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Profiles of phenolic compounds in modern and old common wheat varieties determined by liquid chromatography coupled with time-of-flight mass spectrometry

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ABSTRACT

The health-promoting properties of common wheat (*Triticum aestivum* L.) have been largely attributed to the presence of unique phytochemicals of whole grains. The aim of this study was to profile the phenolic content of 16 old and 6 modern Italian wheat varieties, cropped in the same location and growing season. High variability was observed among the investigated wheat genotypes, both in the free and bound phenolic extracts. The total polyphenol content ranged from 885.5 to 1715.9 μmol GAE/100 g of grain and, on average, the bound fraction contributed for 72.0% to the total phenolic content. As regards the flavonoid content, the free fraction ranged from 50.7 to 106.1 μmol CE/100 g of grain and the bound fraction from 78.3 to 148.9 μmol CE/100 g of grain. Moreover, the interpretation of the mass spectra allowed the characterization of 34 phenolic compounds (104 including isomer forms) belonging to the phenolic acid, flavonoid, coumarin, stilbene, proanthocyanidin and lignan chemical classes. HPLC–ESI–TOF-MS analysis highlighted remarkable differences in the phytochemical fingerprints of old and modern wheat varieties. Six ancient wheat genotypes (Bianco Nostrale, Frassineto, Gentil Rosso, Gentil Rosso Mutico, Marzuolo d'Aqui, Verna) showed phenolic profiles with a number of total compounds and isomer forms much higher than that identified in the modern cultivars. The present findings confirm that ancient wheat may represent a valuable source of biodiversity, especially as regards phenolic compounds. The investigated old wheat genotypes may be successfully used in breeding programs for developing bread wheat varieties with added value in terms of health-promoting phytochemicals.

1. Introduction

Common wheat (*Triticum aestivum* L.) is one of the major crop worldwide and represent the most consumed staple food. Several epidemiological studies indicated that diets rich in whole-grain derived products are associated with decreased incidence of chronic diseases [1–3]. Whole grains contain various phytochemicals in addition to the basic and essential nutrients (proteins, carbohydrates, dietary fiber) and the health benefits have been largely ascribed to the presence of nutraceutics with potential biological activity, such as phenolic compounds. Polyphenols are plant secondary metabolites belonging to the phenylpropanoid pathway that contain one or more aromatic rings and one or more hydroxyl groups, including phenolic acids, coumarins, flavonoids, stilbenes and lignans. Recently, polyphenols have gained attention due to their antioxidant, anti-inflammatory, antimutagenic and anticarcinogenic properties [4,5]. Antioxidants are defined as compounds that, at low concentration, can delay or prevent the oxidative damage of substrates as DNA, enzymes and cell wall molecules, neutralizing free radicals (ROS) that are the cause of many chronic diseases (i.e. cancer and cardiovascular disease). *In vitro* investigations demonstrated that phenolic compounds possess high antiradical power and contribute to most of the total antioxidant activity of wheat grains [6–9]. Indeed, polyphenols are one of the most complex and representative group of phytochemicals in wheat kernel. They are chiefly concentrated in the outer layers of grains (aleurone and bran cells) and exist as soluble free compounds, soluble conjugates esterified to sugars and other low molecular weight molecules, and insoluble forms bound to cell wall components. The latter are crosslinked with cell wall macromolecules (i.e. arabinoxylans) via ester and ether bonds and

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contribute to the total phenolic content for 60–70% [7,9]. Previous studies investigated the phenolic content of whole grains and their different fractions (endosperm, aleurone layer, bran) both free and bound forms, mainly using colorimetric methods as spectrophotometrical analyses [6–8]. Recently traditional methods have been replaced by various separation techniques (HPLC, GC, CE) coupled with mass spectrometry (HPLC–MS, GC–MS, CE–MS) as more sensitive tools for the characterization and identification of individual polyphenols in wheat [10–17]. Phenolic acids are the most representative and investigated class of wheat phenolics and their profile has been thoroughly described in recent years [9,12,18–21]. However, literature lacks information about the complete phenolic composition of wheat and of different wheat varieties as regards flavonoid, anthocyanin, stilbene, coumarin and lignan content. In our previous study, the phenolic profile of old and modern durum wheat varieties was investigated using HPLC coupled with time-of-flight mass spectrometry and high qualitative differences were highlighted among the tested genotypes [22]. In particular, old durum wheat varieties showed a qualitative free and bound phenolics content significantly different than those detected in the modern cultivars. This finding suggested that ancient wheat may represent a valuable source of variability for breeding and eventual commercial production of value-added varieties rich in health-beneficial components. Further studies are needed to better understand how these different classes contribute to the maintenance of human health and the synergistic or additive effect among the different classes of phytochemicals. Few authors reported that several factors as genotype, environmental and growing conditions can influence the presence and distribution of phenolic compounds in wheat grains [7,23–26]. However, most literature data concerning wheat phenolic determinations do not give details about field agronomic conditions and growing locations. The present research was conducted to determine and to compare the phenolic composition of different common wheat varieties, including old and modern Italian genotypes, cropped in the same location and growing season. The investigation aims to contribute to the understanding of wheat whole grain nutraceutical properties and promoting the development of wheat varieties with high level of health-promoting phytochemicals.

2. Materials and methods

2.1. Chemicals

HPLC-grade acetonitrile and methanol was purchased from Labscan (Dublin, Ireland). Acetic acid was of an analytical grade (assay >99.5%) and purchased from Fluka (Switzerland). Water was purified by using a Milli-Q system (Millipore, Bedford, USA). Other reagents unmarked were of an analytical grade.

2.2. Grain samples and sample preparation

Wheat samples included 16 old (Andriolo, Autonomia A, Autonomia B, Benco, Bianco Nostrale, Canove, Carosello, Frassineto, Gentil Bianco, Gentil Rosso, Gentil Rosso Mutico, Inallettabile, Marzuolo d'Aqui, Marzuolo Val Pusteria, Sieve, Verna) and 6 modern cultivars (Bilancia, Bolero, Eureka, Mieti, Nobel, Palesio) of common wheat (*T. aestivum* L.). Seeds from all of the investigated genotypes were grown in the same location at the experimental farm of the University of Bologna, Cadriano (latitude 44°33′N, longitude 11°21′E, 32 m a.s.l.), Italy, during the growing season 2006–2007. The soil at the experimental farm of Cadriano is classified as a fine silty, mixed, mesic, Udic stochrepts, and has a silty loam texture, with 380, 375, and 245 g/kg of sand, silt, and clay, respectively. The pH (1:2.5 soil to water) is 7.9 and organic carbon is 8.5 g/kg. Each genotype was grown in plots (6 m × 5 m) according to a low input agro-technique (nitrogen fertilization with 10 kg NO_3 ha^{-1} applied in pre-sowing and 20 kg NO_3 ha^{-1} applied in leaf sheaths lengthening stage). Weeds were hand controlled and no herbicide (or other pesticide) treatment was applied. Plants were harvested at grain full ripening stage. Whole grain samples were milled to a fine powder, immediately cooled to −20 °C and kept at this temperature until analysis to protect bioactive components from degradation.

2.3. Extraction of soluble and insoluble phenolic compounds

Free phenolic extraction was performed according to the method described previously [7,22] with some modifications. 1 g of whole wheat flour was extracted with 20 mL of 80% chilled ethanol for 10 min. After centrifugation at 2500 × *g* for 10 min, the supernatant was removed and extraction was repeated once. Supernatants were pooled, evaporated to dryness and reconstituted in 10 mL of 80% methanol. The extracts were filtered through a 0.22 μm filter and stored at −20 °C until use. The residue from the free phenolic extraction was subjected to alkaline and acid hydrolysis to recover the bound phenolic compounds as reported by Mattila et al. [18] with some modifications. Briefly, 12 mL of distilled water and 5 mL of 10 M NaOH were added to the residue and stirred overnight at room temperature (about 16 h). The solution was then adjusted to a pH of 2, and liberated phenolics were extracted three times with 15 mL of ethyl acetate by manually shaking and centrifuging. Ethyl acetate layers were combined, evaporated to dryness, and dissolved into 10 mL of methanol. After the above alkaline hydrolysis was completed, an acid hydrolysis was then performed by adding 2.5 mL of concentrated HCl into the test tube and incubating the tube in a water bath (85 °C) for 30 min. After acid hydrolysis, the sample was allowed to cool and the ethyl acetate extraction performed in the same manner as after alkaline hydrolysis. Bound phenolic extracts were filtered through a 0.22 μm filter and stored at −20 °C until use.

2.4. Determination of total polyphenol and flavonoid contents

Free and bound polyphenol content of each wheat sample was determined using the Folin–Ciocalteu procedure described by Singleton et al. [27]. Gallic acid was used as the standard and polyphenol content was expressed as micromoles of gallic acid equivalent (GAE) per 100 g of grain. Free and bound flavonoid content was determined according to a colorimetric method described previously by Adom et al. [7]. Briefly, appropriate dilutions of sample extracts were reacted with sodium nitrite, followed by reaction with aluminium chloride to form a flavonoid–aluminium complex. Solution absorbance at 510 nm was immediately measured and compared to that of catechin standards. Flavonoid content was expressed as micromoles of catechin equivalent (CE) per 100 g of grain. Data are reported as mean ± standard deviation (SD) for six replicates.

