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Differentiation of modern and ancient varieties of common wheat by quantitative capillary electrophoretic profile of phenolic acids

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ABSTRACT

Phenolic compounds have received great attention among the health promoting phytochemicals in common wheat (*Triticum aestivum* L.), mainly because of their strong antioxidant properties. In the present study a simple Capillary Zone Electrophoresis (CZE) method with UV detection was optimized and validated for the quantitation of six of the most important phenolic acids in whole grain *i.e.,* sinapic, ferulic, syringic, p-coumaric, vanillic and p-hydroxybenzoic acid. The separation was achieved in a running buffer composed of sodium phosphate solution (50 mM) in water/methanol 80:20 (v/v) at pH 6.0 and using a fused-silica capillary at the temperature of 30 °C under application of 27 kV. By means of diode array detector, and made possible by the favorable characteristic UV spectra, the quantitation of the solutes was carried out at 200, 220 and 300 nm, in the complex matrices represented by the soluble and bound fractions of wheat flours. The validation parameters of the method *i.e.,* linearity, sensitivity, precision, accuracy and robustness were in line with those obtained by consolidated separation techniques applied for the same purposes (*e.g.,* HPLC-UV), with a significant advantage in term of analysis time (less than 12 min). Ten varieties of soft wheat (five modern Italian and five old Italian genotypes) were analysed and the data were subjected to Principal Components Analysis (PCA). Interestingly, significant differences of the quantitative phenolic acids profile were observed between the modern and the ancient genotypes, with the latter showing higher amount of the main represented phenolic acids.

1. Introduction

Triticum aestivum L. is one of the major crop worldwide and mainly in the Mediterranean-type temperate zones, it is used for production of staple food. Consumption of whole grain food has been consistently associated with reduced risk of developing chronic diseases, including cardiovascular disease, type II diabetes, obesity, cancer. The health benefit is attributed to the unique phytochemical profile which includes phenolics, carotenoids, vitamin E, lignans, β-glucan, inulin, resistant starch, sterols, and phytates [1]. In particular, the disease prevention by daily intake of whole grain food has been linked to the antioxidant properties of phenolic compounds. According to the constitutive phenolic skeleton, natural phenolic compounds are classified as: phenols (phenolic acids and coumarins); quinones and naphtoquinones; flavonoids (flavones, isoflavones, flavanones, flavonols, flavanols, anthocyanins). The most common phenolic compounds in whole grain belong to the classes of phenolic acids (Fig. 1) and flavonoids [1–5]. The amount of these bioactive components is reported to be influenced by types, varieties, and part of the grain sampled [3,5–7]. The majority of phenolic acids are bound as esters to arabinoxylan side chains of hemicellulose of cell wall, constituting the insoluble fraction which still maintains good free radical scavengers activity. On the other hand, the cell vacuoles contain the soluble fraction composed of free phenolic acids (the minor portion) and those esterified to sugars and other low molecular mass compounds [1,5]. Several investigations have been focused on the effects of environment and genotype on phenolic acids profile/content in wheat [8–12] and wheat derived products [13] by quantification in reversed phase HPLC using gradient elution and detection by either UV and ESI-MS. In previous studies, the phytochemical profile of modern and ancient varieties of Italian durum wheat cultivars was assessed using liquid chromatography coupled with time-of-flight mass spectrometry [14,15] and capillary electrophoresis-mass spectrometry CE-MS [16]. Remarkable differences in the qualitative polyphenols profiles of the selected varieties were found; in particular, the ancient wheat samples showed a unique composition with a higher mean number of bioactive compounds. The reported investigations however were not focused on quantitation, which instead is an aspect of paramount importance not only because of the beneficial effects on human health of polyphenols but also for their essential functions in reproduction and growth of wheat as well as for the activity as defense native chemicals against pathogens, parasites and predators [17,18]. Cereal varieties developed in the recent years have been oriented to promote crop yields, height, timing of crop maturation, proteins content, technological characteristics etc..

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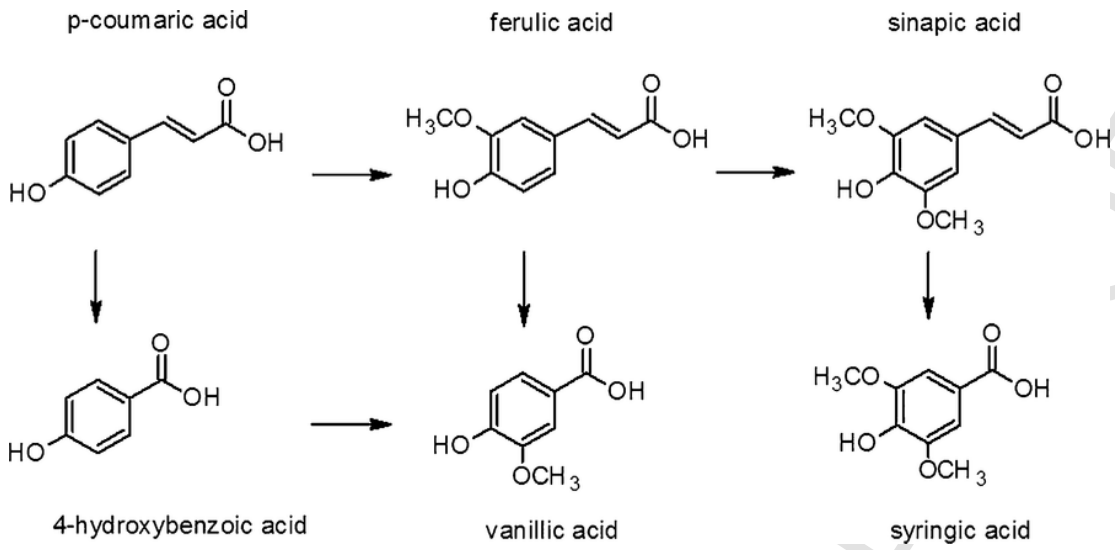


Fig. 1. Chemical structures and biochemical pathway relationships of main phenolic acids in plants.

