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Determination of phenolic compounds in modern and old varieties of durum wheat using liquid chromatography coupled with time-of-flight mass spectrometry

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ABSTRACT

An evaluation of the grain functional components of Italian durum wheat cultivars was conducted. The raw material was obtained from the field trial performed in 2006–2007 at the Experimental Farm of the University of Bologna, (Bologna, Italy). The aim of this study was to define the phytochemical profile of ten varieties, comprised of old and modern durum wheat genotypes, including quantitative and qualitative phenolic and flavonoid content (free and bound forms). The results showed that mean values of total phenolic compound and total flavonoid content in old wheat varieties (878.2 ± 19.0 μmol gallic acid equivalent/100 g of grain and 122.6 ± 25.4 μmol catechin equivalent/100 g of grain, respectively) did not differ significantly from those detected in modern genotypes (865.9 ± 128.9 μmol gallic acid equivalent/100 g and 123.5 ± 20.6 μmol catechin equivalent/100 g, respectively). However, the HPLC–ESI-TOF-MS analysis highlighted remarkable differences between modern and old cultivars. The interpretation of the mass spectra allowed the identification of 70 phenolic compounds, including coumarins, phenolic acids, anthocyanins, flavones, isoflavones, proanthocyanidins, stilbenes and lignans. The free extracts of ancient wheat varieties showed the presence of a mean number of phenolic compounds and isomer forms (8.7 ± 2.5 and 7.7 ± 4.7 respectively) significantly higher than in modern genotypes (4.4 ± 2.9 and 2.0 ± 2.4, respectively). A similar trend was observed also for the bound phenolic fraction. Moreover, the phytochemical profiles showed the presence of unique phenolic compounds in both free and bound fractions of some of the investigated wheat genotypes. Results highlighted that investigated old wheat cultivars may offer unique nutraceutical values for their peculiar contents in bioactive phytochemicals, suggesting their uses into a wide range of regular and specialty products naturally enriched with health-promoting compounds.

1. Introduction

Wheat is a major crop and an important component of the human diet. Research has shown that whole grain consumption helps lower the incidence of chronic diseases such as diabetes [1], cardiovascular disease [2,3], and cancer [2,4–7], and these health benefits have been attributed in part to their unique phytochemical contents. The full characterization of health-beneficial compounds present in whole grain and its products is important for the breeding and marketing of wheat based on its potential to promote health in line with increasing consumer demands for healthier foods [8]. Current interest in the health benefit provided by grain consumption has led to an increased focus on the phytochemical content of different grains and grain varieties. In this context ancient wheat and old wheat cultivars have been recognized to offer unique nutraceutical values for their peculiar contents in health-beneficial phytochemicals [9]. In our previous work the presence of unique lignan compounds (arctigenin, syringaresinol and hinokinin) was exclusively detected in old soft wheat (*Triticum aestivum* L.) genotypes as compared to modern cultivars [10].

Among health-promoting phytochemicals residing in whole grains, phenolic compounds have gained much attention in many scientific research areas as they have strong antioxidant properties and can protect against many degenerative human diseases (i.e. heart disease and cancer) [11].

Phenolics are compounds that possess one or more aromatic rings with one or more hydroxyl groups, and generally are categorised as phenolic acids, flavonoids, stilbenes, coumarins, and

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tannins [12]. Phenolic acids and flavonoids represent the most common form of phenolic compounds found in whole grains, and they are among the major and most complex groups of phytochemicals in the cereal grain, with a number of types that exist as soluble free compounds, soluble conjugates that are esterified to sugars and other low molecular mass components, and insoluble bound forms [13]. The latter are the major form in wheat and are involved in crosslinking polymers, particularly arabinoxylans in the grain cell walls [14].

There are several reports on the content of phenolics in different varieties of wheat grains or their different parts [9,15–20]. More data are, however, needed regarding phenolic profiles in old wheat genotypes, as this could lead to new opportunities for breeding and eventual commercial production of value-added varieties rich in health-beneficial components for making nutraceuticals and other functional foods. Additionally most literature data concerning wheat grain phenolic determinations do not give details about field agronomic conditions and growing locations.

Several analytical methodologies have been reported for the qualitative and quantitative determination of phenolic compounds in various plant extracts. The need for profiling and identifying individual phenolic compounds has seen traditional methods replaced by high-performance chromatographic analyses. Various separation techniques (HPLC, GC, CE), coupled with mass spectrometry (HPLC–MS, GC–MS, CE–MS) [10,21,22] or nuclear magnetic resonance (NMR) [23,24], have been found as valuable tools for the characterization of polyphenols content in wheat samples. The limited volatility of many phenols, the instability of derivatized phenolic compounds and the potential for further chemical modification of the dimers during derivatization have restricted the application of GC to their separation [25]. Nevertheless, HPLC currently represents the most popular and reliable technique for analysis of phenols [26–32]. In recent years, liquid chromatography/time-of-flight mass spectrometry (LC/TOF-MS) has been used to separate and characterize secondary metabolites of some complicated components such as wine antioxidants [33], olive phenolic compounds [34,35], olive secoiridoids [36], anthocyanins [37] and tectorigenin [38]. Benefiting from the increased power of high resolution, accurate mass measurements, LC/TOF-MS provides the elemental compositions of unknown peaks with high accuracy (routinely below 10 ppm) in complex matrices.[37,39]. It has become a competitive technology for the accurate and sensitive characterization of some complicated components in complex matrices [40].

The objective of the present research was to determine the qualitative profiles of phenolic compounds (free and bound fractions) in 10 diverse wheat varieties, comprised of old and modern durum wheat genotypes from Italy, cropped in the same location and growing season.

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

Description of the 10 wheat varieties used in the present study is given in Table 1. The wheat grain samples included two old ("Senatore Cappelli", "Urria"), seven modern ("Anco Marzio", "Claudio", "Iride", "Levante", "Orobel", "Solex", "Svevo") cultivars of durum wheat (*T. turgidum* ssp. *durum*) and a commercial sample of Kamut® (*T. turgidum* ssp. *turanicum*). 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 Ustochrepts, 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 × 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 free and bound phenolic compounds

Free phenolic compounds in wheat flour were extracted according to the method reported by Adom et al. [19] with minor modifications. 1 g of whole wheat flour was mixed 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 at 45 °C to <5 mL, and reconstituted in 10 mL of water. The extracts were stored at −40 °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. [20]. 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 phenolic acids were extracted three times with 15 mL of a mixture of cold diethyl ether (DE) and ethyl acetate (EA, 1:1 v/v) by manually shaking and centrifuging. DE/EA layers were combined, evaporated to dryness, and dissolved into 1.5 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 pH was adjusted to 2. The DE/EA extraction performed was similar to that for alkaline hydrolysis.

2.4. Determination of total phenolic and flavonoid contents

The amount of total phenolics in extracts (free and bound) was determined according to the Folin–Ciocalteu procedure [41]. Results are expressed as micromoles of gallic acid equivalents (GAE) per 100 g of grain.

Total flavonoid content was determined according to a colorimetric method described previously by Adom et al. [19]. 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 equivalents (CE) per 100 g of grain. Data are reported as mean ± standard deviation (SD) for six replicates.

2.5. HPLC–TOF-MS analyses of free and bound fractions of phenolic compounds

2.5.1. Separation by HPLC

Free and bound phenolic extracts were filtered through a 0.22 μm filter before the RRLC analysis. Separation of phenolic com-

Table 1
Description of the 10 tetraploid wheat varieties and their registered pedigrees.

