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**Determination of free soluble phenolic compounds in grains of ancient wheat varieties
(*Triticum* sp. pl.) by liquid chromatography-tandem mass spectrometry**

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Abstract

A liquid chromatography coupled-tandem mass spectrometry method was developed and validated for the determination of free soluble phenolic compounds in eight ancient varieties of wheat (*Triticum* sp. pl.), notably Autonomia, Gentil rosso, Inallettibile, Leone aristato, Mentana, Poulard di Ciano, Risciola and Terminillo. Trace compounds such as two conjugated flavones, vitexin (17.13-34.32 µg/kg) and isovitexin (9.76-30.01 µg/kg), were also determined.

Poulard di Ciano, presumably an autochthonous wheat of the Reggio Emilia province (Northern Italy), showed a peculiar quali-quantitative phenolic profile (7097.03 µg/kg total phenolic sum and 1.97 hydroxycinnamic acids sum to hydroxybenzoic acids sum ratio) along with a tetraploid genome. Terminillo, Risciola, Gentil rosso, Mentana, and Leone aristato showed a hexaploid genome and high concentrations of phenolic compounds (ranged from 6796.12 to 7605.78 µg/kg), also in comparison with two modern varieties of bread wheat, Bolero and Blasco.

The targeted metabolomic approach proved to be effective to determine some secondary metabolites of wheat. The richness in phenolic compounds combined with high rusticity and adaptability to marginal soils showed by ancient wheat varieties make them suitable for sustainable agricultural and organic cultivation.

Keywords: *Triticum* sp.pl., Ancient varieties, Secondary metabolites, Polyphenols, LC-ESI-MS/MS, Genotyping.

38 1. Introduction

39 The human selection of cereal seeds throughout history was aimed at obtaining more productive and
40 resistant plants that better responded to the environmental biotic and abiotic stresses (pests, weeds,
41 drought, and other adverse climatic factors). The ancient varieties and landraces of wheats were
42 characterized by the good adaptation to the local environment and climate of cultivation.¹
43 Moreover, these crops responded to the nutritional needs of the local populations. Some cultivars
44 originated from local landraces due to the work of Italian geneticists, particularly F. Todaro and N.
45 Strampelli, during the first decades of the twentieth century.²

46 After the Second World War, local varieties were replaced by “modern” cultivars and high energy
47 input agriculture.^{2,3} These cultivars were mainly selected for cultivation and/or production related
48 traits and characterized by a relative genetic uniformity.¹ The modern cultivars improved the yields
49 and better complied to the requirements of the baking industry. On the other hand, this process
50 inevitably led to the dramatic loss of agro-biodiversity observed in recent years. A part of the
51 genetic heritage of the ancient cultivars came to us owing to the tenacity of “custodian farmers”,
52 who conserved and made these plant genetic resources available by cultivation over time, along
53 with the higher rusticity and adaptability to marginal.^{4,5}

54 In recent years, a new interest on ancient cereals has been expressed by consumers, manufacturers,
55 farmers, and researchers. The main reasons are related to sensory and nutritional properties of the
56 derived products,⁶ as well as to the good adaptation and rusticity of these plants, which can make
57 their cultivation very attractive in the light of the threat of climate change. In addition, the demand
58 of ancient cereals was fostered by the constant increase of “green-minded” consumers, who are
59 keen on recovering cultural values, traditional, safer, traceable, transparent, whole-wheat, and
60 organic foods, and agriculture of low environmental impact.⁷

61 Wheat and its derivatives (bread and other baked goods) are considered functional foods, based on
62 the correlation between the consumption of products made from whole-wheat and the reduction of
63 chronic diseases (cardiovascular diseases, diabetes, and cancer).^{2,8} These beneficial effects to health

were put in relation to the presence of substances with antioxidant action and the fibrous component.^{9,10}

The ancient grains have a rich heritage of secondary metabolites, so-called “functional” or “bioactive” compounds, which are most lacking in modern cultivars, especially in terms of quality.¹¹ These substances, with antioxidant properties, mainly include phenolic compounds (phenolic acids: hydroxycinnamic acids and hydroxybenzoic acids; flavonoids: flavonols, flavones, flavone-*C*-glycosides, flavone-*O*-glycosides, isoflavones, flavonones, anthocyanidins; other classes: coumarins, stilbenes, proanthocyanidins, lignans), aside from tocopherols and tocotrienols, and carotenoids (typical of the “red” varieties).¹² In addition to performing an antiradical activity, free-radical scavenging, and metal chelating, these compounds are involved in many other metabolic functions and exert important effects on the immune and cardiovascular systems.^{13,14}

Phenolic identification and determination via liquid chromatographic techniques in common wheat (*Triticum aestivum* L.) have been reported in recent years. The identification was performed using LC-ESI-TOF-MS^{8,12} and LC-ESI-MS/MS,¹⁵ while the quantification was carried out using HPLC-UV-Vis,¹⁶ HPLC-DAD,^{10,17-20} and HPLC-UV-MS.²¹ Other studies focused on quantification of phenolics in durum wheat (*T. durum* Desf) and other *Triticum* species using HPLC-DAD.^{22,23}

Although detectors based on UV absorption are still commonly used for the determination of phenolic compounds, they lack in selectivity and sensitivity.²⁴ In particular, for trace components in complex matrices, the impact of impurities on the noise is high, and it results in scarce precision and reliability.

In liquid chromatography, MS detectors equipped with a triple quadrupole analyzer are set to measure only selected reaction monitoring (SRM) transitions for each compound, thus highly improving sensitivity, selectivity, and precision in phenolic identification and quantification. In addition, the chromatographic run can be segmented into different time windows in order to search for each compound solely within its specific window and, in turn, to optimize each individual dwell time.²⁵

A targeted metabolomics approach represents a modern and attractive strategy for crop and food characterization. Indeed, it allows the quantification of a predefined set of metabolites, typically a relatively small number of chemically characterized metabolites. Furthermore, compounds are kept in their native form, thus avoiding any chemical modification of their structure.

The present study aims to identify and quantify free soluble phenolic compounds in ancient varieties of wheat using a sensitive and reliable targeted metabolomics method based on LC-ESI-MS/MS. In this context, quali-quantitative analyses are carried out in eight ancient and two modern varieties, cultivated in the same conditions.

2. Materials and methods

Plant materials

The study included eight ancient varieties of wheat (*Triticum* sp. pl. – family Poaceae), Autonomia, Gentil rosso, Inallettibile, Leone aristato, Mentana, Poulard di Ciano (also known as Turgido Reggiano), Risciola, and Terminillo, and two modern cultivars, Bolero (FPS, 356-Ets Claude Camille Benoist, 159-Venturoli Sementi S.R.L. 1987) and Blasco (FPS, 91-Co.Na.Se. Consorzio Nazionale Sementi S.R.L. 2002). All the cultivars originated from the cereal Germplasm Bank at the Interdepartmental Centre BIOGEST - SITEIA (University of Modena and Reggio). Nowadays, it conserves more than 150 accessions of wheat, including some ancient cultivars.²⁶

Before analysis, the selected varieties have been cultivated to obtain fresh plant material. The experimental field used for the regeneration was located in Gattatico (Reggio Emilia, Northern Italy; GPS coordinates: 44.790318N, 10.498182E), with an area of about 0.01 ha.

The sowing was carried out in autumn, for the vernalization requirement of some no-alternative varieties. The harvest was carried out manually over the course of two days to ensure a good level of maturity of all the cultivated varieties. Spikes were immediately bagged in suitably-labeled paper envelopes and stored at room temperature for a few days in a local laboratory suitable to shelter them from light and moisture.

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Plant growth conditions and DNA extraction

The seeds of all the varieties were germinated in Petri dishes at 25 °C. Once the coleoptiles emerged (growth stage 0.7, according to Zadoks et al.²⁷) seedlings were transferred to polystyrene box cells filled with peat (70% organic matter, 0.6% nitrogen; pH = 6.0 and electric conductivity = 1.33 dS/m). Seedlings were grown for seven days in a growth chamber (Binder KBW 720, Tuttlingen, Germany) under a 16-h photoperiod with an irradiance of 180 µmol/m²/s (white fluorescent tubes Fluora 18W/77, Osram, Munich, Germany), relative humidity of 60% and day/night temperature of 25/15 °C. Young leaves (300 mg) of each cultivar were used for DNA isolation according to Stein et al.²⁸ Concentration and purity of extracted DNAs were measured through a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, U.S.A.).

