24.99		828.33 ± 9.57	745.66 ± 23.10
isoschaftoside	21.25 ± 0.36	15.76 ± 0.12	20.23 ± 0.52	24.28 ± 0.02	10.16 ± 0.24	9.76 ± 0.67	22.17 ± 0.25	17.15 ± 0.16		14.11 ± 0.96	17.19 ± 0.68
schaftoside	21.25 ± 0.32	15.05 ± 0.52	24.27 ± 1.12	24.95 ± 0.25	12.33 ± 0.78	7.42 ± 0.32	18.81 ± 1.13	16.48 ± 0.64		9.41 ± 0.02	20.24 ± 1.62
Total flavonoids	42.50 ± 0.68	30.81 ± 0.64	44.50 ± 1.64	49.23 ± 0.27	22.49 ± 1.02	17.18 ± 0.99	40.98 ± 1.38	33.63 ± 0.80		23.52 ± 0.98	37.43 ± 2.30
Total phenols	1137.52 ± 16.97	901.58 ± 9.66	675.63 ± 18.44	1049.12 ± 35.27	852.33 ± 36.88	811.48 ± 34.93	813.33 ± 24.37	854.19 ± 25.79		851.85 ± 10.55	783.09 ± 25.40

b)

	Ancient wheat									**Modern wheat**		
	µg/g									**µg/g**		
Phenolic compounds	**CAR1**	**CAR2**	**RUS**	**IA1**	**IA2**	**AN**	**SR**	**CA**		**AM**	**AU**	**BOR**
ferulic acid	4.79 ± 0.48	5.46 ± 0.47	1.18 ± 0.26	1.79 ± 0.33	1.67 ± 0.01	7.36 ± 0.69	nd	8.76 ± 0.23		9.45 ± 0.24	4.99 ± 0.01	10.10 ± 0.16
5-nonadecylresorcinol	19.14 ± 0.03	28.16 ± 0.69	31.40 ± 2.12	30.14 ± 0.14	29.27 ± 1.34	24.32 ± 2.04	14.72 ± 1.46	14.94 ± 0.25		22.69 ± 0.19	10.42 ± 0.74	30.47 ± 0.02
cinnamic derivative	22.73 ± 0.68	21.85 ± 0.50	26.77 ± 1.40	17.64 ± 1.29	24.81 ± 2.06	16.61 ± 1.28	12.49 ± 1.20	15.46 ± 0.96		17.36 ± 0.71	9.22 ± 0.94	22.08 ± 0.21
cis ferulic acid	9.40 ± 0.70	7.34 ± 0.24	nd	4.28 ± 0.01	nd	3.26 ± 0.01	nd	11.11 ± 0.03		nd	2.32 ± 0.01	4.79 ± 0.04
methyl hydroxycinnmate	7.86 ± 0.46	4.43 ± 0.01	nd	nd	nd	6.50 ± 0.04	nd	nd		nd	10.75 ± 0.16	nd
methyl ferulate	596.67 ± 0.61	649.46 ± 6.51	597.50 ± 6.81	635.80 ± 11.15	576.33 ± 29.83	667.84 ± 14.30	407.68 ± 0.98	556.68 ± 10.90		669.91 ± 23.18	595.52 ± 22.01	630.87 ± 4.07
dehydrotriferulic acid isomer I	43.76 ± 0.89	36.53 ± 1.48	37.93 ± 0.65	46.06 ± 2.92	34.58 ± 2.10	44.70 ± 1.89	34.58 ± 2.88	61.85 ± 0.03		6.63 ± 0.24	51.75 ± 0.13	32.35 ± 0.29