Genotype	Species	Registration Year	Country	Pedigree
Anco Marzio	*Triticum turgidum* ssp. *durum*	2003	Italy	Stot//Altar84/ALD
Claudio	*Triticum turgidum* ssp. *durum*	1998	Italy	CIMMYT 35/Durango//IS1938/Grazia
Iride	*Triticum turgidum* ssp. *durum*	1996	Italy	Altar84/Ares
Levante	*Triticum turgidum* ssp. *durum*	2002	Italy	G80/Piceno//Ionio
Orobel	*Triticum turgidum* ssp. *durum*	1999	Italy	Composite I.N.R.A.
Senatore Cappelli	*Triticum turgidum* ssp. *durum*	1930	Italy	Strampelli selection from Jennah Khetifa
Solex	*Triticum turgidum* ssp. *durum*	1995	Italy	Creso/Valgerardo
Svevo	*Triticum turgidum* ssp. *durum*	1996	Italy	CIMMYT's Selection/Zenit
Urria	*Triticum turgidum* ssp. *durum*	1900	–	Sicily landrace
Kamut®	*Triticum turgidum* ssp. *turanicum*	–	USA	North African landrace

pounds was performed on an Agilent 1200 series Rapid Resolution LC (Agilent Technologies, CA, USA) consisting of vacuum degasser, autosampler, and a binary pump equipped with a RP C18 analytical column (4.6 × 150 mm, 1.8 μm particle size) from Agilent ZORBAX Eclipse plus. Acidified water (0.5% acetic acid v/v) and acetonitrile were used as the mobile phases A and B, respectively. The gradient elution was programmed as follows: from 5% to 10% B in 5 min; from 10% to 35% B in 35 min; from 35% to 70% 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 analyses. The flow rate was set at 0.50 mL/min throughout the gradient. The effluent from the HPLC column was split 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.

2.5.2. ESI–TOF-MS

The HPLC system was coupled to a microTOF (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer (oaTOF-MS), 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, 21.7 psi.

The accurate mass data of the molecular ions were processed through the 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 formulae. 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 [42]. 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 38 isomers out of the 70 identified compounds. For each of the remaining 32 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. Phenolic and flavonoid content of wheat varieties

In general, cereals are known to be rich in phenolic compounds, and it has been accepted that phenolic compounds may significantly contribute to overall antioxidant capacity of wheat grains [43–45].

Phenolic contents of the tested wheat genotypes are presented in Table 2, expressed as micromoles of gallic acid equivalent (GAE) per 100 g of grain. Significantly different values in free phenolic content were observed for Senatore Cappelli (223.1 ± 6.2 μmol GAE/100 g) and Orobel (230.7 ± 30.1 μmol GAE/100 g) with the highest free phenolic contents and for Iride (67.9 ± 14.9 μmol GAE/100 g) with the lowest free phenolic content among all other wheat samples ($p < 0.05$). No significant differences were observed between the mean values calculated for old (Senatore Cappelli, Urria, Kamut®) and modern wheat varieties (Anco Marzio, Claudio, Iride, Levante, Orobel, Solex, Svevo).

Bound phenolic contents ranged from 773.8 ± 152.2 μmol GAE/100 g in Anco Marzio to 545.3 ± 63.3 μmol GAE/100 g in Orobel and contributed to the total phenolic content for 74–51%. Our results were in agreement with previously reported findings, confirming that phenolic compounds in wheat primarily exist in bound form associated with cell wall materials [9,19,27]. No significant differences were observed neither among wheat varieties

Table 2
Phenolic compound content in cultivar grain, expressed as average μmol gallic acid equivalent per 100 g of whole flour ± standard deviation ($n = 6$).

Cultivar	FPC	BPC	TPC	% BPC
Anco Marzio (M)	189.5 ± 27.5 (ab)[a]	773.8 ± 152.2	963.5 ± 176.3	80.3
Claudio (M)	194.4 ± 38.7 (ab)	744.0 ± 29.2	938.4 ± 13.5	79.3
Iride (M)	67.9 ± 14.9 (b)	545.3 ± 63.4	613.2 ± 68.6	88.9
Kamut® (O)	148.8 ± 18.7 (ab)	732.2 ± 168.9	880.9 ± 212.2	83.1
Levante (M)	205.9 ± 30.0 (ab)	800.6 ± 20.0	1006.5 ± 14.1	79.5
Orobel (M)	230.7 ± 30.1 (a)	616.0 ± 93.4	846.7 ± 93.2	72.8
Senatore Cappelli (O)	223.1 ± 6.2 (a)	634.9 ± 105.6	858.0 ± 140.5	74.0
Solex (M)	188.2 ± 2.5 (ab)	669.1 ± 45.0	857.3 ± 60.1	78.0
Svevo (M)	172.3 ± 2.6 (ab)	663.2 ± 149.9	835.5 ± 198.6	79.4
Urria (O)	173.6 ± 1.25 (ab)	722.2 ± 66.7	895.7 ± 92.6	80.6
Mean modern cultivars	178.4 ± 51.9	687.4 ± 91.0	865.9 ± 128.9	79.4
Mean old cultivars	181.8 ± 37.8	696.4 ± 53.5	878.2 ± 19.0	79.3

Abbreviations: FPC, free phenolic compounds; BPC, bound phenolic compounds; TPC, total phenolic compounds, M, modern cultivar; O, old cultivar.
[a] Means followed by the same letter or no letter for FPC, BPC, TPC and %BPC are not significantly different at $P < 0.05$.

nor between mean values of old and modern genotypes for bound, total phenolics and percentage of bound phenolics.

Flavonoids are an important class of phytochemicals in wheat contributing to its health beneficial properties. They are the predominant class described in phenolic food content investigations, because they account for approximately two-thirds of the dietary phenols [46]. These are known to modulate lipid peroxidation involved in atherogenesis, thrombosis, and carcinogenesis. Known properties of the flavonoids include free radical scavenging, strong antioxidant activity, inhibition of hydrolytic and oxidative enzymes (phospholipase A2, cyclooxygenase, lipoxygenase), and anti-inflammatory action [47].

Flavonoid contents of tested wheat varieties are expressed as micromoles of catechin equivalent (CE) per 100 g of grain (Table 3). Free flavonoid content represented contributions from both free and soluble-conjugated flavonoids and values ranged from 29.08 ± 5.0 μmol CE/100 g in Urria to 75.3 ± 0.3 μmol CE/100 g of Claudio. Significantly different values were observed for Senatore Cappelli (74.5 ± 2.8 μmol CE/100 g) and Claudio (75.3 ± 0.3 μmol CE/100 g) with the highest free phenolic contents among all other wheat varieties. The mean values between old and modern genotypes were not statistically different. The same trend was observed for the total flavonoid content with statistically significant values in Senatore Cappelli (151.0 ± 8.4 μmol CE/100 g) and Claudio (157.4 ± 6.6 μmol CE/100 g) showing the highest total flavonoid content among all other varieties, whereas no significant differences occurred for bound flavonoids. Bound flavonoid contribution to the total flavonoid content ranged from 51% in Senatore Cappelli to 73% in Orobel and Levante but values were not statistically different among genotypes.

Total flavonoid contents for the 10 wheat varieties (90.64–157.41 μmol CE/100 g) were similar to those previously reported by Adom et al. [9] for 11 different wheat varieties and experimental lines.

Our results showed that most of the phenolic phytochemicals (including flavonoids) in wheat occur in the bound form, attached to cell wall material. This is very important when health benefits of whole grains are considered; these phenolics, which exert synergistic antioxidant action with other bioactive compounds, are mainly present in the outer layers of bran [48]. Bound phytochemicals are important functional dietary components as they may survive upper gastrointestinal digestion and be released in the colon through microflora digestion activity [19]. This may partly explain the reduced incidence of colon cancers and other gastrointestinal diseases associated with the consumption of whole wheat and other whole grain-derived products [2–4].

