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Chapter 2

Phenolic Fingerprinting and Glumes Image Analysis as an Effective Approach for Durum Wheat Landraces Identification

Oscar Grillo, Marisol Lo Bianco, Sebastiano Blangiforti, Laura Siracusa, Gianfranco Venora and Giuseppe Ruberto

Additional information is available at the end of the chapter

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Abstract

In the last decade, encouraged by economic, social and nutritional reasons, the trend towards the rediscovery and reuse of durum wheat landraces moved on in Sicily. This growing attention in local wheat landraces made necessary to design new effective and objective identification methods that are able to distinguish landraces. Considering the difficulties coming from the genetic and morphological heterogeneity of a landrace, in this chapter a multidisciplinary approach for durum wheat landraces identification is proposed. Nine Sicilian wheat landraces were investigated from the genotypic and phenotypic point of view, studying their polyphenolic profile, and analyzing the glumes morpho-colorimetric traits, in search of similarities and/or differences. In particular, hydro-alcoholic extracts from whole wheat grains were analyzed by means of HPLC/DAD and HPLC/ESI-MS, revealing 13 metabolites mainly belonging to the classes of hydroxycinnamic acids and flavones *C*-glycosides. The quantitative pattern of the 13 phenolic markers allowed to perfectly identify all the wheat samples, confirming a specific and genotype-dependent pattern of phenolics concentration. Moreover, computerized image analysis techniques were applied to compare the wheat samples on the basis of 138 quantitative morpho-colorimetric variables descriptive of glumes size, shape, color and texture, confirming the possibility to undoubtedly identify wheat samples belonging to local landraces.

Keywords: traceability, biodiversity, local populations, morpho-colorimetric analysis, old varieties, phytochemicals, polyphenols, *Triticum* L

1. Introduction

After the massive use of processed acorns, as food source for prehistoric nomadic populations, the most important food discovery was undoubtedly that of cereals. Even now, wheat (*Triticum*) is one of the main food sources in the world. According to the last FAO report, wheat world production for 2017 was approximately expected in 740 million tons, exceeding the previous last one crop year by 1.2%, and covering about 15% of the world's arable surface [1]. In this scenario, durum wheat production reaches around 30 million tons, accounting approximately for 5–6% of the total world wheat production [2]. Canada, USA, Mexico and Russia are some of the countries around the world where durum wheat is cropped, although the Mediterranean region covers about 60% of world durum wheat production [3], being the EU (Italy, Spain, France and Greece) the leading global producer [2]. South Italy is one of the regions historically most voted to the cereal crops, where the durum wheat varietal biodiversity is particularly high [4].

For geographical position and ecological condition, Sicily represents the perfect environment for the cultivation of cereals, especially for durum wheat. In addition to the pedo-climatic conditions [5], some historical and socio-cultural aspects had also contributed to enrich the varietal heritage, such as the many invasions that characterized the island during the centuries. All these conditions, together with the historically conducted mass selection and the more recent genetic improvement programs based on the artificial crosses, had contributed to build the extremely wide currently existing varietal panorama [4].

In Sicily, old and new durum wheat commercial varieties are currently cropped, but also many ancient landraces or populations characterized by specific bio-morphological traits and qualitative features [6, 7].

In recent years, all over the world, the attention paid to local and traditional productions is growing, especially in the agro-food sector. Maybe, it is due to the impact of globalization and the social and economic changes, but also to the increased consideration to health and nutritional aspects of food. Also in Sicily, this trend has led to the rediscovery and reuse of landraces both of wheat and other crops, responding to requests for more and more demanding market. The rising price of these local productions are contributing to the farmers' satisfaction, changing an unprofitable job in a renewed professional opportunity also for young businessmen. Furthermore, many recent research studies testify the high healthy and nutraceutical value of landraces, both for high amount of antioxidant compounds and for their natural aptitude to organic production [8–11].

This growing interest in local landraces has inspired to find effective and objective identification methods, able to distinguish landraces [12, 13].

In this chapter a multidisciplinary practical approach based on genotype and phenotype characterization of durum wheat Sicilian landraces is proposed. In particular, the polyphenolic profile of whole wheat grains was analyzed by means of HPLC/DAD and HPLC/ESI-MS.

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Moreover, computerized image analysis techniques were applied to compare glume wheat samples, implementing a statistical classificator able to discriminate the landraces.

2. Materials and methods

2.1. Polyphenolic profile analysis

2.1.1. Samples details

Nine durum wheat (*Triticum durum* Desf.) landraces ("Margherito," "Manto di Maria," "Ruscia," "Russello SG8," "Scavuzza," "Tumminia SG3," "Trentino," "Tripolino," "Urria") were selected for phenolics profile evaluation.