This selection has led to changes not only in the composition of primary metabolism (as expected) but also in the quali- quantitative profile of secondary metabolites. Establishing the amount of the individual phenolic acids in ancient and modern wheat varieties can be considered of importance for their characterization addressed to selection and production of cereals with high levels of health-promoting compounds. Accordingly, a compositional approach was for the first time undertaken by Capillary Zone Electrophoresis (CZE), to quantify the six most important phenolic acids *i.e.,* ferulic, sinapic, p-coumaric, vanillic, syringic and p-hydroxybenzoic in five ancient and five modern Italian wheat varieties. Among the separation techniques suitable for quantitation of phenolic acids, CZE is a very convenient approach because of the ionic character and native UV absorption of these solutes [19,20]. The most recent applications of electrokinetic techniques in analysis of phenolic acids in food samples include their determination in tomato by micellar electrokinetic chromatography (MEKC) [21], in fruit juices by MEKC in sweeping injection mode [22], in propolis [23], in *Salvia* species [24] and in other various food matrices, by alkaline CZE conditions [25].

In the present study, CZE was selected for the fast quantitative analysis of the considered phenolic acids focusing on both the soluble and bound fractions obtained by extraction of the flour from wheat. Principal components analysis (PCA) was thus carried out with the aim to determine relationships among investigated wheat samples. Interestingly, significantly different quantitative phenolic acids profiles were found for the modern and the ancient cultivars, with the latter showing higher amount of these bioactive metabolites.

2. Materials and methods

2.1. Chemicals

Ferulic acid, p-coumaric acid, p-hydroxybenzoic acid, sinapic acid, syringic acid, vanillic acid, 4-bromomandelic acid (used as internal standard), sodium phosphate monobasic dihydrate, methanol (HPLC grade), ethanol (96%), ethyl acetate, hydrochloric acid and sodium hydroxide, were from Sigma-Aldrich (Milan, Italy). Deionized water was obtained by a MilliQ apparatus (Millipore, Milford, MA, USA).

2.2. Solutions

Sodium phosphate buffer used as the background electrolyte (BGE) for capillary electrophoresis separations was prepared at the concentration of 50 mM in a mixture of water:methanol (80:20, v/v) by adjusting the pH value at 6.0 using sodium hydroxide solution (1M). Standard stock solutions of the analytes and internal standard were prepared for calibration and quantitation by dissolving the standard compounds in a mixture water:methanol (20:80, v/v); the concentration of sinapic, p-coumaric, vanillic, syringic and p-hydroxybenzoic acid was 0.12 mg/mL, whereas ferulic acid solution was prepared at the concentration of 2.00 mg/mL. A solution of 4-bromomandelic acid at the concentration of 0.5 mg/mL was used as the internal standard. Calibration and working solutions were prepared by properly diluting the stock solutions in water:methanol (20:80, v/v) in the ranges reported in Table 1.

2.3. Wheat samples

Wheat samples consisted of five ancient (Andriolo, Frassineto, Gentilrosso, Inallettabile, Verna) and five modern cultivars (Bolero, Eureka, Mieti, Nobel, Palesio) of Italian common wheat (*Triticum aestivum* L.). Grains were cultivated in the same Italian location (Argelato, latitude 44°39′57″N, longitude 11°19′43″E, 25 m a.s.l.) during the growing season 2014–2015 according to organic agro-techniques.

Whole wheat grains of each variety were ground to flour in a stone mill and the whole flours kept at −20 °C until analysis.

2.4. Phenolic acids extraction

Extraction of phenolic acids was performed following the scheme of Fig. 2, according to previously described methods [9,14,15] with some modifications: an aliquot of 250 mg of whole wheat flour was extracted with 2.5 mL of 80% chilled ethanol spiked with 10 µL of the internal standard solution; the mixture was sonicated at room temperature for 10 min. After centrifugation at 5000 × *g* for 15 min, the supernatant was removed and extraction was repeated once. The combined supernatants were evaporated before the hydrolysis that was carried out with 0.4 mL of NaOH 2M for 4 h. After the acidi-

Table 1
Linearity, sensitivity and system precision (n = 6).