Determination of ploidy level – genotyping

Diploid species belonging to *Triticum* genus have an A genome only, tetraploid species have A and B genomes, while the hexaploid species *Triticum aestivum* L. harbors three genomes: A, B, and D.²⁹ A, B, D genomes specific markers were thus used in the present study to discover the ploidy level of the tested genotypes. While amplification products were expected for all markers for *Triticum aestivum* samples, only A and B specific, and only A specific markers were expected for tetraploid and diploid genotypes, respectively. Following this strategy, six KASP SNP markers (two for each A, B, and D genomes) were selected from CerealsDB 3.0 (<http://www.cerealsdb.uk.net>)³⁰ (Table 1). SNPs detection was performed using the Fluidigm EP1™ system (Fluidigm Corp., San Francisco, CA, U.S.A.). Contig sequences flanking the six SNPs (at least 60 bp before and after the polymorphism) were used by Fluidigm to design and synthesize the primers for the assay. Allele-specific forward primers (ASP1, ASP2) were labelled with the fluorochromes FAM and HEX. Amplifications and data analysis were carried out according to Fluidigm EP1™ system protocols.

142 **Chemicals**

143 All solvents and reference standards were of analytical grade. Acetonitrile, absolute ethyl alcohol,
144 and formic acid were of HPLC grade (VWR International Ltd, Milan, Italy). Pure standard of
145 apigenin, caffeic acid, (+)-catechin, *trans*-cinnamic acid, *p*-coumaric acid, daidzein, *trans*-ferulic
146 acid, formononetin (7-hydroxy-4'-methoxyisoflavone), gallic acid, isovanillin, isovitexin (apigenin-
147 6-*C*-glucoside), orientin (luteolin-8-*C*-glucoside), salicylic acid, sinapic acid, syringic acid, vanillic
148 acid, vanillin, vitexin (apigenin-8-*C*-glucoside), as well as Folin-Ciocalteu reagent and sodium
149 carbonate were purchased from Sigma-Aldrich® (Milan, Italy). Deionized water was obtained
150 through an Elix purification system (Millipore, Milan, Italy).

151

152 **Milling**

153 A gross sample of 10 g of intact and healthy caryopses of each variety was selected and considered
154 as a single batch of raw material. Caryopses were ground using a small electric grinder (MC300,
155 Moulinex), taking small amounts each time to avoid the heating of the sample. Finally, the flour
156 was made finer and more uniform with mortar and pestle.

157

158 **Metabolic characterization**

159 **Optimization of the LC-ESI-MS/MS conditions**

160 Standard stock solutions (50-100 mg/L) of each analyte were prepared using an aqueous solution
161 (hereafter called EAT solution) containing 5% absolute ethanol, 3% acetonitrile, and 0.1%
162 triethylamine, as a solvent.

163 The MS/MS parameters (parent ion, product ions, fragmentor, collision energy, and polarity of ion
164 source) were optimized using standard solutions of each analyte (125 µg/L), introduced individually
165 into the detector. For each analyte, selected reaction monitoring (SRM) transitions of the two most
166 abundant fragments (product ions), were used. The experiment was carried out in a positive-ion
167 mode and fell in the 50-500 *m/z* range. Source parameters were optimized as follows: gas (N₂)

168 temperature, 350 °C; N₂ flow, 11 L/min; nebulizer pressure, 35 psi; capillary, positive 3500 V and
169 negative 4000 V.

170 Peak identification included the comparison of the peak retention times to those obtained with
171 chemical standards and the evaluation of the tandem mass spectroscopy experiments. For each
172 compound, the most intense SRM transition was used as the quantifier ion (QI) and the least intense
173 one as the qualifier ion (qi) to confirm the peak identification. The relative intensity of each peak
174 (qualifier ion SRM to quantifier ion SRM percentage ratio) was also evaluated and verified to be
175 within an uncertainty range of $\pm 10\%$.

176

177 **LC-ESI-MS/MS optimized analytical conditions**

178 Secondary metabolites determination was carried out by liquid chromatography coupled with mass
179 spectrometry using an Agilent Technologies RP-LC-ESI-MS/MS system (Santa Clara, CA, U.S.A.),
180 consisting of an HPLC (Agilent Technologies 1200 Series) equipped with a degasser, a binary
181 pump, an autosampler, and a 6410B triple quadrupole mass spectrometer.

182 After centrifugation (Mini Spin, Eppendorf Italy, Milan, Italy), the supernatant of each sample
183 (5 μ L) was injected into a 100 mm \times 3.0 mm i.d. \times 2.7 μ m particle size RP-phenyl-hexyl column
184 (Poroshell 120Å, Agilent Technologies, D.T.O. Servizi Srl, Spinea, VE, Italy).

185 The solvent system was composed by formic acid 0.1% in water (solvent A) and acetonitrile
186 (solvent B). The elution (25 min of run + 5 min of post-run) was carried out according to the
187 following gradient: 11% B (0.0 min), 11% B (2.0 min), 13% B (5.0 min), 13% B (7.0 min), 16% B
188 (10.0 min), 16% B (12.0 min), 80% B (15.0 min), 80% B (18.0 min), 11% B (18.5 min), 11% B
189 (25.0 min) with a flow rate of 0.4 mL/min at ambient temperature (pressure of about 180 bar at run
190 start). Each chromatogram was segmented into six time windows.

191 Quantification was performed via external standard calibration. The chromatograms were acquired
192 and processed by Quantitative Analysis for QQQ version B.05.00 (MassHunter Workstation
193 software, Agilent Technologies Inc.).

194

195 **Method of extraction, optimization and recovery tests (trueness)**

196 The extraction protocol relied on the method described by Adom et al.,³¹ then modified by Dinelli
197 and et al.,¹² with some adjustments to optimize the yields of extraction.

198 Each sample (2.00 g of finely-ground flour) was introduced into a 50-mL plastic tube together with
199 10 mL of chilled ethyl alcohol (80%). After homogenization through the use of a vortex (150 sec),
200 the tubes were centrifuged (4237R, A.L.C. International Ltd, Cologno Monzese, MI, Italy) at 4000
201 rpm for 5 min at 4 °C. The supernatant was transferred in another tube and stored at -20 °C. Ten mL
202 of chilled ethyl alcohol (80%) were added to the pellet and the entire extraction cycle was repeated
203 three times in total. Finally, all three aliquots were pooled to obtain about 30 mL of extract that was
204 poured into a 100-mL round bottom flask and evaporated to dryness with a rotary evaporator (VV
205 2000, Heidolph Instruments, Kelheim, Germany), using a temperature of the water bath below
206 30 °C. The residue was re-dissolved with EAT solution and made up to 10 mL in a graduated flask
207 with the same solution and, finally, transferred into a test tube. After insufflation of N₂ to prevent
208 oxidation, each tube was closed by a screw cap with Teflon septum and stored at -20 °C until LC-
209 MS/MS determination. The dilution factor of the method was 5. For each real sample, replications
210 (n = 3) were carried out using different batches of raw materials.

211 To establish the optimal number of cycles of extraction, a recovery test was carried out using a
212 homogenous gross sample of Mentana flour (20 g). After each extraction cycle (five cycles in total),
213 carried out on 2.00 g of flour, the extract obtained was checked through the acquisition of the UV-
214 Vis spectrum (using the V-730 UV/VIS spectrophotometer, Jasco Europe Ltd, Cremella, LC, Italy),
215 from 235 to 800 nm. The same samples (n = 5) were also run using the LC-MS/MS method
216 described above to calculate the individual analyte yield. The test was performed in triplicate.

217 The method trueness was also evaluated. Samples of Mentana finely-ground flour (2.00 g) were
218 spiked with a standard mixture of all the analytes. Two fortification levels (three repetitions for
219 each) were tested and the results were compared to those obtained with not-spiked samples. The

220 mixture of pure standards was allowed to stand for 30 min to enhance the interaction with the
221 matrix before the extraction. After each chromatographic run, the column was washed by injecting
222 5 μ L of EAT solution. These samples were subjected to extraction and LC-MS/MS determination as
223 described above.

224

225 **Evaluation of linearity, limit of detection and lower limit of quantification (detectability),**
226 **intra-day and inter-day repeatability (precision).**

227 Solutions of increasing concentration of diluted pure standards were prepared to evaluate the
228 linearity of the instrumental response. For each substance, the range of concentrations was
229 established on the basis of the data present in the literature and by exploring the chromatographic
230 traces of some real samples.

231 In the aqueous reference solution, the instrumental limit of detection (LOD) and the lower limit of
232 quantification (LLOQ) were obtained by applying the equation:³²

233
$$\text{LOD or LLOQ} = (K s_{y/x})/b \quad [\text{Eq. 1}]$$

234 where $s_{y/x}$ and b are the estimated regression standard deviation and the slope of the relevant
235 analytical calibration function, respectively. $K = 3$ and $K = 10$ were chosen in order to obtain the
236 LOD and LLOQ, respectively.³³

237 Precision was evaluated with an intra-day repeatability test on standard mixture solutions containing
238 all the analytes (125 μ g/L), injected five times, and an inter-day repeatability test carried out on
239 samples of Mentana finely-ground flour (2.00 g) injected on five consecutive days in the same
240 conditions. The relative standard deviations were calculated for each substance.