dehydrotriferulic acid isomer II	16.75 ± 1.42	15.36 ± 1.46	14.76 ± 0.02	12.67 ± 0.06	16.78 ± 2.01	22.26 ± 1.35	13.18 ± 0.71	31.78 ± 1.19		34.03 ± 0.50	30.40 ± 0.41	15.57 ± 0.24
other minors cinnamic derivatives	24.61 ± 1.40	21.68 ± 0.26	24.20 ± 0.68	27.74 ± 1.80	21.06 ± 1.30	21.92 ± 0.38	18.66 ± 0.71	34.53 ± 3.17		19.77 ± 2.19	28.35 ± 2.47	19.51 ± 0.01
Total cinnamic derivatives	745.71 ± 6.67	790.27 ± 11.62	733.74 ± 11.94	776.12 ± 17.70	704.50 ± 38.65	814.77 ± 21.98	501.31 ± 7.94	735.11 ± 16.76		779.84 ± 27.25	743.72 ± 26.88	765.74 ± 5.04
schaftoside	46.93 ± 0.32	64.58 ± 0.06	44.49 ± 0.31	40.76 ± 0.46	46.47 ± 0.19	36.58 ± 0.31	59.54 ± 0.29	73.38 ± 0.30		21.50 ± 0.72	35.95 ± 0.62	47.00 ± 0.44
Total flavonoids	46.93 ± 0.32	64.58 ± 0.06	44.49 ± 0.31	40.76 ± 0.46	46.47 ± 0.19	36.58 ± 0.31	59.54 ± 0.29	73.38 ± 0.30		21.50 ± 0.72	35.95 ± 0.62	47.00 ± 0.44
Total phenols	792.64 ± 6.99	854.85 ± 11.68	778.23 ± 12.25	816.88 ± 18.16	750.97 ± 38.84	851.35 ± 22.29	560.85 ± 8.23	808.49 ± 17.06		801.34 ± 27.97	779.67 ± 27.50	812.74 ± 5.48

Table 4. Distribution of steryl ferulates after their relative quantitation in: **a**) ancient and modern Tuscan wheat samples; **b**) Campania wheat samples; (**c**) rice, millet, sorghum and spelt samples; d) ancient and modern pasta samples. Data are expressed in µg/g dry weight as a mean of triplicate. Steryl ferulates were quantified as ferulic acid equivalents applying the specific corrective factors on their MW. nd, not detected. All molecules have been tentatively identified.

GRd2: Gentil Rosso, density 2; VRd2: Verna, density 2; FRd2: Frassineto density 2; BNd2: Bianconostrale density 2; ILd1: Inallettabile density1; ADd2: Andriolo density 2; SVd1: Sieve density 1; GBd2: Gentil Bianco density 2; COd1: Control density 1; BOd2: Bologna density 2; FM: Finger Millet; PM: Pearl Millet; MWP: modern wheat pasta; AWP: ancient wheat pasta; MWWP: modern whole wheat pasta; AWWP: ancient whole wheat pasta.

a)

µg/g					
Ancient wheat	**Δ7-Campestenyl**	**Campesteryl**	**Sitosteryl+ Campestanyl**	**Sitostanyl**	**Total**
GRd2	3.68 ± 0.13	0.50 ± 0.03	20.80 ± 1.16	12.64 ± 1.23	37.62 ± 2.55
VRd2	5.91 ± 0.26	0.82 ± 0.01	31.50 ± 2.25	19.74 ± 0.96	57.97 ± 3.48
FRd2	5.27 ± 0.31	0.32 ± 0.06	23.91 ± 0.01	14.02 ± 0.92	43.52 ± 1.30
BNd2	4.96 ± 0.34	0.55 ± 0.01	21.49 ± 0.63	14.67 ± 0.28	41.67 ± 1.26
ILd1	6.50 ± 0.92	2.70 ± 0.01	27.01 ± 0.78	22.15 ± 0.02	58.36 ± 1.73
ADd2	4.59 ± 0.11	1.01 ± 0.02	27.53 ± 0.12	20.68 ± 1.36	53.81 ± 1.61
SVd1	7.11 ± 0.75	0.80 ± 0.04	32.11 ± 2.25	22.31 ± 0.84	62.33 ± 3.88
GBd2	5.84 ± 0.23	0.93 ± 0.03	31.59 ± 0.36	22.80 ± 1.12	61.16 ± 1.74
Modern wheat					
COd1	5.52 ± 0.17	1.04 ± 0.21	23.31 ± 0.21	18.67 ± 1.29	48.47 ± 1.88
BOd2	4.07 ± 0.12	1.23 ± 0.34	19.22 ± 0.07	15.40 ± 1.39	39.92 ± 1.92

b)