Phenolic acids	Range µg/mL	Equation	r^2	[b]LOQ (RSD%) µg/mL	[b]LOD µg/mL	t_m, (R
Sinapic acid	10–300	[a]$y = 21.977x + 0.0119$	0.998	1.1 (5.6)	0.4	5.9 (1
Ferulic acid	20–1200	$y = 18.004x + 0.0009$	0.998	1.1 (4.8)	0.4	6.7 (2
Syringic acid	3–120	$y = 20.46x + 0.0084$	0.998	0.9 (7.0)	0.3	7.1 (2
Vanillic acid	3-120	$y = 15.589x - 0.0045$	0.998	1.1 (3.4)	0.4	7.9 (2
p-Coumaric acid	1–120	$y = 24.388x - 0.0091$	0.998	0.9 (7.0)	0.3	8.4 (2
p-Hydroxybenzoic acid	1–100	$y = 53.661x - 0.0055$	0.999	0.8 (5.4)	0.3	10 (2

[a] The response *y,* is the peak area ratio (area/migration time) of the analytes versus that of internal standard.

[b] Detection was carried out at 220 nm (sinapic, syringic, vanillic acids and the internal standard); at 300 nm (ferulic and p-coumaric acid); at 200 nm for p-hydroxybenzoic acid.

fication to pH 2 using 80 µL of HCl 12M, the free and conjugated fractions of phenolic acids were extracted twice with 0.5 mL of ethyl acetate. Supernatants were combined, evaporated to dryness and dissolved in 50 µL of water:methanol (20:80, v/v) for the analysis by CE.

The residue from the free phenolic acids extraction was subjected to alkaline and acid hydrolysis to recover the bound phenolic acids as reported by Mattila et al. [26] with some modifications. Briefly, 3 mL of deionized water spiked with 50 µL of the internal standard solution and 1.5 mL of NaOH 10M, were added to the residue from free phenolic acids extraction (obtained as described above) and stirred overnight at room temperature (about 16 h). The solution was then adjusted to pH 2 by adding HCl 6M; after centrifugation, the supernatant was extracted three times with 5 mL of ethyl acetate and centrifuged. The extracts were pooled, whereas the residue was subjected to acid hydrolysis by addition of 0.8 mL of HCl 6M and 2.5 mL of distilled water. The mixture was incubated in a water bath (85 °C) for 30 min and the sample was allowed to cool to room temperature before extraction with 5 mL of ethyl acetate three times. After centrifugation, the ethyl acetate layers were combined with those from alkaline hydrolysis and evaporated to dryness, and reconstituted in 0.250 mL of water:methanol (20:80, v/v) for the analysis by CE.

2.5. Capillary electrophoresis

Electrophoretic experiments were performed by a HP^{3D}CE instrument from Agilent Technologies (Waldbronn, Germany). The data were collected on a personal computer, equipped with the software HP^{3D}CE Chemstation version A 09 (Agilent Technologies). The separations were obtained at a constant voltage of 27 kV and the cartridge temperature was 30 °C. Hydrodynamic injections were per-

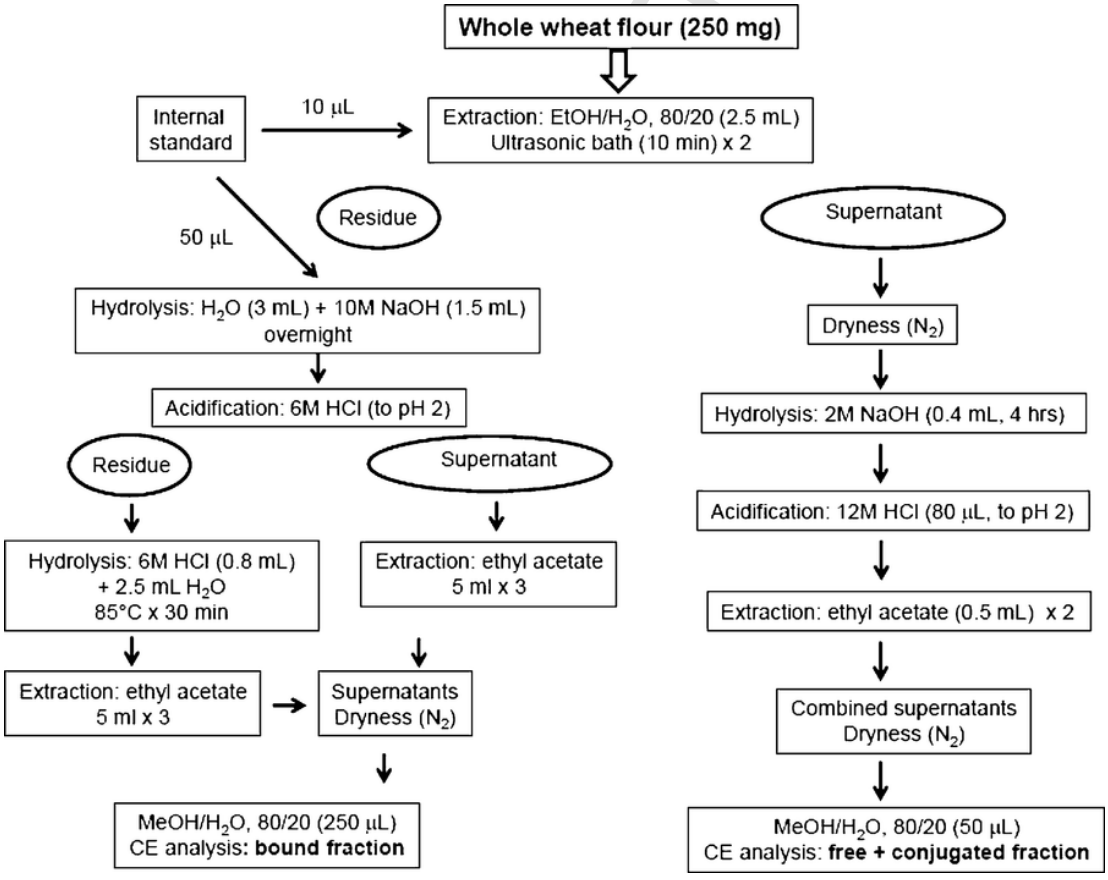


Fig. 2. Scheme of the sample preparation method applied for phenolic acids extraction from wheat.