241

242 **Total phenolic content**

243 The total phenolic content (TPC) was analyzed using the Folin-Ciocalteu method.³⁴ In a 10-mL
244 flask, 6 mL of water, 0.3 mL of the extract obtained through the method described above, and
245 0.5 mL of the Folin-Ciocalteu reagent were added. After 1 min of shaking with a vortex, 2 mL of

15% aqueous sodium carbonate were added and the solution was made up to 10 mL with water. Finally, this solution was mixed and left to stand at ambient temperature for 120 min. Absorbance was read at 750 nm (V-730 UV/Vis spectrophotometer, Jasco Europe Ltd, Cremella, LC, Italy) against a blank represented by the reagents only and compared with a standard gallic acid calibration curve. Results of triplicate analyses on different sample batches are given as mg/g of gallic acid equivalents (GAE).

Statistical analysis

Univariate and multivariate analyses were carried out on the data set. Differences amongst varieties were assessed by analysis of variance (one-way ANOVA) based on three replicates for each sample. When a significant effect (at least $p \leq 0.05$) was showed, comparative analyses were carried out using the post hoc Tukey's multiple comparison test. Correlation matrix analysis and principal component analysis (PCA) were also applied to the whole data set. All tests were performed using Statistica version 8.0 software (Stat 180 Soft Inc., Tulsa, OK, USA).

3. Results and discussion

Determination of ploidy level – genotyping

For nine of tested genotypes, amplification products were obtained for markers designed for A, B, and D genomes and thus proved their hexaploid nature (**Table 1**). Only one genotype – “Poulard di Ciano” – for which no amplification product for markers situated on D genomes was obtained, was identified as tetraploid species.

Determination of the optimal parameters for MS/MS detection

The optimal parameters (fragmentor voltage, V, and collision energy, V) for MS/MS detection, determined through the direct infusion in the spectrometer of a 125 µg/L solution of each pure standard compound, are reported in **Table 2**. For most of the phenolic acids (gallic, caffeic, *p*-

coumaric, *trans*-ferulic, sinapic, and salicylic), the QI corresponded to a loss of water by the parent ion. The dwell-times for each transition were also optimized. High sensitivity was obtained in ESI positive mode for all compounds and the same type of parent ion (protonated molecular ion $[M+H]^+$) of high sensitivity was obtained.

Setup of chromatographic conditions and segmentation of the chromatogram

A satisfactory separation of the analytes was obtained with a double endcapped phenyl-hexyl column. This column was designed for the separation of substances with aromatic rings in their structure, through selective π - π interactions with the aromatic rings of the stationary phase.

The retention times of the analytes are showed in **Table 2**. The chromatographic method was optimized by means of successive tests, carried out by changing the composition of the mobile phase in order to obtain the best profile for peak separation and to reduce the total run time. The separation of the two critical couples of peaks (isovanillin-vanillin and isovitexin-vitexin) was considered. Moreover, inside each couple, the compounds showed the same transitions. The resolution (R) of both the couples of peaks was higher than 1.5, even if the performance of the column decreased during the time, in particular for the couple isovanillin-vanillin. However, isovanillin was never detected in any real sample. In addition, the chromatogram was divided into six time windows to optimize the dwell times of each compound (**Table 2**).

Optimization of the extraction protocol and trueness evaluation

The dissolution of the samples after the evaporation to dryness was a critical step of the method. The chemical nature of the residue needed an alkaline solution to dissolve the polyphenols that contain weak-acids hydroxyls ($pK_a \approx 10$) as substituents, completely. This problem was overcome using triethylamine (0.1%) as an MS-compatible organic base.³⁵ Moreover, the presence of a strong organic base, such as trimethylamine, was able to suppress the formation of sodium and potassium adducts, thus resulting in an improved MS sensitivity.³⁶

298 Repeated extraction cycles were performed on Mentana finely-ground flour to verify the reduction
299 of the absorption at different wavelengths (280, 290, 326, 446, 474 nm), corresponding to the
300 maximum absorptions in the UV-Vis spectrum. Each sample was also analyzed with the optimized
301 LC-MS/MS method.

302 The sum of the first three cycles of extraction accounted for more than 90% of the absorption for all
303 the monitored wavelengths (**Table 3**). In addition, the recovery of the single compounds carried out
304 with LC-MS/MS after three cycles of extraction was considered satisfactory for most of the analytes
305 (**Table 3**). Vanillin was the only compound that showed a recovery lower than 90% in these
306 conditions. The reason is likely related to the chemical nature of this substance. In fact, it is not a
307 phenolic acid, but it contains a less polar aldehyde function ($pK_a \approx 17$). However, it was decided to
308 limit the number of cycles of extraction to three in order to reduce the total time for sample
309 preparation, also.

310 The determination of the recoveries obtained by spiking a known amount of pure compounds into
311 the sample (net of the concentrations found for the same not spiked sample) does not allow the
312 estimation of the extraction yield of the analytes. In fact, the compartmentalization of the
313 compounds inside cell structures needs a number of extraction cycles up to allow the analytes to be
314 quantitatively extracted and dissolved into the solvent. As a matter of fact, the present method does
315 not include the use of tools, such as SPE cartridges and filters that can give a significant reduction
316 of the analytes yield. Nevertheless, a recovery test with two different fortification levels of standard
317 concentration was carried out on the same sample (Mentana), also used for the recovery tests
318 previously described.

319 All the percentages of recovery ranged between 60% and 110% (**Table 3**). Gallic acid, vanillic acid,
320 caffeic acid, syringic acid, *trans*-ferulic acid, sinapic acid, salicylic acid, isovitexin, vitexin,
321 daidzein, *trans*-cinnamic acid, and formononetin showed satisfactory values (between 90% and
322 110%). *p*-Coumaric acid and orientin presented percentages of recovery that were slightly lower
323 than 90%, while (+)-catechin, isovanillin, and vanillin highlighted considerably lower values,

324 especially for the highest fortification level. The method of extraction confirmed to not be properly
325 suitable for the vanillin and its isomer, isovanillin, quantification.

326

327 **Method validation (linearity, detectability, and precision determination)**

328 The ranges of concentration used for calibration are showed in **Table 4**. For all the substances, the
329 coefficient of determination (R^2) was higher than 0.995, except formononetin (0.991), thus showing
330 a satisfactory linear correlation between concentration and response in the range studied.³²

331 LODs and LLOQs are showed in **Table 4**. The results of the detectability evaluation provided LOD
332 values in the standard solutions that were lower than 1 $\mu\text{g/kg}$ for most of the analytes, mainly the
333 trace compounds, and were lower than 10 $\mu\text{g/kg}$ for all the main phenolic acids. However,
334 considering the dilution factor of the method, the detectability showed values ranging from 0.62 and
335 31.8 $\mu\text{g/kg}$.

336 A review of the literature showed that the detectability of the proposed method is very satisfactory
337 when compared with similar studies on the same topic. The ranges of LOD reported are: from 100
338 and 500 $\mu\text{g/kg}$;¹⁷ from 80 and 400 $\mu\text{g/kg}$;²⁰ from 60 and 700 $\mu\text{g/L}$;²² from 220 and 5360, $\mu\text{g/kg}$.²³

339 The intra-day precision was performed on a standard mix extracted and injected five times in the
340 same day. The results were satisfactory (**Table 4**) and all relative standard deviations were
341 contained within 10%, except daidzein (12.5%). The inter-day precision was evaluated for each
342 analyte on a sample of Mentana. RSDs of all the analytes exceeding the LLOQ were below $\pm 10\%$
343 (**Table 4**). For a confidence level of 95% and coverage factor (k) = 2, the method has an uncertainty
344 that is lower than $\pm 20\%$.³²

345

346 **Phenolic determination on real samples**

347 The quantification of the phenolic substances in the real samples is reported in **Table 5**. ANOVA
348 and Tukey's test were applied on the whole data set. Isovanillin was never detected, while (+)-
349 catechin, caffeic acid, and orientin did not show values above the detectability limits in any sample.

350 Formononetin was present with a concentration higher than LLOQ solely in Autonomia. All the
351 other substances showed significantly different concentrations ($p \leq 0.001$) among the varieties.

