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Abstract: This study optimizes the application of portable Near Infrared-Acousto Optically Tunable Filter (NIR) device to meet the increasing demand for cost-effective, non-invasive and easy-to-use methods for measuring physical and chemical properties during olive fruit development. Fruits from different phenotypically cultivars were sampled for firmness, total and specific phenols detection by HPLC, total anthocyanins, chlorophyll and carotenoids detection by spectrophotometry. On the same fruits, a portable NIR device in diffuse reflectance mode was employed for spectral detections. Predictive models for firmness, chlorophyll, anthocyanins, carotenoids and rutin were developed by Partial Least Square analysis. Oleuropein, verbascoside, 3,4-DHPEA-EDA, and total phenols were used to develop a validation model. Internal cross-validation was applied for calibration and predictive models. The standard errors for calibration, cross-validation, prediction, and RPD ratios (SD/SECV) were calculated as references for the model effectiveness. The determination of the optimal harvesting time facilitates the production of high quality extra virgin olive oil and table olives.

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Viterbo, August, 5, 2015

Dear Editor,

I am submitting the manuscript **"On-field monitoring of fruit ripening evolution and quality parameters in olive mutants using portable NIR instrument",** by Cirilli et al., for your consideration as a research article to the journal *Food Chemistry*.

Our article will engage a broad spectrum of interest for plant and food chemicals and technologist as it enlarges and improves the current opportunity to identify the better harvesting time of olive drupe, working also as paradigm for other stone fruits.

In this research, we report the results of spectral NIR-AOTF applications on intact olives of three different cultivars during their ripening evolution, compared with analytical measurements performed by HPLC on total and specific polyphenols, and on chlorophylls, carotenoids, anthocyanins and the important physical property of the drupe: the firmness. The objective is to use the NIR-AOTF for field application to monitor ripening evolution based on phenolic, chlorophylls, carotenoids, anthocyanins content, and firmness. Therefore, this manuscript increases the number of fruit properties that could be detected using a non-destructive procedure to identify the better harvesting time, meantime validating the few properties already used in some olive varieties with similar behavior, as reported in a previous paper (Bellincontro et al. 2012). The use of olive varieties phenotypically divergent for the evolution of the ripening of the drupe reinforces the models obtained. PLS models, robust and reliable in term of accuracy, were developed found validated the prediction of firmness, total chlorophyll, total anthocyanins, total carotenoids, and total and specific phenols in olives for oil production (e.g. oleuropein, verbascoside and 3,4-Dihydroxyphenylethanol-elenolic acid, rutin).

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The experimental procedures used in this work was already experienced thus they can be assumed as consolidate and repeatable.

This manuscript has not been submitted for publication elsewhere. All authors confirm that the data acquisition was not in legal conflict with the authorities where the work was carried out.

Thank you for your consideration and we look forward to hearing from you

Sincerely,

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***Highlights (for review)**

- Feasibility of NIR-AOTF as a spectral tool to predict olive firmness has been established.
- NIR-AOTF portable device can be used on-field non-destructive prediction of olive attributes.
- The knowledge of optimal harvesting time improves high quality extra virgin olive oil production.

On-field monitoring of fruit ripening evolution and quality parameters in olive mutants using a portable NIR instrument

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Abstract

This study optimizes the application of portable Near Infrared-Acousto Optically Tunable Filter (NIR) device to meet the increasing demand for cost-effective, non-invasive and easy-to-use methods for measuring physical and chemical properties during olive fruit development. Fruits from different phenotypically cultivars were sampled for firmness, total and specific phenols detection by HPLC, total anthocyanins, chlorophyll and carotenoids detection by spectrophotometry. On the same fruits, a portable NIR device in diffuse reflectance mode was employed for spectral detections. Predictive models for firmness, chlorophyll, anthocyanins, carotenoids and rutin were developed by Partial Least Square analysis. Oleuropein, verbascoside, 3,4-DHPEA-EDA, and total phenols were used to develop a validation model. Internal cross-validation was applied for calibration and predictive models. The standard errors for calibration, cross-validation, prediction, and RPD ratios (SD/SECV) were calculated as references for the model effectiveness. The determination of the optimal harvesting time facilitates the production of high quality extra virgin olive oil and table olives.

Keywords: olive fruits, total phenols, oleuropein, verbascoside, rutin, NIR-AOTF spectroscopy, firmness, partial least square regression (PLSR)

Chemical compounds studied in this article

Rutin (PubChem CID: 5280805); Oleuropein (PubChem CID: 5281544); Verbascoside (PubChem CID: 5281800).

1. Introduction

Virgin olive oil (VOO) is a key component of the Mediterranean diet, which is associated with a reduced risk of cardiovascular disease as well as colon and prostate cancers (Tuck & Hayball, 2002). Several studies have linked the health-benefits of VOO to its unique characteristics with respect to other vegetables oils, namely the high content of monounsaturated fatty acids, the balanced content of polyunsaturated fatty acid and the presence of at least 30 phenolic compounds having antioxidant and radical scavenging activities (Servili et al., 2009).

In addition to genetic properties, agronomics and environmental factors, the production of high-quality VOO strongly depends on the degree of ripening of olive drupes. Ripening is the process of physiological and biochemical changes by which drupes attain several key quality parameters such as color, texture, flavor and nutritional properties (Conde, Delrot, & Gerós, 2008; García, Seller, & Pérez-Camino, 1996). During ripening, the olive fruit undergoes a color shift owing to a progressive decrease of total chlorophyll and carotenoids followed by the appearance of anthocyanins, hydrophilic pigments, conferring the typical purple/black color of mature drupes (Mínguez-Mosquera, & Gallardo-Guerrero, 1991). In contrast to anthocyanins, chlorophylls and carotenoids are lipid-soluble and, therefore, contribute to olive oil colour (Moyano, Melendez-Martinez, Alba, & Heredia, 2008). Both groups of compounds have functional properties because they affect the oxidative stability of olive oil, and carotenoids are also vitamin-A precursors (Aparicio-Ruiz, Gandul-Rojas, & Roca, 2009). The evolution profile of phenolic compounds during olive fruit maturation has been extensively investigated as their content strongly influences sensorial attributes, shelf life and the nutritional value of olive oil (Ryan, Robards, Lavee, 1999; Alagna et al., 2012).

The secoiridoids oleuropein and ligstroside as well as their aglycon forms are the main phenols present in olive fruit. Their concentrations reach relatively high levels in the earlier stages of drupe growth, after which they sharply decline, particularly during maturation. The extent of decrement varies widely among the cultivars, and it depends strongly on environmental conditions (Romani, Mulinacci, Pinelli, Vinciert, & Cimato, 1999). Olive fruit also contains an appreciable amount of flavonoids, mainly luteolin, apigenin, quercetin-3-rutinoside (rutin) and anthocyanins (Servili & Montedoro, 2002). Some of these compounds are also present in olive oil and may contribute to its antioxidant properties (Brenes, García, García, Rios, & Garrido, 1999;Carrasco-Pancorbo et al., 2006). The leucocarpa variety is a natural mutant producing drupes with an ivory-white color at the ripening, due to the very low or null accumulation of flavonoid compounds (Pasqualone et al., 2012).