formed at 50 mbar for 5 s. The detection was carried out by using the on-line DAD detector for the selective monitoring of the analytes; in details, the quantitation of p-hydroxybenzoic acid and p-coumaric acid was performed at the wavelength of 200 nm and 300 nm, respectively; the quantitation of the other phenolic acids (sinapic acid, vanillic acid, syringic acid and ferulic acid) was performed at 220 nm. Fused-silica capillaries (50 µm i.d., 38.5 cm total length, 30 cm length to the detector) were from Composite Metal Service (Ilkley, UK). New capillaries were conditioned by flushing sequentially NaOH 1M and 0.1M for 10 min each, at 50 °C; a final rinse using deionized water at 30 °C was carried out for 30 min. Between the injections the capillary was rinsed with water and running buffer for 3 min each.

2.6. Statistical analysis

Analysis of sample solutions both in validation experiments and quantitation of wheat extracts were performed on three replicates. Values are represented as means ± SD and values of $p < 0.05$ were considered to be statistically significant. Statistical analyses were performed using STATISTICA Software v. 7.1 (StatSoft, Tulsa, Oklahoma, USA). Levels of statistical significance were evaluated using one-way analysis of variance (ANOVA) followed by Fisher's LSD post test.

3. Results and discussion

3.1. Optimization of CE conditions

Owing to the native absorption spectra as well as for the anionic character, phenolic acids can be conveniently analysed by CZE using UV detection. However, our previous study on the separation of a complex mixture of phenolic acids, showed that CZE conditions using tetraborate buffer at pH 9.2 did not allow separation of the couple of adjacent peaks represented by ferulic and syringic acid; even in the presence of different surfactants (sodium dodecyl sulfate and bile acids salts) in MEKC mode, only a limited separation was achieved [27]. In the present application the described issue is once more complicated because of the mismatch between the amounts of syringic and ferulic acid, being the latter the most abundant compound in the bound fraction of wheat, accounting for up to the 90% of the total phenolic acids content [1,8–13]. Following a simple step-by-step optimization, it was found that a 20 mM solution of sodium phosphate allowed the baseline separation of ferulic and syringic acid as well as the resolution of the other phenolic acids considered in the study. The separation selectivity was effectively tuned by pH variation (Supplementary material Fig. I); in particular, at pH 6.0 the differences of migration times of the analytes was maximized, thus it was selected as the best pH conditions. Further improvement of the separation was achieved by increasing the buffer concentration to 50 mM and by addition to the background electrolyte of 20% (v/v) of methanol. Under the described final conditions, the separation of the solutes was complete within 12 min; the proposed approach is very simple, cheap and of practical application allowing analysis of several samples in short time and thus suitable for the fast screening of wheat extracts. It is noteworthy that similar separations can be obtained in reversed phase HPLC using gradient elution at the expense of analysis time of about 40 min or longer [9,13]. Comparable results in term of analysis time were reported for separation of phenolic acids in beverages by means of a less affordable UPLC approach [28].

3.2. Sample preparation and analysis

Most phenolic acids in cereals primarily occur in bound form and are typically components of complex structures in cell wall linked to hemicelluloses; on the other hand phenolic acids are also contained in the plant cell vacuoles both as free molecules and esterified (conjugated) to sugars and other low molecular mass components [5,8]. According to Adom et al. [6] and based on previous studies [9,14,15,29], the extraction of the soluble fraction of phenolic acids (free and conjugated) from wheat samples was carried out using a mixture ethanol:water 80:20 (v/v). Preliminary experiments showed that in soft wheat flour, the free fraction is contained at very low levels, thus it was established to analyse both the whole components of the soluble fraction (sum of the free and conjugated phenolic acids) and the bound fraction. The extraction procedures adopted is described in experimental section and in the scheme of Fig. 2; even though the applied experimental conditions involve highly concentrated NaOH solutions to release bound analytes, the loss of the six considered phenolic acids has been reported to be very limited and addition of antioxidants (*e.g.*, ascorbic acid) to the sample mixture in order to preserve analytes from oxidation, was not necessary [30]. Since the entire procedure includes a number of steps, the use of an internal standard was adopted to normalize for the loss of the analytes during the samples handling. To this regard, 4-bromomandelic acid, a synthetic compound that is not represented as a metabolite in plant kingdom, was chosen because of the similarity of the pKa and LogP values (3.40 and 1.69 [31], respectively) with those reported for the target analytes. Under the optimized CE conditions, 4-bromomandelic acid was separated from each of the other components of the real samples; in Fig. 3 are shown the electropherograms related to the analysis of the bound (a) and the free plus conjugated (soluble) (b) fractions of the sample Mieti, a modern variety of soft wheat. As it can be seen, by using UV in DAD mode, the selective determination of the analytes was achieved avoiding any interferences from the components of the matrix.