2.5. HPLC–ESI–TOF-MS experimental conditions

HPLC analysis was performed using an Agilent 1200-RRLC system (Agilent Technologies, CA, USA) consisting of a vacuum degasser, autosampler, a binary pump and a UV–vis detector. Phenolic compounds were separated using a RP C18 analytical column (4.6 mm × 150 mm, 1.8 μm particle size) from Agilent ZORBAX Eclipse plus. The mobile phases and gradient program used were as previously described [22]. The gradient elution was performed with mobile phases consisting of water with acetic acid (0.5% acetic acid v/v) (A) and acetonitrile (B) as follows: from 5% to 10% B in 5 min; from 10% to 35% B in 35 min; from 35% to 70%

Table 1
Polyphenol and flavonoid content in cultivar grains, expressed as μmol gallic acid equivalent and μmol catechin equivalent per 100 g of whole flour, respectively.

Cultivar	FPC	BPC	TPC	FFC	BFC	TFC
Andriolo (O)	262.4 (jk)	609.9 (i)	872.2 (j)	86.0 (bc)	97.7 (hi)	183.7 (efg)
Autonomia A (O)	326.1 (ghi)	914.4 (efgh)	1240.5 (fgh)	64.6 (efgh)	103.7 (ghi)	168.3 (fghi)
Autonomia B (O)	307.5 (hi)	793.2 (h)	1100.7 (i)	69.1 (defg)	78.3 (j)	147.4 (j)
Benco (O)	243.0 (k)	642.5 (i)	885.5 (j)	64.8 (efgh)	117.6 (defg)	182.4 (efg)
Bianco Nostrale (O)	326.9 (ghi)	984.3 (de)	1311.2 (efg)	50.7 (i)	103.2 (ghi)	153.8 (ij)
Bilancia (M)	410.8 (de)	832.0 (gh)	1242.8 (fgh)	77.5 (cd)	98.2 (hi)	175.7 (efgh)
Bolero (M)	354.1 (fg)	794.8 (h)	1148.8 (hi)	73.3 (de)	93.2(ij)	166.5 (ghi)
Canove (O)	438.0 (cd)	954.8 (defg)	1392.8 (de)	56.4 (hi)	111.6 (efgh)	168.0 (fghi)
Carosello (O)	470.6 (bc)	1082.2 (bcd)	1552.8 (bc)	62.4 (efgh)	121.1 (def)	183.4 (efg)
Eureka (M)	386.7 (ef)	1187.9 (ab)	1574.5 (bc)	73.0 (de)	134.5 (abcd)	207.6 (bc)
Frassineto (O)	381.3 (ef)	846.0 (fgh)	1227.3 (ghi)	64.6 (efgh)	104.7 (fghi)	169.3 (fghi)
Gentil Bianco (O)	342.4 (ghi)	1044.9 (cde)	1387.3 (de)	63.6 (efgh)	124.1 (cde)	187.7 (def)
Gentil Rosso (O)	419.3 (de)	1226.7 (a)	1646.0 (ab)	63.3 (efgh)	148.9 (a)	212.3 (bc)
Gentil Rosso Mutico (O)	442.6 (cd)	1009.2 (cde)	1451.8 (cde)	92.2 (b)	142.5 (ab)	234.7 (a)
Inallettabile (O)	344.0 (fgh)	1060.4 (bcd)	1404.4 (de)	77.0 (cd)	129.5 (bcd)	206.6 (bcd)
Marzuolo d'Aqui (O)	450.4 (cd)	1125.7 (abc)	1576.1 (bc)	90.0 (b)	123.1 (cde)	213.0 (bc)
Marzuolo Val Pusteria (O)	292.7 (ij)	1060.4 (bcd)	1353.1 (defg)	72.1 (def)	139.5 (abc)	211.5 (bc)
Mieti (M)	381.3 (ef)	985.9 (de)	1367.1 (def)	58.6 (ghi)	103.2 (ghi)	161.8 (hij)
Nobel (M)	316.0 (ghi)	1237.6 (a)	1553.6 (bc)	61.4 (fgh)	132.5 (abcd)	193.9 (cde)
Palesio (M)	346.3 (fgh)	1007.6 (cde)	1353.9 (defg)	69.1 (defg)	97.7 (hi)	166.8 (ghi)
Sieve (O)	498.6 (b)	964.1 (def)	1462.7 (cd)	92.7 (b)	100.7 (ghi)	193.4 (cde)
Verna (O)	579.4 (a)	1136.6 (abc)	1715.9 (a)	106.1 (a)	107.7 (efghi)	213.8 (b)
Mean value	378.2	977.3	1355.5	72.2	114.2	186.4

Abbreviations: FPC, free phenolic compounds; BPC, bound phenolic compounds; TPC, total phenolic compounds; FFC, free flavonoid compounds; BFC, bound flavonoid compounds; TFC, total flavonoid compounds; M, modern cultivar; O, old cultivar.
Means followed by the same letter or no letter are not significantly different at $P < 0.05$.

B in 20 min; from 70% to 95% B in 2 min; from 95% to 5% B in 2 min. An 8 min re-equilibration time was used after each analysis. The flow rate was set at 0.50 mL/min throughout the gradient. The effluent from the HPLC column was splitted using a T-type phase separator before being introduced into the mass spectrometer (split ratio = 1:3). Thus in this study the flow which arrived into the MS detector was 0.125 mL/min. The column temperature was maintained at 40 °C and the injection volume was 10 μL. The HPLC system was coupled to a microTOF (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer (oaTOFMS), equipped with an ESI interface. Parameters for analysis were set using negative ion mode with spectra acquired over a mass range from *m*/*z* 50 to 1000. The optimum values of the ESI–MS parameters were: capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 7.0 L/min; and nebulizing gas pressure, 2 bar. The accurate mass data of the molecular ions were processed through the newest software Data Analysis 4.0 (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formula by using the Smart Formula Editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm. We also have to say that even with very high mass accuracy (<1 ppm) many chemically possible formulae are obtained depending on the mass regions considered. So, high mass accuracy (<1 ppm) alone is not enough to exclude enough candidates with complex elemental compositions. The use of isotopic abundance patterns as a single further constraint removes >95% of false candidates. This orthogonal filter can condense several thousand candidates down to only a small number of molecular formulae. During the development of the HPLC method, external instrument calibration was performed using a Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, passing a solution of sodium formate cluster containing 5 mM sodium hydroxide in the sheath liquid of 0.2% formic acid in water/isopropanol 1:1 (v/v). Using this method, an exact calibration curve based on numerous cluster masses each differing by 68 Da ($NaCHO_2$) was obtained. Due to the compensation of temperature drift in the microTOF, this external calibration provided accurate mass values (better 5 ppm) for a complete run without the need for a dual sprayer setup for internal mass calibration.

2.6. Statistical analysis

One-way analysis of variance (ANOVA, Tukey's honest significant difference multiple comparison) was evaluated using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA). Phenolic compound data were processed according to the correspondence analysis [28]. Correspondence analysis is a statistical visualization method for picturing the association between the levels of a two-way contingency table. The contingency table was prepared excluding the isomers out of the 104 identified compounds. For each of the remaining 34 compounds the relative isomer abundance in the wheat varieties was computed. The plotting in the first two dimensions of the coordinates of row (wheat genotypes) and column (phytochemicals) variables permitted to have a global view of the correspondence between variety distribution and the factor axis. In the biplot only the phytochemicals with high variation distribution were represented. The first and second dimensions explained 38 and 32% of total variability, respectively.