352 A concentration of gallic acid above 300 $\mu\text{g/kg}$ was highlighted only in Mentana. Vanillic acid was
353 present in significantly higher concentrations (around 2000 $\mu\text{g/kg}$) in Gentil rosso, Terminillo,
354 Mentana, and Leone aristato, while syringic acid showed the significantly highest concentrations in
355 Mentana, Risciola and Leone Aristato.

356 Risciola showed a high content of vanillin, followed by Inallettibile, even though the significant
357 highest concentration was found in the modern cultivar Bolero. Terminillo showed the statistically
358 highest concentration of *trans*-ferulic acid, followed by Risciola, Mentana, Gentil Rosso, Poulard di
359 Ciano, and Leone Aristato, while the other varieties remained under 2000 $\mu\text{g/kg}$. Poulard di Ciano
360 showed an extremely higher concentration of sinapic acid (above 1300 $\mu\text{g/kg}$) in comparison with
361 all the other varieties.

362 Salicylic acid, isovitexin, vitexin, and daidzein were not found in concentrations above the LLOQ
363 in some samples. In general, the concentrations of vitexin were higher than isovitexin, while
364 daidzein was present in very tiny concentrations. Poulard di Ciano had the highest concentrations of
365 both isovitexin and vitexin, closely followed by Gentil rosso. Terminillo, Leone aristato, Risciola
366 and Gentil rosso showed the highest concentrations of daidzein, as well as of *trans*-cinnamic acid.

367 Finally, Risciola, Inallettibile and Terminillo highlighted the highest concentrations of apigenin.

368 Mentana and Terminillo showed the significant highest hydroxybenzoic acids sum, followed by
369 Leone aristato, Risciola and Gentil rosso. Poulard di Ciano showed the significant highest values
370 for hydroxycinnamic acids sum along with Terminillo. Risciola and Gentil rosso, followed by
371 Terminillo and Poulard di Ciano, highlighted the highest concentrations for flavones and
372 isoflavones sum.

373 Poulard di Ciano showed a ratio between hydroxycinnamic acids sum and hydroxybenzoic acids
374 sum (HC/HB) as high as 1.97, while all the other varieties had values that ranged from 1.07 to 1.32,

except Blasco, which was the only variety with an amount hydroxybenzoic acids higher than hydroxycinnamic ones.

In recent studies aimed at differentiating certain modern and ancient cultivars of common wheat, Gentil rosso showed the highest total amount of phenolic substances, a significantly higher concentration of *trans*-ferulic acid than Inallettibile and higher concentrations of sinapic, syringic, and vanillic acids in comparison with Inallettibile and Bolero.³⁷ Autonomia and Bolero varieties showed a similar concentration of *trans*-ferulic acid in a study carried out on wheat flour.⁴ However, the comparison of data obtained in the present study with those presented in other papers is quite complex, due to the different extractive-quantitative approach herein applied.

Total phenolic content

The TPC of every sample is showed in **Table 6**. The one-way ANOVA showed statistically-significant differences ($p \leq 0.001$) among the varieties. The highest figure was found in Poulard di Ciano, followed by Risciola and then Leone aristato, Terminillo and Mentana, while the lowest values were found in Inallettibile, Autonomia, as well as in the two modern varieties, Bolero and Blasco.

Apart from Bolero, data on TPC were found in literature solely for Autonomia, Inallettibile and Gentil rosso.^{12,38,39} Data on Gentil rosso, Bolero and Blasco were also available for wheat bread.⁴⁰ In agreement with the results of the present work, the TPC relative amount found in Gentil rosso was higher than in Autonomia and Inallettibile.^{12,38,39} The TPC values obtained in this study for the modern cultivars were relatively lower, in accordance with those reported by several authors.^{12,38,39,40}

Correlation matrix analysis

The relationships between the main parameters were studied by the linear correlation matrix (**Table 7**). Concentrations lower than the LLOQs of some parameters did not permit their inclusion in this

study. TPC was mainly correlated ($p \leq 0.001$) with the individual hydroxycinnamic acids (sinapic, *p*-coumaric, *trans*-ferulic, and *trans*-cinnamic) and their sum. It also showed a linear correlation ($p \leq 0.05$) with gallic acid, hydroxybenzoic acids sum, and flavones and isoflavones sum. The hydroxybenzoic acids (gallic acid, vanillic acid, and syringic acid) showed a significant linear correlation amongst them, as well as with their sum, but also with some hydroxycinnamic acids and their sum. Vanillin did not show any significant linear correlation, neither with *trans*-ferulic acid (its precursor), nor with vanillic acid (its derivative).⁴¹ *p*-Coumaric acid was significantly correlated only with hydroxycinnamic acids, as well as with flavones and isoflavones sum and total sum. Conversely, *trans*-ferulic acid, sinapic acid, and *trans*-cinnamic acid, aside from the significant correlations with hydrocinnamic-like compounds, also showed a correlation with hydroxybenzoic-like compounds, as well as the flavones and isoflavones sum. Apigenin showed a significant correlation only with flavones and isoflavones sum ($p \leq 0.001$). The flavones and isoflavones sum was significantly correlated with hydrocinnamic-like compounds, due to their biochemical origin from 4-hydroxycinnamoyl-CoA.⁴¹

Principal component analysis

A principal component analysis (PCA) was carried out on the autoscaled values to explore part of data set (only the parameters that did not show concentrations lower than the LLOQs) and to achieve information about the relationship amongst the variables and the overall distribution of the samples on the score plot.

The first four principal components (PCs), all with eigenvalues > 1.0 , explained 88.57% of the total variance. All factors with eigenvalues < 1.0 were discarded, according to the Kaiser's criterion.⁴²

All the considered parameters (except vanillin) weighed on PC1 (52.94% of the total variance) with a negative sign (**Fig. 1A**). The hydroxybenzoic-like compounds were grouped together and weighed with a positive sign on the PC2 (18.44% of the total variance), while the hydroxycinnamic-like compounds were not grouped in a similar way. Total phenolic content weighed on PC2 with a

negative sign and showed a linear correlation with sinapic acid. Apigenin weighed on PC3 (10.08% of the total variance) with a positive sign, as did flavones and isoflavones sum and vanillin (**Fig. 1B**). Vanillin weighed also on PC4 (7.12% of the total variance) with a negative sign.

Samples of Poulard di Ciano were clearly separated on the PC2 in the lower part of the plot (**Fig. 2A**), due to the high concentration of sinapic acid, *p*-coumaric acid, total phenolic content, and HC/HB. All the other samples were located on the PC1 according to the different amounts of the total sum, as well as the concentrations of *trans*-ferulic acid and *trans*-cinnamic acid, and hydroxybenzoic-like compounds. Inallettibile and Autonomia were located in the positive quadrant of the PC1, while Risciola and Terminillo, Gentil rosso and Leone aristato, due to their higher concentrations, were set in the negative quadrant of the PC1. Mentana samples were rather separated on the PC2, due to the high concentration of hydroxybenzoic-like compounds. The two modern varieties were located in the positive quadrant of PC1 and PC2 and were mainly characterized by the high concentration of vanillin (Bolero) and the low concentration of HC (Blasco).

Inallettibile was discriminated on PC3 for its high concentrations of vanillin and apigenin (**Fig. 2B**), while the PC4 permitted the separation of Bolero, due to its high concentration of vanillin.

443

In conclusion, this study permitted the determination of free soluble phenolic compounds in ancient varieties of wheat using a sensitive and reliable method based on LC-ESI-MS/MS. For the first time, applying this new metabolomic quantitative approach, polyphenolic compounds, such as vitexin and isovitexin, were determined in wheat.

Interesting phenolic profiles, in terms of amount and peculiarity, emerged. Poulard di Ciano, probably an autochthonous wheat of the Reggio Emilia province, showed a tetraploid genome. It furthermore displayed a characteristic high hydroxycinnamic acids/hydroxybenzoic acids ratio, which could be proposed as its peculiar biomarker. Its interesting profile certainly deserves a more thorough investigation.

453 Terminillo, Risciola, Gentil rosso, Mentana and Leone aristato showed high concentrations of
454 phenolic compounds, with a balanced amount of hydroxycinnamic acids and hydroxybenzoic acids.
455 Although a more extensive comparison is needed to formulate wider conclusions, these varieties
456 highlighted higher concentrations of secondary metabolites in comparison with the two modern
457 varieties Bolero and Blasco.

458 The results of this study pick out the importance to work in the direction of recovery and reuse of
459 ancient varieties, not only to safeguard the agro-biodiversity, but also for their advantageous
460 characteristics. In particular, the great rusticity and adaptability to marginal soils that may therefore
461 ensure stability of both yield and quality, the suitability for organic cultivation, along with their
462 richness in secondary metabolites.