Olive drupe maturation is associated with changes in the cell wall structure and composition that lead to a modification of the fruit texture as well as a progressive loss of firmness (Prasanna, Prabha, & Tharanathan, 2007) due to enzymatic activity involved in the degradation of cell wall polysaccharides (Jiménez et al., 2001a). The major textural changes, which generally occur concomitantly with color appearance, are driven by the solubilization of pectins and the reduction of tightly bound hemicelluloses (Jiménez et al., 2001b). Firmness correlates with drupe resistance to mechanical damage, an important parameter for storage and processing (García, Seller, & Pérez-Camino, 1996). It has been demonstrated in many studies on grape berries that changes in the textural characteristics during maturation strongly affect the extractability of phenolic compounds and other metabolites during winemaking (Rolle, Torchio, Zeppa, & Gerbi, 2009). The importance of textural characteristics are also well-known in the olive oil industry, which has long introduced enzymatic preparations during milling process, which aids in degrading olive

fruit cell-wall and improves oil yield and phenol extraction (Servili et al., 1992; Vierhuis et al., 2001).

Several indices have been developed to evaluate the degree of olive drupe ripening, with the goal of establishing an optimum balance between olive yield and quality (Famiani, Proietti, Farinelli, & Tombesi, 2002). The most widely used indices are based on simple and easily detectable parameters such as color, firmness, oil content and sugar content (García, Seller, & Pérez-Camino, 1996; Uceda & Frias, 1975). However, the application of these indices is affected by many factors in the ripening process, including the properties of different cultivars. The use of NIR spectroscopy possesses many advantages over traditional destructive approaches, including simplicity, sensitivity and high-throughput. NIR spectroscopy allows simultaneous monitoring of several parameters as well as repeated analysis of the same samples (Gabioud et al., 2008), which can be used to obtain good predictive models for olive moisture, dry matter, oil content and free acidity (Cayuela & Pérez-Camino, 2010). Marquez, Diáz and Reguera, (2005) applied an NIR sensor during olive processing for real-time evaluation of oil acidity, bitter taste and fatty acids composition. NIR spectroscopy has also been applied successfully to detect the fraudulent addition of other vegetable oils to the olive oil (Wesley, Barnes, & McGill, 1995) and to determine geographic origin (Galtier, et al., 2007). In a recent paper, Bellincontro et al. (2012) applied NIR-AOTF spectroscopy to the on-field measurement of the evolution of the total phenolic profile and other specific metabolites during olive fruit ripening, obtaining good predictive models. In horticultural foods, fruit firmness is measured by puncture-based tests following the Magness-Taylor procedure or using a texture analyzer or hand-held penetrometer to measure the maximum penetration force and other related parameters (Chen, & Opara, 2013). The application of NIR spectroscopy for the analysis of textural parameters has often led to unsatisfactory results in other fruits (Nicolai et al.,

2007). Difficulties arise from several factors, including the high instrumental error of puncture-based tests, the variability of firmness values and, in general, the development of a calibration model to predict an index that is not directly associable with a chemical species. In olive fruit, Kavdir et al. (2009) applied NIRS for predicting firmness using the Magness-Taylor (MT) maximum force as reference measure, obtaining a barely acceptable R^2 value of approximately 0.7 in cross-validation. Beghi et al. (2013) obtained a similar value, where the predictive model was developed using a portable penetrometer as the reference measure.

The purpose of this work was to develop a NIRS-based approach for on-field monitoring of olive drupe physical properties (i.e., texture, total chlorophylls, total carotenoids, total anthocyanin, total and specific phenolic compounds) during ripening in three cultivars with extremely different genetic and phenotypic properties. The three cultivars considered were Leccino and Buscionetto, known in olive oil production as high and low phenolic content fruits, respectively (Alagna et al., 2012; Bartolini, 2015), and the cv. Leucocarpa mutant, which synthesize very low amount of flavonoids (Pasqualone et al., 2012).

All of the results obtained were combined and used as reference data to compare with NIR spectra, with the aim of developing accurate predictive models. These models could be used to implement a rapid and functional method for determining, through a multiparametric approach, the most advantageous harvesting time for high quality VOO production.

2. Materials and Methods

2.1 Plant material

Olive plants of from the cvs Leccino and Leucocarpa were cultivated at the

156 experimental farm of the University of Tuscia (42°250’ N, 12°080’ E), whereas those of
157 the cv. Buscionetto were at the ARSIAL field collection, located at Montopoli in Sabina
158 (42°12' N, 12°38' E). The plants were rain fed and fertilized in the spring, receiving a total
159 of approx. 90 g of N, P_2O_5 and K_2O. Drupes of the cv. Leccino are categorized as fruit with
160 ‘high phenolic content,’ whereas those of Buscionetto are considered to have a ‘low
161 phenolic content’ the drupes of the cv. Leucocarpa are defined as fruit without any
162 accumulation of anthocyanin compounds (Pasqualone et al., 2012). Drupes were randomly
163 harvested from those positioned in the equatorial part of the entire canopy for three plants
164 from each cultivar. The fruits were sampled according to phenological observations during
165 the ripening process. At each sampling time point, a total number of 30 drupes were
166 collected and split into three aliquots of 10 drupes each. Texture analysis and NIR spectra
167 acquisition were rapidly performed on the collected fruit. The drupes were then
168 immediately frozen in liquid nitrogen and stored at -80°C until destructive analysis was
169 performed.

170 *2.2 Ripeness Index*

171 The ripening index (RI) was determined according to the method described by the
172 International Olive Oil Council (Salvador, Aranda, & Fregapane, 2001), and the ripening
173 developmental period was split into four stages according to the work of Conde, Delrot &
174 Gerós (2008) and Cimato, Baldini, & Moretti (2001). For this purpose, 100 drupes were
175 randomly sampled as previously described and divided into color groups according to the
176 spread of pigmentation on the pericarp and mesocarp of the fruit. The scale for color
177 grouping varied from 0 (intense green) to 7 (100% colored of pericarp and mesocarp). The
178 index was calculated as the weighted average number of drupes within each subset of
179 samples.

180 *2.3 Fruit firmness measurement*

Olive firmness was estimated using a deformation test carried out on an Instron Universal Testing Machine - model 5900 (Instron Inc., Canton, MA, USA). Each entire drupe was placed on the flat surface support and pressed vertically in the middle part of the drupe using a flat 35 mm probe, with a load of deformation equal to 5 N and a bar speed of 25 mm min^{-1}. This fixed load value was determined after assessing the damage to several fruit peels and pulps under different load values as well as the reliability of the response, which was reported as fruit deformation (mm).

2.4 Total chlorophyll quantification

The total chlorophyll amount was determined in the olive fruit as described by Moran (1982), with slight modification. Briefly, the total chlorophylls were extracted by incubating 100 mg of drupe tissue in *N*,*N*-dimethylformamide (Sigma-Aldrich, Milano, Italy), using a 1:10 volume/weight ratio, for 24 hours at 4°C. The liquid phase was filtered and the absorbance was measured at 625, 647 and 664 nm using a spectrophotometer (Thermo Scientific, Milano, Italy) and 1 cm quartz cuvettes. The total chlorophyll concentration was determined by the equation Chl_{tot} = 7.04 (A_{664}) + 20.27 (A_{647}) and expressed as mg g^{-1} of fresh tissue. Analyses were performed in triplicate.

2.5 Total carotenoid quantification

Carotenoids were extracted by incubating 100 mg of drupe tissues in 1 mL of 100% (v/v) acetone for 24 hours at 4°C. The total carotenoid amount (xanthophylls plus carotenes) was determined by measuring thee absorbance at wavelengths of 470, 645 and 663 nm, using the equation $C_{(x+c)}$ = (1000 A_{470} – 2.27 Chl_a - 81.4 Chl_b)/227 (Lichtenthaler & Wellburn, 1983). The total carotenoid content was expressed as mg g^{-1} of fresh tissue. Analyses were performed in triplicate.