3. Results and discussion

3.1. Polyphenol and flavonoid content of wheat varieties

Polyphenols are a large group of phytochemicals including flavonoids, the most representative in wheat kernel. Phenolics are chiefly concentrated in the outer layers of wheat grains (bran and aleurone) and contribute to the wheat flour nutraceutical value arising from their antioxidant, anti-inflammatory and anticancer properties [8]. The determination of phenolic content of whole grains evidenced high variability among the 16 old and 6 modern investigated wheat genotypes. The polyphenol content (free and bound fractions) of each wheat variety is presented in Table 1

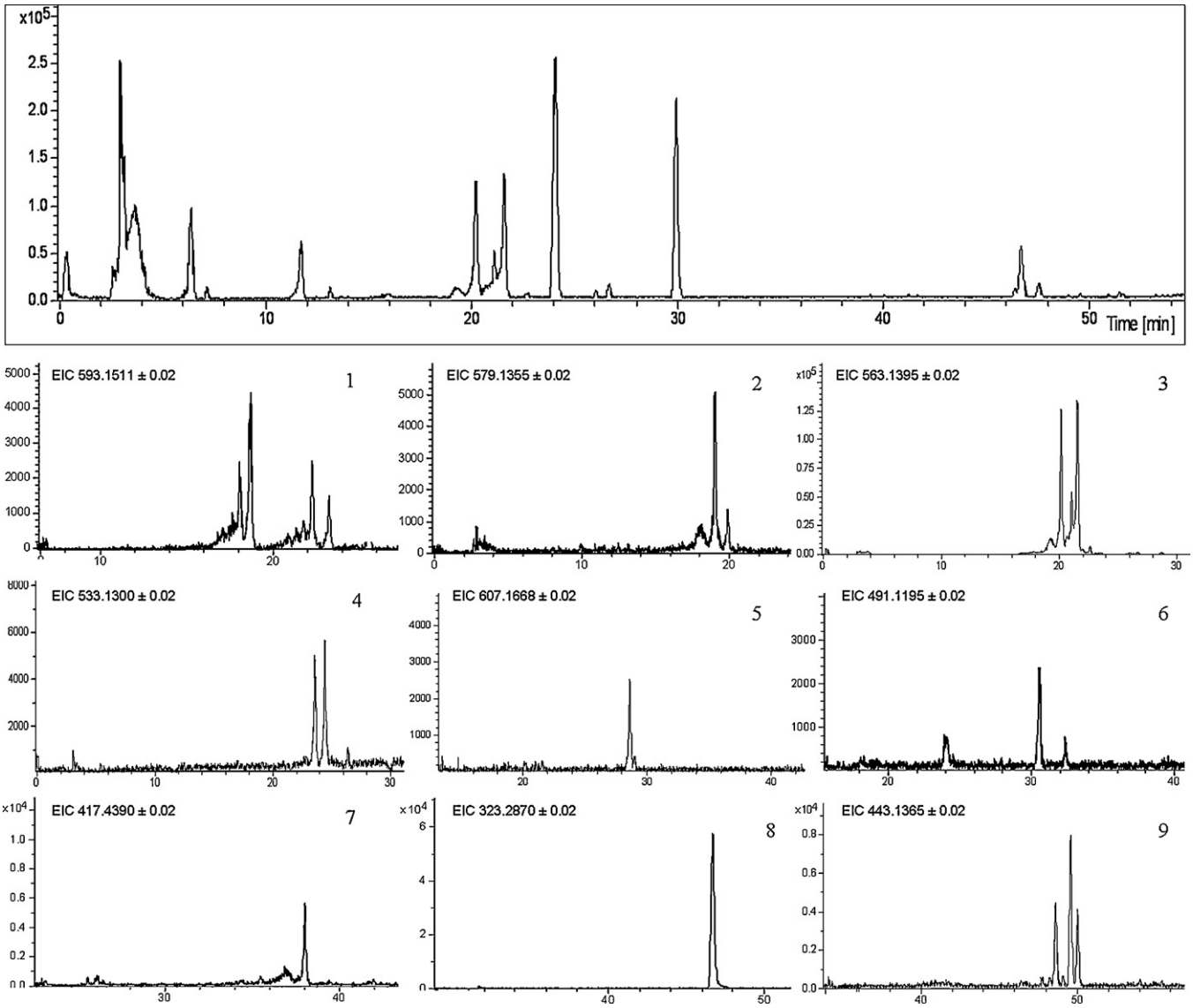

Fig. 1. Base Peak Chromatogram (BPC) obtained by HPLC–ESI–TOF-MS in Marzuolo d'Aqui free fraction and Extracted Ion Chromatograms (EICs) ± 0.02 of well-known detected compounds: (1) vicenin-2 (apigenin-6,8-di-*C*-glucoside), (2) lucenin-1/3 (luteolin-6/8-*C*-xyloside-8/6-*C*-glucoside), (3) apigenin-6-*C*-arabinoside-8-*C*-hexoside (schaftoside/isoschaftoside), (4) glycosylated and acetylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone, (5) methylisoorientin-2″-O-rhamnoside, (6) glycosylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone, (7) syringaresinol, (8) anthocyanidin (cyanidin chloride), (9) formononetin (glycosylated and methylated).

and expressed as micromoles of gallic acid equivalent (GAE) per 100 g of grain. Free polyphenol content ranged from 579.4 μmol GAE/100 g of grain in Verna to 243.0 μmol GAE/100 g of grain in Benco, and a mean value of 378.2 μmol GAE/100 g was obtained (Table 1). Along with Verna, other five old wheat varieties (Canove, Carosello, Gentil Rosso, Gentil Rosso Mutico, Sieve) showed an elevated free polyphenol content, higher than the obtained mean value. As regards the bound fraction, phenolic content ranged from 1237.6 μmol GAE/100 g of grain (Nobel) to 609.9 μmol GAE/100 g of grain (Andriolo). The old genotypes Carosello, Gentil Rosso, Inallettabile, Marzuolo d'Aqui, Marzuolo Val Pusteria and Verna were the highest in terms of bound polyphenol content, along with the modern varieties Eureka and Nobel. On average, the bound fraction contributed to the total phenolic content for 72.0%, confirming previous findings that in wheat kernel polyphenols primarily exist in the bound form, associated with cell wall components [7,9,29]. Flavonoid content of the wheat samples is presented in Table 1 and expressed as micromoles of catechin equivalent (CE) per 100 g of grain. Significantly different values were observed among the 22 tested wheat varieties in the free, bound and total flavonoid fractions. Free flavonoid content ranged from 106.1 ± 0.4 (Verna) to 50.7 ± 6.3 (Bianco Nostrale) μmol CE/100 g of grain. The old genotypes Andriolo, Gentil Rosso Mutico, Marzuolo d'Aqui, Sieve and Verna showed the higher free flavonoid content. The bound flavonoid content ranged from 148.9 ± 4.2 (Gentil Rosso) to 78.3 ± 0.1 (Autonomia B) μmol CE/100 g of grain. The highest values of bound flavonoids were observed for the old genotypes Gentil Rosso, Gentil Rosso Mutico, Inallettabile, Marzuolo d'Aqui and Marzuolo Val Pusteria, along with the modern varieties Eureka and Nobel. The bound fraction contributed to the total flavonoid content for 61.2%. Previous studies investigated the free and bound phenolic content in diverse common wheat varieties. In our study, high variability was observed among the 22 wheat samples and total polyphenol and flavonoid amounts of several genotypes resulted higher compared to data previously reported, especially as regards the bound fraction [7,9,30]. Both genotype and environmental conditions have been demonstrated to affect the phenolic content of wheat grains. Previous investigations reported on highly significant differences of polyphenol content among different wheat varieties, suggesting the genotype-specificity of this characteristic. More-

over the comparison of wheat cultivars grown at different locations showed that environmental and growing conditions may have a certain effect on the biosynthesis and accumulation of phenolic compounds [23–26]. Further studies are necessary to better understand the interaction between genotype and environment with the aim to identify wheat varieties that, at certain growing conditions, may provide wheat-based food products with high nutraceutical value.

3.2. HPLC–ESI–TOF-MS optimization and identification of phenolic compounds

The free phenolic extract of the Marzuolo d'Aqui variety was used to optimize the chromatographic and MS conditions.

Several preliminary experiments were performed testing different mobile phases. A solvent system consisting of acetonitrile of 0.5% acetic acid aqueous solution and acetonitrile was ultimately selected, providing lower pressure, greater baseline stability and higher ionization efficiency. Flow rate is a key factor for separation when using short columns packed with 1.7–2.5 μm particles. Selection of optimum flow rate is based on a compromise between the speed, separation efficiency, peak width and column backpressure. The flow rate of 0.5 mL/min adopted in this method produced a relative short analytical time of less than 50 min and moderate column pressure at about 125 bar for the Marzuolo d'Aqui sample.

Many phenolic compounds in wheat have isomers and are difficult to be separated due to their extremely similar structures. The chromatographic separation of those compounds, having the same molecular weight, is important as single-stage TOF/MS does not distinguish coeluting compounds. Thus, gradient elution was applied to improve the separation of the extracts by varying the solvent strength during the elution process and the optimum gradient was finally picked out through a large number of empirical attempts.

Tentative characterization of phenolic compounds, free and bound, were generated based on elemental composition data determined from accurate mass measurements and comparison with literature data. Fig. 1 shows the base peak chromatogram (BPC) for the free phenolic fraction of the wheat sample Marzuolo d'Aqui and the extracted ion chromotograms (EICs) for the main characterized compounds. All the polyphenols detected for Marzuolo d'Aqui free extract are summarized in Table 2. This table includes selected ion, tolerance (ppm) in generated molecular formula, molecular formula, *m*/*z* experimental, error, sigma values and retention time. The characterization by TOF (MS) was carried out using the Generate Molecular Formula Editor. First of all, a low tolerance was chosen (5 ppm). After that, options with a low sigma value (<0.05) and a low error (<5 ppm) were taken into account in most cases. Finally, the position of the molecular formula in the list of possible compounds was considered. Table 3 lists the phenolic compounds detected in the free and bound extracts of all the wheat varieties. The interpretation of mass spectra allowed the identification of 34 phenolic compounds (104 including isomer forms) in the 22 investigated genotypes. Most of the detected polyphenols were previously described in wheat in other reports [13,14,16,17,31,32]. As outlined below, compounds were grouped into chemical classes and structural formulae of representative compounds are shown in Fig. 2. The phenolic compounds (including isomers) were numbered according to the retention time and their occurrence in the common wheat genotypes is presented in Table 3.