463 It is thus important to start up new genetic improvement programs aimed at obtaining a new wheat
464 ideotype, harboring a right combination of technological and nutritional characteristics. This is
465 particularly important for wheat, which is at the base of the diet of a large part of the world
466 population, both in view of a further notable demographic growth and in light of the much-feared
467 climatic changes, capable of affecting the agricultural production. Finally, the reintroduction of
468 ancient grains could contribute to the diffusion of a more sustainable agricultural model, able to
469 provide farmers with a greater added value to their production.

470

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477

478 Abbreviations used

479 A, apigenin; ANOVA, analysis of variance; ASP, allele-specific forward primers; Au, Autonomia;
480 Bl, Blasco; Bo, Bolero; CE, collision energy; EAT, solvent solution containing absolute ethanol
481 (5%), acetonitrile (3%), and triethylamine (0.1%); ESI, electrospray ionization; FI, flavones and
482 isoflavones sum; FV, fragmentor voltage; GA, gallic acid; GAE, gallic acid equivalents; GPS,
483 global positioning system; GR, Gentil rosso; HB, hydroxybenzoic acids sum; HC, hydroxycinnamic
484 acids sum; HC/HB, hydroxycinnamic acids sum to hydroxybenzoic acids sum ratio; HPLC, high
485 performance liquid chromatography; KASP, competitive allele specific PCR; In, Inallettibile; La,
486 Leone aristato; LLOQ, lower limit of quantification; LOD, limit of detection; Me, Mentana; MS,
487 mass spectrometry; MS/MS, tandem mass spectrometry; MW, molecular weight; m/z , mass-to-
488 charge ratio; PdC, Poulard di Ciano; PC, principal component; PCA, principal component analysis;
489 pCA, *p*-coumaric acid; R, resolution; R^2 , coefficient of determination; Ri, Risciola; RSD, relative
490 standard deviation; QQQ, triple quadrupole; QI, quantifier ion; qi, qualifier ion; qi/QI, qualifier ion
491 SRM to quantifier ion SRM percentage ratio; SA, syringic acid; SinA, sinapic acid; SNP, single
492 nucleotide polymorphisms; SRM, selected reaction monitoring; tCA, *trans*-cinnamic acid; Te,
493 Terminillo; tFA, *trans*-ferulic acid; TPC, total phenolic content; t_R , retention times; TS, total sum;
494 UV, ultraviolet; V, vanillin; VA, vanillic acid.

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658 **Figure captions**

659

660 **Figure 1.**661 PC1 vs. PC2 (**A**) and PC1 vs. PC3 (**B**) loadings plots of the main parameters.

662 TPC, total polyphenols content; GA, gallic acid; VA, vanillic acid; SA, syringic acid; V, vanillin;
663 pCA, *p*-coumaric acid; tFA, *trans*-ferulic acid; SinA, sinapic acid; tCA, *trans*-cinnamic acid; A,
664 apigenin; HB, hydroxybenzoic acids sum; HC, hydroxycinnamic acids sum; FI, flavones and
665 isoflavones sum; TS, total sum; HC/HB, hydroxycinnamic acids sum to hydroxybenzoic acids sum
666 ratio.

667

668 **Figure 2.**669 PC1 vs. PC2 (**A**) and PC1 vs. PC3 (**B**) score plots of the samples.

670 Au, Autonomia; Bl, Blasco; Bo, Bolero; GR, Gentil rosso; In, Inallettibile; La, Leone aristato; Me,
671 Mentana; PdC, Poulard di Ciano; Ri, Risciola; Te, Terminillo.

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Table 1

Six Single nucleotide polymorphisms (SNP) markers used for genotyping assays and their properties. Experiment results are reported for each genotype.

		SNP IDs					
		BS00047241	BS00000006	BS00022342	BS00046601	BS00097126	BS00023046
Genotypes	Chromosome	5A	5A	1B	2B	2D	4D
	Allele X	g	g	a	g	t	g
	Allele Y	c	t	t	a	c	a
	Autonomia	XX	YY	YY	XY	XX	XX
	Gentil Rosso	XX	XX	XX	XY	XX	YY
	Inallettabile	XX	YY	XX	XX	XX	XX
	Leone Aristato	XY	XX	XX	XX	XX	XX
	Mentana	XX	XX	YY	XX	XX	XX
	Poulard di Ciano	YY	XY	YY	XY	No Call	No Call
	Risciola	XX	XX	XX	XX	XX	XX
	Terminillo	XY	YY	YY	XX	XX	XX
	Bolero	YY	YY	XX	XY	XX	YY
	Blasco	XX	YY	YY	XY	XX	XX

682 **Table 2**

683 List of the retention times (t_R), time windows, molecular weights (MW), parent and product ions obtained after MS parameters optimization and
 684 main MS parameters optimized for each compound.

685

Analyte	t_R (min)	Time window (initial min)	MW (g/mol)	Parent ion (m/z)	Product ions		Dwell times		FV ^c (V)	CE ^d		qi/QI ^e
					QI ^a (m/z)	qi ^b (m/z)	QI (msec)	qi (msec)		QI (V)	qi (V)	
Gallic acid	1.89	1 (1.50)	170.12	171.0 [M + H] ⁺	153.1	81.1	600	350	89	6	26	94.8
(+)-Catechin	5.38	2 (3.00)	290.27	291.1 [M + H] ⁺	139.1	123.1	150	50	98	10	30	48.6
Vanillic acid	5.80	2	168.14	169.0 [M + H] ⁺	65.1	93.1	150	50	83	26	10	95.5
Caffeic acid	6.30	2	180.16	181.0 [M + H] ⁺	163.1	89.1	150	50	71	6	34	58.0
Syringic acid	6.51	2	198.17	199.1 [M + H] ⁺	140.1	155.1	150	50	95	14	6	61.5
Isovanillin	8.97	3 (7.40)	152.15	153.1 [M + H] ⁺	65.1	93.1	200	70	92	26	10	83.8
Vanillin	9.36	3	152.15	153.1 [M + H] ⁺	65.1	93.1	200	70	83	26	14	79.1
<i>p</i> -Coumaric acid	10.35	3	164.16	165.06 [M + H] ⁺	147.1	91.1	200	70	86	6	26	62.1
<i>trans</i> -Ferulic acid	12.76	4 (11.60)	194.18	195.1 [M + H] ⁺	177.1	89.1	200	70	95	6	34	55.1
Sinapic acid	13.06	4	224.21	225.1 [M + H] ⁺	207.2	91.1	200	70	80	2	26	42.4
Orientin	13.22	4	448.38	449.1 [M + H] ⁺	329.2	299.2	200	70	146	30	40	80.5
Salicylic acid	15.24	5 (13.90)	138.12	139.0	121.1	65.1	200	70	71	10	26	50.1

				[M + H] ⁺ 433.1								
Isovitexin	15.45	5	432.38	[M + H] ⁺ 433.1	313.1	283.1	200	70	122	26	26	81.1
Vitexin	15.75	5	432.38	[M + H] ⁺ 433.1	313.1	283.2	200	70	150	30	26	81.1
Daidzein	16.82	6 (16.50)	254.23	[M + H] ⁺ 255.1	91.1	199.1	150	50	145	40	26	92.1
<i>trans</i> -Cinnamic acid	16.99	6	148.16	[M + H] ⁺ 149.1	103.1	131.1	150	50	77	18	6	95.9
Apigenin	17.10	6	270.24	[M + H] ⁺ 271.1	153.0	91.1	150	50	155	34	40	45.8
Formononetin	17.38	6	268,26	[M + H] ⁺ 269.1	197.1	253.2	150	50	149	40	30	57.0

^aQI: quantifier ion. ^bqi: qualifier ion. FV^c: Fragmentor voltage. CE^d: Collision energy. qi/QI^e: qualifier ion SRM to quantifier ion SRM percentage ratio.

691 **Table 3**692 **Recovery tests.** Sum of the first three cycles of extraction (%) carried out through spectrophotometric reads at different wavelengths and through

693 LC-MS/MS injections of the same samples.

694 **Method trueness.** Comparison of recoveries (%) of each analyte obtained from samples spiked with two fortification levels of pure standards.