Although the range in mean values of free, bound and total phenolic contents among old and modern varieties did not vary greatly, some statistically significant differences were observed between genotypes. Several studies in literature reported that polyphenols content varies depending on wheat cultivars and agronomic conditions [15–17]. Further investigations on the relationship among phytochemical content, environmental conditions and genotype should be carried out in order to support breeding programs aimed at developing wheat varieties with enhanced health and nutritional benefits.

3.2. HPLC–ESI-TOF-MS optimization

The free phenolic extract of the Senatore Cappelli 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 and 0.5% acetic acid aqueous solution 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 Senatore Cappelli sample.

Many phenolic compounds in wheat have isomers and are difficult to be separated due to their extremely similar structures. The chromatographic separation of these compounds of the same molecular weight is important, because it is impossible for single-stage TOF/MS to distinguish these 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.

3.3. Identification of phenolic compounds in wheat extracts

Tentative identification of phenolic compounds in both free and bound fraction extracts 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) of the free phenolic fraction of the wheat sample Senatore Cappelli and the extracted ion chromatograms (EICs) for identified main compounds.

All the phenolic compounds identified in Senatore Cappelli sample in the free fraction are summarized in Table 4. This table includes molecular formula, selected ion, calculated and experimental m/z, error, sigma values, tolerance (ppm) in generated molecular formula, retention time, classification order, number of

Table 3
Flavonoid compound content in cultivar grain, expressed as average μmol catechin acid equivalent per 100 g of whole flour ± standard deviation ($n = 6$).

Cultivar	FFC	BFC	TFC	% BFC
Anco Marzio (M)	48.1 ± 7.5 (b)[a]	66.6 ± 13.1	114.7 ± 8.0 (b)	58.0
Claudio (M)	75.3 ± 0.3 (a)	82.1 ± 5.0	157.4 ± 6.6 (a)	52.1
Iride (M)	38.2 ± 5.9 (b)	52.4 ± 1.9	90.6 ± 5.8 (b)	57.9
Kamut® (O)	53.9 ± 3.8 (b)	59.1 ± 9.4	113.0 ± 8.0 (b)	52.3
Levante (M)	36.4 ± 1.6 (b)	101.1 ± 15.7	137.5 ± 19.9 (b)	73.5
Orobel (M)	31.1 ± 1.6 (b)	86.9 ± 4.4	118.0 ± 4.0 (b)	73.7
Senatore Cappelli (O)	74.5 ± 2.8 (a)	77.0 ± 8.8	151.5 ± 8.4 (a)	50.8
Solex (M)	32.8 ± 1.6 (b)	89.2 ± 6.3	122.0 ± 6.6 (b)	73.1
Svevo (M)	42.1 ± 0.9 (b)	81.8 ± 33.2	124.0 ± 45.6 (b)	66.0
Urria (O)	29.1 ± 5.0 (b)	74.4 ± 3.8	103.5 ± 1.8 (b)	71.9
Mean modern cultivars	43.4 ± 15.2	80.0 ± 15.9	123.5 ± 20.6	64.9
Mean old cultivars	52.5 ± 22.7	70.2 ± 9.7	122.6 ± 25.4	58.4

Abbreviations: FFC, free flavonoid compounds; BFC, bound flavonoid compounds; TFC, total flavonoid compounds, M, modern cultivar; O, old cultivar.
[a] Means followed by the same letter or no letter for FFC, BFC, TFC and %BFC are not significantly different at $P < 0.05$.

possibilities and possible compounds. The tentative identification by MS-TOF was carried out using the Generate Molecular Formula Editor. First a low tolerance was chosen (in most cases 5 ppm) and subsequently options with a low sigma value (<0.05) and a low error (<5 ppm) were considered. The last step was to consider the position of the molecular formula in the table of possible compounds (most of the identified compounds are in position number 1 in Table 4).

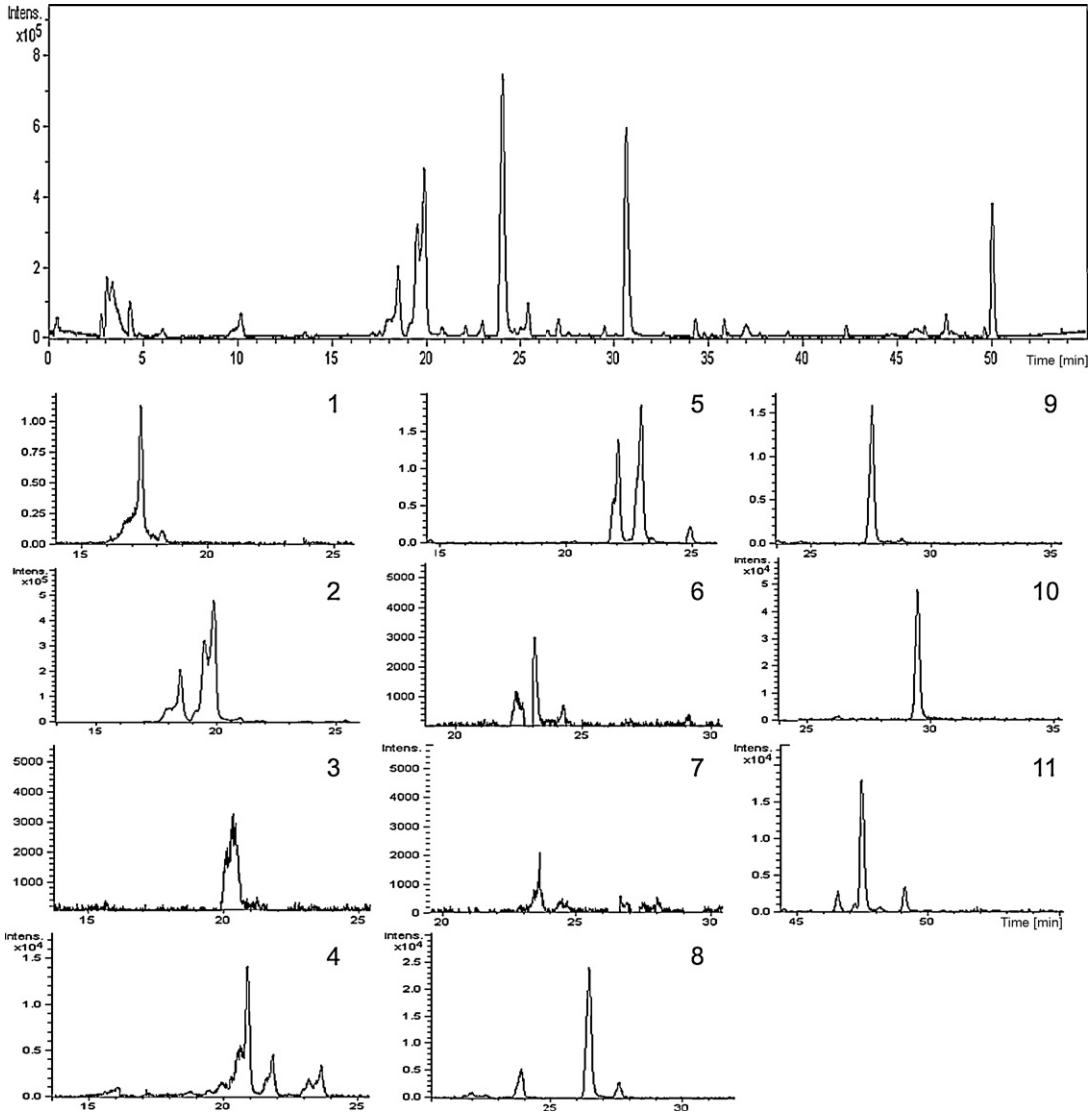

Fig. 1. Base peak chromatogram (BPC) obtained by HPLC–ESI-TOF-MS in Senatore Cappelli free fraction and extracted ion chromatograms (EICs) ± 0.02 of detected well-known compounds: (1) lucenin-1/3 (luteolin-6/8-*C*-xyloside-8/6-*C*-glucoside), (2) apigenin-6-*C*-arabinoside-8-*C*-hexoside (schaftoside/isoschaftoside), (3) vanillin, (4) apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside), (5) glycosylated and acetylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone, (6) pinosylvin (double glycosylation), (7) apigenin, (8) isovitexin-2″-*O*-rhamnoside, (9) methylisoorientin-2″-*O*-rhamnoside, (10) glycosylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone, (11) formononetin (glycosylated and methylated).