3.2.1. Phenolic acids

Several studies investigated the phenolic acid composition of wheat grains and of different seed parts (endosperm, bran and aleurone layer) [7,10,12,18–20,29,33]. In wheat, ferulic acid is the predominant compound of the class and accounts for 70–90% of total phenolic acid content [18,24]. Ferulic acid can be found in

Table 2
Polyphenols detected in the free phenolic extract of Marzuolo d'Aqui variety.

No.	Molecular formula	Selected ion	*m*/*z* experimental	*m*/*z* calculated	Error (ppm)	Sigma value	Tolerance (ppm)	Retention time (min)	Classification order (number of possibilities)	Compound	Ref.
1	$C_{27}H_{30}O_{15}$	$[M-H]^-$	593.1512	593.1511	−1.5	0.0396	5	17.00	1° (10)	Vicenin-2 (apigenin-6,8-di-C-glucoside)	[13]
2	$C_{26}H_{28}O_{15}$	$[M-H]^-$	579.1356	579.1355	−1.7	0.0088	5	19.10	1° (9)	Lucenin-1/3 (luteolin-6/8-C-xyloside-8/6-C-glucoside)	[13]
3	$C_{26}H_{28}O_{14}$	$[M-H]^-$	563.1396	563.1395	1.9	0.0066	5	20.25	1° (7)	Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside)	[13]
4	$C_{25}H_{26}O_{13}$	$[M-H]^-$	533.1300	533.1300	0.2	0.0246	5	22.94	2° (7)	Glycosylated and acetylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone	[13]
5	$C_{28}H_{32}O_{15}$	$[M-H]^-$	607.1640	607.1668	4.7	0.0354	5	28.68	1° (11)	Methylisoorientin-2″-O-rhamnoside	[13]
6	$C_{23}H_{24}O_{12}$	$[M-H]^-$	491.1205	491.1195	−2.1	0.0289	5	30.57	2° (6)	Glycosylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone	[13]
7	$C_{22}H_{26}O_8$	$[M-H]^-$	417.1555	417.4390	3.5	0.0365	5	38.55	1° (3)	Syringaresinol	[14]
8	$C_{15}H_{11}O_6Cl$	$[M-H]^-$	285.0394	323.2870	−0.8	0.0188	5	46.65	1° (2)	Anthocyanidin (cyanidin chloride)	[16]
9	$C_{23}H_{24}O_9$	$[M-H]^-$	436.2347	443.1365	−3.9	0.0209	5	48.58	1° (3)	Formononetin (Glycosylated and methylated)	[17]

Table 3
Phenolic compounds detected by HPLC–ESI–TOF-MS in the free and bound extracts of wheat varieties.

No.	Retention time (min)	Molecular formula	m/z calculated	Compound	Class	Sample		Reference
						Free extract	Bound extract	
1	10.92	$C_9H_{10}O_5$	197.0455	Syringic acid isomer	Phenolic acids	BO		[31]
2	12.73	$C_{10}H_{10}O_4$	193.0506	**Ferulic acid**	Phenolic acids	AA AB CAR	AN AB CAR GR NO	[31]
3	13.58	$C_{10}H_{10}O_4$	193.0506	Ferulic acid isomer	Phenolic acids		AN AA AB CAR NO	[31]
4	15.43	$C_8H_8O_4$	167.0349	**Vanillic acid**	Phenolic acids	BE CAR EU	AB BE BN CAR GR GRM MA	[31]
5	16.23	$C_{27}H_{30}O_{15}$	593.1511	Vicenin-2 (apigenin-6,8-di-C-glucoside) isomer	Flavone-C-glycoside	GR		[13]
6	16.69	$C_9H_{10}O_5$	197.0455	**Syringic acid**	Phenolic acids	BO	AN AA AB BN BI BO CAN CAR EU FR GR GRM IN PA	[31]
7	17.00	$C_{27}H_{30}O_{15}$	593.1511	Vicenin-2 (apigenin-6,8-di-C-glucoside) isomer	Flavone-C-glycoside	GR MA		[13]
8	17.32	$C_7H_6O_2$	121.0290	**p-Hydroxybenzaldehyde**	Phenolic acids		BI FR IN MA MVP PA	[31]
9	17.44	$C_{26}H_{28}O_{15}$	579.1355	Lucenin-1/3 (luteolin-6/8-C-xyloside-8/6-C-glucoside) isomer	Flavone-C-glycoside	BI GR		[13]
10	17.83	$C_8H_8O_4$	167.0349	Vanillic acid isomer	Phenolic acids	EU		[31]
11	18.10	$C_{27}H_{30}O_{15}$	593.1511	Vicenin-2 (apigenin-6,8-di-C-glucoside) isomer	Flavone-C-glycoside	MA VE		[13]
12	18.22	$C_{26}H_{28}O_{15}$	579.1355	Lucenin-1/3 (luteolin-6/8-C-xyloside-8/6-C-glucoside) isomer	Flavone-C-glycoside	GR		[13]
13	18.51	$C_{27}H_{30}O_{15}$	593.1511	Vicenin-2 (apigenin-6,8-di-C-glucoside) isomer	Flavone-C-glycoside	GRM MVP		[13]
14	18.57	$C_{26}H_{28}O_{14}$	563.1395	Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside) isomer	Flavone-C-glycoside	BI GR	BI	[13]
15	18.80	$C_{27}H_{30}O_{15}$	593.1511	Vicenin-2 (apigenin-6,8-di-C-glucoside) isomer	Flavone-C-glycoside	GRM MA MVP		[13]
16	19.10	$C_{26}H_{28}O_{15}$	579.1355	**Lucenin-1/3 (luteolin-6/8-C-xyloside-8/6-C-glucoside)**	Flavone-C-glycoside	GRM MA MVP VE	BN FR	[13]
17	19.90	$C_{26}H_{28}O_{14}$	563.1395	Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside) isomer	Flavone-C-glycoside	GR	BI	[13]
18	19.97	$C_{26}H_{28}O_{15}$	579.1355	Lucenin-1/3 (luteolin-6/8-C-xyloside-8/6-C-glucoside) isomer	Flavone-C-glycoside	VE		[13]
19	20.25	$C_{26}H_{28}O_{14}$	563.1395	Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside) isomer	Flavone-C-glycoside	GR GRM MA MVP VE	EU FR GR MA MVP SI VE	[13]
20	20.62	$C_{27}H_{30}O_{15}$	593.1511	Vicenin-2 (apigenin-6,8-di-C-glucoside) isomer	Flavone-C-glycoside	GR		[13]
21	20.90	$C_{27}H_{30}O_{15}$	625.1411	**Apigenin-6/8-C-pentoside-8/6-C-hexoside**	Flavone-C-glycoside		BI	[13]
22	21.14	$C_{26}H_{28}O_{14}$	563.1395	Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside) isomer	Flavone-C-glycoside	MA MVP VE		[13]
23	21.60	$C_{26}H_{28}O_{14}$	563.1395	**Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside)**	Flavone-C-glycoside	FR GRM MA MVP PA VE	EU FR GB MA PA SI	[13]
24	21.80	$C_{27}H_{30}O_{15}$	593.1511	Vicenin-2 (apigenin-6,8-di-C-glucoside) isomer	Flavone-C-glycoside	GR MVP VE		[13]
25	22.08	$C_{25}H_{26}O_{13}$	533.1300	Glycosylated and acetylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone isomer	Flavone-O-glycoside	GR		[13]
26	22.27	$C_{27}H_{30}O_{15}$	593.1511	Vicenin-2 (apigenin-6,8-di-C-glucoside) isomer	Flavone-C-glycoside	FR MVP		[13]
27	22.68	$C_{26}H_{28}O_{14}$	563.1395	Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside) isomer	Flavone-C-glycoside	GRM PA		[13]
28	22.94	$C_{25}H_{26}O_{13}$	533.1300	**Glycosylated and acetylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone**	Flavone-O-glycoside	GR MA VE	BO NO	[13]
29	22.98/23.00	$C_8H_8O_3$	151.0400	**Vanillin**	Phenolic acids		CAN CAR EU GRM IN MA SI	[31]
30	23.09	$C_{26}H_{32}O_{12}$	535.1821	**Pinosylvin (double glycosylation)**	Stilbenoids	GR		[17]
31	23.20	$C_9H_6O_2$	145.0295	**Coumarin**	Coumarins (lactones)		GRM MA	[31]
32	23.30	$C_{27}H_{30}O_{15}$	593.1511	Vicenin-2 (apigenin-6,8-di-C-glucoside) isomer	Flavone-C-glycoside	MA MVP VE		[13]
33	23.63	$C_9H_8O_3$	163.0400	**p-Coumaric acid**	Phenolic acids		AN AA AB BN BO CAN GRM IN MA SI	[31]
34	24.25	$C_9H_{10}O_4$	181.0506	**Syringaldehyde**	Phenolic acids		AN AA BO CAN CAR GRM IN VE	[31]
35	24.30	$C_{26}H_{32}O_{12}$	535.1821	Pinosylvin (double glycosylation) isomer	Stilbenoids		GR	[17]
36	24.50	$C_{25}H_{26}O_{13}$	533.1300	Glycosylated and acetylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone isomer	Flavone-O-glycoside	MA VE		[13]
37	24.88	$C_{21}H_{20}O_{10}$	431.0983	**Vitexin/Isovitexin**	Flavone-C-glycoside	GRM SI	BN BI MVP NO	[13]
38	24.92	$C_{10}H_{10}O_4$	193.0506	Ferulic acid isomer	Phenolic acids	AA AB CAR	AN AA AB BE BN BI BO CAN CAR GR GRM MA MI NO PA	[31]
39	25.00	$C_{27}H_{30}O_{15}$	593.1511	Vicenin-2 (apigenin-6,8-di-C-glucoside) isomer	Flavone-C-glycoside	VE GR	CAN CAR EU FR SI	[13]
40	25.20	$C_{27}H_{30}O_{15}$	593.1511	**Vicenin-2 (apigenin-6,8-di-C-glucoside)**	Flavone-C-glycoside	VE GR	CAN CAR EU FR SI	[13]
41	25.67	$C_{10}H_{10}O_4$	193.0506	Ferulic acid isomer	Phenolic acids	AB	CAN NO	[31]
42	25.71	$C_{11}H_{12}O_5$	223.0612	**Sinapic acid**	Phenolic acids		CAR	[31]