695 All the tests were repeated three times using samples of Mentana (2.00 g) in the same conditions.

696

697

Recovery test				Method trueness			
UV reads		LC-MS/MS		LC-MS/MS			
Wavelength (nm)	Sum of the first 3 cycles of extraction (%)	Analyte	Sum of the first 3 cycles of extraction (%)	Spiked concentration (µg/L)	Recovery (%)	Spiked concentration (µg/L)	Recovery (%)
280	95	Gallic acid	99	172	105	689	98
290	94	(+)-Catechin	n.d. ^a	13	65	51	61
326	93	Vanillic acid	98	715	93	2860	107
446	100	Caffeic acid	n.d.	26	93	103	90
474	100	Syringic acid	99	345	104	1379	99
		Isovanillin	n.d.	25	79	99	66
		Vanillin	82	204	88	817	67
		<i>p</i> -Coumaric acid	91	86	84	345	81
		<i>trans</i> -Ferulic acid	99	1280	95	5120	90
		Sinapic acid	99	345	95	1378	98
		Orientin	100	13	87	50	89
		Salicylic acid	100	116	110	463	108

Isovitexin	100	13	99	50	90
Vitexin	100	12	97	48	91
Daidzein	100	12	108	47	91
<i>trans</i> -Cinnamic acid	100	31	90	125	93
Apigenin	100	13	99	51	80
Formononetin	100	6	101	22	98

698
699 ^an.d.: not detected.
700

701

702 **Table 4**

703 Main parameters of method validation for standard compounds. Evaluation of linearity (R^2) for each range of concentration, straight line equations,
 704 limits of detection (LOD), lower limits of quantification (LLOQ), precision (relative standard deviation, RSD), in a real sample and in a standard
 705 mixture are reported.

706

Analyte	Range of concentration ($\mu\text{g/kg}$)	Linearity		Detectability		Precision (RSD %)	
		R^2	Equations	LOD ($\mu\text{g/kg}$)	LLOQ ($\mu\text{g/kg}$)	Intra-day on a mix of standard ($n=5$)	Inter-day on a real sample (Mentana) ($n=5$)
Gallic acid	2.06÷492.00	0.995	$y = 103.41 x - 1718.00$	4.1 (20.7)	13.8 (69.0)	9.1	3.6
(+)-Catechin	2.13÷508.00	0.999	$y = 351.65 x - 1863.72$	0.25 (1.3)	0.85 (4.2)	2.6	<LOD
Vanillic acid	3.00÷715.00	0.999	$y = 426.75 x - 1554.03$	2.2 (11.1)	7.4 (37.1)	3.3	3.3
Caffeic acid	2.16÷515.50	0.999	$y = 323.83 x - 2757.26$	6.4 (31.8)	21.2 (106.0)	3.1	<LOD
Syringic acid	2.06÷492.50	0.999	$y = 579.43 x - 2263.85$	0.89 (4.5)	3.0 (14.9)	2.5	3.4
Isovanillin	2.07÷494.00	0.999	$y = 3802.64 x - 13338.11$	0.29 (1.5)	1.0 (4.9)	3.7	n.d.
Vanillin	2.14÷510.35	0.999	$y = 3725.13 x - 17591.79$	0.30 (1.5)	1.0 (5.0)	4.2	0.6
<i>p</i> -Coumaric acid	2.06÷493.00	0.999	$y = 550.26 x - 401.23$	5.5 (27.5)	18.3 (91.7)	3.5	2.3
<i>trans</i> -Ferulic acid	4.15÷990.00	0.998	$y = 1058.46 x - 16444.28$	0.51 (2.6)	1.7 (8.6)	3.1	2.1

Sinapic acid	8.88÷2120.00	0.995	$y = 728.08 x - 22693.25$	1.4 (7.0)	4.7 (23.3)	6.3	6.3
Orientin	0.42÷100.00	0.996	$y = 302.51 x - 5.45$	0.21 (1.1)	0.71 (3.6)	2.4	<LOD
Salicylic acid	3.87÷925.00	0.996	$y = 548.10 x + 275.19$	3.6 (18.2)	12.1 (60.6)	2.2	10.1
Isovitexin	0.42÷100.00	0.999	$y = 214.38 x - 73.69$	0.37 (1.9)	1.2 (6.2)	0.8	8.0
Vitexin	2.00÷475.00	0.998	$y = 208.11 x - 645.64$	0.32 (1.6)	1.1 (5.4)	2.0	<LLOQ
Daidzein	1.95÷465.00	0.995	$y = 665.91 x - 342.83$	0.12 (0.62)	0.41 (2.1)	12.5	<LLOQ
<i>trans</i> -Cinnamic acid	2.08÷498.00	0.999	$y = 390.64 x - 745.45$	0.64 (3.2)	2.1 (10.6)	3.5	3.2
Apigenin	2.14÷510.00	0.997	$y = 558.59 x - 21.96$	0.19 (0.97)	0.65 (3.2)	1.4	8.9
Formononetin	0.18÷44.00	0.991	$y = 297.77 x + 345.33$	0.42 (2.1)	1.4 (7.0)	7.9	<LOD

707
708

709 **Table 5**710 Concentrations of the phenolic substances ($\mu\text{g/kg}$ FW) in the real samples (modern varieties are reported in italics).

711

		Gallic acid		(+) -Catechin		Vanillic acid	
ANOVA ^a		F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
		53.5*	0.000			9.9*	0.000
Accession number	Variety	Mean	Standard deviation (\pm)	Mean	Standard deviation (\pm)	Mean	Standard deviation (\pm)
1	Mentana	358.61 f	34.61	<LOD ^b		1929.18 c	128.00
10	<i>Bolero</i>	209.56 bc	3.79	<LOD		1464.17 abc	33.04
19	<i>Blasco</i>	167.55 ab	5.46	<LOD		1526.02 bc	91.99
23	Autonomia	148.74 a	23.67	<LOD		1275.60 ab	86.51
24	Inallettibile	148.07 a	8.92	<LOD		908.09 a	44.61
145	Poulard di Ciano	206.05 bc	19.92	<LOD		1487.58 bc	82.67
146	Terminillo	266.43 de	6.18	<LOD		1993.10 c	410.94
148	Leone aristato	227.44 cd	6.40	<LOD		1840.14 c	120.62
149	Risciola	298.61 e	13.76	<LOD		1728.52 bc	371.99
150	Gentil rosso	188.67 abc	7.72	<LOD		2000.77 c	101.41
		Caffeic acid		Syringic acid		Isovanillin	
ANOVA		F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
				23.6*	0.000		
Accession number	Variety	Mean	Standard deviation (\pm)	Mean	Standard deviation (\pm)	Mean	Standard deviation (\pm)
1	Mentana	<LOD		725.05 e	64.34	n.d. ^d	

10	Bolero	<LOD		426.36 abc	14.05	n.d.	
19	Blasco	<LOD		381.79 abc	17.40	n.d.	
23	Autonomia	<LOD		429.43 abc	25.07	n.d.	
24	Inallettibile	<LOD		272.42a	8.00	n.d.	
145	Poulard di Ciano	<LOD		296.71 ab	26.66	n.d.	
146	Terminillo	<LOD		498.10 cd	72.88	n.d.	
148	Leone aristato	<LOD		628.21 de	43.08	n.d.	
149	Risciola	<LOD		666.26 e	112.05	n.d.	
150	Gentil rosso	<LOD		447.28 bc	61.84	n.d.	
		Vanillin		<i>p</i> -Coumaric acid		<i>trans</i> -Ferulic acid	
ANOVA		F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
		7.6*	0.000	90.2*	0.000	11.1*	0.000
Accession number	Variety	Mean	Standard deviation (±)	Mean	Standard deviation (±)	Mean	Standard deviation (±)
1	Mentana	407.17 a	30.17	173.52 a	25.57	2469.24 cd	307.16
10	Bolero	851.56 d	96.35	184.47 a	32.11	1899.97 abc	172.44
19	Blasco	620.37 abcd	39.61	132.17 a	8.92	1184.18 a	23.02
23	Autonomia	433.74 ab	22.76	326.60 b	48.03	1557.59 ab	315.60
24	Inallettibile	669.96 bcd	16.59	115.90 a	9.04	1192.14 a	106.05
145	Poulard di Ciano	557.49 abc	11.75	642.82 d	42.10	2241.81 bcd	78.60
146	Terminillo	579.22 abc	84.64	379.35 bc	24.62	2883.20 d	551.52
148	Leone aristato	477.30 abc	54.40	434.11 c	4.95	2178.10b cd	103.66
149	Risciola	697.15 cd	142.77	388.72 bc	47.61	2658.46 cd	594.10
150	Gentil rosso	578.14 abc	163.32	335.98 b	10.42	2277.36 bcd	134.09
		Sinapic acid		Orientin		Salicylic acid	