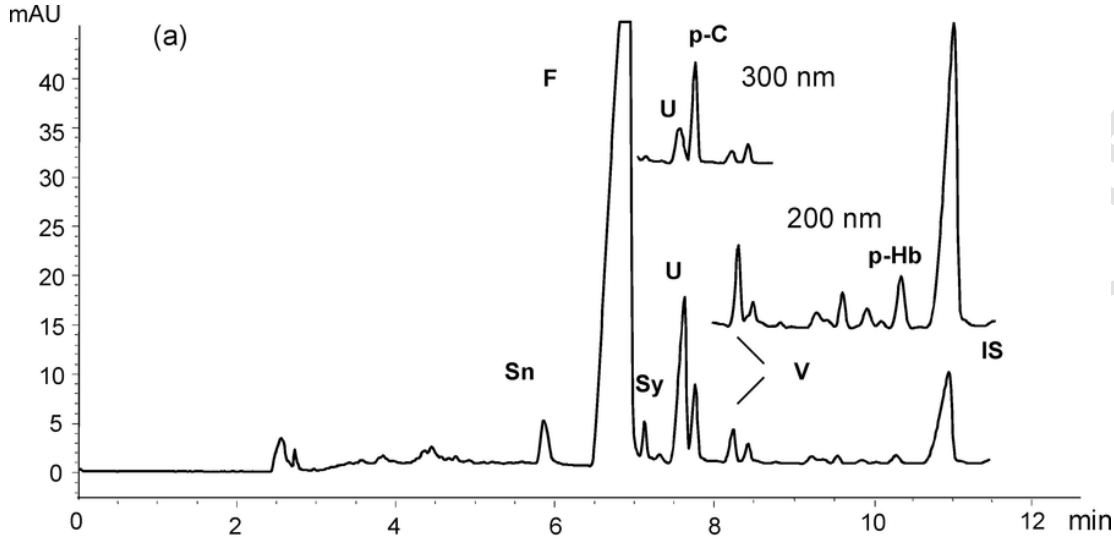
3.3. Validation

Method validation was performed by estimating linearity, sensitivity, system precision and repeatability, accuracy and robustness, according to ICH (International Council for Harmonization) guidelines [32].

3.3.1. Linearity and sensitivity

The linearity ranges were selected to include the concentrations of each of the phenolic acids in soluble and bound fractions. Equations of the corresponding calibration curves were obtained by plotting the corrected peak area (peak area/migration time) ratio of the analytes to the internal standard, versus the concentration (µg/mL). The data are reported in Table 1: the r^2 values obtained by linear regression analysis were at least 0.998 and no trends for residual distribution were observed.

Limit of detection (LOD) and limit of quantitation (LOQ) were within 0.3–0.4 and 0.8–1.1 µg/mL, respectively and are reported in detail in Table 1; the values were estimated by the signal-to-noise ratio defined as 3 (LOD) and 10 times (LOQ) the noise, assumed as the distribution of the response at zero analyte concentration. The data obtained in the present study are comparable with those reported by applying similar approaches for analysis of phenolic acids in food matrices [21,23,25]. Higher sensitivity by CE methods was reported by application of on-line preconcentration techniques such as electro-



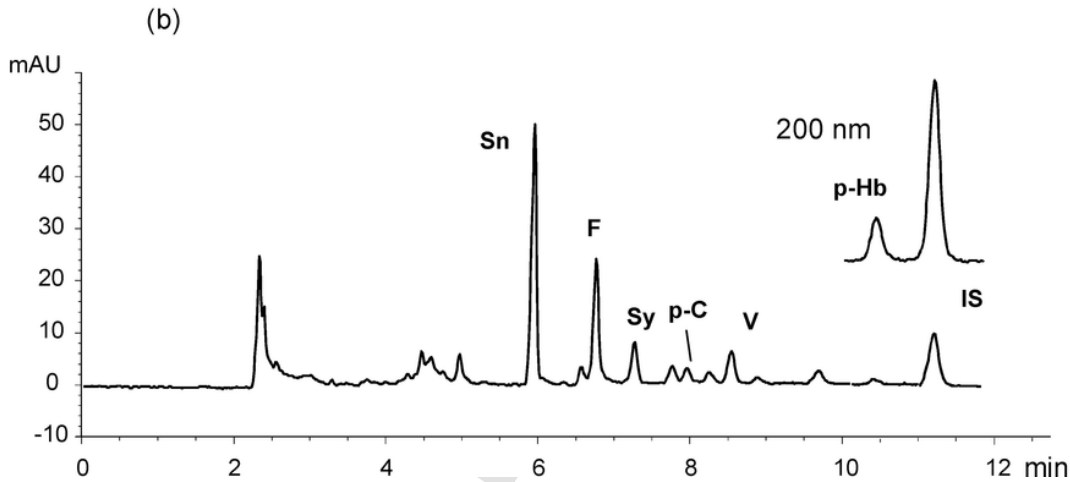


Fig. 3. Electropherograms of the bound (a) and soluble (b) fractions of Mieti wheat sample under optimized CE conditions: 50 mM phosphate buffer pH 6.0 containing 20% (v/v) methanol. Voltage 27 kV, temperature 30 °C, hydrodynamic injection at 50 mbar × 5 s. Detection was performed at 220 nm (entire electropherograms), and at 300 nm and 200 nm, as labeled. Symbols: Sinapic acid (Sn); Ferulic acid (F)*; Syringic acid (Sy); Vanillic acid (V), p-Coumaric acid (p-C); p-Hydroxybenzoic acid (p-Hb); internal standard (IS); Unknown compound (U). *The peak of F in electropherogram (a) is out of the response scale.