43	25.90	$C_{22}H_{23}O_{11}Cl$	461.1089	**Peonidin-3-glucoside**	Anthocyanin	SI		
44	26.00	$C_{33}H_{38}O_{21}$	769.1821	**Apigenin-6-C-beta-galactosyl-8-C-beta-glucosyl-O-glucuronopyranoside**	Flavone-C-glycoside	AN AA AB BN NO		[13]
45	26.60	$C_{33}H_{38}O_{21}$	769.1821	Apigenin-6-C-beta-galactosyl-8-C-beta-glucosyl-O-glucuronopyranoside isomer	Flavone-C-glycoside	AN AA AB BE BN NO		[13]
46	26.65	$C_{21}H_{21}O_{10}Cl$	433.2710	**Pelargonidin-3-glucoside (callistephin)**	Anthocyanin		AN AA FR	[16]
47	26.70	$C_{15}H_{10}O_5$	269.0455	**Apigenin**	Flavone	BE	BI BN	[13]
48	26.75	$C_{21}H_{20}O_{10}$	431.0983	Vitexin/Isovitexin isomer	Flavone-C-glycoside	GB	BN MA	[13]
49	27.50	$C_{10}H_{10}O_4$	193.0506	Ferulic acid isomer	Phenolic acids		CAN NO	[31]
50	27.56	$C_{28}H_{32}O_{15}$	607.1668	Methylisoorientin-2″-O-rhamnoside isomer	Flavone-C-glycoside	BE BO GR		[13]
51	27.90	$C_{27}H_{30}O_{14}$	577.1562	**Isovitexin-2″-O-rhamnoside**	Flavone-C-glycoside	VE		[13]
52	28.00	$C_{15}H_{10}O_5$	269.0455	Apigenin isomer	Flavone	EU IN	IN	[13]
53	28.12	$C_{22}H_{26}O_8$	417.4390	Syringaresinol isomer	Lignans	NO PA	GRM IN	[14]
54	28.63	$C_{21}H_{22}O_8$	401.1241	**Glycosylated pinosylvin**	Stilbenoids		EU FR GB GR GRM IN MA MVP PA VE	[17]
55	28.68	$C_{28}H_{32}O_{15}$	607.1668	**Methylisoorientin-2″-O-rhamnoside**	Flavone-C-glycoside	MA SI VE	BE FR	[13]
56	28.72	$C_{33}H_{38}O_{21}$	769.1821	Apigenin-6-C-beta-galactosyl-8-C-beta-glucosyl-O-glucuronopyranoside isomer	Flavone-C-glycoside	AN BI NO		[13]
57	29.00	$C_{21}H_{20}O_{11}$	447.3800	**Orientin/Isoorientin**	Flavone-C-glycoside	VE		[13]
58	29.07	$C_{21}H_{21}O_{11}Cl$	447.0932	**Cyanidin-3-glucoside (kuromanin)**	Anthocyanin	AB BN	FR GB MA SI	[16]
59	29.12	$C_{20}H_{18}O_8$	385.0928	Dihydroferulic acid isomer	Phenolic acids		AB BI BN EU FR GB GR IN MA PA SI VE	[31]
60	29.50	$C_{23}H_{24}O_{12}$	491.1195	Glycosylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone isomer	Flavone-O-glycoside	GR MVP	FR	[13]
61	29.68	$C_{33}H_{38}O_{21}$	769.1821	Apigenin-6-C-beta-galactosyl-8-C-beta-glucosyl-O-glucuronopyranoside isomer	Flavone-C-glycoside	AA AB BN		[13]
62	30.57	$C_{23}H_{24}O_{12}$	491.1195	**Glycosylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone**	Flavone-O-glycoside	MA MVP	MA MVP VE	[13]
63	31.57	$C_{15}H_{10}O_5$	269.0455	Apigenin isomer	Flavone	BE		[13]
64	32.39	$C_{23}H_{24}O_{12}$	491.1195	Glycosylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone isomer	Flavone-O-glycoside	GB IN MA MVP	IN AB	[13]
65	32.42	$C_{15}H_{10}O_5$	269.0455	Apigenin isomer	Flavone	BE GB	BN	[13]
66	32.70	$C_{30}H_{26}O_{12}$	577.1351	Procyanidin B-3 isomer	Proanthocyanidin		FR GB GR MA MVP	[32]
67	32.88	$C_{20}H_{18}O_8$	385.0928	Dihydroferulic acid isomer	Phenolic acids		BI GR	[31]
68	33.97	$C_{10}H_{10}O_4$	193.0506	Ferulic acid isomer	Phenolic acids		GR	[31]
69	34.00	$C_{30}H_{26}O_{12}$	577.1351	Procyanidin B-3 isomer	Proanthocyanidin		FR GB MVP PA VE	[32]
70	34.06	$C_{26}H_{28}O_{14}$	563.1395	Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside) isomer	Flavone-C-glycoside		AN AA	[13]
71	34.32	$C_{21}H_{22}O_8$	401.1241	Glycosylated pinosylvin isomer	Stilbenoids		GRM VE	[17]
72	34.98	$C_{20}H_{18}O_8$	385.0928	**Dihydroferulic acid**	Phenolic acids		AN BN BI BO EU FR GB GR IN MA MVP NO PA SI VE	[31]
73	35.57	$C_{26}H_{28}O_{14}$	563.1395	Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside) isomer	Flavone-C-glycoside		AA BE	[13]
74	36.36	$C_{10}H_{10}O_4$	193.0506	Ferulic acid isomer	Phenolic acids		BI	[31]
75	37.14	$C_{20}H_{18}O_6$	353.1030	**Hinokinin**	Lignans		BN CAR EU MA	[14]
76	37.28	$C_{21}H_{22}O_8$	401.1241	Glycosylated pinosylvin isomer	Stilbenoids		GRM IN VE	[17]
77	37.37	$C_{33}H_{38}O_{21}$	769.1821	Apigenin-6-C-beta-galactosyl-8-C-beta-glucosyl-O-glucuronopyranoside isomer	Flavone-C-glycoside		MVP PA	[13]
78	37.82	$C_{20}H_{18}O_8$	385.0928	Dihydroferulic acid isomer	Phenolic acids		AN BN BI BO CAR EU FR GR GRM IN MA MI PA SI VE	[31]
79	38.20	$C_{30}H_{26}O_{12}$	577.1351	Procyanidin B-3 isomer	Proanthocyanidin		BI FR GR MVP	[32]
80	38.26	$C_{10}H_{10}O_4$	193.0506	Ferulic acid isomer	Phenolic acids		AA BN BE BO CAN GRM MA MI NO PA	[31]
81	38.38	$C_{20}H_{18}O_8$	385.0928	Dihydroferulic acid isomer	Phenolic acids		BI EU IN MVP SI VE	[31]
82	38.55	$C_{22}H_{26}O_8$	417.4390	**Syringaresinol**	Lignans	EU GB IN MA MVP	GB IN MA NO PA	[14]
83	39.45	$C_{33}H_{38}O_{21}$	769.1821	Apigenin-6-C-beta-galactosyl-8-C-beta-glucosyl-O-glucuronopyranoside isomer	Flavone-C-glycoside		EU GB MVP PA	[13]
84	39.66	$C_{20}H_{18}O_8$	385.0928	Dihydroferulic acid isomer	Phenolic acids		BO CAR EU FR IN MVP MI PA VE	[31]

Table 3 (*Continued*)