	µg/g				
Ancient wheat	**Δ7-Campestenyl**	**Campesteryl**	**Sitosteryl+ Campestanyl**	**Sitostanyl**	**Total**
CAR1	1.85 ± 0.03	0.41 ± 0.06	10.97 ± 0.01	8.44 ± 0.06	21.67 ± 0.16
CAR2	1.71 ± 0.06	0.40 ± 0.01	10.43 ± 0.36	7.65 ± 0.28	20.19 ± 0.71
RUS	6.52 ± 0.42	2.50 ± 0.01	24.62 ± 1.23	20.50 ± 0.34	54.14 ± 2.00
IA1	10.46 ± 0.85	0.75 ± 0.02	17.34 ± 0.85	8.59 ± 0.01	37.14 ± 1.73
IA2	3.23 ± 0.26	0.29 ± 0.01	8.93 ± 0.21	5.50 ± 0.01	17.95 ± 0.49
AN	6.27 ± 1.01	0.81 ± 0.02	18.96 ± 0.69	12.21 ± 0.35	38.25 ± 2.07
SR	1.01 ± 0.01	0.10 ± 0.01	8.91 ± 0.02	4.52 ± 0.01	14.54 ± 0.05
CA	1.17 ± 0.01	0.31 ± 0.01	11.78 ± 0.04	6.37 ± 0.01	19.63 ± 0.07
Modern wheat					
AM	3.51 ± 0.03	1.52 ± 0.03	15.74 ± 0.06	9.71 ± 0.24	30.48 ± 0.36
AU	3.22 ± 0.04	1.32 ± 0.02	11.81 ± 0.03	7.33 ± 0.78	23.68 ± 0.87
BOR	4.44 ± 0.52	0.61 ± 0.01	30.59 ± 0.18	20.96 ± 1.14	56.60 ± 1.85

c)

					µg/g						
	Δ7-Stigmastenyl	**Stigmasteryl**	**Cycloartenyl**	**Methylencycloartanyl**	**Δ7-Campestenyl**	**Campesteryl**	**Sitostenyl**	**Sitosteryl**	**Campestanyl**	**Sitostanyl**	**Total**
Rice	2.71 ± 0.01	2.90 ± 0.05	27.26 ± 0.29	26.18 ± 0.65	12.34 ± 0.07	4.26 ± 0.01	nd	6.41 ± 0.02	4.83 ± 0.02	2.23 ± 0.01	89.12 ± 1.13
	Δ7-Stigmastenyl	**Stigmasteryl**	**Caffeoyl phytosterol**	**Methylencycloartanyl**	**Δ7-Campestenyl**	**5*s**	**Sitostenyl**	**Sitosteryl+ Campestanyl**		**Sitostanyl**	
Millet											

FM	nd	4.21 ± 0.01	12.62 ± 0.03	nd	nd	14.60 ± 0.26	nd	34.46 ± 0.78		23.97 ± 0.65	89.86 ± 1.73
PM	nd	1.22 ± 0.01	5.22 ± 0.01	nd	nd	2.52 ± 0.01	nd	6.01 ± 0.01		1.83 ± 0.01	16.80 ± 0.05
	Caffeoyl phytosterol	**Stigmasteryl**	**Caffeoyl phytosterol**	**Methylencycloartanyl**	**Campestenyl**	**Campesteryl**	**Sitostenyl**	**Sitosteryl**	**Campestanyl**	**Sitostanyl**	
Sorghum	1.54 ± 0.01	nd	1.39 ± 0.01	nd	nd	2.01 ± 0.01	nd	1.86 ± 0.01	nd	nd	6.80 ± 0.04
	Δ7-Stigmastenyl	**Stigmasteryl**	**Cycloartenyl**	**Methylencycloartanyl**	**Δ7-Campestenyl**	**Campesteryl**	**Sitostenyl**	**Sitosteryl+ Campestanyl**		**Sitostanyl**	
Spelt	nd	nd	nd	nd	0.52 ± 0.01	Nd	nd	2.73 ± 0.01		1.28 ± 0.01	4.53 ± 0.03

d)

	µg/g				
	Δ7-Campestenyl	**Campesteryl**	**Sitosteryl+ Campestanyl**	**Sitostanyl**	**Total**
MWP	nd	nd	nd	nd	0
AWP	nd	1.72 ± 0.02	14.61 ± 0.45	7.10 ± 0.03	23.43 ± 0.50
MWWP	nd	2.15 ± 0.04	18.22 ± 1.17	8.84 ± 0.08	29.21 ± 1.29
AWWP	1.82 ± 0.01	9.48 ± 0.28	29.44 ± 1.26	16.43 ± 0.48	57.17 ± 2.03