Grains were cropped, in three plots of 10 m^2 each, using 350 viable seeds/m^2, during three consecutive years (2012, 2013, 2014), in the fields of the Stazione Sperimentale di Granicoltura per la Sicilia, sited in Santo Pietro - Caltagirone [37°07′12″N; 14°31′17″E; 313 m a.s.l.] (CT, Sicily, Italy). 40 kg N/ha and 90 kg P_2O_5/ha were supplied at sowing carried out at the beginning of December; nitrogen fertilization with 50 kg N/ha were applied before the beginning of stem elongation stage (20–30 code in the BBCH-scale for cereals). Mechanical weed control methods were carried out in spring time and harvest was performed when physiological maturity of each genotype was reached.

Whole grain samples were milled to a fine powder by a laboratory mill (1093 Cyclotec Sample Mill, Tecator Foss, Hillerød, Denmark) equipped with a 1 mm sieve, immediately cooled to −20°C and kept at this temperature until analysis to protect bioactive components from degradation [14].

2.1.2. Chemicals

All solvents and reagents used in this study were high purity laboratory solvents by Carlo Erba (Milano, Italy); HPLC grade water and acetonitrile were obtained from VWR (Milano, Italy). Pure vitexin (apigenin 8-*C*-glucoside) and orientin (luteolin 8-*C*-glucoside) were provided by Extrasynthese (Lyon, France) whilst vanillic acid, ferulic acid, p-coumaric acid and caffeic acid were purchased from Sigma (Sigma-Aldrich s.r.l., Milano, Italy).

2.1.3. Extraction of free and bound phenolic compounds

Phenolic acids and flavonoids represent the most common form of phenolic compounds found in whole grains, existing as soluble free compounds, soluble conjugates esterified to sugars and other low molecular mass components, and insoluble bound forms either encapsulated in the cell-wall structures or chemically bound at molecular level [15].

According to Lo Bianco et al. [11], free phenolics were recovered by applying the method proposed by Dinelli et al. [14] with few changes. In brief, 1 g of whole wheat flour was mixed

under vigorous stirring for 10 min with 20 mL of an acidic aqueous methanol solution (80% methanol, 19% water, 1% formic acid). The resulting heterogeneous mixture was transferred into standard glass sample tubes and centrifuged at 2500 g/min for 10 min. After that, the supernatant was removed and the extraction was repeated. Collected supernatants were pooled, evaporated to dryness, and then stored at −20°C until use.

The solid residue from the free phenolic extraction was subjected to alkaline hydrolysis to recover the bound phenolic compounds, according to Mattila et al. [16]. Distilled water (12 mL) and 5 mL of 10 M NaOH were added to the residue and stirred overnight at room temperature. The mixture was acidified to pH = 2 and then extracted three times with 15 mL of a 1:1 (v/v) mixture of cold diethyl ether and ethyl acetate by manually shaking and centrifuging. Organic layers were combined, evaporated to dryness, and dissolved into 2 mL of the aqueous methanol solution to analytical determinations.

2.1.4. HPLC/DAD quantitative analyses

For HPLC/DAD analyses dry extracts were reconstituted in 3 mL of the extracting solvent and immediately analyzed. Quantitative analyses were carried out on a UltiMate3000 "UHPLC focused" instrument equipped with a binary high pressure pump, a Photodiode Array detector, a Thermostatted Column Compartment and an Automated Sample Injector (Thermo Scientific, Italy). Collected data were processed through a Chromeleon Chromatography Information Management System v. 6.80. Chromatographic runs were all performed using a reverse-phase column (Gemini C_{18}, 250 × 4.6 mm, 5 μm particle size, Phenomenex, Italy) equipped with a guard column (Gemini C_{18} 4 × 3.0 mm, 5 μm particle size, Phenomenex, Italy). Wheat polyphenols were eluted with the following gradient of B (formic acid, 2.5% solution in acetonitrile) in A (2.5% solution of formic acid in water): 0 min: 5% B; 10 min: 15% B; 30 min: 25% B; 35 min: 30% B; 50 min: 90% B; then kept for 7 min at 100% B. The solvent flow rate was 1 mL/min and. Quantifications were carried out at 350 nm using orientin (R^2 = 0.9999) as external standard; the detector was set at 280 nm to build the calibration curve for vanillic acid (R^2 = 0.9997), whilst vitexin (R^2 = 0.9999), caffeic acid and ferulic acid were quantified at 330 nm using the corresponding reference substances (R^2 = 0.9999 and R^2 = 0.9998, respectively). The same reference wavelength was used for the quantification of coumarins against p-coumaric acid (R^2 = 0.9998). All analyses were carried out in triplicate.