2.6 Total anthocyanin quantification

Total anthocyanins were quantified using the protocol described by Martinelli & Tonutti (2012). Briefly, 100 mg of fruit tissue was ground with pre-chilled mortar and pestle, extracted with 5 mL of a methanol:HCl (1%) solution and incubated overnight at 4°C in darkness. The supernatant was obtained by centrifugation at 5000 RCF and filtered. Spectroscopic analysis was performed by measuring the absorbance at 530 nm. Serial dilutions of a cyanidin-3-glucoside standard (SIGMA, Italy) were used to generate a reference curve, and anthocyanin concentration was expressed as mg g^{-1} of fresh weight.

2.7 Sample preparation and HPLC analysis

Fruit were frozen in liquid nitrogen, stored at −80°C, and successively used to determine the phenol content. Phenols were extracted from the olive pulp according to the procedure previously published by Bellincontro et al. (2012) with slight modification. Briefly, 10 g of frozen olive pulp was homogenized with 100 mL of 80% methanol containing 20 mg L^{-1} butylated hydroxytoluene (BHT); this extraction was performed in triplicate. After methanol removal, the aqueous extract was used for the extraction of phenols by solid-phase separation (SPE). The SPE procedure was applied by loading a 1000 mg Bond Elute Jr-C18 cartridge (Agilent Technologies, USA) with 1 mL of sample and using 50 mL of methanol as the eluting solvent. After solvent removal under vacuum at 30°C, the phenolic extract was recovered, then dissolved in methanol (1 mL), and filtered through a polyvinylidene fluoride (PVDF) syringe filter (0.2 μm). HPLC analyses for oleuropein, verbascoside and 3,4-DHPEA-ED were then conducted according to the procedure of Selvaggini et al. (2006) using a reversed-phase column on an Agilent Technologies system Model 1100 (Agilent Technologies, Santa Clara, CA, USA) equipped with a vacuum degasser, a quaternary pump, an autosampler, a thermostated column compartment, a diode-array detector (DAD) and a fluorescence detector (FLD). The C18 column used in this study was a Spherisorb ODS-1 250 x 4.6 mm with a particle size of 5

μm (Waters, Milford, MA, USA); the injected sample volume was 20 μL. The mobile phase consisted of 0.2% acetic acid (pH 3.1) in water (solvent A) / methanol (solvent B) at a flow rate of 1 mL min^{-1} and the gradient was as follows: 95% (A) / 5% (B) for 2 min, 75% (A) / 25% (B) in 8 min, 60% (A) / 40% (B) in 10 min, 50% (A) / 50% (B) in 16 min and 0% (A) / 100% (B) in 14 min. This composition was maintained for 10 min, returned to the initial conditions and equilibrated for 13 min, giving a total running time of 73 min. Phenol detection was performed using the DAD set at 278 nm. The oleuropein, verbascoside and quercetin-3-O-rutinoside (rutin) were purchased from Extrasyntethese (Genay, France). 3,4-DHPEA-EDA was extracted from virgin olive oil using a procedure previously reported by Montedoro et al. (1993). The purity of this compound was tested by analytical HPLC, and NMR test (Montedoro et al., 1993) verified its chemical structure.

The HPLC analyses of rutin were conducted with the same instrumentation reported above. The C18 column used was Inertsil ODS-3, 150 m with a particle size of 5 mm (GL Sciences Inc.). The volume of injected sample was 20 mL. The mobile phase was 5% formic acid in water (A) / acetonitrile (B) at a flow rate of 0.9 mL min^{-1}. The total running time was 64 min, and the gradient was as follows: 95% (A) / 5% (B) for 5 min, 35% (A) / 65% (B) in 50 min, 0% (A) / 100% (B) in 3 min, return to initial conditions in 2 min, and hold for 4 min. Rutin was detected by the DAD at 360 nm.

2.8 NIR spectra collection

A laminar 5030 miniature Hand-held NIR Analyzer (Brimrose Corporation, Baltimore, 92 MD, USA), based on the Acousto-Optical Tunable Filter (AOTF) NIR principle, was used for spectral detection. This instrument is a portable device that can be used directly in the field on tree, although in this experimental work the spectral acquisition was performed under laboratory conditions. Two distinct measurements were performed on each intact olive through contact between the external gun of the NIR device

and the pericarp of the fruit using the diffuse reflectance method of detection, whereas the raw spectra were detected and recorded in transmittance, as reported by Santos & Kaye (2005). Detection was conducted over the 1100-2300 nm range using 2 nm wavelength increments and ten spectra per average, which represented a single measurement. The average of the two measurements was regarded as the spectral response of the fruit.

2.9 Near infrared spectroscopy analysis and chemometrics

The raw spectra were statistically pre-treated for absorbance (log 1/T) transformation using SNAP 2.03 software (Brimrose, Crop, Baltimore, MD, USA). Before calibration and developing the predictive models, the spectral variation in the data sets was analyzed using Principal Component Analysis (PCA). The absorbance spectra, obtained as the spectral average for each olive subset, were used as X-variables in the final models. Partial Least Squares (PLS) models were obtained on the full spectrum observed, considering the spectrally significant variables at specific wavelength intervals. The mean values and the standard deviation (SD) values obtained by analyzing the HPLC measurements were used as Y-variables in the PLS matrices, in which they were contrasted with the averaged spectra, as previously reported. Models were developed for the specific phenols as well as for total phenols, calculated as the sum of the measured compounds. Models were constructed by combining data from all three cultivars and the total sample set of data (n = 33). No outlier identification or elimination was applied. The following statistical indices were used to determine the significance of the calculations: R^2 (coefficient of multiple determination) in calibration, cross-validation and prediction; Root Mean Standard Error in Calibration, Cross-Validation and Prediction (RMSEC, RMSECV, RMSEP); and bias. PCA, statistical pretreatments, and PLS models were performed using Unscrambler v9.7 software (CAMO ASA, Oslo, Norway). Graphs, score plots and scatter

plots were generated after data exportation from Unscrambler using SigmaPlot v. 10.0 (Systat Software Inc., San Jose, CA, USA).

3. Results and Discussion

The onset and length of the ripening period for the olive fruit was different among the three cultivars. The green stage (V-I) was reached approximately 130 days after bloom (DAFB) in cv. Leccino, whereas it occurred later in cvs Leucocarpa and Buscionetto, at 155 and 158 DAFB, respectively. The ripening process lasted for four weeks in the drupes of Leccino and Leucocarpa and ended at 155 and 180 DAFB, respectively, whereas it was significantly shorter in Buscionetto, lasting for three weeks and ending at 175 DAFB. As shown in Table 1, the Ripening Index increased during olive fruit development differently between the colored Leccino and Buscionetto, reaching values of 3.76 and 2.4, respectively. This parameter was not determinable in Leucocarpa due to the lack of fruit pigmentation (Figure 1). The RI value determined in Leccino is often indicated in the literature as the optimum harvest period (Rotondi et al., 2004), corresponding to the stage V-IV of ripeness. At this stage, the pigmentation on the pericarp tissue of the drupes is spread differently among the three cultivars, varying from the purple-black color of Leccino to the reddish and white-ivory of Buscionetto and Leucocarpa, respectively (Figure 1). Moreover, a different pigmentation was also visible in mesocarp tissues: a complete white color was present in that of cv. Leucocarpa; white with a layer of reddish tissue near the pericarp tissue in Buscionetto; and green-white in the mesocarp of Leccino (Figure 1). The diverse pattern of pericarp and mesocarp pigmentation of the drupes could reflect differences in the pattern of synthesis and accumulation of total chlorophylls, carotenoids and anthocyanins between the three cultivars.