kinetic supercharging which allowed 1000-fold better sensitivity in analysis of some phenolic acids in fruit juices [22] and environmental (water) samples [33]. Analysis of wheat extracts has been conventionally carried out by HPLC showing in general, slightly better sensitivity than CE determinations as due to the favourable path-length of the HPLC-UV detection flow cell; in the case of p-hydroxybenzoic, vanillic and syringic acid, the sensitivity obtained in the present study was comparable to those reported by HPLC in analysis of durum wheat and derived products [13].

3.3.2. System precision and repeatability

System precision was assessed by repeated injections (n = 6) of a standard mixture of the analytes (concentration at LOQ values). The RSD% values of migration time as well as those of the estimated LOQs, are reported in Table 1 showing that the instrumental variability at low concentration of the analytes is adequate and consistent with those obtained by CE methods applied to similar samples [21,23,25]. The contribution of the sample preparation procedure to the whole precision method (repeatability) was estimated by quantifying the six considered phenolic acids in both the fractions (soluble and bound) of three independent samples of Verna soft wheat. The RSD% (n = 3) values were found to do not exceed 7% (Supplementary material Table I), thus in line with those reported in analysis of wheat samples by HPLC, showing RSDs of about 10% [13] and 1–18% [8,9], depending on the considered analyte.

3.3.3. Accuracy

Accuracy was estimated by recovery experiments performed on both bound and soluble fractions on the representative sample Verna soft wheat. In details, each of the phenolic acids was spiked to the real sample at two levels corresponding to about 20% and 40% of the analytes native content, before the extraction processes. The results are reported in Supplementary material Table II; the recovery ranged

within 84–105.4%, consistently to the data reported in similar studies carried out by HPLC separation [9,11,13].

3.3.4. Robustness

The electrophoretic method was tested for robustness by evaluating its capability to remain unaffected by small variations of the optimised parameters. In particular, concentration of the phosphate buffer used as the BGE, its pH and the applied voltage, were the factors to be considered because of their influence on the separation of the analytes. The robustness study was carried out by following a "one-by-one" approach, in which the individual parameters were changed over an established range around the optimized values. Phosphate buffer was varied within 50 ± 2 mM; the pH value was varied within 5.9–6.1 and the applied voltage within 27 ± 1 kV. The resolutions between ferulic/syringic acid and between the unknown peak/p-coumaric acid (see Fig. 3) in a real sample represented by a bound extract of soft wheat, were maintained at levels allowing for the selective peak area integration. The obtained results showed that the quantitation of these critical analytes is still possible even in the case of small fluctuations of the optimized parameters. In general, the method robustness was found to be good maintaining the resolution between the other couples of adjacent peaks higher than 2.0, in each of the tested conditions.

In conclusion, the validation parameters assessed in this study showed that the proposed CE method fits the requirements for the analytical quality control of bio-samples [34].

3.4. Method application: analysis of modern and ancient wheat samples

By the validated CZE method, the selected six phenolic acids were quantified across the ten considered wheat genotypes; in particular each of the samples was analysed (three independent determinations) for the content of phenolic acids in both soluble and bound fraction. The contents (soluble plus bound amount) of each of the compounds in the wheat varieties, are reported in Table 2. For each individual phenolic acid, significant variations ($p < 0.01$) were observed among all the varieties. Considering all modern and ancient genotypes, ferulic, sinapic and p-coumaric acids were the most abundant. Notably, these compounds are derivatives of hydroxycinnamic acid and were previously found to be mainly present as linked to cell wall structural components [5,8,35], constituting the most abundant phenolic acids in the whole-wheat grain [9]. In the present investigation, ferulic acid was the most represented ranging from 862.3 ± 43.2 μg/g in Gentilrosso to 458.6 ± 15.3 μg/g in Palesio. Ndolo and Beta [36] reported a mean concentration of ferulic acid of 689 μg/g in whole wheat kernels, which is perfectly in line with the mean value observed in the present study (665 μg/g). In contrast, the total ferulic acid levels observed in 120 soft wheat varieties by Li et al. [9] ranged from 181 to 742 μg/g (mean content equal to 395 μg/g) and do not completely match with the range of our investigation (458–862 μg/g). Actually, significant differences in the amount of ferulic acid have been found among the wheat varieties, as reported by Lempereur et al. [37] who suggested a high influence of the genetic components on the presence of this metabolite in wheat kernels. Second and third for abundance, sinapic and p-coumaric acids, accounted for 53.5 and 21.4 μg/g, respectively. Minor components were represented by syringic, vanillic and p-hydroxybenzoic acids, whose mean contents were 15.5, 15.2 and 7.9 μg/g, respectively. Moore et al. [38] and Li et al. [9] reported ranges and average levels for total vanillic acid, syringic acid and p-coumaric acid in soft wheat consistently with those of the present study.