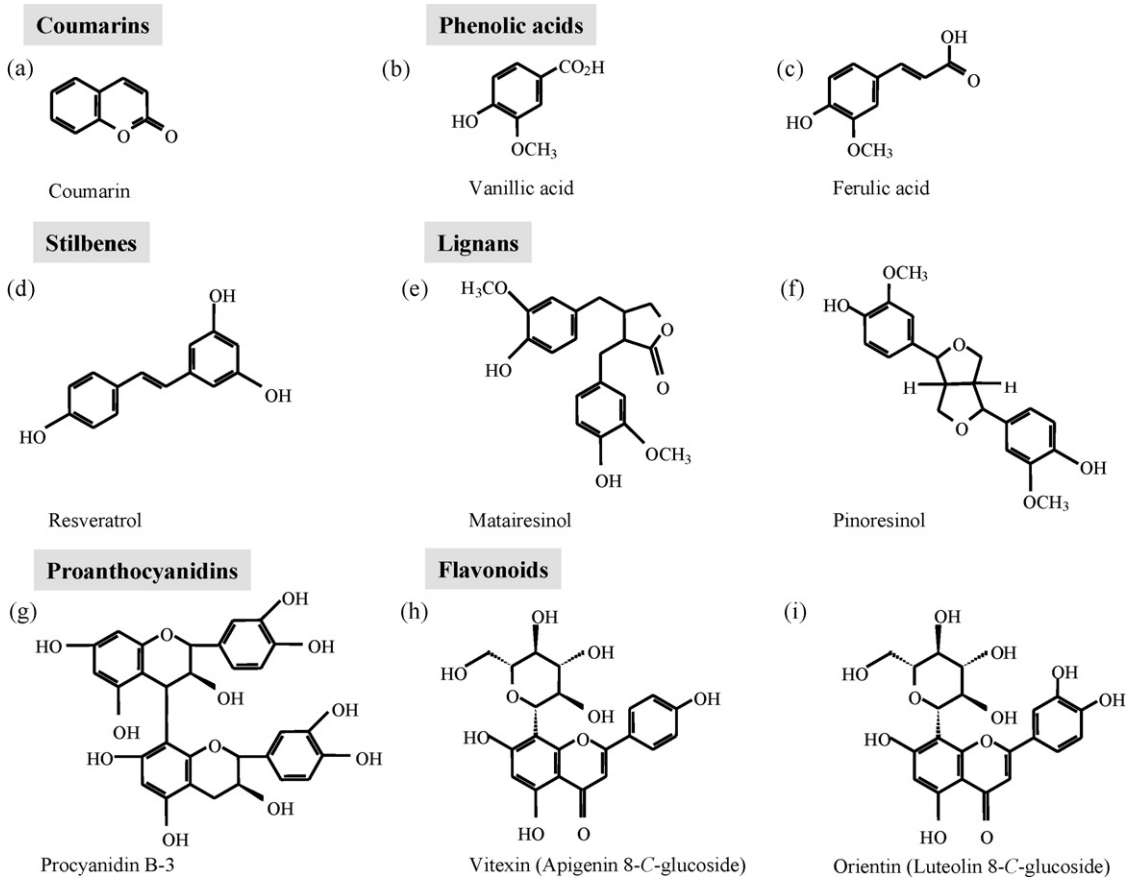

Fig. 2. Structural formulae of representative compounds for the investigated chemical classes.

Most of the phenolic compounds found in this study have been previously described in wheat [10,20,49–54]. A total of 70 phenolic compounds were identified and grouped into chemical classes as outlined below. The structural formulae of representative compounds for each considered chemical class are shown in Fig. 2. Compound identification (numbered according to their retention times) as well as their occurrence in investigated durum wheat cultivars is presented in Table 5.

3.3.1. Coumarins

Coumarins belong to a group of compounds known as the benzopyrones, all of which consist of a benzene ring joined to a pyrone. They have a variety of bioactivities including anticoagulant, estrogenic, dermal photosensitising, antimicrobial, vasodilator, molluscacidal, antithelmintic, sedative and hypnotic, analgesic and hypothermic activity [55,56].

Wheat grains have been shown to contain various coumarins, among which the compound coumarin and its hydroxylated derivatives [57]. In our findings compound **20** (mass 145.0295, $C_9H_6O_2$), tentatively identified as coumarin, was detected only in the free phenolic fraction of the old wheat genotype Kamut®.

3.3.2. Phenolic acids

Phenolic acids are derivatives of benzoic and cinnamic acids and occur in cereals as both free and bound forms [58]. Free phenolic acids are located in the outer layer of the pericarp, whereas bound phenolic acids are esterified to cell walls [11]. Ferulic, *p*-coumaric, and vanillic acids are the most dominant phenolic acids in cereals and are found together with other phenolics including caffeic, chlorogenic, gentisic, syringic, and *p*-hydroxybenzoic acids [58]. In our study ferulic (isomer compounds **24**, **26**, **32**, **56**), dihydroferulic (isomer compounds **40**, **48**, **49**, **54**, **57**, **58**) vanillic (isomer compounds **2**, **8**), syringic (compound **4**), sinapic (compound **21**), *p*-coumaric (compound **12**) acids, *p*-hydroxybenzaldehyde (isomer compounds **6**, **10**) and syringaldehyde (compound **16**). were detected in both free and bound phenolic fractions of most of the investigated wheat varieties. These compounds were also recorded in wheat by Lam et al. [49], even if in plant internodes and not directly in grains. Additionally, vanillin (compound **11**, mass 151.0400, $C_8H_8O_3$) was found exclusively in the free phenolics of Senatore Cappelli grain extract. Vanillin is known for its strong flavouring power and it can be hypothesized that its occurrence in an old wheat genotype may concur at conferring the peculiar sensory properties to ancient wheat-derived products [59].

3.3.3. Proanthocyanidins

Proanthocyanidins, also called procyanidins or condensed tannins, consist of polymerized flavanol units and they contribute to astringency in food. They have high antioxidant activity compared to monomeric phenolic compounds [60]. Proanthocyanidins have been detected in several cereals, including wheat, and in caryopses they act as protective agents because of their resistance to microbial degradation [61].

Two proanthocyanidins were detected in the present study: compound **17**, with mass 593.1300 (C_{30} $H_{26}O_{13}$) was tentatively identified as prodelphinidin B-3 and was present in the free phenolic fraction of Solex, Levante, Urria and Kamut® samples; compound **55**, with mass 577.1351 (C_{30} $H_{26}O_{12}$) was assigned as procyanidin B-3 and occurred in the free phenolics of Iride and Kamut®. These observation were in line with findings of McCallum and Walker [53] who detected these metabolites, along with other proanthocyanidins, in red-grained wheat bran.

Table 4
Phenolic compounds detected by HPLC-ESI-TOF-MS in the free phenolic extract of Senatore Cappelli wheat grains.