During ripening, drupe fresh weight increased until stage V-II in Leccino and then decreased. The dynamics of fruit growth was different in the two other cultivars. Fruit growth increased until full ripeness in Leucocarpa, whereas it slightly decreased in Buscionetto during the ripening period (Table 1). The firmness values of the drupes decreased during the ripening of fruits, although the softening process appeared to be cultivar-dependent (Figure 2). Indeed, firmness dropped rapidly in Leucocarpa compared to Buscionetto and Leccino, and in the last cultivar, the firmness was consistently the highest until harvesting time. As expected, the total chlorophyll and carotenoid content decreased during the ripening progress, although the rate of chlorophyll degradation was higher. The extent of decrement was cultivar-dependent as the mature drupe of Leccino retained nearly double the chlorophyll content of Leucocarpa and Buscionetto (Table 2). Total anthocyanins were higher in the drupes of Leccino than in those of Buscionetto, and only trace amounts were detected in the drupes of Leucocarpa. A high total phenol content was detected in the drupes of cv. Leucocarpa and cv. Leccino, and the values were comparable to those reported in the literature for the same as well as other cultivars (Alagna et al., 2012; Pasqualone et al., 2012; Esti, Cinquanta, & La Notte, 1998). Typical of low-phenol cultivar, a significantly lower total phenol content was detected in the fruits of Buscionetto, which had the lowest value among the three cultivars studied. The amount of phenolic compounds showed a decreasing trend during the ripening period. However, the dynamics of the decrement were quite different among the cultivars as the decrease was more accentuated in Leccino and Buscionetto than Leucocarpa (Table 2).

Qualitative analysis of single phenolic compounds also highlighted important differences between the cultivars, i.e., the compound verbascoside was undetectable in the drupes of Buscionetto, whereas rutin compound was undetectable in Leucocarpa. According to other research reported in the literature (Alagna et al., 2012), the amount of

each phenolic compound decreases during ripening (Table 2). At harvest time (stage V-VI), the content of oleuropein, verbascoside and 3.4, DHPEA-EDA was higher in the drupe of Leucocarpa than in the other two cultivars. In particular, at stage V-IV the oleuropein and verbascoside content was equal to or higher than that at the stage V-I of the ripening period of the Leucocarpa drupe (Table 2). The qualitative and quantitative variability in the phenolic composition of the olive fruit among the cultivars is particularly interesting, considering that a widespread variability of data is favorable for generating a model by multivariate regression.

Many wavelengths of the NIR spectrum affect the PLS modelling. Thus, the entire spectrum (1100-2300 nm) was monitored to build a calibration model for each class of compounds and for the firmness parameter. As shown in Figure 3, the principal component analysis (PCA) calculated for all spectral datasets discriminated the three cultivars, and significant separation was obtained for Buscionetto. In particular, the variance was well explained by PC1 and PC2 and accounted for approximately 98% of the observed variability. The ability of NIR spectra to discriminate cultivars was previously reported by Bellincontro et al. (2012).

The accuracy of the PLS was described by the coefficient of determination in calibration (R^2) and cross-validation or prediction (R^2cv, R^2p), the root mean square error of calibration (RMSEC) and the root mean square of cross-validation (RMSECV) or prediction (RMSEP). The number of latent variables (LVs) was selected to minimize the RMSECV or RMSEP. In general, fitted models are characterized by high R^2 and by low RMSEC and RMSEP values but with small differences to each other. Indeed, elevated differences between RMSEC and RMSEP indicate the introduction of too many latent variables in the model. Excluding the PLS model of total chlorophylls, which had a value of $R^2 = 0.86$, the other PLS models had values close to or higher than 0.9, indicating valid

353 quantitative information in the detected results (Table 3, 4). The values for calibration and
354 cross-validation of the physical and biochemical parameters in the olive drupes during the
355 ripening period are presented in Table 3, whereas the values of biochemical parameters
356 that were used to validate the previous calculations already performed for the other
357 cultivars are presented in Table 4 (Bellincontro et al., 2012). The highest correlation (R^2 =
358 0.997) value was obtained for firmness (Fig. 4), whereas slightly lower values were
359 obtained for total phenol and verbascoside content (R^2 = 0.965 for both; Fig. 5a and Fig.
360 5c, respectively), 3,4-DHPEA-EDA (R^2 = 0.934; Fig. 5b), rutin (R^2 = 0.925; Fig. 5e) and
361 total anthocyanins (R^2 = 0.910; Fig. 5f). Lower but still acceptable R^2 were obtained for the
362 calibration models for oleuropein (R^2 = 0.897; Fig. 5d), total carotenoids (R^2 = 0.887; Fig.
363 5g) and total chlorophylls (R^2 = 0.868; Fig. 5h). The RMSEC index, expressed as
364 milligrams per gram of fresh weight, varied from the lowest value of 0.002 for total
365 carotenoids to the highest of 1.44 for 3,4-DHPEA-EDA, whereas the number of LVs was
366 in the range of 4-8, except for verbascoside, where it was of 10. The leave-one-out cross-
367 validation method was used to evaluate the predictive ability of the PLS models. This
368 method is considered appropriate for a limited sample data set (Dardenne, 2010).
369 The cross-validation of PLS models was characterized by a reduction of the R^2cv
370 coefficient, particularly for total anthocyanins and rutin (R^2cv = 0.80 and R^2cv = 0.83,
371 respectively). However, the RMSEC and RMSECV indices for total chlorophyll and
372 carotenoids had very similar values, indicating that an optimum number of factors were
373 included in the models. Interestingly, the cross-validated model for the firmness parameter
374 still had a high R^2cv value (0.99) and low error. Oleuropein, verbascoside, 3,4-DHPEA-
375 EDA and total phenols were validated using PLS models already created by Bellincontro et
376 al.[36] in the cvs Moraiolo, Dolce d'Andria and Nocellara Etnea. The validation showed a
377 substantial reduction in the R^2 value and an approximately 2-fold increase in the error of

RMSEP (Table 4). The R^2p ranged from the lowest value of 0.74 for oleuropein and was highest for total phenols, 3,4-DHPEA-EDA and verbascoside (R^2p = 0.85, 0.84 and 0.82, respectively). The observed reduction in the determination coefficient using the PLS model obtained from different cultivars highlights the necessity of developing specific models for each cultivar to improve the predictive ability of NIR. Residual predictive deviation (RPD), which is the ratio between the standard deviations of reference measures and the standard error of prediction, was also calculated for all models. Except for oleuropein, which had value that was not sufficient, the RPD values for the other models indicated a discrete discrimination ability. Firmness parameters were highly discriminant, showing a very high value of 13.86 (Table 3, Table 4).

Although the increased expectations of consumers for food products that are of high quality and safety necessitate accurate quality determination, many agronomical and food process decisions are based on fast of determination these characteristics. New techniques, therefore, become necessary to enable control over the quality parameters to meet requirements during handling, storage and acceptability by the consumer (Chen & Opara, 2013). In the olive, the identification of the optimum harvesting time of the fruit through accurate, rapid and cost-effective methods is a new challenge for producing extra virgin high-quality oils enriched with phenolic compounds (Bonoli, Bendini, Cerretani, Lercker, & Gallina-Toschi, 2004). The intrinsic variability of the olive fruit ripening process, which is influenced by genetic, environmental and agronomic factors, requires intensive and accurate monitoring of compounds to determine oil quality. Pigments and phenolic compounds affect important quality attributes of VOO, such as color, stability, sensory profile and nutritional properties (Inglese et al., 2011). The firmness of the olive drupe should also be considered an important marker as it has practical implications during olive fruit processing for the extraction yield of oil and phytochemicals as well as for oil quality

403 (García, Seller, & Pérez-Camino, 1996; Servili et al. 1992; Mínguez-Mosquera, Gallardo-
404 Guerrero, & Roca, 2002). Kavdir et al. (2009) and, more recently Beghi et al. (2013), have
405 correlated olive firmness measured with a portable penetrometer to reflectance spectra; in
406 the first case the spectra were detected by a FT-NIR spectrometer (ranging from 800 to
407 2500 nm), whereas a vis/NIR spectrophotometer (ranging from 400 to 1000 nm) was used
408 in the second. In Kavdir's work[40] the R^2 results obtained in calibration and in cross-
409 validation were of 0.75 and 0.68, respectively, whereas they were equal to 0.68 and 0.66,
410 respectively, in Beghi's work (2013).