No.	Retention time (min)	Molecular formula	m/z calculated	Compound	Class	Sample		Reference
						Free extract	Bound extract	
85	40.02	$C_{20}H_{18}O_8$	385.0928	Dihydroferulic acid isomer	Phenolic acids		AA EU FR GB GR GRM IN MA MI PA VE	[31]
86	40.25	$C_{30}H_{26}O_{12}$	577.1351	**Procyanidin B-3**	Proanthocyanidin		EU FR GB MA MVP NO SI	[32]
87	41.01	$C_{22}H_{26}O_8$	417.4390	Syringaresinol isomer	Lignans		GB VE	[14]
88	42.20	$C_{30}H_{26}O_{12}$	577.1351	Procyanidin B-3 isomer	Proanthocyanidin		FR GB SI	[32]
89	43.63	$C_{33}H_{38}O_{21}$	769.1821	Apigenin-6-C-beta-galactosyl-8-C-beta-glucosyl-O-glucuronopyranoside isomer	Flavone-C-glycoside		FR VE	[13]
90	44.00	$C_{20}H_{18}O_8$	385.0928	Dihydroferulic acid isomer	Phenolic acids		AA AB BE FR MVP VE	[31]
91	44.06	$C_{10}H_{10}O_4$	193.0506	Ferulic acid isomer	Phenolic acids		AA BO GR	[31]
92	44.37	$C_{33}H_{38}O_{21}$	769.1821	Apigenin-6-C-beta-galactosyl-8-C-beta-glucosyl-O-glucuronopyranoside isomer	Flavone-C-glycoside		FR VE	[13]
93	44.85	$C_{20}H_{22}O_6$	357.1343	**Pinoresinol**	Lignans	CAR	BN CAR FR GB GR GRM IN MI NO SI VE	[14]
94	45.28	$C_{15}H_{10}O_5$	269.0455	Apigenin isomer	Flavone	BE CAR GB SI	BI BN CAR EU IN	[13]
95	45.37	$C_{26}H_{28}O_{14}$	563.1395	Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside) isomer	Flavone-C-glycoside		AB	[13]
96	45.85	$C_{17}H_{14}O_7$	329.0666	**5,7,4′-trihydroxy-3′,5′-dimethoxy-flavone (tricin)**	Flavone	CAN CAR EU GB GR GRM NO	CAR EU GRM MI PA	[13]
97	46.65	$C_{15}H_{11}O_6Cl$	323.2870	**Anthocyanidin (cyanidin chloride)**	Anthocyanidin	MA	CAN EU MI	[16]
98	46.98	$C_{33}H_{38}O_{21}$	769.1821	Apigenin-6-C-beta-galactosyl-8-C-beta-glucosyl-O-glucuronopyranoside isomer	Flavone-C-glycoside		EU FR IN	[13]
99	47.02	$C_{26}H_{28}O_{14}$	563.1395	Apigenin-6-C-arabinoside-8-C-hexoside (Schaftoside/Isoschaftoside) isomer	Flavone-C-glycoside		AB BO	[13]
100	47.59	$C_{23}H_{24}O_9$	443.1347	**Formononetin (Glycosylated and methylated)**	Isoflavone	GB	GB MA PA	[17]
101	48.58	$C_{23}H_{24}O_9$	443.1347	Formononetin (Glycosylated and methylated) isomer	Isoflavone	GR MA MI VE		[17]
102	48.67	$C_{30}H_{26}O_{12}$	577.1351	Procyanidin B-3 isomer	Proanthocyanidin		GB MA	[32]
103	49.46	$C_{23}H_{24}O_9$	443.1347	Formononetin (Glycosylated and methylated) isomer	Isoflavone	AN GB MA NO VE		[17]
104	49.90	$C_{23}H_{24}O_9$	443.1347	Formononetin (Glycosylated and methylated) isomer	Isoflavone	AN MA MVP VE		[17]

Abbreviations: AN, Andriolo; AA, Autonomia A; AB, Autonomia B; BE, Benco; BI, Bilancia; BO, Bolero; CAN, Canove; CAR, Carosello; EU, Eureka; FR, Frassineto; GB, Gentil Bianco; GR, Gentil Rosso; GRM, Gentil Rosso Mutico; IN, Inallettabile; MA, Marzuolo d'Aqui; MVP, Marzuolo Val Pusteria; MI, Mieti; NO, Nobel; PA, Palesio; SI, Sieve; VE, Verna.

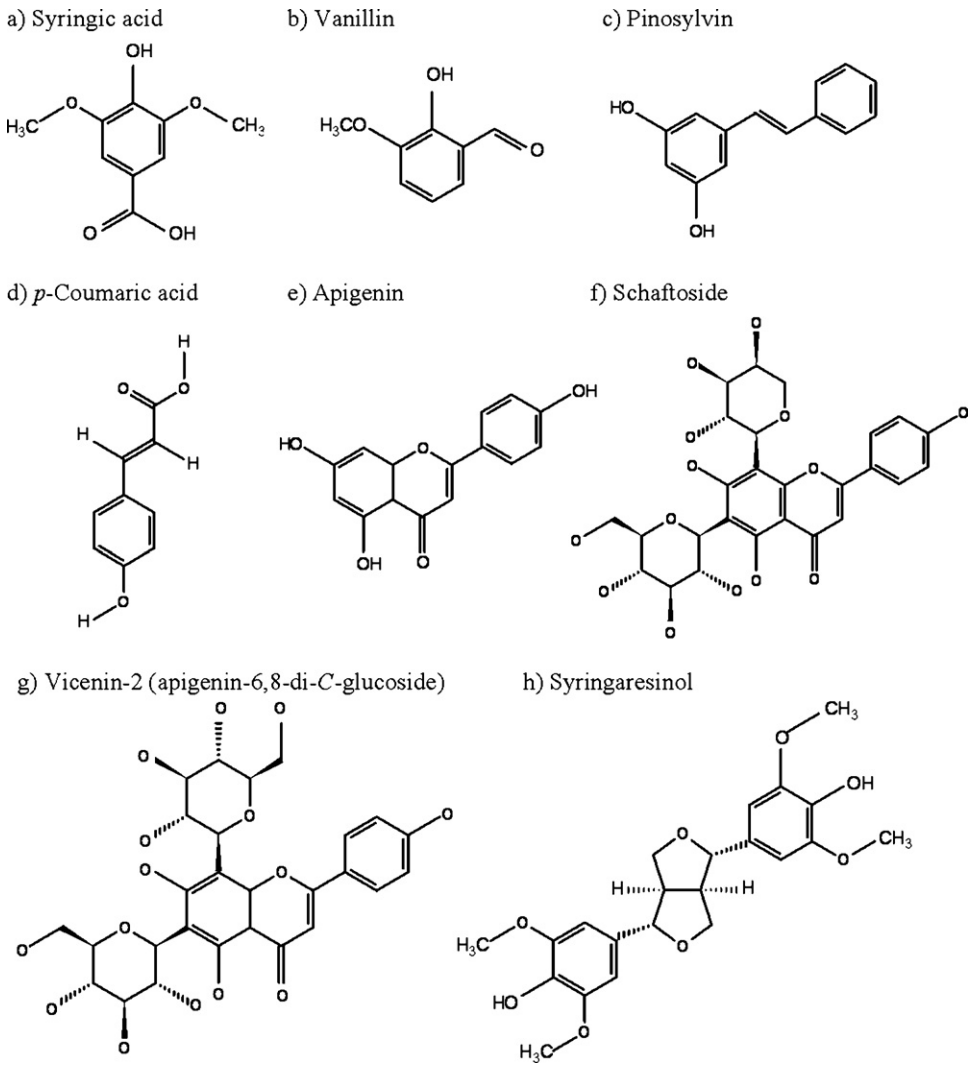

Fig. 2. Structural formulae of representative phenolic compounds detected in the wheat genotypes.

both free and bound forms; however, it is chiefly abundant in the aleurone layer cross-linked with cell wall components (i.e. arabinoxylans) via ester and ether bonds [34]. Ferulic acid was previously described in wheat grains along with other benzoic and cinnamic acid derivatives as dihydroferulic, sinapic, caffeic, vanillic, syringic, p-coumaric and p-hydroxybenzoic acids [7,12,18,20,31]. In our study dihydroferulic (isomer compounds 59, 67, 72, 78, 81, 84, 85, 90), ferulic (isomer compounds 2, 3, 38, 41, 49, 68, 74, 80, 91), syringic (isomer compounds 1 and 6), vanillic (isomer compounds 4 and 10) and *p*-coumaric acid (compound 33) were detected in most of the investigated varieties, mainly in the bound fractions. *p*-hydroxybenzaldehyde (compound 8), vanillin (compound 29) and syringaldehyde (compound 34) were found in the bound extracts of some of the analyzed genotypes. Moreover, the old wheat variety Carosello showed the presence of compound 42 with mass 224.0612 ($C_{11}H_{12}O_5$) in the bound extract, tentatively identified as sinapic acid. Vanillin was recorded in six old wheat varieties (Canove, Carosello, Gentil Rosso Mutico, Inallettabile, Marzuolo d'Aqui, Sieve) and in the modern cultivar Eureka. This finding is intriguing for the development of wheat-based food with peculiar sensory features since, even at low concentrations, vanillin contributes to the flavor and aroma properties of wheat flours and derived foods (bread and bakery products) [35].