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	Reference
$C_{26}H_{28}O_{15}$	$[M-H]^-$	579.1346	579.1355	1.7	0.0250	5	17.43	1° (9)	Lucenin-1/3 (luteolin-6/8-*C*-xyloside-8/6-*C*-glucoside)	[21]
$C_{26}H_{28}O_{14}$	$[M-H]^-$	563.1326	563.1406	3.6	0.0098	5	19.49	1° (1)	Apigenin-6-*C*-arabinoside-8-*C*-hexoside (schaftoside and isoschaftoside)	[21]
$C_8H_8O_3$	$[M-H]^-$	151.0396	151.0400	3.2	0.0163	5	20.41	1° (1)	Vanillin	[49]
$C_{27}H_{30}O_{15}$	$[M-H]^-$	593.1512	593.1511	2.6	0.0097	5	20.89	1° (12)	Apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside	[21]
$C_{25}H_{26}O_{13}$	$[M-H]^-$	533.1320	533.1300	−3.7	0.0046	5	22.08	1° (7)	Glycosylated and acetylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone	[20]
$C_{26}H_{32}O_{12}$	$[M-H]^-$	533.1297	535.1821	0.6	0.0318	5	23.11	3° (7)	Pinosylvin (double glycosylation)	[50]
$C_{15}H_{10}O_5$	$[M-H]^-$	269.0445	269.0455	3.7	0.0457	5	23.59	1° (1)	Apigenin	[50]
$C_{27}H_{30}O_{14}$	$[M-H]^-$	577.1570	577.1562	−1.2	0.0110	5	26.46	2° (8)	Isovitexin-2″-*O*-rhamnoside	[21]
$C_{28}H_{32}O_{15}$	$[M-H]^-$	607.1698	607.1668	−4.9	0.0237	5	27.52	3° (12)	Methylisoorientin-2″-*O*-rhamnoside	[21]
$C_{23}H_{24}O_{12}$	$[M-H]^-$	491.1192	491.1195	0.6	0.0092	5	31.01	3° (6)	Glycosylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone	[20]
$C_{23}H_{24}O_9$	$[M-H]^-$	443.1367	443.1347	−4.4	0.0071	5	47.41	1° (3)	Formononetin (glycosylated and methylated)	[50]

3.3.4. Flavonoids

Flavonoids are compounds with a C6–C3–C6 skeleton that consists of two aromatic rings joined by a three-carbon link; they include anthocyanins, flavones, flavanones, and flavonols [11]. They are reported to have antioxidant, anticancer, anti-allergic, anti-inflammatory, anticarcinogenic and gastroprotective properties [62–64]. In the present study metabolites belonging to anthocyanin and flavone (including isoflavone) classes were detected in grains of the investigated durum wheat varieties.

Anthocyanins are water-soluble pigments that contribute to the blues, purples, and reds in plant foods and are among the major flavonoids studied in cereals. Although pigments exist in wheat grains at very low concentrations, anthocyanins have been reported in the pericarp of pigmented varieties of barley, maize, rice, rye and wheat [65,66]. For example, cyanidin 3-glucoside and peonidin 3-glucoside have been shown to be the major anthocyanins in purple and blue wheat [67,68].

In the present study compounds **30** and **35**, with mass of 433.2710 and deduced molecular formula $C_{21}H_{21}O_{10}Cl$ (pelargonidin-3-glucoside), were pair of isomers detected only in the bound phenolic fractions of Claudio, Senatore Cappelli and Urria samples. Compound **39** was identified as cyanidin 3-glucoside and occurred in the free and bound phenolic fraction of Svevo and Orobel, respectively. Compound **65** was identified as cyanidine chloride in the bound phenolics of both Orobel and Svevo.

In recent years, scientific and public interest in flavones has grown enormously due to their putative beneficial effects against atherosclerosis, osteoporosis, diabetes mellitus and certain cancers [69].A total of 32 flavones were detected in the investigated wheat varieties, comprised of 21 *C*-glycosidic forms, 7 *O*-glycosidic derivatives and 4 aglycone compounds. Although a large number of conjugates could be identified, these were based on a restricted range of flavones, namely 5,7,4′-trihydroxyflavone (apigenin) and 5,7,3′,4′,-tetrahydroxyflavone (luteolin) (Table 6). In wheat, these flavones accumulate as their respective 6-*C* and/or 8-*C*-glycosidic conjugates. The 8-*C*-glucosides of apigenin and luteolin are historically named as vitexin and orientin, respectively, while the respective 6-*C*-glucosides are known as isovitexin and isoorientin, respectively [61,70]. In common with previous studies, apigenin (three isomer compounds **22**, **31**, **44**), vitexin/isovitexin (compound **36**), apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside) (five isomer compounds **3**, **5**, **13**, **14**, **19**) and lucenin 1/3 (luteolin 6/8-*C*-xyloside-8/6-*C*-glucoside) (two isomer compounds **7**, **51**) were identified in the investigated wheat samples. Several variations in the sugar substitutions of the two invariant flavones (apigenin and luteolin) have been described for wheat in the literature [70–72]. Besides vitexin, isovitexin and vicenin, other apigenin derivatives were vicenin-2 (apigenin-6,8-di-*C*-glucoside (compound **25**), apigenin-6-*C*-B-galactosyl-8-*C*-b-glucosyl-*O*-glucopyranoside (isomer compounds **27**, **29**, **38**, **41**), isovitexin-2″-*O*-rhamonoside (compound **28**) and apigenin-6/8-*C*-arabinoside-8/6-*C*-hexoside (schaftoside/isoschaftoside) (isomer compounds **9**, **46**, **47**, **50**, **63**, **66**) Analogously, other luteolin *O*-derivatives were identified such as methylisoorientin-2″-*O*-rhamnoside (isomer compounds **1**, **34**). All these metabolites were also detected in winter and spring wheat (*T. aestivum* L.), but they were part of the phenolic complex of leaves [21,73] and, as far as we are aware, no reports are available in literature about their occurrence in wheat grains.

Three additional flavones, not bearing apigenin or luteolin backbones, were detected in the wheat samples: compound **64**, with mass 329.0666 and deduced molecular formula $C_{17}H_{14}O_7$ was assigned as 3′,4′,5,-trihydroxy-3,7-dimethoxyflavone. Masses 491.1195 ($C_{23}H_{24}O_{12}$) (isomer compounds **42**, **43**) and 533.1300 ($C_{25}H_{26}O_{13}$) (isomer compounds **15**, **23**, **45**, **61**, **69**) were putatively identified as glycosylated forms of 3′,4′,5,-trihydroxy-

Table 5
Peak assignments for HPLC-ESI-TOF-MS analyses of free and bound phenolic extracts in the investigated durum wheat varieties. The main compounds (excluding isomers) are in bold highlighted.