For ferulic acid, p-hydroxybenzoic acid and vanillic acid, significantly higher values were observed in the considered ancient varieties than in modern ones: the total content of phenolic acids was found to be approximately 1.33 times higher.

The screening carried out by Li et al. [9] on 120 soft wheat varieties, showed multifold differences in total phenolic content among various genotypes, suggesting the possible development of varieties enriched in phenolics for the benefit of health consumers; thus, the considered genotypes were clustered in eight groups on the basis of the total phenolic content. According to the proposed classification, we found that the modern wheat varieties analysed in our investigation may be included in the group 3 (range 500–600 μg/g) while the ancient ones fall in group 5 (700–800 μg/g). Thus, the latter can be considered a rich genetic resource for breeding programs aimed at improving the content of phenolics in soft wheat varieties. In addition to their antioxidant properties and anti-ageing effects, ferulic, p-hydroxybenzoic and vanillic acid, are also characterized by strong flavour. Therefore, the different composition of phenolic acids in ancient and modern wheat can have not only diversified health effects on consumers but also affect their perception of the related transformed products (*e.g.,* bread). The results of the present investigation provide evidences for prompting reintroduction in farming, of neglected ancient wheat varieties.

Table 2
Amount of phenolic acids (sum of soluble and bound fractions) found in the studied wheat varieties expressed as average values ± standard deviation (μg/g DW).

	Ferulic acid **	p-Coumaric acid **	p-Hydroxybenzoic acid **	Sinapic acid **	Syringic acid **	Vanillic acid **	Total **
Ancient Wheat							
Andriolo	772.4 ± 14.1 (b)	27.1 ± 1.5 (a)	8.1 ± 0.1 (c)	48.9 ± 1.4 (c)	17.0 ± 0.4 (bc)	16.7 ± 0.4 (b)	890.2 ± 17.1 (b)
Frassineto	670.1 ± 3.4 (cd)	20.8 ± 0.7 (bc)	8.2 ± 0.3 (c)	32.3 ± 0.2 (d)	17.9 ± 0.5 (b)	17.0 ± 0.1 (b)	766.2 ± 3.2 (b)
Gentilrosso	862.3 ± 43.2 (a)	25.4 ± 1.0 (a)	11.3 ± 0.3 (a)	62.9 ± 5.2 (b)	20.7 ± 0.2 (a)	21.4 ± 1.2 (a)	1004.0 ± 45.8 (a)
Inalettabile	711.3 ± 5.0 (c)	20.2 ± 1.0 (bc)	9.0 ± 1.4 (bc)	53.2 ± 7.5 (c)	12.9 ± 0.6 (de)	15.5 ± 1.6 (bc)	822.2 ± 7.8 (bc)
Verna	817.5 ± 8.0 (ab)	20.9 ± 0.4 (bc)	9.7 ± 0.3 (b)	71.9 ± 3.1 (ab)	16.1 ± 0.1 (bc)	22.4 ± 0.9 (a)	958.4 ± 3.2 (a)
Modern Wheat							
Bolero	618.5 ± 28.2 (de)	25.9 ± 2.5 (a)	5.7 ± 0.5 (ef)	53.0 ± 4.0 (c)	13.6 ± 1.7 (d)	13.1 ± 1.0 (bcd)	729.8 ± 38.0 (bcd)
Eureka	650.7 ± 33.5 (d)	21.6 ± 1.1 (b)	7.7 ± 0.1 (cd)	74.6 ± 3.9 (a)	20.2 ± 1.4 (a)	13.0 ± 0.1 (bcd)	787.7 ± 40.0 (bcd)
Mieti	574.2 ± 42.6 (e)	17.8 ± 0.9 (c)	5.0 ± 0.4 (f)	46.8 ± 3.8 (c)	10.7 ± 1.1 (f)	8.7 ± 5.1 (d)	663.2 ± 41.9 (d)
Nobel	479.7 ± 20.1 (f)	20.5 ± 2.7 (bc)	6.7 ± 0.7 (de)	46.3 ± 5.6 (c)	10.9 ± 0.7 (ef)	12.5 ± 1.3 (bcd)	576.5 ± 25.7 (bcd)
Palesio	458.6 ± 15.3 (f)	14.3 ± 1.1 (d)	8.1 ± 0.3 (c)	44.9 ± 0.2 (c)	15.1 ± 1.3 (cd)	12.0 ± 0.5 (cd)	552.9 ± 18.7 (cd)
Ancient	766.7 (a) **	22.9 ns	9.3 (a) *	53.8 ns	16.9 ns	18.6 (a) **	888.2 (a) **
Modern	556.3 (b) **	20.0 ns	6.6 (b) *	53.1 ns	14.1 ns	11.9 (b) **	662.0 (b) **

Mean values followed by different letters are statistically different at $P < 0.05$. Ns, not significant; *$P < 0.05$; **$P < 0.01$.