2.1.5. Identification of main components via HPLC/ESI-MS

In order to unambiguously identify the chromatographic signals and/or to confirm peak assignments, a series of HPLC/ESI/MS analyses were performed on wheat samples. In this case, variable aliquots (1.0–1.5 mL) of the above mentioned hydro-alcoholic solutions coming from quantitative analyses (see previous paragraph) were transferred into standard laboratory vials and brought to dryness *in vacuo* with a rotary evaporator (Heidolph Laborota 400). The resulting yellowish residues were then re-dissolved in 500 μL of the original hydroalcoholic solution and submitted to qualitative analyses. The HPLC apparatus used was the same described above, whilst ESI mass spectra were acquired by a Thermo Scientific Exactive Plus Orbitrap MS (Thermo Fisher Scientific, Inc., Milan, Italy), using a heated electrospray ionization (HESI II) interface. Mass spectra were recorded operating in negative ion mode in the *m*/*z*

range 120–1800 at a resolving power of 25,000 (full-width-at-half-maximum, at *m/z* 200, RFWHM), resulting in a scan rate of >1.5 scans/s when using automatic gain control target of 1.0×10^6 and a C-trap inject time of 250 ms, under the following conditions: capillary temperature 300°C, nebulizer gas (nitrogen) with a flow rate of 60 arbitrary units; auxiliary gas flow rate of 10 arbitrary units; source voltage 3 kV; capillary voltage 82.5 V; tube lens voltage 85 V. The Orbitrap MS system was tuned and calibrated in positive modes, by infusion of solutions of a standard mixture of sodium dodecyl sulfate (Mr 265.17 Da), sodium taurocholate (Mr 514.42 Da) and Ultramark (Mr 1621 Da). Data acquisition and analyses were performed using the Xcalibur software.

2.2. Glume image analysis

2.2.1. Samples details

For the glumes image analysis, ears of the same ten wheat landraces were reaped, at the time of maximum ripening, in order to include a widest morphological and environmental variability, the wheat ears were collected during three consecutive years (2012, 2013, 2014).

From three to six ears were sampled and from two to four glumes were removed from the spikelets of the ear middle section and from the both sides of each ear. The glumes were stored at room temperature under controlled conditions (20°C and 50% RH).

2.2.2. Images acquisition

Digital images of glumes samples were acquired using a flatbed scanner (ScanMaker 9800 XL, Microtek Denver, CO), applying the same resolution and scanning area conditions reported in Grillo et al. [4]. As suggested by Venora et al. [17], before digital image capture, the scanner was standardized according to the calibration protocol proposed by Shahin and Symons [18]. Morpho-colorimetric features were only measured for sound intact glumes, rejecting that ones with broken beak or shoulder, distinguishing in right and left side of the ear. A total of 902 wheat glumes were analyzed (**Table 1**).

Code	Variety/landrace	Sample amount
mar6	Margherito 06	97
mm1	Manto di Maria 01	95
rsc9	Ruscia 09	97
russg8	Russello 13 SG8	97
sca1	Scavuzza 01	95
tre2	Trentino 02	119
tri2	Tripolino 02	80
tumsg3	Tumminia SG3	94
urr1	Urrìa 01	88

Table 1. List of the ten different wheat local varieties studied.

2.2.3. Image processing and analysis

All the images were processed and analyzed using the software package KS-400 V. 3.0 (Carl Zeiss, Vision, Oberkochen, Germany). The same macro used by Grillo et al. [4], specifically developed for the characterization of wheat glumes was applied to perform automatically all the morpho-colorimetric measurements on the glume samples of the present study.

The macro allowed to compute 138 quantitative variables measured for each analyzed left and right glume (**Tables 2** and **3**). In particular, it was possible to measure 18 parameters descriptive of the glume size and shape and 20 features descriptive of the glume surface color. Afterwards, applying the same procedure reported by Orrù et al. [19], 78 quantitative Elliptic Fourier Descriptors (EFDs) were used to describe the shape of the glume. Finally, the macro was kitted to compute 11 Haralick's descriptors including the relative standard deviations, as reported in Lo Bianco et al. [20].

$$G = \begin{bmatrix} p(1,1) & p(1,2) & \cdots & p(1,N_g) \\ p(2,1) & p(2,2) & \cdots & p(2,N_g) \\ \vdots & \vdots & \ddots & \vdots \\ p(N_g,1) & p(N_g,2) & \cdots & p(N_g,N_g) \end{bmatrix} \quad (1)$$