No.	Retention time (min)	Molecular formula	$[M-H]^-$	Compound	Class	Sample		Reference
						Free extract	Bound extract	
1	12.20	$C_{28}H_{32}O_{15}$	607.1668	Methylisoorientin-2″-*O*-rhamnoside isomer	Flavone-*C*-glycoside	IR SO LE KM		[21]
2	15.43	$C_8H_8O_4$	167.0349	**Vanillic acid**	Phenolic acids		AM OR SC UR KM	[49]
3	16.32	$C_{27}H_{30}O_{15}$	593.1511	Apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside isomer	Flavone-*C*-glycoside	SC		[21]
4	16.69	$C_9H_{10}O_5$	197.0455	**Syringic acid**	Phenolic acids		CL IR LE SC	[49]
5	16.95	$C_{27}H_{30}O_{15}$	593.1511	Apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside isomer	Flavone-*C*-glycoside	OR CL SC		[21]
6	17.32	$C_7H_6O_2$	121.0290	***p*-Hydroxybenzaldehyde**	Phenolic acids		SC	[49]
7	17.43	$C_{26}H_{28}O_{15}$	579.1355	**Lucenin-1/3 (luteolin-6/8-*C*-xyloside-8/6-*C*-glucoside)**	Flavone-*C*-glycoside	OR IR SC	LE UR	[21]
8	17.83	$C_8H_8O_4$	167.0349	Vanillic acid isomer	Phenolic acids	OR	UR	[49]
9	19.49	$C_{26}H_{28}O_{14}$	563.1395	Apigenin-6-*C*-arabinoside-8-*C*-hexoside (schaftoside and isoschaftoside) isomer	Flavone-*C*-glycoside	SC	SC	[21]
10	20.18	$C_7H_6O_2$	121.0290	*p*-Hydroxybenzaldehyde isomer	Phenolic acids		AM SC	[49]
11	20.41	$C_8H_8O_3$	151.0400	**Vanillin**	Phenolic acids	SC		[49]
12	20.51	$C_9H_8O_3$	163.0400	***p*-Coumaric acid**	Phenolic acids		OR IR LE SC UR KM	[49]
13	20.89	$C_{27}H_{30}O_{15}$	593.1511	**Apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside**	Flavone-*C*-glycoside	OR SC		[21]
14	21.76	$C_{27}H_{30}O_{15}$	593.1511	Apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside isomer	Flavone-*C*-glycoside	OR SC		[21]
15	22.08	$C_{25}H_{26}O_{13}$	533.1300	**Glycosylated and acetylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone**	Flavone-*O*-glycoside	OR SC UR		[50]
16	22.20	$C_9H_{10}O_4$	181.0506	**Syringaldehyde**	Phenolic acids		OR IR SC	[49]
17	22.26	$C_{30}H_{26}O_{13}$	593.1300	**Prodelphinidin B-3**	Proanthocyanidin	SO LE UR KM		[53]
18	23.11	$C_{26}H_{32}O_{12}$	535.1821	**Pinosylvin (double glycosylation)**	Stilbenoids	SC		[50]
19	23.17	$C_{27}H_{30}O_{15}$	593.1511	Apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside isomer	Flavone-*C*-glycoside	OR SC UR		[21]
20	23.21	$C_9H_6O_2$	145.0295	**Coumarin**	Coumarins (lactones)	KM		[57]
21	23.57	$C_{11}H_{12}O_5$	223.0612	**Sinapic acid**	Phenolic acids		SC	[20]
22	23.59	$C_{15}H_{10}O_5$	269.0455	**Apigenin**	Flavone	SC		[51]
23	24.49	$C_{25}H_{26}O_{13}$	533.1300	Glycosylated and acetylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone isomer	Flavone-*O*-glycoside	AM OR CL SC UR	LE UR KM	[50]
24	24.92	$C_{10}H_{10}O_4$	193.0506	**Ferulic acid**	Phenolic acids	KM	IR SV KM OR	[49]
25	25.17	$C_{27}H_{30}O_{15}$	593.1511	**Vicenin-2 (apigenin-6,8-di-*C*-glucoside)**	Flavone-*C*-glycoside	CL	SO	[21]
26	25.82	$C_{10}H_{10}O_4$	193.0506	Ferulic acid isomer	Phenolic acids	KM	AM SV KM	[49]
27	26.01	$C_{33}H_{38}O_{21}$	769.1821	**Apigenin-6-*C*-B-galactosyl-8-*C*-B-glucosyl-*O*-glucuronopyranoside**	Flavone-*C*-glycoside	AM CL SO SV UR		[21]
28	26.46	$C_{27}H_{30}O_{14}$	577.1562	**Isovitexin-2″-*O*-rhamnoside**	Flavone-*C*-glycoside	OR SC		[21]
29	26.65	$C_{33}H_{38}O_{21}$	769.1821	Apigenin-6-*C*-B-galactosyl-8-*C*-B-glucosyl-*O*-glucuronopyranoside isomer	Flavone-*C*-glycoside	AM CL IR SO SV LE UR		[21]
30	26.79	$C_{21}H_{21}O_{10}Cl$	433.2710	**Pelargonidin-3-glucoside (callistephin)**	Anthocyanidin (*O*-glycoside)		CL SC UR	[52]
31	26.80	$C_{15}H_{10}O_5$	269.0455	Apigenin isomer	Flavone	CL LE SC	CL	[51]
32	27.12	$C_{10}H_{10}O_4$	193.0506	Ferulic acid isomer	Phenolic acids	KM	AM SO SV KM OR SC	[49]
33	27.43	$C_{21}H_{22}O_8$	401.1241	**Glycosylated pinosylvin**	Stilbenoids		OR SC KM	[50]
34	27.52	$C_{28}H_{32}O_{15}$	607.1668	**Methylisoorientin-2″-*O*-rhamnoside**	Flavone-*C*-glycoside	AM OR SC KM	SO	[21]
35	27.96	$C_{21}H_{21}O_{10}Cl$	433.2710	Pelargonidin-3-glucoside (callistephin) isomer	Anthocyanidin (*O*-glycoside)		UR	[52]
36	28.11	$C_{21}H_{20}O_{10}$	431.0983	**Vitexin/isovitexin**	Flavone-*C*-glycoside		CL SC KM	[21]
37	28.71	$C_{21}H_{22}O_8$	401.1241	Glycosylated pinosylvin isomer	Stilbenoids		AM OR CL SO KM	[50]
38	28.74	$C_{33}H_{38}O_{21}$	769.1821	Apigenin-6-*C*-B-galactosyl-8-*C*-B-glucosyl-*O*-glucuronopyranoside isomer	Flavone-*C*-glycoside	AM		[21]
39	29.02	$C_{21}H_{21}O_{11}Cl$	447.0932	**Cyanidin-3-glucoside (kuromanin)**	Anthocyanidin (*O*-glycoside)	SV	OR	[52]
40	29.15	$C_{20}H_{18}O_8$	385.0928	Dihydroferulic acid isomer	Phenolic acids		AM IR	[20]
41	29.70	$C_{33}H_{38}O_{21}$	769.1821	Apigenin-6-*C*-B-galactosyl-8-*C*-B-glucosyl-*O*-glucuronopyranoside isomer	Flavone-*C*-glycoside	AM IR KM		[21]