3.5. Data analysis

Principal Components Analysis (PCA) was carried out with the aim to determine relationships among investigated soft wheat cultivars (Fig. 4). The correlation method was preferred over covariance since PCA on the covariance matrix is not invariant to a component-wise change of scale [39,40]. The original space for variable measurements was projected down onto two low-dimensional subspaces. One of these was case-related (ten common wheat genotypes), the other was variable-related (content of ferulic, p-coumaric, p-hydroxybenzoic, sinapic, syringic and vanillic acids). The variable-related subspace was analyzed (factor loading) to understand the correlation between the variables and factors (principal component). In the Principal Component 1 (PC1), explaining 62% of the total variability, the four variables ferulic, sinapic, vanillic, syringic acid were loaded (−0.92, −0.66, −0.86, −0.76, respectively). In contrast, in the PC2 explaining 15% of total variability, p-coumaric and p-hydroxybenzoic acid were loaded (−0.78, 0.53, respectively). Consequently PC1 can be labelled as ferulic acid derivatives, whereas PC2 represents ferulic acid precursors (Fig. 1). Except for the cultivar Eureka, the modern cultivars (Bolero, Nobel, Mieti and Palesio) were grouped in the North-East and South-West quadrants of the Cartesian plan, while the ancient varieties were placed in the opposite quadrants (North-West and South-West), forming thus two sharply separated clusters (Fig. 4). In summary: i) in all wheat genotypes, the most abundant phenolics compounds were found to be ferulic, sinapic and p-coumaric acids; ii) for the first time, it was observed that in the considered ancient varieties, the content of ferulic acid, p-hydroxybenzoic acid and vanillic acid was significantly higher with respect to the content in modern genotypes; iii) PCA, allowed the visualization of relatedness amongst the cultivars, showing two distinct clusters including respectively, ancient and modern varieties.

4. Conclusion

A simple CZE method was optimized and validated for the analysis of six phenolic acids and it was applied to their quantitation in real samples (soluble and bound fractions) represented by different cultivars of soft wheat. Significant differences of the quantitative phenolic acids profile was observed between the modern and the ancient cultivars, with the latter showing higher phenolic acids content. Even though a limited number of varieties was screened, it is possible to argue that the modern approaches of wheat breeding oriented to promote grain characteristics such as crop yields, timing of crop maturation, proteins content etc., might have hindered wheat types with higher bioactive phenolic acids content. The present study, focused on quantitation of phenolic acids in modern and ancient wheat varieties suggests for the first time the evidence that the ancient wheat could be considered a rich genetic resource for breeding programs aimed at improving the content of phenolic acids in soft wheat varieties.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2017.11.058

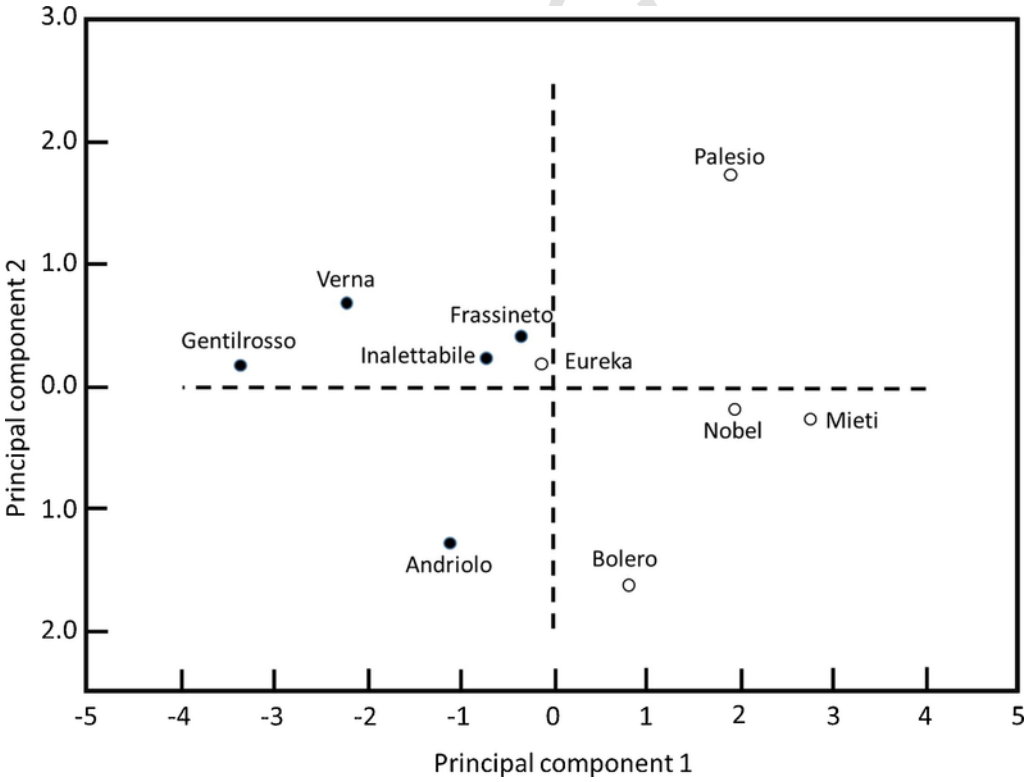

Fig. 4. Relationships among the investigated soft wheat cultivars (● = ancient varieties; ○ = modern varieties) obtained by PCA based on phenolic acids content. The two Principal Components accounted for 77% of the total variation.

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