411 In the present study, non-destructive NIR-AOTF technology provides a suitable
412 method for the on field monitoring of the maturation process. The firmness R^2 values for
413 calibration and cross-validation were as high as 0.99. The R^2 values for total chlorophyll,
414 total carotenoids, total anthocyanins and rutin ranged from 0.86 to 0.92 for calibration and
415 from 0.80 and 0.85 for cross-validation. This experience gave us the opportunity to
416 develop a predictive model of firmness for intact drupes with high degree of fitness and
417 statistical significance and low RMSEC/RMSECV ratio. Recently, Giovenzana et al.
418 (2015) described models for the prediction of texture, using vis/NIR and NIR spectroscopy
419 on Moraiolo and Frantoio olive drupes directly at the mill, just before the oil extraction
420 process, and obtained calibration and validation R^2 values ranging from 0.86 to 0.88 for
421 spectroscopic techniques.

422 The obtained results confirmed the ability of NIRS-AOTF to predict total phenol
423 content and specific metabolites, as previously reported in other cultivars. The use of fruits
424 collected from the plants of cultivars defective in specific phenotypical characters and,
425 therefore, considered as natural mutants allowed for the robustness of the procedure to be
426 assessed. The cultivars diverged in phenol composition: Leccino fruit contains a high
427 amount of phenols and a complete phenolic composition, Leucocarpa fruit does not contain

any flavonoids and Buscionetto fruit contains a low amount of phenols and does not contain verbascoside. These fruit properties contributed to the validation of the NIRS-AOTF as a non-destructive method for estimating the phenolic content in olive fruit during ripening (Bellincontro et al., 2012). In fact, a large degree of phenolic variability is included in the pool of those cultivars, resembling the variability that is found in the olive fruit (Alagna et al., 2012). This strategy was interesting as it also allowed us to elaborate satisfactory models for the prediction of chlorophyll and carotenoid content.

Interestingly, a very high correlation with a low RMSEC/RMSECV ratio was also found for firmness prediction. Indeed, the use of a non-destructive compression-test and accurate instruments for texture analysis to obtain reference measures appear to have improved the predictive capabilities of NIR. Although an additional number of samples will be required to improve the model's robustness, the results are particularly encouraging, especially considering that the application of NIR spectroscopy for firmness prediction has encountered considerable difficulties.

Knowledge of the optimal ripening stage of the olive fruit is a strategic point for producing high quality virgin olive oil. In addition to some important compounds and their evolution during the ripening process, the firmness of the drupe was also considered, which is also an important parameter necessary for predicting bruising damage during and between harvesting as well as during olive processing (García & Yousfi, 2006). Furthermore, avoiding physical and biological deterioration of the fruit is a goal for the production of both high quality virgin oil and high quality table olives. This importance of this goal might be accentuated by the total mechanization of farming, from planting to harvesting, and the need to characterize new cropping systems (Camposeo, Vivaldi, & Gattullo, 2013). The accumulation of anthocyanin compounds increases during the ripeness, except in the null mutant Leucocarpa, and this behavior is counterpoised to that

of total chlorophyll, phenolic and carotenoid compounds. Thus, anthocyanins can be considered an important analytical marker for determining the best ripening stage of fruit, in combination with traditional indices such as oil accumulation. The results obtained from the natural mutant for the accumulation of phenols and from the cv. Leccino define and validate the rapid method for evaluating phenolic compounds directly in olives using a non-destructive technology such as NIR-AOTF spectroscopy. The use of the natural mutants improved the robustness of the predicting models by taking a large biological variability into account. This technology has the important advantage that it can be used on field, even for measuring firmness and total anthocyanin in null mutants and specific phenolic compounds in the cultivars.

4. Conclusion

We studied the applicability of NIR-AOTF spectroscopy as a rapid and inexpensive technique, using a portable instrument for physical and chemical analysis of olive properties during ripening and at maturation, just before oil extraction. The obtained results for some parameters enabled us to develop specific models that can be used as predictive systems, even for other cultivars. In the meantime, the accumulation of data here improved the predictive power and robustness of models previously developed for other cultivars. The asynchronous maturation of the fruit causes extreme variability in the evolution of physical and chemical properties among the fruits of a canopy. Therefore, the opportunity to overcome the difficulty of estimating a ripeness index that is not directly correlated with specific chemicals using reference data from many physical and chemical properties of a single drupe will allow for good results to be obtained for the development of maturation models for olive fruit by optical, non-destructive systems. The accumulation of experience and data as well as the selection of specific wavelength ranges for spectral analyses will be

helpful for improving the portable inexpensive device and the overall program to monitor physical and chemical properties of fruit. Understanding the firmness and quality properties of olive drupes, which can develop differently into various fruits of a canopy, is key to developing novel approaches that will advance our ability to identify and characterize the stages of ripeness, detect the optimal harvesting time and, ultimately, produce high quality extra virgin olive oil and table olives.

Abbreviations and Nomenclature

AOTF, Acousto Optically Tunable Filter; cv., cultivar; DAD, diode-array detector; FLD, fluorescence detector; NIR, Near Infrared; PCA, Principal Component Analysis; PLS, Partial Least Square; RMSEC, Root Mean Standard error in Calibration; RMSECV, Root Mean Standard error in Cross-Validation; RMSEP, Root Mean Standard error in Prediction; RPD, Residual predictive deviation; 3,4-DHPEA-EDA, 3,4-DHPEA-Elenolic acid Di-Aldehyde (Oleuropein-aglycone di-aldehyde).

Conflict of interest

The authors declare no conflicts of interest

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Figure captions

Olive fruits overview.

Figure 1. Overview showing the diffusion of pigmentation on the pericarp and mesocarp of the mature drupes of Leucocarpa, Leccino and Buscionetto olive cultivars at ripening stage V-IV.

Fruit firmness

Figure 2. Firmness evolution during drupe development in the ripening stages in Leccino, Leucocarpa and Buscionetto olive cultivars. Firmness is expressed in N/mm of deformation under a constant load force of 5 N. Values are the mean of three biological replicates (10 drupes per replication) ± standard deviation. Asterisks indicate a statistically significant difference with $p<0.05$; ns, not significant.

PCA analysis of NIR-AOTF

Figure 3. Three-dimensional score plot of the principal component analysis (PC1 vs PC2 vs PC3) conducted on the absorbance NIR-AOTF spectra of grouped samples coming from all three olive cultivars (Leccino, Leucocarpa and Buscionetto). The percent of the explained variance is reported in parentheses on the axes.

Predictive model of firmness

Figure 4. Scatter plot for the drupe firmness compared to the predictive model for the global data set of olive samples (sum of the three cultivars). For each compound measured, experimental values are plotted versus predicted values. Calibration and validation data sets are also grouped and reported. Leucocarpa values are shown with white symbols,

while the values of Buscionetto and Leccino are shown with light gray and dark gray symbols, respectively.

Predictive model of metabolite compounds

Figure 5. Scatter plots compared to the predictive models for total phenols (**a**), DHPEA-EDA (**b**), verbascoside (**c**), oleuropein (**d**), rutin (quercetin-3-O-rutinoside) (**e**), total chlorophylls (**f**), total anthocyanins (**g**) and total carotenoids (**h**) for the global data set of olive samples (sum of the three cultivars). For each compound measured, experimental values are plotted versus predicted values. Calibration and validation data sets are also grouped and reported. Leucocarpa values are shown with white symbols, while the values of Buscionetto and Leccino are shown with light gray and dark gray symbols, respectively.