42	30.48	$C_{23}H_{24}O_{12}$	491.1195	**Glycosylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone**	Flavone-*O*-glycoside	SV SC		[50]
43	31.01	$C_{23}H_{24}O_{12}$	491.1195	Glycosylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone isomer	Flavone-*O*-glycoside	SC		[50]
44	32.47	$C_{15}H_{10}O_5$	269.0455	Apigenin isomer	Flavone	UR KM	CL	[51]
45	33.95	$C_{25}H_{26}O_{13}$	533.1300	Glycosylated and acetylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone isomer	Flavone-*O*-glycoside		SO LE UR KM	[50]
46	34.00	$C_{26}H_{28}O_{14}$	563.1395	Apigenin-6-*C*-arabinoside-8-*C*-hexoside (schaftoside/isoschaftoside) isomer	Flavone-*C*-glycoside		CL IR UR KM	[23]
47	34.04	$C_{26}H_{28}O_{14}$	563.1395	**Apigenin-6-*C*-arabinoside-8-*C*-hexoside (schaftoside/isoschaftoside)**	Flavone-*C*-glycoside	CL OR SC	IR SO LE SC UR KM	[21]
48	34.23	$C_{20}H_{18}O_8$	385.0928	Dihydroferulic acid isomer	Phenolic acids		AM UR KM	[20]
49	35.02	$C_{20}H_{18}O_8$	385.0928	**Dihydroferulic acid**	Phenolic acids		AM IR SO LE SC KM OR CL UR	[20]
50	35.57	$C_{26}H_{28}O_{14}$	563.1395	Apigenin-6-*C*-arabinoside-8-*C*-hexoside (schaftoside/isoschaftoside) isomer	Flavone-*C*-glycoside	OR SC	AM IR SO LE	[21]
51	36.89	$C_{26}H_{28}O_{15}$	579.1355	Lucenin-1/3 (luteolin-6/8-*C*-xyloside-8/6-*C*-glucoside) isomer	Flavone-*C*-glycoside	OR SC KM	OR IR SO LE UR KM	[21]
52	37.18	$C_{20}H_{18}O_6$	353.1030	Hinokinin isomer	Lignans		SO SC	[10]
53	37.32	$C_{21}H_{22}O_8$	401.1241	Glycosylated pinosylvin isomer	Stilbenoids		SC UR KM	[50]
54	37.87	$C_{20}H_{18}O_8$	385.0928	Dihydroferulic acid isomer	Phenolic acids	SO	AM OR CL SV SC UR KM	[20]
55	38.16	$C_{30}H_{26}O_{12}$	577.1351	**Procyanidin B-3**	Proanthocyanidin	IR KM	AM OR SC	[53]
56	38.30	$C_{10}H_{10}O_4$	193.0506	Ferulic acid isomer	Phenolic acids	KM	CL SO SV LE SC UR KM	[49]
57	38.74	$C_{20}H_{18}O_8$	385.0928	Dihydroferulic acid isomer	Phenolic acids		AM CL IR SC UR KM	[20]
58	39.70	$C_{20}H_{18}O_8$	385.0928	Dihydroferulic acid isomer	Phenolic acids		AM CL IR OR SC SV UR KM	[20]
59	42.80	$C_{20}H_{18}O_6$	353.1030	**Hinokinin**	Lignans		SC	[10]
60	43.27	$C_{20}H_{22}O_6$	357.1343	Pinoresinol isomer	Lignans		SC KM	[54]
61	44.64	$C_{25}H_{26}O_{13}$	533.1300	Glycosylated and acetylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone isomer	Flavone-*O*-glycoside		LE KM	[50]
62	44.87	$C_{20}H_{22}O_6$	357.1343	**Pinoresinol**	Lignans		AM OR	[10]
63	45.41	$C_{26}H_{28}O_{14}$	563.1395	Apigenin-6-*C*-arabinoside-8-*C*-hexoside (schaftoside/isoschaftoside) isomer	Flavone-*C*-glycoside		UR	[23]
64	45.88	$C_{17}H_{14}O_7$	329.0666	**3′,4′,5′-trihydroxy-3,7-dimethylflavone**	Flavone		AM	[50]
65	46.57	$C_{15}H_{11}O_6Cl$	323.2870	**Anthocyanidin (cyanidin chloride)**	Anthocyanidin		OR SV	[52]
66	47.05	$C_{26}H_{28}O_{14}$	563.1395	Apigenin-6-*C*-arabinoside-8-*C*-hexoside (schaftoside/isoschaftoside) isomer	Flavone-*C*-glycoside		AM LE SV	[21]
67	47.41	$C_{23}H_{24}O_9$	443.1347	**Formononetin (glycosylated and methylated)**	Isoflavone	OR SC UR KM		[50]
68	48.53	$C_{23}H_{24}O_9$	443.1347	Formononetin (glycosylated and methylated) isomer	Isoflavone	OR SC UR KM		[50]
69	48.79	$C_{25}H_{26}O_{13}$	533.1300	Glycosylated and acetylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone isomer	Flavone-*O*-glycoside		LE UR KM	[50]
70	49.91	$C_{23}H_{24}O_9$	443.1347	Formononetin (glycosylated and methylated) isomer	Isoflavone	SC UR KM		[50]

Abbreviations: AM, Anco Marzio; CL, Claudio; IR, Iride; LE, Levante; OR, Orobel; SC, Senatore Cappelli; SO, Solex; SV, Svevo; UR, Urria; KM, Kamut®.

Table 6
Total compounds, excluding isomers, total isomers and unique compounds detected in free and bound phenolic fractions for each wheat genotype. The mean values (±standard deviation) for modern and old cultivars are reported.

Cultivar	Free phenolic			Bound phenolic		
	Total compounds[a]	Total isomers	Unique compounds	Total compounds[a]	Total isomers	Unique compounds
Anco Marzio (M)	3	3	0	9	7	1
Claudio (M)	5	1	1	8	4	1
Iride (M)	4	1	0	7	5	0
Kamut® (O)	9	6	2	10	13	0
Levante (M)	4	0	0	8	5	0
Orobel (M)	8	7	1	12	4	1
Senatore Cappelli (O)	11	13	2	16	7	1
Solex (M)	4	1	1	10	1	2
Svevo (M)	3	1	1	5	3	0
Urria (O)	6	4	0	10	14	1
Mean modern cultivars	4.4 ± 1.7 (b)[b]	2.0 ± 2.4 (b)	0.6 ± 0.8	8.4 ± 2.2 (b)	4.1 ± 1.9 (b)	0.7 ± 0.8
Mean old cultivars	8.7 ± 2.5 (a)	7.7 ± 4.7 (a)	1.3 ± 1.2	12.0 ± 3.5 (a)	11.3 ± 3.8 (a)	0.7 ± 0.6

Abbreviations: M, modern cultivar; O, old cultivar.
[a] Excluding isomers.
[b] Means followed by the same letter or no letter are not significantly different at $P < 0.05$.

3,7-dimethoxyflavone, with mass 533.1300 as the acetylated form of mass 491.1195. These metabolites appeared to be all derivatives of the flavonol quercetin ($C_{15}H_{10}O_7$) as recently proposed by Matus-Cádiz et al. [50].

Isoflavones were also present in the free phenolic fractions of some of the investigated wheat varieties. Mass 443.1347 ($C_{23}H_{24}O_9$; isomer compounds **67**, **68**, **70**) appeared to be glycosylated and methylated forms of formononetin. These metabolites were detected in all three old genotypes (Urria, Senatore Cappelli, Kamut®) and in one modern cultivar (Orobel). Glycosylated and methylated formononetin has been also detected in hard white wheat (*T. aestivum* L.) and it has been postulated that they may derive from the isoflavone daidzein, through successive steps of methylation and glycosylation [50].

3.3.5. Stilbenes

Stilbenes are small molecular weight (~200–300 g/mol), naturally occurring compounds and are found in a wide range of plant sources. They act as natural protective agents to defend the plant against viral and microbial attack, excessive ultraviolet exposure, and disease. One stilbene, resveratrol, has been extensively studied and has been shown to possess potent anticancer, anti-inflammatory and anti-oxidant activities [74].

Two phenolic compounds (**18** and **33**) of masses 535.1821 and 401.1241 with deduced molecular formulas $C_{26}H_{32}O_{12}$ (double glycosylated pinosylvin) and $C_{21}H_{22}O_8$ (glycosylated pinosylvin) were detected in the free (Senatore Cappelli) and in the bound phenolic fractions (Senatore Cappelli, Orobel and Kamut®), respectively. Other two compounds (**37** and **53**) were assigned as isomers of compound **33** and occurred in the bound phenolics of all three old varieties (Urria, Kamut®, Senatore Cappelli) and in four modern cultivars (Anco Marzio, Orobel, Claudio, Solex). These antioxidant metabolites are generally found in great amounts in pigmented vegetables (mainly grapes and blueberry) and have been shown to possess various medicinal properties [75]. Up to now very few are the reports about stilbene occurrence in cereals. Among these, a recent study of Matus-Cádiz et al. [50] reported the presence of double glycosylated pinosylvin in hard white wheat (*T. aestivum* L.) but the detected amounts in grains resulted to be very low.