2.3. Statistics

The data, obtained from chemical and image analysis, were used to build a global database. Statistical elaborations were executed using SPSS software package release 16.0 (SPSS Inc. for Windows, Chicago, Illinois, USA), and the stepwise Linear Discriminant Analysis (LDA) method was applied to identify and discriminate among the investigated wheat samples [23]. This approach is commonly used to classify/identify unknown groups characterized by quantitative and qualitative variables [24–27], finding the combination of predictor variables with the aim of minimizing the within-class distance and maximizing the between-class distance simultaneously, thus achieving maximum class discrimination [28–31]. Then, the stepwise procedure, carried out as explained in [4], identifies and selects the most statistically significant features among the chemical metabolites and the 138 traits measured on each glume. Finally, a cross-validation procedure was applied to verify the performance of the identification system, testing individual unknown cases and classifying them on the basis of all others [32].

All the raw data were standardized before starting any statistical elaboration. Moreover, in order to evaluate the quality of the discriminant functions achieved for each statistical comparison, the Wilks' Lambda, the percentage of explained variance and the canonical correlation between the discriminant functions and the group membership, were computed. The Box's M test was executed to assess the homogeneity of covariance matrices of the features chosen by the stepwise LDA while the analysis of the standardized residuals was performed to verify the homoscedasticity of the variance of the dependent variables used to discriminate among the groups' membership [33]. Kolmogorov-Smirnov's test was performed to compare the empirical distribution of the discriminant functions with the relative cumulative distribution function of the reference probability distribution, while the Levene's test was executed to assess the equality of variances for the used discriminant functions calculated for groups membership [34].

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	Feature	Equation
Har 1	Angular second moment	$\sum_i \sum_j p(i,j)^2$
Har 2	Contrast	$\sum_{n=0}^{N_g-1} n^2 \left\{ \sum_{i=1}^{N_g} \sum_{j=1}^{N_g} p(i,j) \right\}, \lvert i,j \rvert = n$
Har 3	Correlation	$\frac{\sum_i \sum_j (ij) p(i,j) - \mu_x \mu_y}{\sigma_x \sigma_y}$ where μ_x, μ_y, σ_x and σ_y are the means and the standard deviations of p_x and p_y.
Har 4	Sum of square: variance	$\sum_i \sum_j (i-\mu)^2 p(i,j)$
Har 5	Inverse difference moment	$\sum_i \sum_j \frac{1}{1+(i-j)^2} p(i,j)$
Har 6	Sum average	$\sum_{n=2}^{2N_g} i p_{x+y}(i)$ where x and y are the coordinates (row and column) of an entry in the co-occurrence matrix, and $p_{x+y}(i)$ is the probability of co-occurrence matrix coordinates summing to $x + y$.
Har 7	Sum variance	$\sum_{i=2}^{2N_g} (i - f_8)^2 p_{x+y}(i)$
Har 8	Sum entropy	$-\sum_{i=2}^{2N_g} p_{x+y}(i) \log\{p_{x+y}(i)\} = f_8$
Har 9	Entropy	$-\sum_i \sum_j p(i,j) \log[p(i,j)]$
Har 10	Difference variance	$\sum_{n=0}^{N_{g-1}} i^2 p_{x-y}(i)$
Har 11	Difference entropy	$-\sum_{n=0}^{N_{g-1}} p_{x-y}(i) \log\{p_{x-y}(i)\}$

The basis for these features is the gray-level co-occurrence matrix (G in Eq. (1)). This matrix is square with dimension Ng, where Ng is the number of gray levels in the image. Element [i,j] of the matrix is generated by counting the number of times a pixel (p) with value i is adjacent to a pixel with value j and then dividing the entire matrix by the total number of such comparisons made. Each entry is therefore considered to be the probability that a pixel with value i will be found adjacent to a pixel of value j.

Table 2. Haralick's descriptors measured as reported in Haralick et al. [21].