3.2.2. Flavonoids

Flavonoids are a large class of phenolic compounds with a C6–C3–C6 skeleton and consist of two aromatic rings joined by a three-carbon bridge. Several studies reported on the health-promoting effects of flavonoids as antioxidant, anticancer, anti-allergic, anti-inflammatory, anticarcinogenic and gastroprotective agents [36–38]. Flavonoids are classified in different classes according to diverse chemical structures and characteristics, including flavonols, flavones, flavonones, anthocyanidins and isoflavones. In wheat grains, flavonoids are mainly located in the pericarp layers and exist in the free and bound forms, usually occurring as glycosides [8,21]. In our study, flavones resulted the most representative flavonoid group of the investigated wheat grains, comprising of 2 aglyconic forms, 9 *C*-glycosylated and 2 *O*-glycosylated compounds. In wheat, the glycosidic forms are mainly apigenin (5,7,4′-trihydroxyflavone) and luteolin (5,7,3′,4′-tetrahydroxyflavone) derivatives and accumulate as their respective 6-*C* and/or 8-*C*-glycosidic conjugates [13,17]. The interpretation of the mass spectra allowed the detection of two aglyconic flavones, identified in the free and bound extracts of the wheat genotypes as apigenin (isomer compounds 47, 52, 63, 65, 94) with mass 270.0455 ($C_{15}H_{10}O_5$) and 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone (tricin) (compound 96) with mass 330.0666 ($C_{17}H_{14}O_7$). In common with previous studies, lucenin-1/3 (luteolin-6/8-

C-xyloside-8/6-*C*-glucoside) (isomer compounds 9, 12, 16, 18), vicenin-2 (apigenin-6,8-di-*C*-glucoside) (isomer compounds 5, 7, 11, 13, 15, 20, 24, 27, 32, 39, 40), apigenin-6-*C*-beta-galactosyl-8-*C*-beta-glucosyl-*O*-glucuronopyranoside (isomer compounds 44, 45, 56, 61, 77, 83, 89, 92, 98), methylisoorientin-2″-*O*-rhamnoside (isomer compounds 50, 55), vitexin/isovitexin (isomer compounds 37, 48) and apigenin-6-*C*-arabinoside-8-*C*-hexoside (schaftoside/isoschaftoside) (isomer compounds 14, 17, 19, 22, 23, 27, 70, 73, 95, 99) were found in most of the investigated wheat genotypes, both in free and bound fractions (Table 3). Compound 21 with molecular mass 625.1411 ($C_{27}H_{30}O_{17}$) was tentatively identified as apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside and detected only in the bound extract of the modern variety Bilancia. Compounds 51 and 57, identified as isovitexin-2″-*O*-rhamnoside ($C_{27}H_{30}O_{14}$, mass 578.1562) and orientin/isoorientin ($C_{21}H_{20}O_{11}$, mass 448.3800), respectively, were found exclusively in the free fraction of the old genotype Verna. Two flavone-*O*-glycosides were found in the free and bound extracts of the wheat samples, with masses 492.1195 and 534.1300, putatively identified as glycosylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone (isomer compounds 60, 62, 64) and glycosylated and acetylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone (isomer compounds 25, 28, 36), respectively. All the flavones detected in the free and bound extracts of the 22 common wheat varieties were previously described in wheat bran and whole grains [13,17,39]. Isoflavones are part of the flavonoid group with the B-ring attached at C3 rather than the C2 position. These secondary metabolites have been widely investigated in leguminous plants (i.e. soybean), in which they occur in high concentrations, mainly as daidzein and genistein derivative compounds [40]. Matus-Cádiz et al. [17] reported on the presence of formononetin (the glycosilated and methylated daidzein derivative) in wheat. In our study, compound 100 and relating isomers (101, 103, 104) with mass 443.1347 ($C_{23}H_{24}O_9$), tentatively identified as formonotenin, were detected in the free and bound extracts of 9 wheat varieties (Table 3).

Anthocyanidins are secondary metabolites belonging to the flavonoid class and playing an important physiological role in UV protection of plant tissues. Moreover, several studies reported on the health-promoting effects of anthocyanidins as antioxidants and anti-inflammatory compounds [16,41,42]. In wheat, anthocyanidins are found in different parts of the plant (coleoptile, culm, leaves) and in the pericarp and aleurone layer of the caryopsis. Although in common wheat grains pigments are generally present at very low concentration, they were detected in higher amounts in the outer layers of blue, purple and red wheat cultivar kernels [15,16,43,44]. Wheat anthocyanidins are mainly found as cyanidin, peonidin and pelargonidin derivatives in the glycosylated forms (anthocyanins), linked with glucose, galactose and arabinose [16]. Compound 46 with mass 434.2710 and deduced molecular formula $C_{21}H_{21}O_{10}Cl$ was found in the bound extracts of Andriolo, Autonomia A and Frassineto varieties and assigned as pelargonidin-3-glucoside (callistephin). A cyanidin glycosylated derivative (mass 448.0932, $C_{21}H_{21}O_{11}Cl$) was detected in the free and bound extracts of few old genotypes and putatively identified as cyanidin-3-glucoside (kuromanin) (compound 58). Peonidin-3-glucoside (compound 43), with mass 462.1089 and deduced molecular formula $C_{22}H_{23}O_{11}Cl$, was found exclusively in the free phenolic fraction of the old variety Sieve. Along with anthocyanins, an anthocyanidin aglycone was detected in the wheat varieties Canove, Eureka, Marzuolo d'Aqui and Mieti and identified as cyanidin chloride (mass 324.2870, $C_{15}H_{11}O_6Cl$) (compound 97).

3.2.3. Other chemical classes (coumarins, stilbenes, proanthocyanidins, lignans)

Besides phenolic acids and flavonoids which are the most representative and investigated polyphenol classes, whole wheat grains contain different phenolic compounds belonging to the groups of coumarins, stilbenes, proanthocyanidins and lignans.

Coumarins are a class of lactones with a 2*H*-1-benzopyran-2-one nucleus. These phenolic compounds are used clinically as antineoplastic and for the treatment of lymphedema and venous insufficiency [45,46]. Coumarin (1,2-benzopyrone) represents the simplest compound of the class, consisting of an aromatic ring fused to a condensed lactone ring, and possesses aroma properties. Previous studies described the presence of coumarins in common wheat caryopses in the free, carboxylated and hydroxylated forms [31]. Compound 31 with mass 146.0295 and deduced molecular formula $C_9H_6O_2$ was tentatively identified as coumarin. It was detected exclusively in the bound extracts of Gentil Rosso Mutico and Marzuolo d'Aqui, thus representing a peculiarity of the two old genotypes.

Stilbenes are a small family of phenolics implicated in plant disease resistance, thus classified as phytoalexins. These secondary metabolites act as natural protective agents against pests and diseases as they possess antiviral, antifungal and antimicrobial activity, and their production increases in case of biotic and abiotic stresses. In addition, numerous studies investigated the pharmacological activity of stilbenes, in particular the antioxidant, anti-inflammatory and anticarcinogenic effects of resveratrol and pinosylvin [47–51]. Although very few studies reported on stilbene occurrence in wheat, two pinosylvin derivatives were described by Matus-Cádiz et al. [17]. The glycosylated form of pinosylvin (mass 402.1241, $C_{21}H_{22}O_8$) was assigned as compound 54 (isomer forms 71, 76) and detected in the bound extracts of 10 investigated wheat varieties. Isomer compounds 30 and 35 with mass 536.1821 and deduced molecular formula $C_{26}H_{32}O_{12}$ were tentatively identified as double glycosylated pinosylvin and detected exclusively in the free and bound extracts of the old wheat variety Gentil Rosso.

Proanthocyanidins are a class of colorless phenolics characterized by an oligomeric or polymeric structure, based on flavanol units. Several studies demonstrated the proanthocyanidin health benefits due to their antioxidant activity, modulation of immune function and anti-thrombotic effect [52,53]. Catechin and epicatechin are the constitute units of procyanidins, a group of proanthocyanidins described previously in red-grained wheat bran [32]. Compound 86 (isomer forms 69, 79, 88, 102) with mass 578.1351 and deduced molecular formula $C_{30}H_{26}O_{12}$ was assigned as procyanidin B-3 and detected in the bound extracts of the wheat genotypes.

Lignans are a group of phenolics classified as phytoestrogens. Although they are non-steroidal compounds, lignans are structurally similar to endogenous estrogens and exhibit anticancerogenic, antioxidant, anti-proliferative, pro-apoptotic and antiangiogenic properties [54]. As reported in our previous study, different lignan compounds are present in wheat whole grains, with a total amount ranging from 2.6 to 5.0 μg per gram of dry weight [14]. Compound 75 with mass 353.1030 and deduced molecular formula $C_{20}H_{18}O_6$ was tentatively identified as hinokinin and detected in the bound extracts of Bianco Nostrale, Carosello, Eureka and Marzuolo d'Aqui varieties. Two other lignan compounds were identified both in the free and bound extracts of the wheat samples and assigned as syringaresinol (isomer compounds 53, 82, 87) with mass 417.4390 ($C_{22}H_{26}O_8$) and pinoresinol (compound 93) with mass 357.1343 ($C_{20}H_{22}O_6$).