ANOVA		F	p	F	p	F	p
		55.6*	0.000			169.7*	0.000
Accession number	Variety	Mean	Standard deviation (±)	Mean	Standard deviation (±)	Mean	Standard deviation (±)
1	Mentana	671.16 b	151.45	<LOD		126.73 b	12.02
10	Bolero	227.36 a	4.82	<LOD		<LLOQ a	
19	Blasco	164.11 a	14.01	<LOD		<LLOQ a	
23	Autonomia	344.22 a	109.70	<LOD		<LLOQ a	
24	Inallettibile	201.44 a	7.27	<LOD		<LLOQ a	
145	Poulard di Ciano	1343.47 c	94.16	<LLOQ ^c		183.88 d	7.95
146	Terminillo	604.42 b	19.16	<LLOQ		234.12 e	13.17
148	Leone aristato	726.81 b	74.31	<LLOQ		155.42 c	3.69
149	Risciola	677.67 b	110.04	<LLOQ		186.91 d	20.44
150	Gentil rosso	631.95 b	70.34	<LLOQ		222.52 e	8.69
		Isovitexin		Vitexin		Daidzein	
ANOVA		F	p	F	p	F	p
		13.8*	0.000	23.7*	0.000	20.2*	0.000
Accession number	Variety	Mean	Standard deviation (±)	Mean	Standard deviation (±)	Mean	Standard deviation (±)
1	Mentana	17.16 ab	3.07	21.95 bc	1.15	<LLOQ a	
10	Bolero	<LLOQ a		<LOD a		2.29 b	0.16
19	Blasco	<LLOQ a		17.13 b	1.76	2.23 b	0.51
23	Autonomia	9.76 b	0.37	22.28 bc	1.92	<LLOQ a	
24	Inallettibile	<LLOQ a		18.53 b	1.02	<LLOQ a	
145	Poulard di Ciano	30.01 c	2.43	34.32 d	1.65	4.53 c	0.38
146	Terminillo	10.45 b	2.42	24.36 bc	1.25	4.65 c	0.24

148	Leone aristato	11.48 b	0.35	22.42 bc	0.17	4.32 c	0.38
149	Risciola	13.08 b	1.83	24.94 bc	0.62	4.43 c	0.71
150	Gentil rosso	27.53 bc	11.72	27.43 cd	7.49	4.09 c	0.98
		<i>trans</i> -Cinnamic acid		Apigenin		Formononetin	
ANOVA		F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
		15.9*	0.000	23.9*	0.000		
Accession number	Variety	Mean	Standard deviation (±)	Mean	Standard deviation (±)	Mean	Standard deviation (±)
1	Mentana	57.45 bc	12.98	10.33 a	1.25	<LOD a	
10	Bolero	58.78 bc	4.57	6.42 a	0.82	<LOD a	
19	Blasco	42.68 ab	12.24	7.16 a	0.82	<LOD a	
23	Autonomia	49.92 bc	14.23	16.07 ab	0.37	8.30 b	0.47
24	Inallettabile	20.51 a	4.72	45.85 cd	4.31	<LOD a	
145	Poulard di Ciano	60.92 bcd	5.04	7.43 a	0.65	<LOD a	
146	Terminillo	83.07 d	6.51	45.32 cd	7.21	<LOD a	
148	Leone aristato	69.16 cd	0.45	21.20 ab	6.66	<LOD a	
149	Risciola	72.52 cd	1.93	46.97 d	11.10	<LOD a	
150	Gentil rosso	84.55 d	8.03	29.06 bc	11.07	<LOD a	
		Hydroxybenzoic acids sum		Hydroxycinnamic acids sum		Flavones and isoflavones sum	
ANOVA		F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
		16.7*	0.000	20.9*	0.000	40.4*	0.000
Accession number	Variety	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation

			(±)		(±)		(±)
1	Mentana	3139.58 d	347.45	3371.37 cd	474.00	49.45 b	4.78
10	<i>Bolero</i>	2100.09 b	44.83	2370.59 abc	206.11	8.70 a	0.73
19	<i>Blasco</i>	2075.37 b	107.43	1523.15 a	39.38	26.52 a	0.99
23	Autonomia	1853.78 ab	128.73	2278.34 ab	458.87	56.41 bc	2.54
24	Inallettibile	1328.57 a	52.26	1529.99 a	119.91	64.38 bcd	3.50
145	Poulard di Ciano	2174.22 bc	117.27	4289.02 d	184.63	76.30 cde	3.59
146	Terminillo	2991.75 d	501.61	3950.03 d	593.57	84.78 de	8.49
148	Leone aristato	2851.20 cd	145.97	3408.19 cd	71.43	59.42 bc	6.17
149	Risciola	2880.31 cd	515.28	3797.38 d	704.97	87.43 e	12.87
150	Gentil rosso	2859.24 cd	64.53	3329.83 bcd	182.38	88.11 e	13.76
		Total sum		HC/HB			
ANOVA		F	p	F	p		
		12.7*	0.000	38.5*	0.000		
Accession number	Variety	Mean	Standard deviation (±)	Mean	Standard deviation (±)		
1	Mentana	6967.56 bc	729.11	1.07 b	0.07		
10	<i>Bolero</i>	5330.94 ab	213.46	1.13 bc	0.12		
19	<i>Blasco</i>	4245.41 a	184.79	0.73 a	0.02		
23	Autonomia	4622.26 a	585.08	1.23 bc	0.20		
24	Inallettibile	3592.90 a	172.41	1.15 bc	0.08		
145	Poulard di Ciano	7097.03 bc	295.78	1.97 d	0.03		
146	Terminillo	7605.78 c	1171.90	1.32 c	0.05		
148	Leone aristato	6796.12 bc	254.76	1.20 bc	0.04		
149	Risciola	7462.27 c	1347.99	1.32bc	0.04		
150	Gentil rosso	6855.34 bc	407.21	1.16 bc	0.04		

712 ^aResults of the one-way ANOVA and the Tukey's test are reported as F_{values} and lower case letters, respectively. Different letters identify samples
713 significantly different ($p \leq 0.05$). * = $p \leq 0.001$. ^bLOD: limit of detection. ^cLLOQ: lower limit of quantification. ^dn.d.: not detected.

714

715 **Table 6**

716 Total polyphenols content determined by Folin-Ciocalteu assay (three replicates) and expressed as mg of gallic acid equivalents (GAE)/g FW.

717 Modern varieties are reported in italics.

718

719

Accession number	Variety	Mean (mg/g GAE)	Standard deviation (\pm)	ANOVA ^a (F_{values})
				7.0 ($p \leq 0.001$)
1	Mentana	0.68 ^{abc}	0.08	
10	<i>Bolero</i>	0.53 ^a	0.09	
19	<i>Blasco</i>	0.53 ^a	0.05	
23	Autonomia	0.52 ^a	0.04	
24	Inallettibile	0.46 ^a	0.08	
145	Poulard di Ciano	0.95 ^c	0.15	
148	Leone aristato	0.70 ^{abc}	0.18	
146	Terminillo	0.70 ^{abc}	0.06	
149	Risciola	0.88 ^{bc}	0.14	
150	Gentil rosso	0.60 ^{ab}	0.08	

720

721 ^aResults of the one-way ANOVA and the Tukey's test are reported as F_{values} and lower case letters, respectively. Different letters identify samples722 significantly different ($p \leq 0.05$).