	Feature	Description
A	Area	Seed area (mm^2)
P	Perimeter	Seed perimeter (mm)
P_{conv}	Convex Perimeter	Convex perimeter of the seed (mm)
P_{Crof}	Crofton Perimeter	Crofton perimeter of the seed (mm)
P_{conv}/P_{Crof}	Perimeter ratio	Ratio between convex and Crofton's perimeters
D_{max}	Max diameter	Maximum diameter of the seed (mm)
D_{min}	Min diameter	Minimum diameter of the seed (mm)
D_{min}/D_{max}	Feret ratio	Ratio between minimum and maximum diameters
Sf	Shape factor	Seed shape descriptor = $(4 \times \pi \times \text{area})/\text{perimeter}^2$ (normalized value)
Rf	Roundness factor	Seed roundness descriptor = $(4 \times \text{area})/(\pi \times \text{max diameter}^2)$ (normalized value)
Ecd	Eq. circular diameter	Diameter of a circle with equivalent area (mm)
F	Fiber length	Seed length along the fiber axis
C	Curl degree	Ratio between $\boldsymbol{D_{max}}$ and $\boldsymbol{F}$
$Conv$	Convessity degree	Ratio between $\boldsymbol{P_{Crof}}$ and $\boldsymbol{P}$
Sol	Solidity degree	Ratio between $\boldsymbol{A}$ and convex area
Com	Compactness degree	Seed compactness descriptor = $[\sqrt{(4/\pi)}\,\boldsymbol{A}]/\boldsymbol{D_{max}}$
EA_{max}	Maximum ellipse axis	Maximum axis of an ellipse with equivalent area (mm)
EA_{min}	Minimum ellipse axis	Minimum axis of an ellipse with equivalent area (mm)
R_{mean}	Mean red channel	Red channel mean value of seed pixels (gray levels)
R_{sd}	Red std. deviation	Red channel standard deviation of seed pixels
G_{mean}	Mean green channel	Green channel mean value of seed pixels (gray levels)
G_{sd}	Green std. deviation	Green channel standard deviation of seed pixels
B_{mean}	Mean blue channel	Blue channel mean value of seed pixels (gray levels)
B_{sd}	Blue std. deviation	Blue channel standard deviation of seed pixels
H_{mean}	Mean hue channel	Hue channel mean value of seed pixels (gray levels)
H_{sd}	Hue std. deviation	Hue channel standard deviation of seed pixels
L_{mean}	Mean lightness ch.	Lightness channel mean value of seed pixels (gray levels)
L_{sd}	Lightness std. dev.	Lightness channel standard deviation of seed pixels
S_{mean}	Mean saturation ch.	Saturation channel mean value of seed pixels (gray levels)
S_{sd}	Saturation std. dev.	Saturation channel standard deviation of seed pixels
D_{mean}	Mean density	Density channel mean value of seed pixels (gray levels)
D_{sd}	Density std. deviation	Density channel standard deviation of seed pixels
S	Skewness	Asymmetry degree of intensity values distribution (gray levels)
K	Kurtosis	Peakness degree of intensity values distribution (densit. units)
H	Energy	Measure of the increasing intensity power (densitometric units)

Table 3. List of morphometric features measured on seeds, excluding the elliptic Fourier descriptors (EFDs) calculated according to Hâruta [22] and the Haralick's descriptors reported in **Table 2**.

To graphically highlight the differences among groups, multidimensional plots were drawn using the first three discriminant functions.

3. Results and discussion

3.1. Phenolic profile in wheat landraces

Phenolics are mainly concentrated in the outer layers of kernel and contribute to the wheat flour nutraceutical value owing to their antioxidant, anti-inflammatory and anticancer properties [35]. In literature, ca. 70 different phenolic compounds, including coumarins, phenolic acids, anthocyanins, flavones, isoflavones, proanthocyanidins, stilbenes and lignans, were identified in durum wheat genotypes [14].

Referring to flavones, whose interest has grown enormously due to their putative beneficial effects against atherosclerosis, osteoporosis, diabetes mellitus and certain cancers [36] 5,7,4′-trihydroxyflavone (apigenin) and 5,7,3′,4′-tetrahydroxyflavone (luteolin) are the main representatives in wheat, where they accumulate as 6-*C* and/or 8-*C*-glycosidic conjugates. The 8-*C*-glucosides of apigenin and luteolin are also known as vitexin and orientin, respectively.

Hydro-alcoholic extracts from wheat grains were exhaustively analyzed by means of HPLC/DAD and HPLC/ESI-MS. Although the major portion of phenolics in grains exist in the bound form [37], there is a general trend for studying polyphenols in the free form when dealing with chemotaxonomic studies [38, 39]. The chromatograms relating to free phenolics profile of durum wheat grains showed ca. 20 different signals, eluting in the range from 7 to 30 min. Among these, 13 signals were tentatively identified: a preliminary analysis of the UV–VIS (in terms of spectrum shape and absorption maximum, see **Table 4**) spectra of the peaks revealed the presence of compounds belonging to the chemical subclasses of hydroxycinnamic acids and organic acids; several peaks showing the typical spectrum of apigenin derivatives were also detected.

The use of mass spectrometry as detector was helpful in tentatively identifying wheat metabolites (**Table 4**); peak assignments were further confirmed by comparison with literature data [14, 40, 41] and co-injection with pure reference standards when available (see material and methods).