Table 1

Table 1. Pomological characteristics of the olive cultivars analyzed, evaluated at different ripening stages. The color index was not determined for Leucocarpa, as the typical pericarp red color did not develop in these drupes. Superscript letters for the DAFB values indicate the ripening developmental stage: [a] stage V-I (green-ripe stage), [b] stage V-II (veraisòn), [c] stage V-III (full veraisòn), and [d] stage V-IV (ripe fruit). The reported values are the mean of three biological replicates (10 drupes per replicate) ± standard deviation.

Cultivar	Drupe Sampling (DAFB)	Ripening Index (0-7)	Fresh Weight (g)	Polar Diameter (mm)	Transverse Diameter (mm
Leccino	130[a]	0.26	2.89±0.15	24.60±0.69	16.49±0.36
	140[b]	1.97	3.01±0.21	24.53±0.53	16.41±0.33
	147[c]	3.28	2.84±0.11	23.89±0.26	16.29±0.18
	155[d]	3.68	2.73±0.18	24.28±0.22	16.67±0.40
Leucocarpa	155[a]	-	1.57±0.25	18.16±0.31	10.82±0.26
	166[b]	-	1.69±0.12	19.32±0.42	10.93±0.35
	173[c]	-	1.78±0.16	20.86±0.38	11.08±0.38
	180[d]	-	1.83±0.16	19.79±0.67	11.17±0.21
Buscionetto	158[a]	0.38	5.23±0.44	26.87±0.81	21.54±0.52
	166[c]	2.06	5.07±0.28	27.29±0.75	21.85±0.64
	175[d]	2.45	5.02±0.31	26.45±0.62	21.49±0.49

8

Table 2

Table 2. Content of total chlorophylls, total carotenoids, total anthocyanins, total phenols and principal phenol compounds in the drupes of cvs Leccino, Leucocarpa and Buscionetto, as detected at different stages of ripening. Superscript letters for the DAFB values indicate the ripening developmental stage according to Conde et al. 2008 and Cimato et al. 2011: [a] stage V-I (green-ripe stage), [b] stage V-II (veraisòn), [c] stage V-III (full veraisòn), and [d] stage V-IV (ripe fruit). The values, which are expressed as mg/g of fresh weight, are the mean of three biological replicates (10 drupes per replicate) ± standard deviation.

Cultivar	Sampling Stage (DAFB)	Deformation (mm)	Total Chlorophyll (mg g-1)	Total Carotenoids (mg g-1)	Total Anthocyanins (mg g-1)	Oleuropein (mg g-1)	Verbascoside (mg g-1)	Rutin (mg g-1)	3,4-DHPEA-EDA (mg g-1)	Total Phenols (mg g-1)
Leccino	130[a]	0.379±0.015	0.100±0.008	0.024±0.007	0.008±0.001	9.20±0.08	1.75±0.04	1.12±0.05	9.38±0.10	24.6±0.15
	140[b]	0.457±0.021	0.059±0.004	0.015±0.001	0.028±0.003	4.51±0.02	0.29±0.01	0.61±0.01	8.45±0.07	16.3±0.08
	147[c]	0.514±0.061	0.043±0.004	0.010±0.001	0.200±0.029	1.48±0.04	0.97±0.02	0.70±0.01	2.86±0.11	8.43±0.21
	155[d]	0.873±0.083	0.039±0.005	0.008±0.001	0.297±0.018	1.58±0.01	0.91±0.02	0.70±0.02	3.20±0.26	8.70±0.30
Leucocarpa	155[a]	0.326±0.011	0.104±0.010	0.030±0.007	0.010±0.003	3.94±0.03	0.69±0.04	0.0	16.8±0.07	22.3±0.01
	166[b]	0.443±0.056	0.063±0.003	0.023±0.001	0.012±0.003	3.35±0.03	0.56±0.01	0.0	13.7±0.04	18.5±0.1
	173[c]	0.821±0.59	0.023±0.002	0.014±0.001	0.005±0.001	1.45±0.05	0.55±0.02	0.0	12.9±0.07	15.6±0.1
	180[d]	1.035±0.033	0.012±0.001	0.007±0.001	0.008±0.001	3.27±0.05	1.13±0.11	0.0	6.63±0.22	12.7±0.3
Buscionetto	158[a]	0.559±0.030	0.069±0.007	0.019±0.002	0.007±0.001	3.84±0.09	0.0	0.30±0.01	0.60±0.03	5.67±0.12
	166[c]	0.644±0.048	0.021±0.001	0.011±0.002	0.022±0.003	1.22±0.03	0.0	0.41±0.01	0.21±0.02	3.39±0.05
	175[d]	1.118±0.014	0.011±0.002	0.007±0.001	0.091±0.009	0.46±0.01	0.0	0.38±0.01	0.50±0.02	1.80±0.02
	Mean	0.65	0.049	0.015	0.062	3.12	0.62	0.38	6.85	12.57
	SD	0.26	0.031	0.007	0.094	2.34	0.53	0.36	5.69	7.37
	Min	0.32	0.009	0.006	0.004	0.45	0	0	0.19	1.77
	Max	1.12	0.115	0.031	0.317	9.28	1.80	1.17	16.91	24.82

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Table 3.

Calibration and cross-validation results in the PLS models for total chlorophylls, total carotenoids, total anthocyanins, quercetin-3-O-rutinoside (rutin) and firmness, calculated using the whole data set from all stages of ripening for Leccino, Leucocarpa and Buscionetto olive cultivars.

Compound	n	Calibration				Cross-Validation		
		R^2	RMSEC	LVs	Bias	R^2	RMSECV	RPD
Total chlorophylls	33	0.868	0.011	5	$-1.411\ e^{-09}$	0.828	0.013	2.45
Total carotenoids	33	0.887	0.002	4	$-1.016\ e^{-09}$	0.853	0.003	2.50
Total anthocyanins	33	0.910	0.027	6	$-1.814\ e^{-08}$	0.805	0.042	2.25
Rutin	33	0.925	0.098	6	$-1.066\ e^{-07}$	0.835	0.14	2.59
Firmness	33	0.997	0.015	4	$-9.031\ e^{-09}$	0.995	0.019	13.86

Table 4

Table 4. Calibration and cross-validation results in the PLS models for oleuropein, verbascoside, 3,4-DHPEA-EDA and total phenols, calculated using the whole data set from all stages of ripening for Leccino, Leucocarpa and Buscionetto olive cultivars.