3.3. Phenolic profiles of old and modern wheat varieties

On the basis of the similarity among each phytochemical profile, the 16 old wheat genotypes were classified in two groups as indicated in Table 4 and compared to the 6 modern wheat varieties. The number of detected free polyphenols resulted significantly higher in group 2 containing the old varieties Bianco Nostrale, Frassineto,

Table 4
Total compounds, including isomers, total isomers and unique compounds detected in free and bound phenolic fractions in the wheat samples. The mean values for modern and old varieties (group1, group 2) are reported.

Cultivar	Free phenolic			Bound phenolic		
	Total compounds[a]	Total isomers	Unique compounds	Total compounds[a]	Total isomers	Unique compounds
Modern varieties	3.5 (b)[b]	0.8 (b)	0.2 (a)	13.3 (b)	4.5 (b)	0.3 (a)
Old varieties (group 1)	5.8 (ab)	2.1 (ab)	0.2 (a)	12. 3 (b)	4.0 (b)	0.2 (a)
Old varieties (group 2)	11.0 (a)	5.1 (a)	0.6 (a)	18.6 (a)	7.0 (a)	0.5 (a)

Modern varieties: Bilancia, Bolero, Eureka, Mieti, Nobel, Palesio; Goup 1: Andriolo, Autonomia A, Autonomia B, Benco, Canove, Carosello, Gentil Bianco, Inallettabile, Marzuolo Val Pusteria, Sieve; Group 2: Bianco Nostrale, Frassineto, Gentil Rosso, Gentil Rosso Mutico, Marzuolo d'Aqui, Verna.

[a] Including isomers.

[b] Means followed by the same letter or no letter are not significantly different for $P < 0.05$.

Gentil Rosso, Gentil Rosso Mutico, Marzuolo d'Aqui and Verna (11.0) than those found in the modern cultivars (3.5). Moreover the mean number of isomer forms was approximately six times higher in group 2 (5.1) than in modern genotypes (0.8). No significant differences were observed between group 1 and modern cultivars, in terms of both total polyphenols and isomer forms. As regards the bound fraction, not statistically different mean values were observed for modern and group 1 varieties (13.3 and 12.3 phenolic compounds, respectively). Group 2 showed a mean value of bound phenolics (18.6) and isomer forms (7.0) significantly higher than all the other investigated genotypes. The interpretation of the mass spectra highlighted the presence of phenolic compounds detected exclusively in some of the investigated genotypes. However, no significant differences were found as regards the mean number of unique compounds among the three groups, both for free and bound fractions. To show the relationships among the investigated wheat genotypes, the whole set of data was elaborated for the correspondence analysis as indicated in the biplot of Fig. 3. In the biplot six old varieties (Gentil Bianco, Gentil Rosso, Frassineto, Marzuolo d'Aqui, Marzuolo Val Pusteria, Verna) formed a separated cluster that can be associated with apigenin-6-C-arabinoside-8-C-hexoside (compound 23), vicenin-2 (apigenin-6,8-di-C-glucoside) (compound 40), glycosylated pinosylvin (compound 54), dihydro-ferulic acid (compound 72) and procyanidin B-3 (compound 86). The six old genotypes were characterized by an elevated number of isomer forms for all these compounds. Moreover, in this cluster, the presence of unique compounds influenced the position of the old varieties Gentil Rosso (double glycosilated pinosylvin, compound 30) and Verna (isovitexin-2″-O-rhamnoside, compound 51 and orientin/isoorientin, compound 57). Another group, clustering in the North-East quadrant of the plot, included both old and modern varieties (Andriolo, Autonomia A, Autonomia B, Bolero, Canove, Mieti, Nobel) sharing the presence of phenolic acids as syringic acid (compound 6), p-coumaric acid (compound 33) and syringaldehyde (compound 34). The old varieties Andriolo, Autonomia A and Canove were positioned in the upper part of the quadrant probably due to the common presence of pelargonidin-3-glucoside (compound 46). The modern cultivar Palesio and the old variety Gentil Rosso Mutico occupied an intermediated position between two clusters previously described, indicating similarities with both groups. The position of three old (Bianco Nostrale, Benco, Carosello) and one modern varieties (Eureka) in the South-East quadrant, did not form a compact cluster. However, this could be ascribed to the high number of isomers for compound 47 (apigenin) and the common presence of vanillic acid (compound 4) and hinokinin (compound 75). Sinapic acid (compound 42), detected exclusively in the old variety Carosello, affected the separated position of the wheat genotype within the South-East

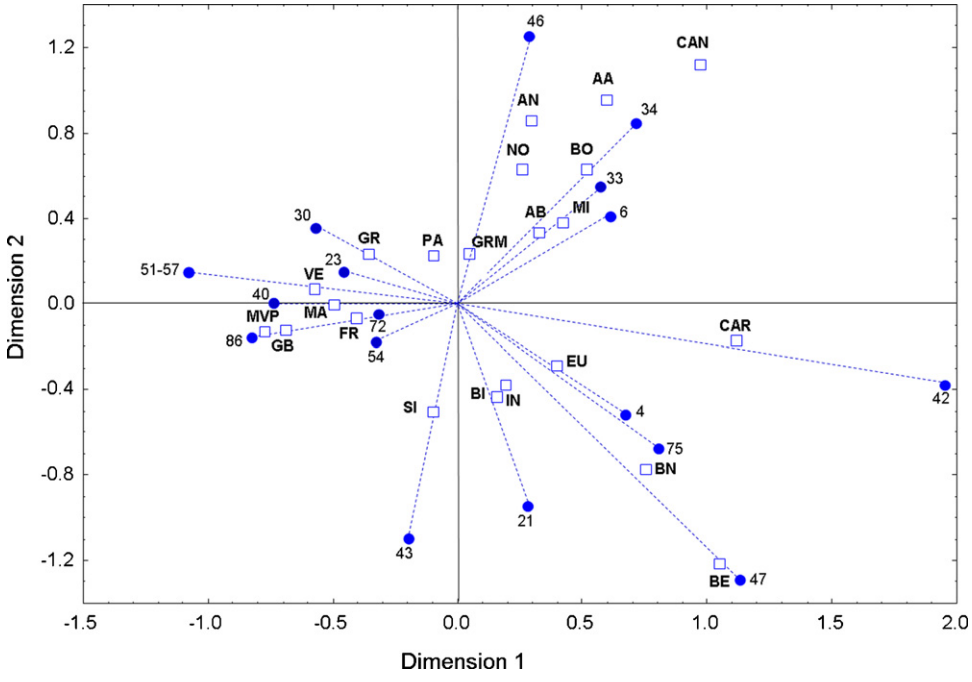

Fig. 3. Biplot of the correspondence analysis between wheat varieties (empty squares) and phenolic compounds (solid circles). Compound numbers are as in Table 3. In the graph only 17 phenolics with the highest variation were projected. The relative importance of single phenolic compounds is illustrated by the length of their corresponding centrifugal lines (dotted lines).

quadrant. The modern variety Bilancia and ancient genotypes Sieve and Inallettabile were placed in an intermediate position between the cluster of Gentil Bianco and that of Bianco Nostrale. The Sieve position was strongly influenced by the presence of peonidin-3-glucoside (compound 43) as unique compound, while apigenin-6/8-C-pentoside-8/6-C-hexoside (compound 21) highly affected the relative position of Inallettabile and Bilancia.

4. Concluding remarks

The present work aimed at giving a pioneering insight on the phenolic-based phytochemical composition of old and modern common wheat varieties from Italy. By using a powerful HPLC–ESI–TOF-MS analytical method, a total of 104 compounds (including isomer forms) have been separated and tentatively identified in the free and bound phenolic fractions of whole grains. The obtained metabolomic fingerprints showed the presence of several classes of health-promoting compounds, phenolic acids and flavonoids being the most representative groups, along with coumarins, stilbenes, proanthocyanidins and lignans.

Remarkable quantitative and qualitative differences were detected among the 16 old and the 6 modern investigated wheat genotypes. The highest total polyphenol content was detected in the old variety Verna, followed by other three old genotypes, Gentil Rosso, Gentil Rosso Mutico, Marzuolo d'Aqui, and the two modern cultivars Nobel and Eureka. A high variability among genotypes was also observed for the total flavonoid content: Verna, Gentil Rosso, Gentil Rosso Mutico and Marzuolo d'Aqui, along with the old genotypes Inallettabile and Sieve were the richest varieties, whereas among the modern ones, Eureka and Nobel showed again the highest amounts.

As regards the qualitative phenolic composition the fingerprints of most of the old genotypes revealed the presence of a number of total compounds and total isomers much higher than that identified in the modern cultivars, confirming that ancient grains may represent a rich source of biodiversity, especially as regards phenolic compounds. The present findings may be successfully used in breeding programs for developing bread wheat varieties with added value in terms of health-promoting phytochemicals.

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