According to Lo Bianco et al. [11], three hydroxycinnamic acids were identified in wheat grains: caffeic acid (peak 4), ferulic acid (peak 10) and another member of this class (peak 1) for which unfortunately the MS spectrum was not determined. Vanillic acid (peak 3) was identified for its diagnostic UV–VIS and mass spectrum; the assignment was confirmed with co-injection with the corresponding standard. Peaks 2 and 6, showing almost identical UV–VIS spectra (a symmetrical absorption with λ max = 317 nm) were tentatively identified as coumarins; furthermore, peak 2 showed a clear mass spectrum with a pseudomolecular ion at 145.14 m/z $(M\text{-}H)^-$. Presence of coumarins in durum wheat has been reported by other authors [14, 40]. The UV–VIS spectrum of peak 5 (λ max = 268, 35 nm) was typical of that of luteolin derivatives; the corresponding mass spectrum exhibited a base peak of 609.52 m/z (pseudomolecular ion) with no signals ascribable to fragments generated by the loss of sugars. The peak corresponding to luteolin aglycone was

Peak #	Retention time (min)	λ ass. (nm)	Selected ion	*m/z* calculated	Tentative identification	Phenolic subclass
1	13.66	295(sh), 316		n.d	Hydroxycinnamic acid	Hydroxycinnamic acid
2	14.24	317	$[M-H]^-$	145.14	Coumarin	Coumarin
3	14.57	258, 291	$[M-H]^-$	167.04	Vanillic acid	Hydroybenzoic acid
4	14.98	290 (sh), 323	$[M-H]^-$	179.16	Caffeic acid	Hydroxycinnamic acid
5	16.57	268, 348	$[M-H]^-$	609.52	Luteolin di-*C*-hexoside (lucenin-2 isomer)	Flavone-*C*-glycoside
6	17.10	316		n.d	Coumarin	Coumarin
7	17.99	270, 334	$[M-H]^-$	563.14	Apigenin *C*-hexoside-*C*-pentoside	Flavone-*C*-glycoside
8	18.95	270, 335	$[M-H]^-$	563.14	Apigenin *C*-hexoside-*C*-pentoside	Flavone-*C*-glycoside
9	19.52	271, 335	$[M-H]^-$	563.14	Apigenin *C*-hexoside-*C*-pentoside	Flavone-*C*-glycoside
10	22.54	295 (sh), 323	$[M-H]^-$	193.05	Ferulic acid	Hydroxycinnamic acid
11	25.18	272, 332	$[M-H]^-$	769.18	Apigenin *C*-hexoside-*C*-hexoside *O*-glucuronide	Flavone-*C*-glycoside
12	26.00	272, 332	$[M-H]^-$	769.18	Apigenin *C*-hexoside-*C*-hexoside *O*-glucuronide	Flavone-*C*-glycoside
13	28.99	270, 334	$[M-H]^-$	431.10	Apigenin *C*-hexoside	Flavone-*C*-glycoside

(sh) is for shoulder.

Table 4. Phenolic compounds detected in the free form extracts from durum wheat grains.

absent as well. These data are usually diagnostic of the presence of *C*-bound glycosides; the peak was then tentatively identified as luteolin di-*C*-hexoside (lucenin-2 isomer). This is in discordance to what was reported by Dinelli et al. [14, 40] who found in durum wheat grains several isomers of lucenin 1/3, the *C*-hexoside-*C*-pentoside derivative of luteolin. Peaks 7, 8, 9, 11, 12 and 13 showed UV–VIS spectra whose shapes and absorption maxima clearly recalled apigenin (**Table 4**); in this case mass analysis was determinant in the assignments. Peaks 7, 8 and 9 all exhibited a mass spectrum with a base peak of 563.14 m/z units, corresponding to the pseudomolecular ion of an hexoside- pentoside derivative; absence of intermediate fragments lead us to assign the peaks as *C*-hexoside *C*-pentoside derivatives of apigenin (**Table 4**). Similarly, peak 13 was tentatively identified as apigenin *C*-hexoside, whilst peaks 11 and 12, both showing a base peak of 769.18 m/z units, were tentatively identified as apigenin *C*-hexoside *C*-hexoside *O*-glucuronide.

3.2. Phenolic content in wheat landraces

The determination of free phenolics in whole grains extracted by a hydroalcoholic solution (see experimental) was carried out through calibration curves obtained via HPLC/DAD triplicate injection of standard solutions. In **Table 5** the concentration of 13 phenolic markers and total free phenolics for the all investigated wheat genotypes is given.

Some of the phenolic markers identified in the free form, were quantitatively quite different among the genotypes studied. For example, coumarin (peak 6), ranging from 2.57 μg/g in Tumminia SG3 to 0.09 μg/g in Tripolino, and vanillic acid (peak 3) from 1.34 μg/g in Manto di Maria to 0.25 μg/g in Tumminia SG3. The apigenin *C*-hexoside-*C*-pentoside (peak 7) content was significantly different among wheat grains, recording values above 21 μg/g for Tumminia SG3 and about 6 μg/g for Trentino.