Compound	n	Calibration				Prediction		
		R^2	RMSEC	LVs	Bias	R^2	RMSEP	RPD
Oleuropein	33	0.897	0.74	8	$-1.210\ e^{-07}$	0.746	1.2	1.95
Verbascoside	33	0.965	0.09	10	$-6.954\ e^{-08}$	0.824	0.23	2.32
3,4-DHPEA-EDA	33	0.934	1.44	7	$-1.350\ e^{-07}$	0.848	2.28	2.49
Total phenols	33	0.965	1.35	5	$-1.210\ e^{-07}$	0.858	2.82	2.61

Figure 1

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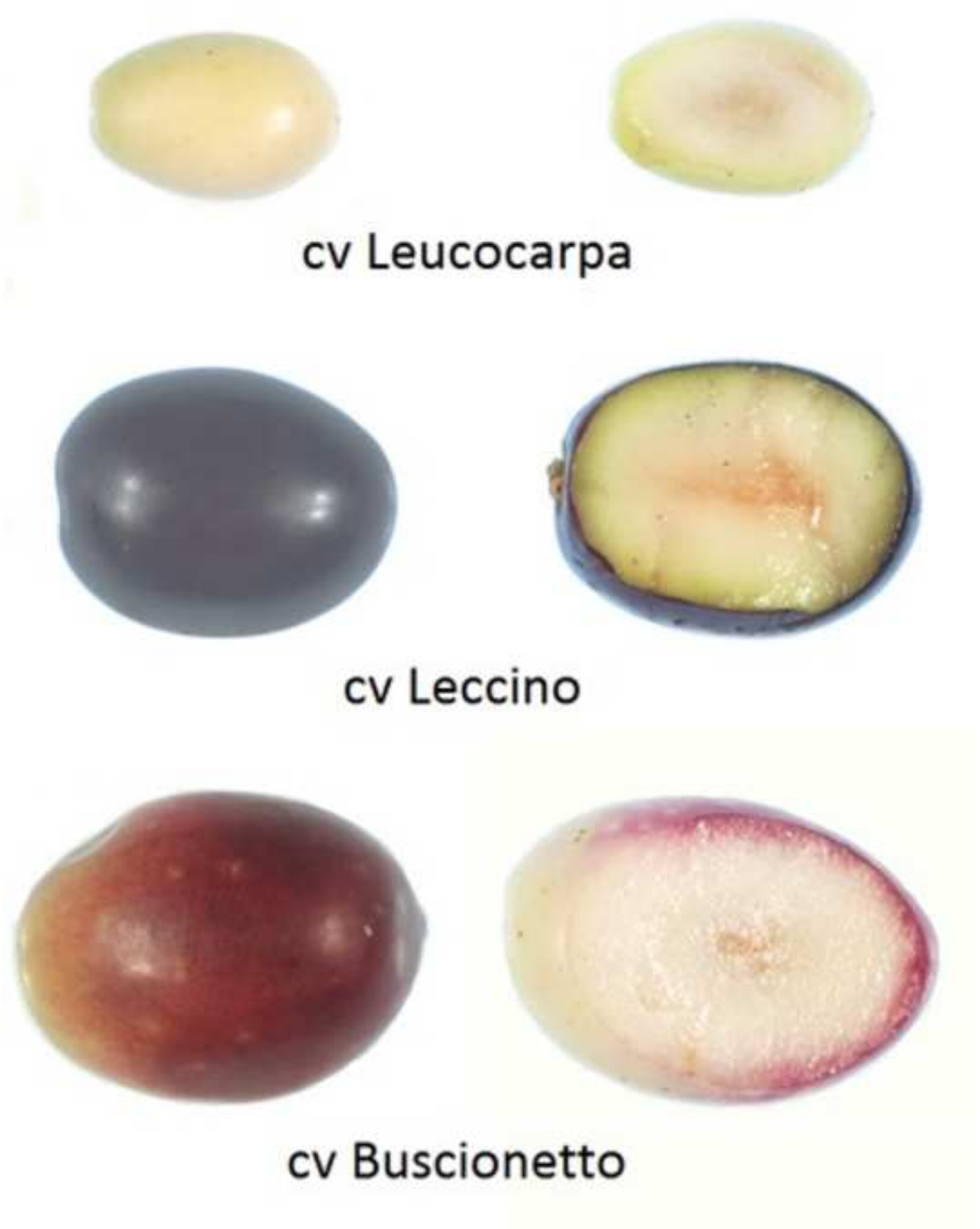

Figure 2

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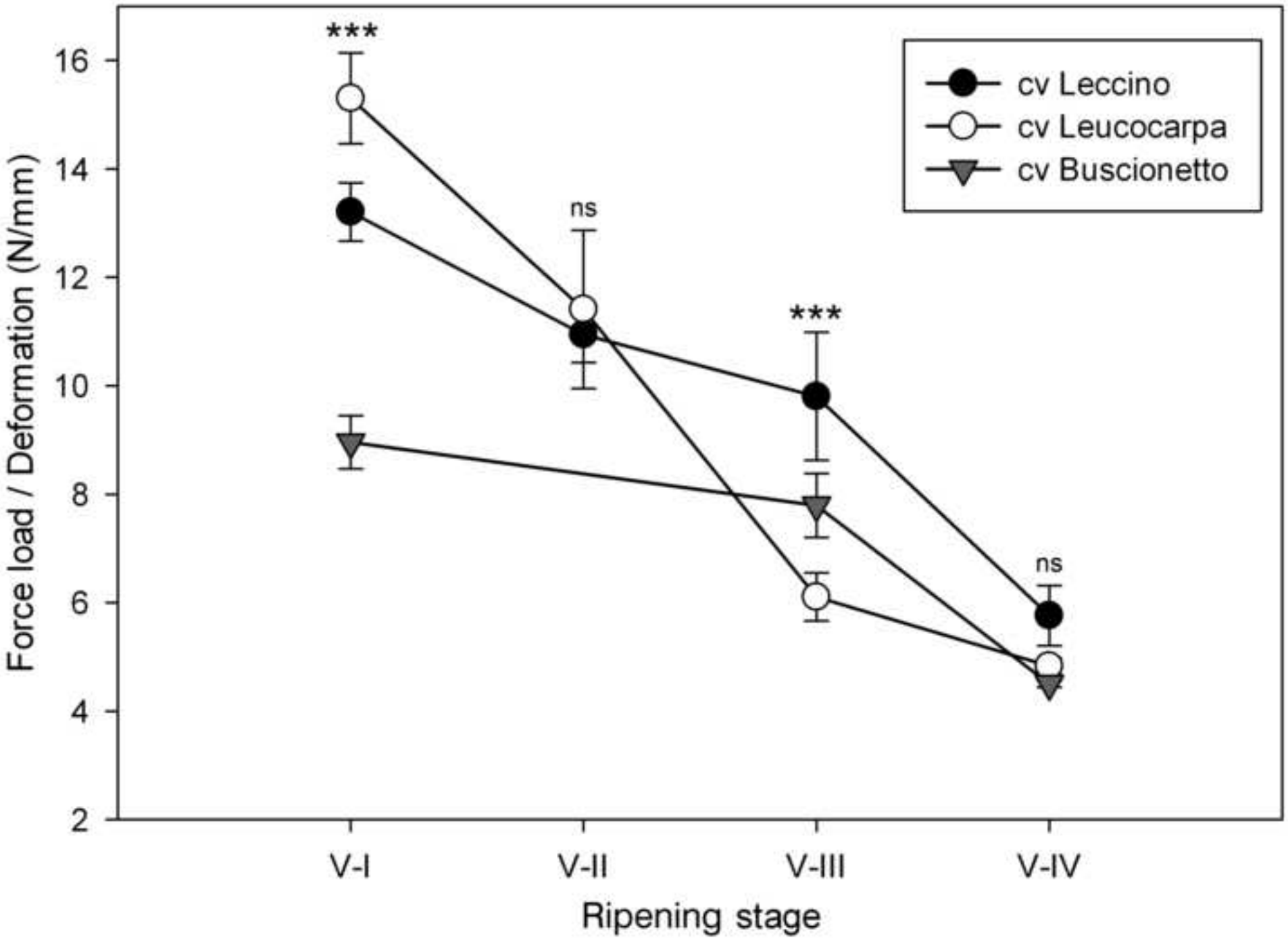