723

Table 7

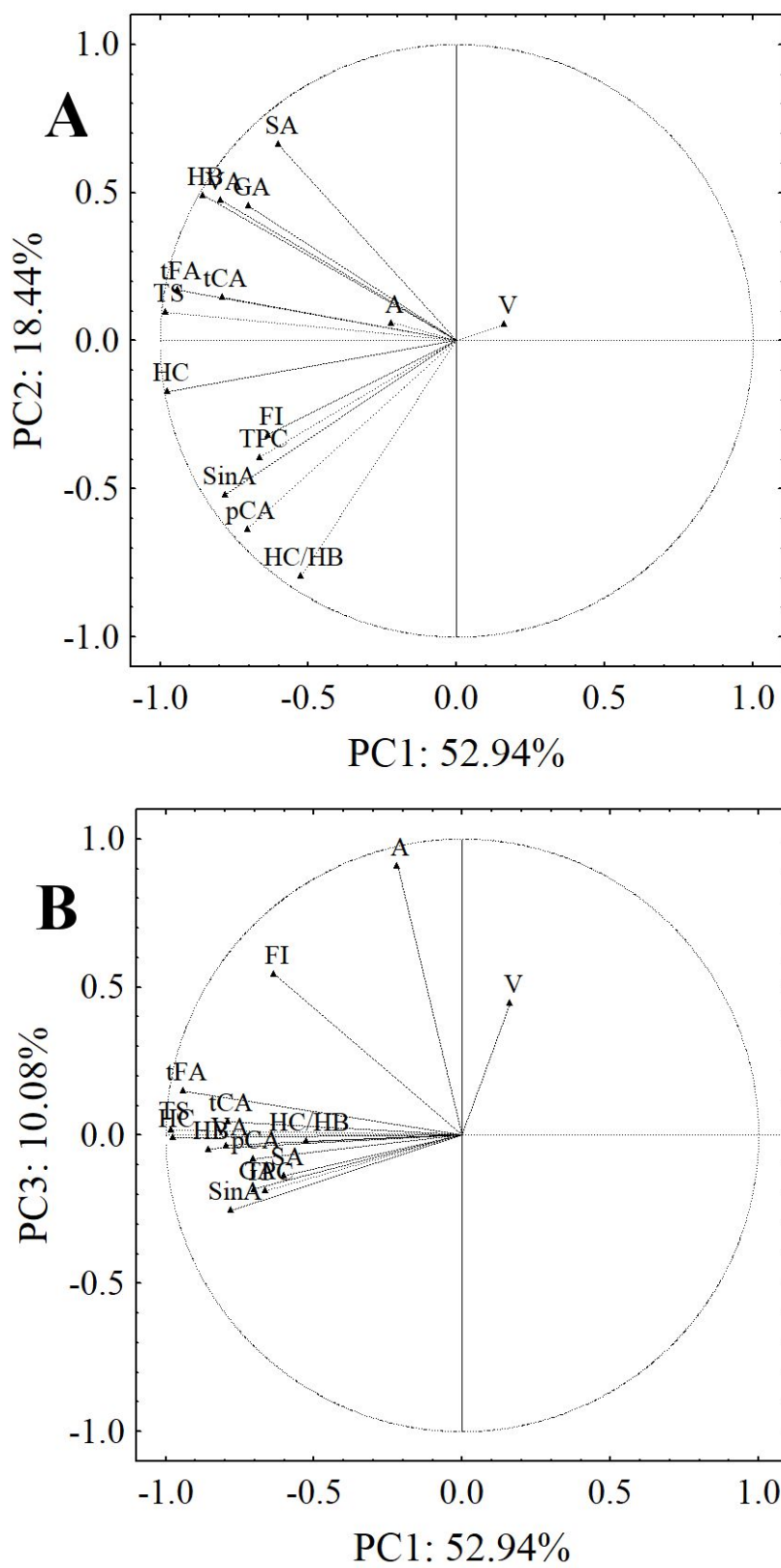
Significant correlation matrix of the main parameters data set^a.

	^b TPC	^c GA	^d VA	^e SA	^f V	^g pCA	^h tFA	ⁱ SinA	^j tCA	^k A	^l HB	^m HC	ⁿ FI	^o TS	^p HC/HB
TPC															
GA	0.44														
VA		<u>0.62</u>													
SA		<u>0.81</u>	<u>0.69</u>												
V															
pCA	<u>0.69</u>														
tFA	<u>0.47</u>	<u>0.74</u>	<u>0.82</u>	<u>0.63</u>		0.53									
SinA	<u>0.73</u>	0.39	0.38			<u>0.87</u>	<u>0.59</u>								
tCA	0.38	0.44	<u>0.78</u>	0.44		<u>0.52</u>	<u>0.77</u>	0.46							
A															
HB	0.37	<u>0.78</u>	<u>0.97</u>	<u>0.82</u>			<u>0.87</u>	0.43	<u>0.76</u>						
HC	<u>0.66</u>	<u>0.61</u>	<u>0.70</u>	0.46		<u>0.80</u>	<u>0.92</u>	<u>0.85</u>	<u>0.73</u>		<u>0.75</u>				
FI	0.45					<u>0.58</u>	<u>0.56</u>	<u>0.55</u>	0.43	<u>0.67</u>	0.39	<u>0.63</u>			
TS	<u>0.57</u>	<u>0.72</u>	<u>0.85</u>	<u>0.62</u>		<u>0.64</u>	<u>0.97</u>	<u>0.72</u>	<u>0.79</u>		<u>0.89</u>	<u>0.96</u>	0.55		
HC/HB	<u>0.59</u>					<u>0.85</u>	0.42	<u>0.82</u>				<u>0.68</u>	0.50	0.46	

^a $p \leq 0.05$; bold $p \leq 0.01$; bold and underlined $p \leq 0.001$. ^bTPC: total polyphenols content. ^cGA: gallic acid. ^dVA: vanillic acid. ^eSA: syringic acid. ^fV: vanillin. ^gpCA: *p*-coumaric acid. ^htFA: *trans*-ferulic acid. ⁱSinA: sinapic acid. ^jtCA: *trans*-cinnamic acid. ^kA: apigenin. ^lHB: hydroxybenzoic acids sum. ^mHC: hydroxycinnamic acids sum. ⁿFI: flavones and isoflavones sum. ^oTS: total sum. ^pHC/HB: hydroxycinnamic acids sum to hydroxybenzoic acids sum ratio.

733 **Figure 1.**

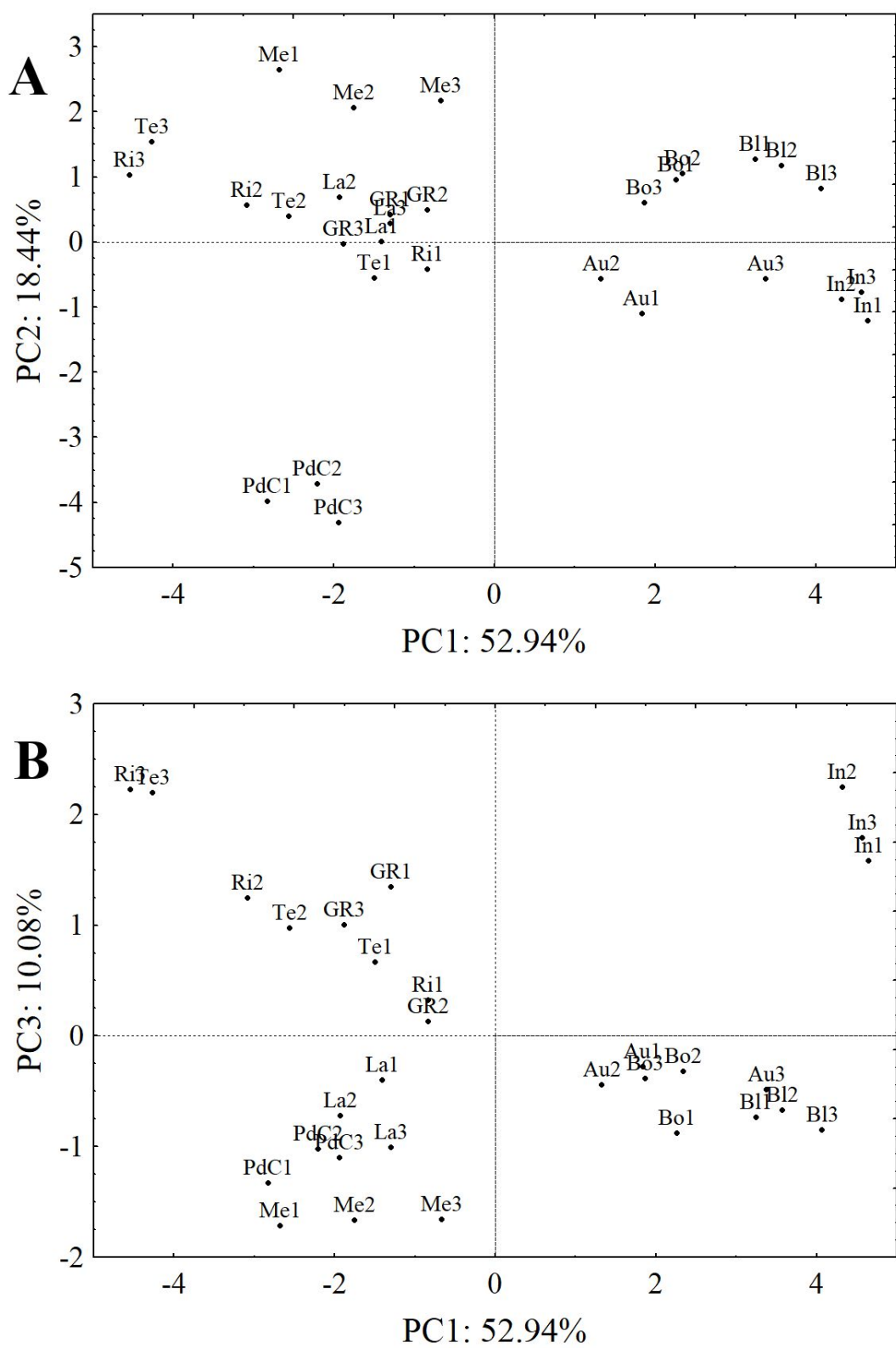
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736

737 **Figure 2.**



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741 **For Table of Contents Only**

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