Luteolin di-*C*-hexoside (lucenin-2 isomer), present in all genotypes in low concentration (mean value 0.33 μg/g, excluding the extremes of the interval, Tumminia SG3 and Manto di Maria) was about 50-times more abundant in Tumminia SG3 (18.15 μg/g) than in other genotypes.

Free ferulic acid content resulted almost 3-times higher in Tumminia SG3 (5.81 μg/g) with respect to the mean value (1.84 μg/g).

Total phenolics concentration ranged from 65.65 μg/g of grain in Russello SG8 to 104.84 μg/g of grain in Scavuzza, and a mean value of 82.78 μg/g was recorded. Three landraces (Tumminia SG3, Tripolino, Scavuzza) showed a content higher than the average.

In general, genotype has been demonstrated to affect the phenolic content of wheat grains. Previous investigations reported on highly significant differences of polyphenol content among different wheat cultivars, suggesting the genotype-specificity of this characteristic [9, 14]. Moreover, the comparison of wheat cultivars grown at different locations showed that environmental and growing conditions may have a certain effect on the biosynthesis and accumulation of phenolic compounds [42].

With regard to the bound phenolic fraction subjected to alkaline hydrolysis, the main component is undoubtedly ferulic acid, as already observed by other authors [43], and confirmed by co-injection with the corresponding analytical standard; this metabolite is present ubiquitously in all the genotypes considered with a mean value of 543.20 μg/g. The landrace Ruscia showed the highest level of ferulic acid content (673.58 μg/g), while Scavuzza the lowest (375.13 μg/g) (**Table 5**).

3.3. Landraces statistical comparison

In order to discriminate among the studied wheat landraces, a statistical classification system was implemented using the data from the 15 analyzed chemical variables and the 138 measured morpho-colorimetric parameters. An overall percentage of correct identification of 100.0% was achieved, proving the peculiarity of the nine studied Sicilian wheat landraces and, on the other hand, the absolute effectiveness of the proposed method (**Table 6**).

Finally, in the evaluation of the parameters that more than other influenced the discrimination process of the studied landraces, none of the assessed variables chosen by the stepwise LDA highlighted particular statistical weight, proving that a high amount of quantitative information is necessary to distinguish and characterize botanical entities so heterogeneous, under chemical, phenotypical and genetic profile, such as landraces.

This work represent the first attempt of wheat landraces identification based on glume phenotypic characters, applying image analysis techniques, coupled with phenolic fingerprinting.