Figure 3

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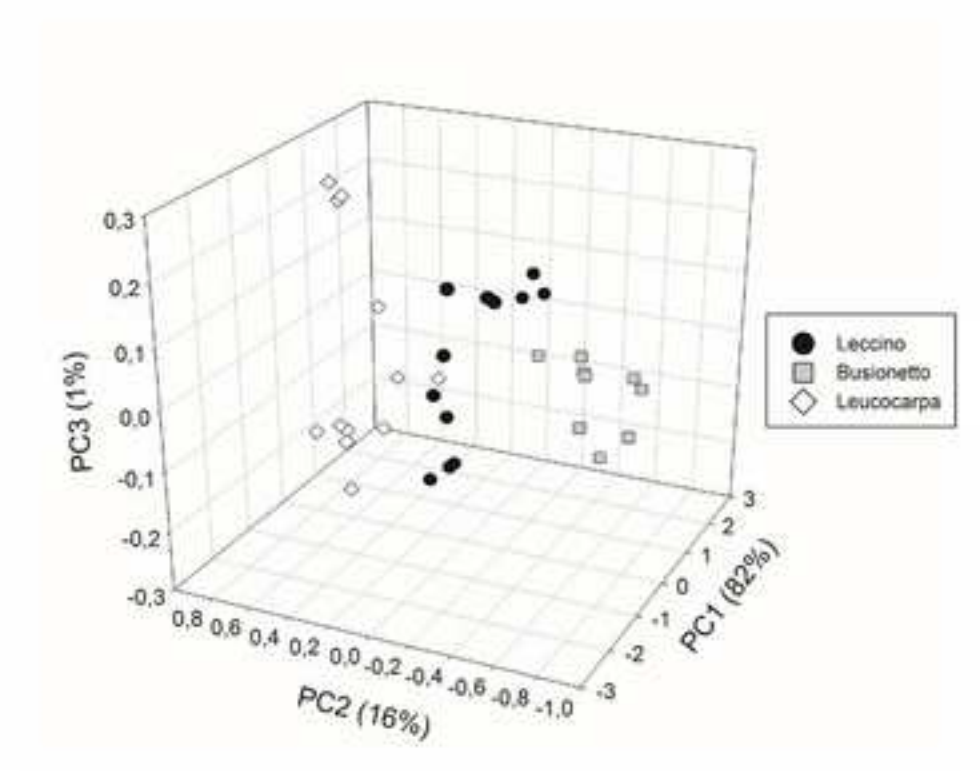

Figure 4

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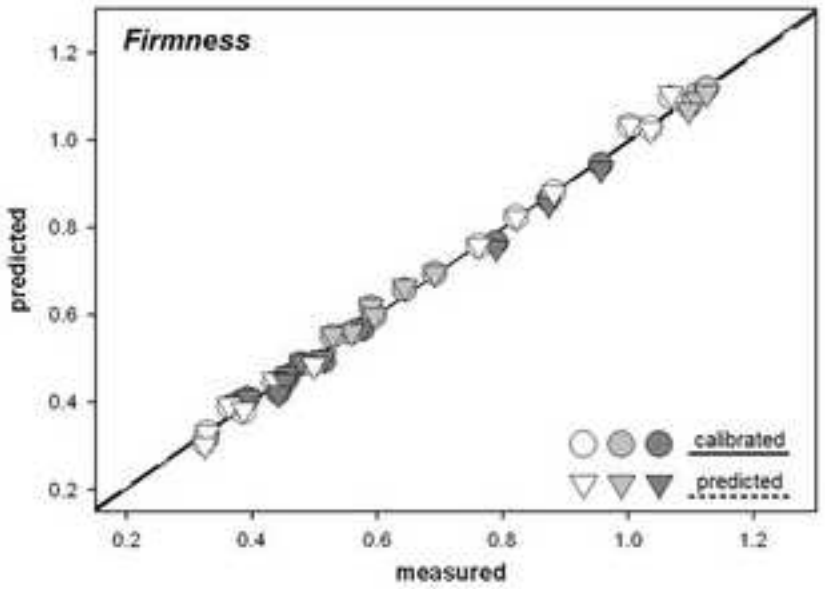

Figure 5

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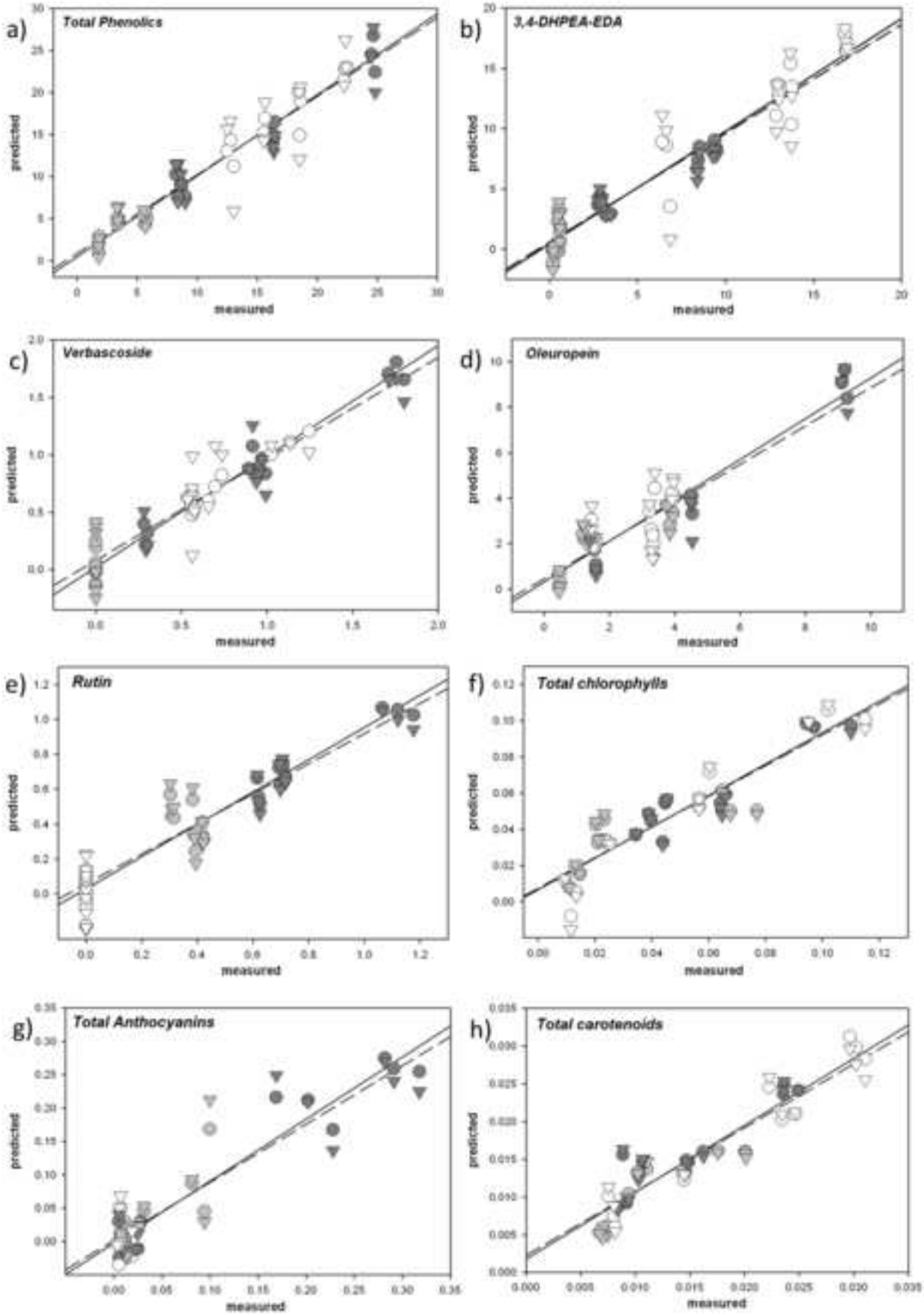

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