3.3.6. Lignans

Lignans are a group of dietary phytoestrogen compounds with significant pharmacological activities including antitumor [76,77], anti-inflammatory, immunosuppressive, cardiovascular [78], antioxidant [79–82] and antiviral actions. It has also been suggested that lignan-rich diets could have a protective effects against estrogen-related diseases such as osteroporosis [79].

Among the investigated wheat varieties two lignans were detected in the bound phenolic fractions of two old (Senatore Cappelli and Kamut®) and two modern (Anco Marzio, Solex) cultivars. Compounds **59** and **52** (mass 353.1030, $C_{20}H_{18}O_6$) were assigned as hinokinin pair of isomers, compounds **60** and **62** (mass 357.1343, $C_{20}H_{18}O_6$) appeared to be isomers of the lignan pinoresinol. It is to highlight that hinokinin was detected exclusively in the old genotype Senatore Cappelli: this is in line with our previous findings on lignan content of soft wheat varieties showing that hinokinin was peculiar to old genotypes [10].

3.4. Phytochemical profiles of old and modern durum wheat genotypes

The number of detected free phenolic compounds, excluding isomers, ranged between 3 and 8 for modern varieties, and between 6 and 11 for old genotypes (Table 6). The mean number of free phenolic compounds, excluding isomers, was approximately two times higher in old wheat cultivars (8.7 ± 2.5) than in modern genotypes (4.4 ± 1.7). Significant differences were also observed for the mean number of free isomers observed in modern and old varieties. Except for Anco Marzio (3 isomers) and Orobel (7 isomers), all the remaining modern cultivars (Claudio, Iride, Levante, Solex, Svevo) exhibited no or one isomer form. The highest number of isomers has been found in the old genotype Senatore Cappelli (13 isomers), while the old genotypes Kamut® and Urria were characterized by six and four free isomers, respectively. A similar trend was observed for the bound phenolic fraction. The mean number of bound phenolic compounds and isomers was significantly higher in old wheat genotypes than in modern cultivars (Table 6). The phytochemical profiles highlighted the presence of phenolic compounds exclusively detected in some of the investigated genotypes. The analysis of the free fractions evidenced four unique compounds detected only in the old genotypes Senatore Cappelli (compounds **11** and **18**) and Kamut® (compound **20** and **26**), while four modern varieties (Claudio, Orobel, Solex, Svevo) were characterized by one unique compound (compounds **25**, **8**, **54**, **39**, respectively). As regards bound extracts, the modern genotype Solex showed two unique phenolics (compounds **25** and **34**), whereas the modern cultivars Anco Marzio (compound **64**), Claudio (compound **31**) and Orobel (compound **39**), and the old ones Senatore Cappelli (compound **21**) and Urria (compound **63**) exhibited one unique phenolic compound. For free and bound extracts no statistical difference was

observed between old and modern wheat genotypes for the mean number of detected unique compounds (Table 6).

The whole set of data dealing with the phytochemical profile of free and bound extracts was employed for the correspondence analysis aimed at evidencing the relationships between the investigated wheat genotypes and the phenolic compounds (Fig. 3). In the biplot four modern cultivars (Claudio, Solex, Anco Marzio and Iride) formed a separated cluster. Their position in the plot was associated with apigenin-6-*C*-B-galactosyl-8-*C*-B-glucosyl-*O*-glucuronopyranoside (compound **27**), methylisoorientin-2″-*O*-rhamnoside (compound **34**) and dihydroferulic acid (compound **49**). Another cluster grouped together the two old varieties Kamut® and Urria which shared prodelphinidin B-3 (compound **17**) and glycosylated and acetylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone (compound **15**). The modern variety Levante occupied an intermediate position between the two previously mentioned clusters, indicating some communalities with both modern and old genotypes. The two modern varieties Orobel and Svevo and the old variety Senatore Cappelli did not clusterise. In particular Svevo was positioned in the North-East quadrant between the compounds glycosylated 3′,4′,5′-trihydroxy-3,7-dimethylflavone (compound **42**) and apigenin-6-*C*-B-galactosyl-8-*C*-B-glucosyl-*O*-glucuronopyranoside (compound **27**): this could be ascribed to the lower free and bound phenolic compound occurrence than all other varieties and to the absence of association with the considered variables. Senatore Cappelli and Orobel were positioned in the West quadrants of the graphic because of their similar composition in apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside (compound **13**), syringaldehyde (compound **16**) and formononetin (glycosylated and methylated) (compound **67**) but they differ mainly for hinokinin (compound **59**) and *p*-hydroxybenzaldehyde (compound **6**).

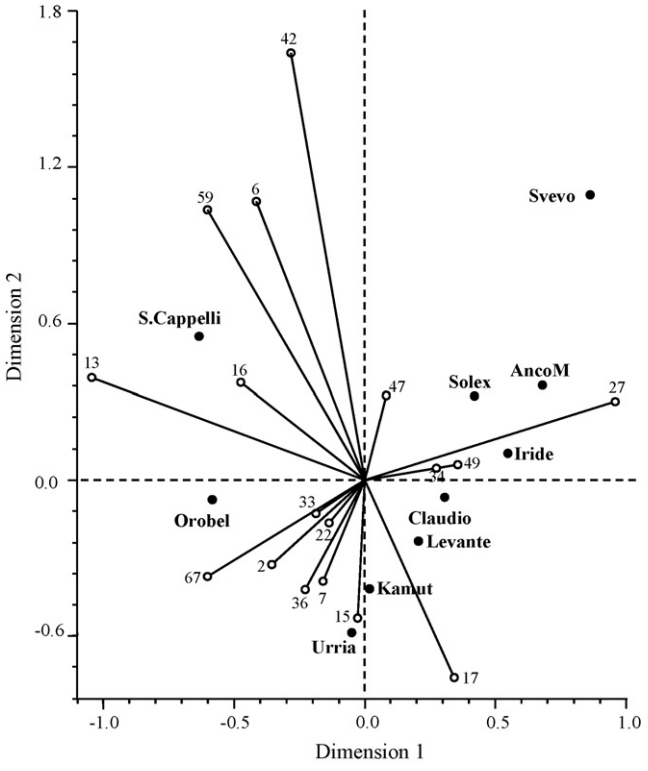

Fig. 3. Biplot of the correspondence analysis between wheat varieties (solid circles) and phenolic compounds (empty circles). Compound numbers are as in Table 5. In the graph only 17 phenolics with the highest variation were projected. The relative importance of single phenolic compounds is illustrated by the length of their corresponding centrifugal lines (dotted lines).

4. Concluding remarks

To the best of our knowledge this is the first time that HPLC coupled with TOF-MS was used to study the phytochemical profile of the phenolic fractions (free and bound) in wheat grains. The developed analytical method allowed the tentative identification of 70 phenolic compounds, including coumarins, phenolic acids, anthocyanins, flavones, isoflavones, protoanthocyanidins, stilbenes and lignans.

Besides no significant differences among investigated cultivars were detected as regards the amounts of total phenolic and flavonoid compounds, the qualitative phytochemical profile between old and modern varieties was remarkably diverse. In particular, the mean numbers of total compounds and total isomers (in both free and bound fractions) were significantly higher in old wheat genotypes than in modern cultivars. The peculiar and varied phytochemical profile of investigated old wheat genotypes confirmed that ancient grains may represent a rich source of genetic diversity, especially with regard to functional properties [10]. Our results may concur at developing breeding programs aimed at producing value-added wheat varieties for higher concentration and better composition of health-beneficial phytochemicals.

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