Peak #	Peak ID	Tumminia SG3	Russello SG8	Manto di Maria	Margherito	Ruscìa	Tripolino	Scavuzza	Trentino	Urrìa
1	Hydroxycinnamic acid	n.d.	0.06 ± 0.00	0.48 ± 0.02	n.d.	n.d.	0.20 ± 0.01	n.d.	0.05 ± 0.00	0.16 ± 0.01
2	Coumarin	1.51 ± 0.06	0.52 ± 0.02	1.06 ± 0.04	0.47 ± 0.02	1.28 ± 0.05	0.84 ± 0.03	1.76 ± 0.07	1.29 ± 0.05	1.39 ± 0.06
3	Vanillic acid	0.25 ± 0.01	0.86 ± 0.03	1.34 ± 0.05	0.51 ± 0.02	0.42 ± 0.02	0.58 ± 0.02	1.05 ± 0.04	0.94 ± 0.04	0.91 ± 0.04
4	Caffeic acid	0.11 ± 0.01	0.17 ± 0.01	0.41 ± 0.02	0.07 ± 0.00	0.63 ± 0.03	0.25 ± 0.01	1.29 ± 0.05	0.31 ± 0.01	1.28 ± 0.05
5	Luteolin di-*C*-hexoside	18.15 ± 0.72	0.41 ± 0.02	n.d.	0.20 ± 0.01	0.25 ± 0.01	1.01 ± 0.04	0.22 ± 0.01	0.07 ± 0.00	0.15 ± 0.01
6	Coumarin	2.57 ± 0.10	0.84 ± 0.03	1.28 ± 0.05	0.34 ± 0.01	0.87 ± 0.03	0.09 ± 0.00	0.17 ± 0.01	0.54 ± 0.02	0.32 ± 0.01
7	Apigenin *C*-hexoside-*C*-pentoside	21.46 ± 0.85	7.91 ± 0.31	12.15 ± 0.48	9.25 ± 0.36	11.67 ± 0.46	15.89 ± 0.63	18.81 ± 0.74	5.86 ± 0.23	11.80 ± 0.47
8	Apigenin *C*-hexoside-*C*-pentoside	3.76 ± 0.15	4.83 ± 0.19	3.09 ± 0.12	5.12 ± 0.20	3.97 ± 0.16	6.82 ± 0.27	5.90 ± 0.23	5.19 ± 0.20	4.84 ± 0.19
9	Apigenin *C*-hexoside-*C*-pentoside	10.38 ± 0.41	26.13 ± 1.03	22.78 ± 0.90	28.06 ± 1.11	24.30 ± 0.96	27.71 ± 1.09	29.42 ± 1.16	23.92 ± 0.94	35.32 ± 1.39
10	Ferulic acid	5.81 ± 0.23	0.88 ± 0.04	1.28 ± 0.05	1.04 ± 0.04	1.47 ± 0.06	0.90 ± 0.04	1.88 ± 0.07	1.53 ± 0.06	1.81 ± 0.07
11	Apigenin *C*-hexoside-*C*-hexoside *O*-glucuronide	7.22 ± 0.29	4.26 ± 0.17	10.81 ± 0.43	7.41 ± 0.29	9.40 ± 0.37	13.79 ± 0.54	16.92 ± 0.67	6.32 ± 0.25	5.48 ± 0.22
12	Apigenin *C*-hexoside-*C*-hexoside *O*-glucuronide	17.51 ± 0.69	14.98 ± 0.59	18.26 ± 0.72	22.66 ± 0.89	17.88 ± 0.70	18.67 ± 0.74	21.51 ± 0.85	18.21 ± 0.72	15.07 ± 0.60
13	Apigenin *C*-hexoside	4.87 ± 0.19	3.79 ± 0.15	5.36 ± 0.21	7.01 ± 0.28	3.11 ± 0.12	2.90 ± 0.11	5.91 ± 0.23	5.03 ± 0.20	7.76 ± 0.31
—	Total phenolics	93.61 ± 3.69	65.65 ± 2.59	78.30 ± 3.08	82.14 ± 3.23	75.26 ± 2.96	89.64 ± 3.53	104.84 ± 4.13	69.26 ± 2.73	86.30 ± 3.4
—	Ferulic acid in the bound phenolic fraction	522.30 ± 20.56	516.73 ± 20.34	603.90 ± 23.78	577.40 ± 22.73	673.58 ± 26.52	558.92 ± 22.01	328.67 ± 12.94	560.45 ± 22.07	546.83 ± 21.53

Table 5. Phenolics detected in the durum wheat landraces extracts.

	Margherito	Manto di Maria	Ruscia	Russello SG8	Trentino	Tripolino	Tumminia SG3	Urrìa	Total
Margherito	100.0% (192)	—	—	—	—	—	—	—	100.0% (192)
Manto di Maria	—	100.0% (192)	—	—	—	—	—	—	100.0% (192)
Ruscia	—	—	100.0% (192)	—	—	—	—	—	100.0% (192)
Russello SG8	—	—	—	100.0% (192)	—	—	—	—	100.0% (192)
Trentino	—	—	—	—	100.0% (282)	—	—	—	100.0% (282)
Tripolino	—	—	—	—	—	100.0% (192)	—	—	100.0% (192)
Tumminia SG3	—	—	—	—	—	—	100.0% (192)	—	100.0% (192)
Urrìa	—	—	—	—	—	—	—	100.0% (192)	100.0% (192)
Overall									100.0% (1626)

Percentages refer to the classification performance; in parentheses, the number of analyzed glumes.

Table 6. Percentages identification among the studied landraces.

The achieved results here discussed allowed to demonstrate the usefulness of this discrimination system for the identification and classification wheat landraces, notoriously very difficult to do. The technique here proposed, conveniently sustained by a conspicuous database, can be undoubtedly considered a helpful identification tool both for commercial varieties and for no genetically defined samples, such as populations or landraces.

Considering the heterogeneous nature of the wheat landrace samples used in this study, in order to validate these preliminary achievements, further trials will have to be conducted focusing on the collection of new data, enriching the database with new and accurate information, allowing to the system to give results more and more reliable.

Author details

Oscar Grillo[1]*, Marisol Lo Bianco[1], Sebastiano Blangiforti[1], Laura Siracusa[2], Gianfranco Venora[1] and Giuseppe Ruberto[2]

*Address all correspondence to: oscar.grillo.mail@gmail.com

1 Stazione Sperimentale di Granicoltura per la Sicilia, Caltagirone (CT), Italy

2 Italian National Research Council - Institute of Biomolecular Chemistry ICB, Catania, Italy

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